Identification et rôle in vitro de la chemerine, résistine et vistafine dans l'ovaire humain et bovin

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Identification et rôle *in vitro* de la chemerine, résistine et visfatine dans l'ovaire humain et bovin

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Résumé

Les adipocytokines sont des cytokines majoritairement produites par le tissu adipeux impliquées dans les mécanismes d'insulino-résistance et d'obésité. Si ces adipocytokines jouent un rôle clé dans la régulation des fonctions métaboliques, elles semblent être aussi importantes dans le contrôle des fonctions de la reproduction. Dans ce contexte, nous avons choisi de rechercher la présence au niveau de l'ovaire et d'étudier l'effet et les mécanismes moléculaires *in vitro* de trois adipocytokines appelées, chemerine, visfatine et résistine sur la stéroïdogenèse et la prolifération des cellules de la granulosa humaine. Nous avons effectué le même travail dans l'ovaire de vache pour la chemerine et la visfatine, la résistine étant déjà caractérisée dans cet organe chez cette espèce. En revanche, nous avons étudié la concentration plasmatique de la résistine et l'expression et le rôle de cette adipocytokine dans le tissu adipeux de vache au cours de la lactation.

Nous montrons que la chemerine, la résistine et la visfatine ainsi que et les récepteurs de la chemerine sont présents dans l'ovaire de femme et de vache, et plus particulièrement dans les follicules (cellules de la granulosa, cellules de la thèque et complexe cumulo-ovocytaire) et le corps jaune chez la vache. Dans les cellules de la granulosa, l'expression des adipocytokines étudiées est régulée *in vitro* par l'insuline, l'IGF-1 et les agents insulino-sensibilisateurs metformine et rosiglitazone. Dans les cellules humaines, cette régulation par la metformine s'exerce via la voie de signalisation AMPK/SIRT1. Dans les cellules bovines, l'expression du gène de la chemerine est régulée positivement par l'insuline, l'IGF-1, la metformine et la rosiglitazone alors que l'expression de ces trois récepteurs CMKLR1, GPR1 et CCRL2 est diminuée en réponse à ces différents agents. De plus, le TNF-α augmente l'expression de la chemerine, de l'adiponectine et celle de CMKLR1. L'expression du gène de la visfatine n'est pas significativement régulée par les précédents acteurs.

Chez la femme, la chemerine, la résistine et la visfatine modulent *in vitro* la sécrétion de stéroïdes par les cellules de la granulosa. La chemerine et la résistine diminuent la production de P4 et d'E2 en réponse à l'IGF-1 alors que la visfatine l'augmente. Pour induire de tels effets, la chemerine et la résistine inhibent l'expression des enzymes clés de la stéroïdogenèse comme la P450 aromatase et la P450scc ainsi que la phosphorylation de la sous-unité beta du récepteur de l'IGF-1 et la voie de signalisation MAPK ERK1/2 en réponse à l'IGF-1. L'effet des adipocytokines sur la prolifération des cellules de la granulosa est variable, en effet la chemerine diminue la prolifération cellulaire induite par IGF-1 alors que la visfatine l'augmente et la résistine n'a aucun effet. A l'état basal (sans stimulation en absence d'IGF-1 ou FSH), la chemerine, la visfatine et la résistine modulent non seulement la phosphorylation des MAPK ERK1/2 mais aussi celle d'Akt et des MAPK-P38. La phosphorylation de l'AMPK est activée seulement par la chemerine.

Chez la vache, la chemerine et la visfatine modulent aussi *in vitro* la stéroïdogenèse des cellules de la granulosa. La chemerine diminue la sécrétion de P4 et d'E2 à l'état basal et en réponse à l'IGF-1 ou à la FSH et la visfatine l'augmente en absence ou en présence de l'IGF-1. Dans ce modèle, la chemerine affecte la sécrétion de stéroïdes en diminuant la teneur en cholestérol, la quantité de la protéine de transport du cholestérol StAR, les protéines P450 aromatase et HMGCR et le niveau de phosphorylation de la voie de signalisation MAPK-ERK1/2 en présence ou en absence d'IGF-1 et FSH. Tous ces effets observés de la chemerine impliquent le récepteur CMKLR1. La visfatine quant à elle induit ces effets probablement via la voie de signalisation SIRT1. Ces deux adipocytokines n'affectent dans nos conditions ni la viabilité, ni la prolifération des cellules de la granulosa bovine. Dans les complexes cumulo-ovocytaires bovin, la chemerine bloque la majorité des ovocytes au stade GV *in vitro* et ce résultat est associé avec une diminution de la sécrétion de P4 par les cellules du cumulus et de la phosphorylation de la voie de signalisation MAPK-ERK1/2 dans l'ovocyte et les cellules du cumulus.

Chez la vache laitière, un dosage de la résistine plasmatique a été réalisé en utilisant un ELISA commercial spécifique bovin. La résistinémie est faible avant vêlage puis augmente jusqu'à atteindre un pic une semaine après vêlage, avant de diminuer progressivement pour atteindre la concentration d'avant vêlage, six semaines après la parturition. La concentration plasmatique de résistine est corrélée positivement avec le niveau plasmatique d'AGNE et négativement avec la production laitière, la matière sèche ingérée et la balance énergétique entre les semaines 1 et 22 après parturition. De plus, dans le tissu adipeux les niveaux d'expression (messager et protéine) de résistine sont plus élevés une semaine après parturition qu'à 5 mois de gestation. Enfin, la résistine augmente la libération de glycérol et le niveau d'ARNm de HSL et ATGL dans des explants de tissu adipeux suggérant un rôle de la résistine dans la lipolyse du tissu adipeux chez le bovin.

L'ensemble de ces résultats suggèrent un rôle des trois adipocytokines, chemerine, résistine et visfatine dans les fonctions ovariennes chez la femme et la vache. Il reste maintenant à déterminer l'importance de cette production locale des adipocytokines au sein de l'ovaire et à élargir les recherches au niveau de l'axe hypothalamo-hypophysaire pour mieux comprendre leur importance dans les fonctions de la reproduction chez la femelle.

Abstract

Adipocytes are cytokines primarily produced by adipose tissue and implicated in insulin resistance and obesity. If these adipocytes play a key role in the regulation of metabolic function, they also seem to be implicated in reproductive functions. In this context, we have chosen to identify the presence of three adipokines; chemerin, visfatin and resistin in the ovary and their molecular mechanisms *in vitro* during human granulosa cell steroidogenesis and proliferation. We have carried out the same work in the bovine ovary for chemerin and visfatin as resistin had been already characterized in this species. However, we studied the plasma concentration of resistin, its expression and its role in adipose tissue of the cow during lactation.

We show that chemerin, resistin and visfatin, together with chemerin receptors are present in the ovary of humans and cows, and particularly in the follicle (granulosa cells, theca cells and in the cumulus-oocyte complex), and in the corpora lutea of the cow. In granulosa cells, the expression of adipocytokines in the present study is regulated *in vitro* by insulin, IGF1 and the insulin sensitizers, metformin and rosiglitazone. In human cells, this regulation by metformin is mediated by the AMPK/SIRT1 signalling pathway. In the cow, the expression of the chemerin gene is positively regulated by insulin, IGF1, metformin and rosiglitazone so that the expression of the receptors CMKLR1, GPR1 and CCRL2 is diminished in response to these different agents. In addition, TNF-α increases the expression of chemerin, adiponectin and CMKLR1. The expression of the visfatin gene is not significantly regulated by these factors.

In the female, chemerin, resistin and visfatin modulate *in vitro* the secretion of steroids by the granulosa. Chemerin and resistin reduce the production of P4 and E2 in response to IGF1 whereas visfatin increases it. To induce these effects, chemerin and resistin inhibit the expression of key steroidogenic enzymes such as P450 aromatase and P450scc and the phosphorylation of the beta sub-unit of the IGF1 receptor and the MAPK ERK1/2 signalling pathways in response to IGF1. The effect of adipocytokines on the proliferation of granulosa cells is variable. In effect, chemerin reduces cellular proliferation induced by IGF1 whereas visfatin increase proliferation. There is no effect of resistin. Basal state (in the absence of IGF1 or FSH), chemerin, visfatin and resistin modulate not only the phosphorylation of

MAPK ERK1/2 but also that of AKT and MAPK-P38. Phosphorylation of AMPK is activated only by chemerin.

In the cow, chemerin and visfatin also modulates *in vitro* granulosa cell steroidogenesis. Chemerin reduces P4 and E2 secretion at basal levels and in response to IGF1 or FSH. Visfatin increases steroidogenesis in the absence or presence of IGF1. In this model, chemerin affects the secretion of steroids and reduces cholesterol content, the protein quantity of the cholesterol transporter StAR, the proteins P450 aromatase and HMGCR and the level of phosphorylation of the MAPK-ERK1/2 signalling pathways in the presence or absence of IGF1 and FSH. All the observed effects of chemerin implicate the receptor CMKLR1. Visfatin induces its effects probably by the SIRT1 signalling pathway. Under our conditions, these two adipocytokines do not affect the viability or proliferation of bovine granulosa cells. In the cumulus-oocyte complex of the bovine, chemerin arrests the majority of oocytes at the GV stage *in vitro* and this is associated with a reduction in P4 secretion by the cumulus cells and the phosphorylation of the MAPK-ERK1/2 signalling pathway in the oocyte and cumulus cells.

In the dairy cow, plasma resistin was carried out by a commercially available, bovine specific ELISA. Resistin is low before calving then increases until it reaches a peak one week after parturition, before a gradual reduction to pre-calving levels six after calving. Plasma concentrations of resistin are positively correlated with plasma FFA and negatively with milk production, dry matter intake and energy balance between 1 – 22 weeks after parturition. Furthermore in adipose tissue, mRNA and protein levels of resistin are higher one week after parturition than at 5 months of gestation. Finally, resistin increases the release of glycerol and the mRNA expression of HSL and ATGL in tissue explants of adipose tissue, suggesting a role for resistin in lipolysis of adipose tissue in the cow.

Together, these results suggest a role for the three adipocytokines; chemerin, resistin and visfatin in ovarian function in both the human and cow. It remains to be determined the importance of the local production of adipocytokines within the ovary. Expanding research to the hypothalamic-pituitary axis to better understand their importance in reproductive function in the female is required.

Liste des publications à comité de lecture

Articles originaux :

-Cloix L, **Reverchon M**, Cornuau M, Froment P, Ramé C, Costa C, Froment G, Lecomte P, Chen W, Royère D, Guerif F, Dupont J. Expression and Regulation of INTELECTIN1 (ITLN1) in Human Granulosa-Lutein Cells: Role in IGF1-Induced Steroidogenesis Through NAMPT. *Biol Reprod*. 2014 Jun 18.

-Reverchon M, Bertoldo MJ, Ramé C, Froment P, Dupont J. CHEMERIN (RARRES2) decreases *in vitro* granulosa cell steroidogenesis and blocks oocyte meiotic progression in bovine species. *Biol Reprod*. 2014 May;90(5):102.

-Reverchon M, Ramé C, Cognié J, Briant E, Elis S, Guillaume D, Dupont J. Resistin in dairy cows: plasma concentrations during early lactation, expression and potential role in adipose tissue. *PLoS One.* 2014 Mar 27;9(3):e93198.

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Revue :

-Dupont J, Scaramuzzi RJ, **Reverchon M**. The effect of nutrition and metabolic status on the development of follicles, oocytes and embryos in ruminants. *Animal.* 2014 Apr 28:1-14.

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Présentations affichées ou orales à comité de lecture

Poster :

-Cloix, L. ; **Reverchon, M**. ; Rame, C. ; Cornuau, M. ; Royère, D. ; Guérif, F. ; Dupont, J. Caractérisation et rôle de l'omentine dans les cellules de la granulosa humaine ? 25ème Colloque Biotechnocentre (2012-10-11-2012-10-12) Seillac (FRA)

-**Reverchon, M**. ; Rame, C. ; Dupont, J. Expression of chemerin and its receptor, CMKLR1, in bovine ovary: role in granulosa cells? 63. Annual Meeting of the European Federation of Animal Science (2012-08-27-2012-08-31) Bratislava (Slovaquie). In : 63rd Annual Meeting of the European Federation of Animal Science. Wageningen (Pays Bas) : Wageningen Academic Publishers (EAAP Book Abstracts, 18), 2012. 177.

-**Reverchon, M**. ; Ramé, C. ; Dupont, J. Expression et rôle de la chemerine et de son récepteur CMKLR1 dans les cellules de la granulosa bovine30. Congrès de la Société Française d'Endocrinologie (2013-10-02) Paris (FRA) In : 30ème Congrès de la Société Française d'Endocrinologie. Paris (FRA) : Elsevier Masson SAS Editeur (Annales d'Endocrinologie, 74 (4)), 2013. 317.

-**Reverchon M**, Rame C, Dupont J. Caractérisation et effet de la visfatine dans les fonctions ovariennes bovines. 30. Congrès de la Société Française d'Endocrinologie (2013-10-02) Paris (FRA) In : 30ème Congrès de la Société Française d'Endocrinologie. Paris (FRA)

-Cloix, L. ; **Reverchon, M**. ; Cornuau, M. ; Ramé, C. ; Guerif, F. ; Royère, D. ; Dupont, J. Expression et rôle de l'omentine dans les cellules de la granulosa humaine. 30. Congrès de la Société Française d'Endocrinologie (2013-10-02-2013-10-05) Paris (FRA). In : 30ème Congrès de la Société Française d'Endocrinologie. Paris (FRA) : Elsevier Masson SAS Editeur (Annales d'Endocrinologie, 74 (4)), 2013. 317

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Présentation orale :

- **Reverchon, M.** ; Cornuau, M. ; Rame, C. ; Cloix, L. ; Royère, D. ; Guérif, F. ; Dupont, J. La vistafine est exprimée par les cellules de la granulosa humaine : Régulation par la metformine via la voie AMPK/SIRT1 et rôle dans la stéroïdogenèse ? 25. Colloque Biotechnocentre (2012-10-11) Seillac (FRA). In : 25ème Colloque Biotechnocentre. 2012. 32.

-**Reverchon, M**.; Rame, C.; Dupont, J. Expression of chemerin and its receptor, CMKLR1, in bovine ovary: A potential role in granulosa cell steroidogenesis and lipid metabolism. 46. Annual Meeting of the Society for the Study of Reproduction (SSR) (2013-07-22-2013-07-26) Montréal (Canada).In : 46th Annual Meeting of the Society for the Study of Reproduction (SSR). Madison (USA) : Society for the Study of Reproduction (Biology of Reproduction, Supplément), 2013. 33

Table des matières

Liste des abréviations

AICAR : 5-aminoimidazole-4-carboxamide ribonucléotide
AC: Adénylate Cyclase
ACC : Acétyl CoA Carboxylase
ADNc: Acide DésoxyriboNucléique complémentaire
AdipoR1/2: Récepteur adiponectine ½
AgRP : Agouti Related Protein
AGNE : Acide Gras Non Esterifiés
AKT ou PKB: Protéine Kinase B
AMPc: Adénosine MonoPhosphate cyclique
AMPK: AMP-activated protein Kinase
AMH : Anti-Mullérienne Hormone
APPL : Adaptor protein containing Pleckstrin homology domain, Phosphotyrosine-binding domain and a Leucine zipper motif
ARN: Acide Ribonucléique
ARNm: ARN messager
ATGL: Adipose triglyceride lipase
Bax : Bcl-2–associated X protein
BCL2 : B-cell lymphoma 2
BMP15 : Bone morphogenetic protein 15
bFGF : Basic Fibroblast Growth Factor
3betaHSD : 3-beta–hydroxysteroid dehydrogenase
CCRL2 : C-C chemokine receptor-like 2
CDK: Cyclin Dependent Kinase
ChemR23: Récepteur de la Chemerine R23
COCs : Complexe Cumulo-ovocytaires
CMKLR1 : Chemokine-like receptor 1
Cox2: cyclo-oxygénase-2
CPM: Coup Par Minute
CREB: cAMP response element-binding protein
Cx: Connexine
DMEM: Dulbecco's Modified Eagle's Medium
DAG: Diacylglycérol
cEBP: enhancer binding protein
ECL: Enhanced CheLuminescence
EDTA: Ethylene-Diamine-Tetra Acetic acid
Eff : Efficacité
EGF : Epidermal growth factor
E2 : Oestradiol
ERK1/2: Extracellular signal-Regulated Kinase1/2
FSH: Follicle Stimulating Hormone
GDF9: Growth differentiation factor-9
GDM: Gestiationnal diabete metillus
GH: Growth Hormone
GnRH: Gonadotropin Releasing Hormone
GPR1: G protein-coupled receptor 1
GPR54: G-Protein Coupled Receptor 54
GVBD: germinal vesicle breakdown
HDL: high density lipoprotein
HSL: Hormone-Sensitive Lipase
HHG: Hypothalamo-Hypophyso-Gonadotrope
HMGCR ou HMGCoA réductase: 3-hydroxy-3-methyl-glutaryl-CoA reductase
HMW: High Molecular Weight
HRP: HorseRadish Peroxidase
IBMX: 3-isobutyl-1-methylxanthine
IGF: Insulin Like Growth Factor
IGF-BP: Insulin Like Growth Factor Binding Protein
IL: Interleukine

IR: Insulino-Résistance
IRS: Insulin Receptor Substrate
ITLN: Intelectin
Jak: Janus kinase
kDa: Kilo Dalton
LDL: low density lipoprotein
LH: Luteinizing Hormone
LPL: Lipoprotéine lipase
MAPK: Mitogen Activated Protein Kinase
MMLV: Moloney Murine Leukemia Virus reverse transcriptase
MPF: maturation-promoting factor
NAD: Nicotinamide Adenine Dinucléotide
Nampt: Nicotinamide Phosphoribosyltransférase
NPY: Neuropeptide Y
p38: Protéine 38
P4: Progestérone
Pb : Paire de Base
PBEF: Pre B-cell Colony Enhancing Factor
P450scc : P450 side-chain cleavage
P450arom : P450 aromatase
PE: Pre-éclampsie
PI3K : Phosphatidylinositide 3-kinase
PKC : Protéine kinase C
PKA : Protéine kinase A
PLC : Phospholipase C
PPAR : Peroxisome proliferator-activated receptors
qPCR : quantitative Polymerase Chain Reaction
RARRES2: Retinoic acid receptor responder (tazarotene induced) 2
RIA: Radio Immuno Assay
Rpm: Rotation Par Minute
SF1: steroidogenic factor 1
SHC: Src Homology Collagen protein
SIRT: Sirtuines
SOPK: Syndrome des Ovaires PolyKystiques
StAR: Steroidogenic acute regulatory protein
STAT: Signal Transducer and Activator of Transcription
SREBP: Sterol Regulatory Element-Binding Proteins
TBE: Tris Borate EDTA
TIG2: Tazarotene Induced Gene 2 Protein
TGFbeta: Transforming Growth Factor β
TNF-α: Tumor Necrosis Factor α
VEGF : Vascular endothelial growth factor
VLDL: Very-low-density lipoprotein
VG: Vésicule germinale
ZP: Zone Pellucide

Liste des tableaux

Liste des figures

Introduction

I) L'axe hypothalamo-hypophyso-ovarien

A) L'hypothalamus

L'hypothalamus est une petite région du cerveau (quelques centimètres cubes) formée de groupements de noyaux constitués de neurones. Il est situé à la base du diencéphale et forme le plancher et les parois du troisième ventricule, il est délimité par le chiasma optique en partie frontale et par le mésencéphale et les tubercules mamillaires en parties postérieures (Figure 1). L'hypothalamus influence la régulation de plusieurs grandes fonctions comme la prise alimentaire et la prise d'eau, le sommeil, la température corporelle, le comportement sexuel et les émotions. Ce sont les noyaux de la région préoptique qui sont notamment impliqués dans les fonctions de la reproduction et leur taille diffère chez l'homme et la femme.

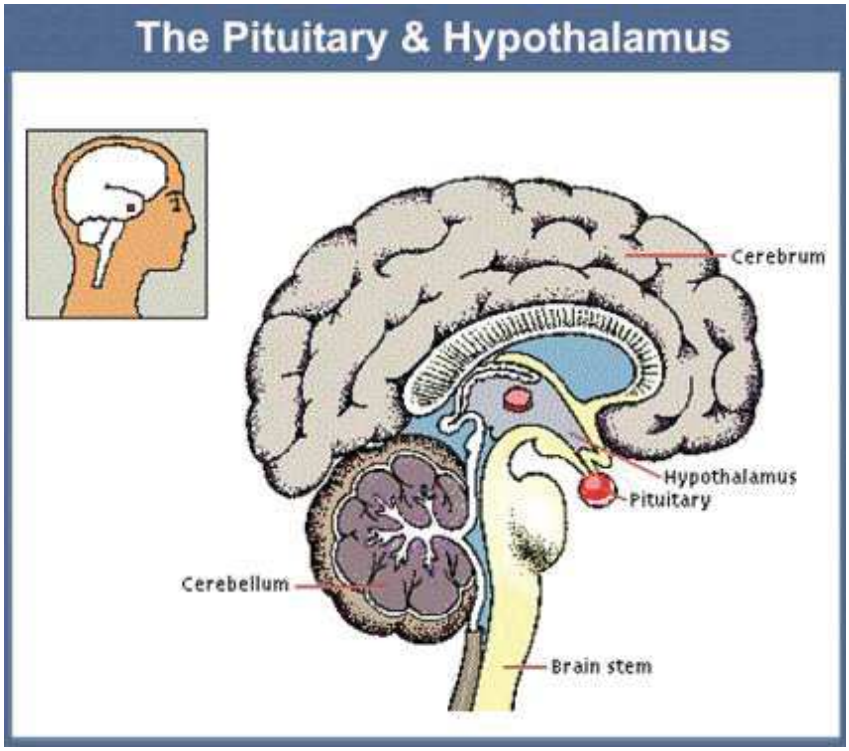


Figure 1 : Coupe longitudinale et frontale du cerveau, hémisphère gauche

D'après Type free Diabete le 06/05/14 à 11h ; http://www.typefreediabetes.com/Symptoms-of-Diabetes-Insipidus-TypeFree-Diabetes-s/11816.htm

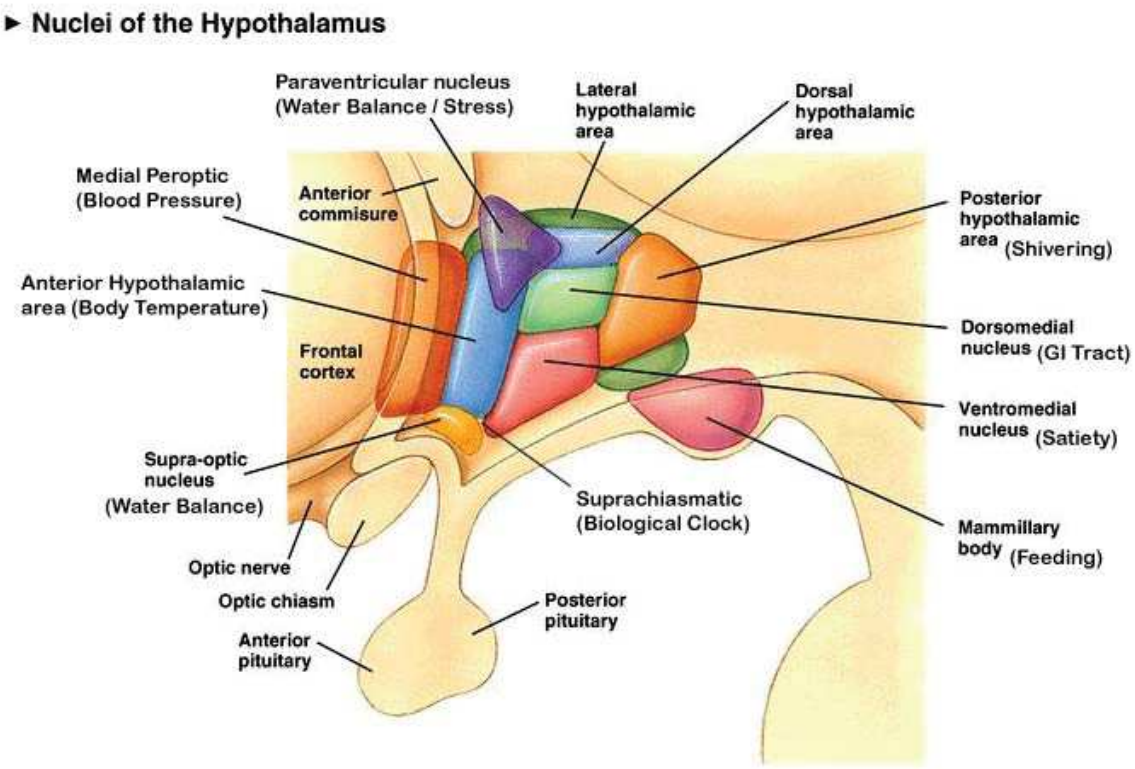


Figure 2: Les différents noyaux de l'aire hypothalamique
adapté de Kurnool Medical College, Kurnool, Andhra Pradesh, India.

Les fonctions endocrines de l'hypothalamus conduisent à la régulation des fonctions de l'hypophyse, une glande située juste en dessous. Ces deux glandes sont reliées entre elles par la tige pituitaire qui est composée d'axones et d'un réseau de capillaires sanguins. Les neurones de l'hypothalamus synthétisent différentes hormones qui vont être libérées dans un réseau de capillaires appelé le système porte hypothalamo-hypophysaire. (Figure 3)

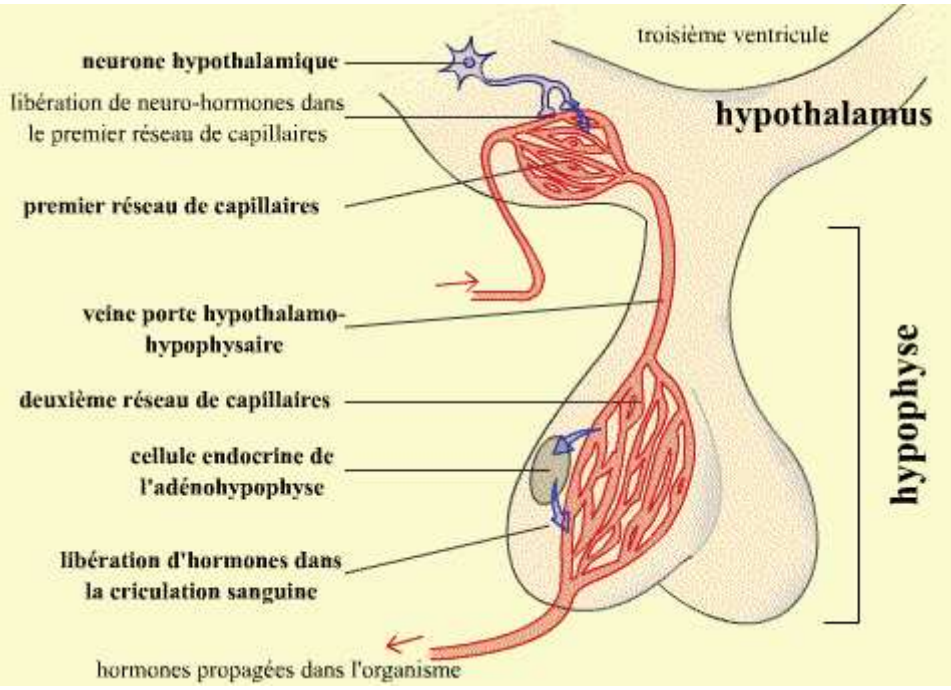


Figure 3: Hypophyse: lobe antérieur (adénohypohyse), lobe postérieur (neurohypophyse) et système porte hypophysaire
D'après cours licence biologie Université de Caen. 2011.

Deux de ces hormones sont stockées dans la neurohypophyse (post-hypophyse) avant d'être libérées dans la circulation ; l'hormone antidiurétique (vasopressine) impliquée dans la rétention d'eau et l'ocytocine qui active les contractions utérines durant l'accouchement.

L'hypothalamus sécrète d'autres « releasing » hormones ou libérines qui vont contrôler les sécrétions de l'adénohypophyse ; la corticolibérine, la thyréolibérine, la gonadolibérine, et des inhibiting hormones ou statine, la somatocrinine, la somatostatine et la dopamine. Enfin, l'hypothalamus sécrète du GnRH (Gonadotropin Releasing Hormone), un décapeptide synthétisé par les neurones du noyau arqué, qui va aller se fixer à ses récepteurs sur l'adénohypophyse et stimuler la production et la libération dans le sang des gonadotrophines, la LH (Luteinizing Hormone) et la FSH (Folliculo-Stimulating Hormone) qui vont réguler le fonctionnement des gonades. Ces différentes neurohormones ont une demi-vie très courte. Le GnRH agit via un récepteur spécifique à 7 domaines transmembranaires couplé aux protéines G. Le GnRH ne peut être mesuré dans la circulation périphérique mais seulement dans le système porte hypothalamo-hypophysaire, sa pulsatilité est en concordance parfaite avec la sécrétion de LH dans la veine jugulaire chez l'ensemble des mammifères (Kah et al., 2004). Depuis fin 2003, de nombreuses études ont démontré que le système kisspeptide/GPR54 est essentiel à l'augmentation de la libération pulsatile de GnRH/LH qui survient au moment de

la puberté et qui conditionne la maturation des gonades. Des données plus récentes suggèrent également un rôle important du couple kisspeptide /GPR54 dans le soutien d'une libération de GnRH en période pré-natale (Kauffman et al., 2007) et, chez la femelle adulte, dans la genèse du pic pré-ovulatoire de GnRH/LH (Kinoshita et al., 2005).

B) L'hypophyse

L'hypophyse également appelée glande pituitaire est située chez l'humain sous l'hypothalamus dans une fossette de la face supérieure du corps du sphénoïde ; la selle turcique. L'hypophyse est composée de 2 lobes, un lobe postérieur la neurohypophyse et un lobe antérieur l'adénohypophyse. La neurohypophyse est un lieu de stockage et de sécrétion des neurohormones synthétisées par les neurones hypothalamiques, la vasopressine et l'ocytocine. L'adénohypophyse sécrète 6 hormones. La thyréotrophine (Thyroïd Stimulating Hormone), la corticotrophine (AdrenoCorticoTropic Hormone) et les deux gonadotrophines LH et FSH. Deux autres hormones sont également sécrétées, il s'agit de la somatotrophine une hormone de croissance (Growth Hormone) et de la prolactine impliquée dans la croissance des glandes mammaires et la synthèse du lait (Figure 4) (Junnila et al., 2011). La LH et la FSH jouent un rôle important dans la communication entre le système central et les gonades. Chez la femelle ces hormones vont agir au niveau de l'ovaire et sont impliquées dans la maturation folliculaire, l'ovulation et le maintien du corps jaune. La LH et la FSH régulent la production de stéroïdes (œstrogène et P4), les différentes concentrations des hormones ovariennes au cours du cycle féminin vont être à l'origine de rétro-contrôles négatif et positif sur les sécrétions du complexe hypothalamo-hypophysaire, par exemple l'E2 va réguler la sécrétion de GnRH. Au début du cycle, la faible teneur en stéroïdes va exercer un rétro-contrôle négatif au niveau hypothalamo-hypophysaire. En milieu de cycle, les concentrations croissantes d'œstrogènes vont exercer un rétro-contrôle positif sur la sécrétion de GnRH par l'hypothalamus induisant une augmentation de la sécrétion de LH par l'hypophyse provoquant ainsi l'ovulation du follicule mature lors du pic de LH qui suit de très près le pic d'E2.

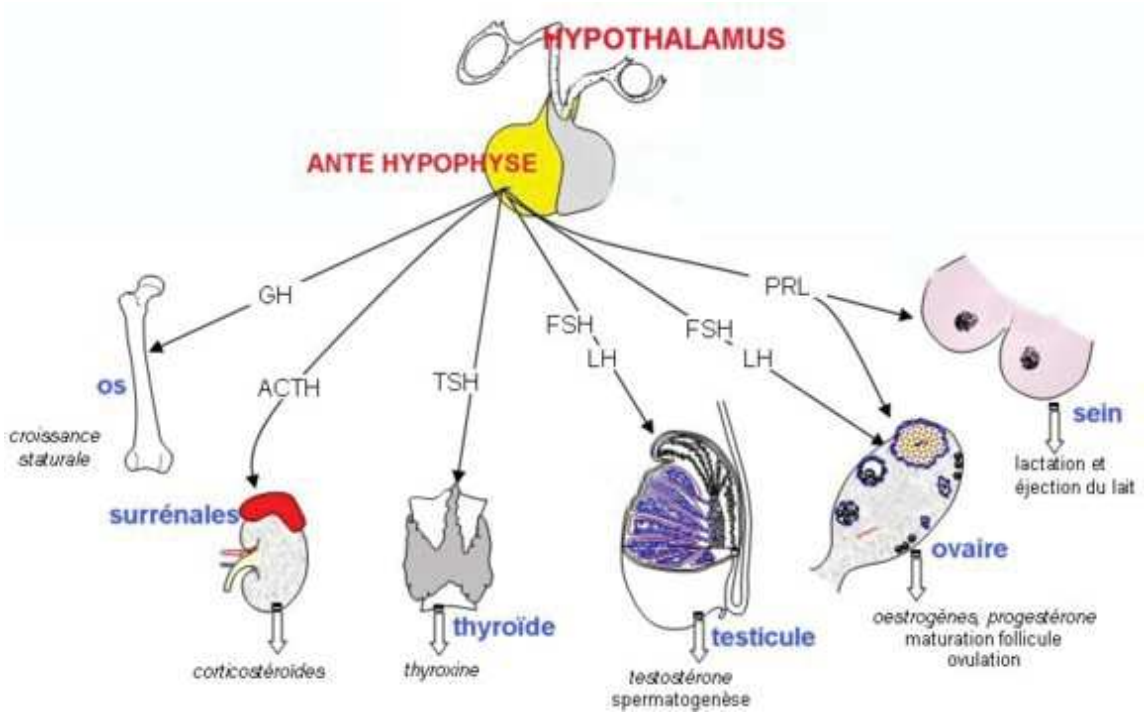


Figure 4: Les hormones hypophysaires

Adapté de cours de Master de biologie de la reproduction, Faculté François Rabelais, Tours, 2010.

C) L'appareil reproducteur femelle : l'ovaire

L'ovaire est une glande génitale paire située entre l'utérus et la paroi du bassin dans la grande cavité péritonéale contre la paroi pelvienne. Les ovaires ont une forme d'amande d'environ 4 cm de longueur pour 2 cm de largeur et 1 cm d'épaisseur chez la femme. Les ovaires sont maintenus en place dans l'abdomen grâce à ses ligaments suspenseurs, sa face interne est accolée au pavillon de la trompe de Fallope (Levasseur et al, 2001). L'ovaire est composé de deux parties distinctes avec des fonctions bien précises. La zone médullaire centrale, composée de tissu conjonctif dense et fibreux, est le siège de l'innervation et de l'irrigation ovariennes (présence de vaisseaux sanguins et lymphatiques) (Bazot et al., 2004) (Figure 5A). Leur irrigation est alimentée par l'intermédiaire des artères ovariennes issues de l'aorte abdominale elle-même provenant de l'aorte thoracique. La zone corticale périphérique, composée de tissu conjonctif lâche est le lieu de l'activité folliculaire, elle représente les 2/3 des ovaires (Figure 5A). Les follicules ovariens sont composés de plusieurs types cellulaires variant suivant leurs stades de maturation : l'ovocyte entouré des cellules du cumulus *oophorus*, les cellules de la granulosa, les cellules de la thèque interne et les cellules de la thèque externe (Figure 5B). Enfin, la surface de l'ovaire est bordée par une membrane fibreuse, une couche conjonctive dense sous épithéliale (la membrane est aussi sous

épithéliale) appelée l'albuginée (Levasseur et al., 2001). L'ovaire possède deux fonctions principales. **La première fonction est exocrine**, elle consiste en la production de cellules germinales femelles matures (ovocytes) aptes à être fécondées et à permettre un développement embryonnaire et fœtal précoce. Cette première fonction se divise en deux phases : la **folliculogenèse** et **l'ovogenèse**. Ces phases débutent durant la vie fœtale et s'arrêtent à la **ménopause**. A la naissance les ovaires détiennent un stock défini (environ 2 millions) d'ovocytes qui sont bloqués au stade de prophase méiose I et dont la majorité va involuer. A la puberté il ne reste que 400 000 ovocytes en stock qui n'ont pas la capacité de se multiplier et seulement 400 arriveront à une maturation complète. **La deuxième fonction de l'ovaire est endocrine**, elle consiste en la production de stéroïdes [**œstrogène (E2) et P4 (P4)**], ces deux hormones vont influencer l'activité sexuelle, l'ovulation et le maintien de l'embryon.

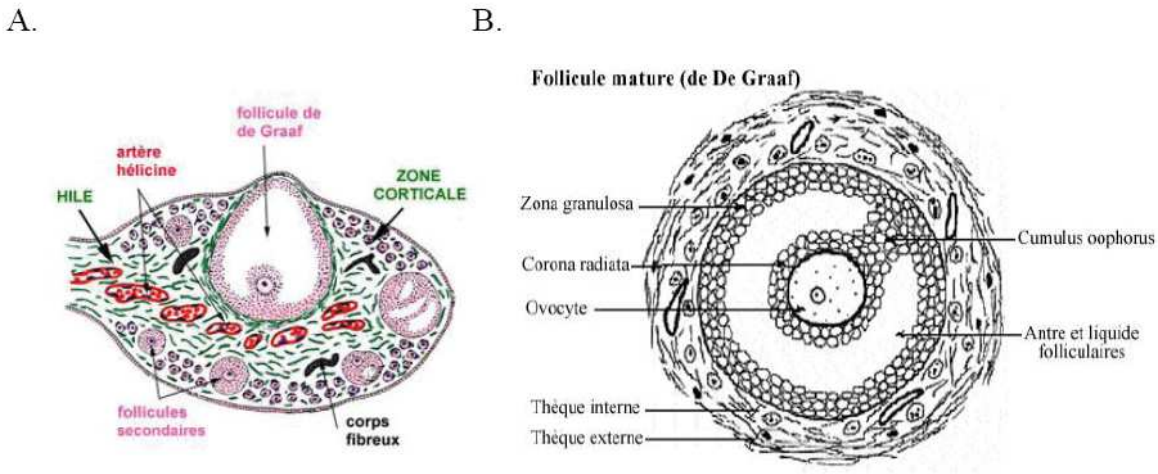


Figure 5A et B : A. Représentation schématique d'une coupe d'ovaire et B. détail d'un follicule de Graaf

D'après (Abdennebi et al., 1999)**.**

C1) La folliculogenèse

La folliculogenèse correspond à la **croissance** et à la **maturation** des follicules, c'est-à-dire aux transformations que subit le follicule primordial pour devenir un follicule mûr jusqu'à l'ovulation. Il s'agit de la **phase folliculaire** dont la durée varie selon les espèces, la fin de cette phase va déterminer l'ovulation. Cette phase dure plus de 3 mois chez la femme (Monniaux et al., 1997) et se compose de plusieurs phases : tout d'abord une phase de **croissance basale** qui est le recrutement initial, cette étape est lente et continue (Figure 6).

Elle est suivie par la **croissance terminale** des follicules qui est contrôlée par les gonadotrophines produites par la LH et la FSH hypophysaires (Figure 6).

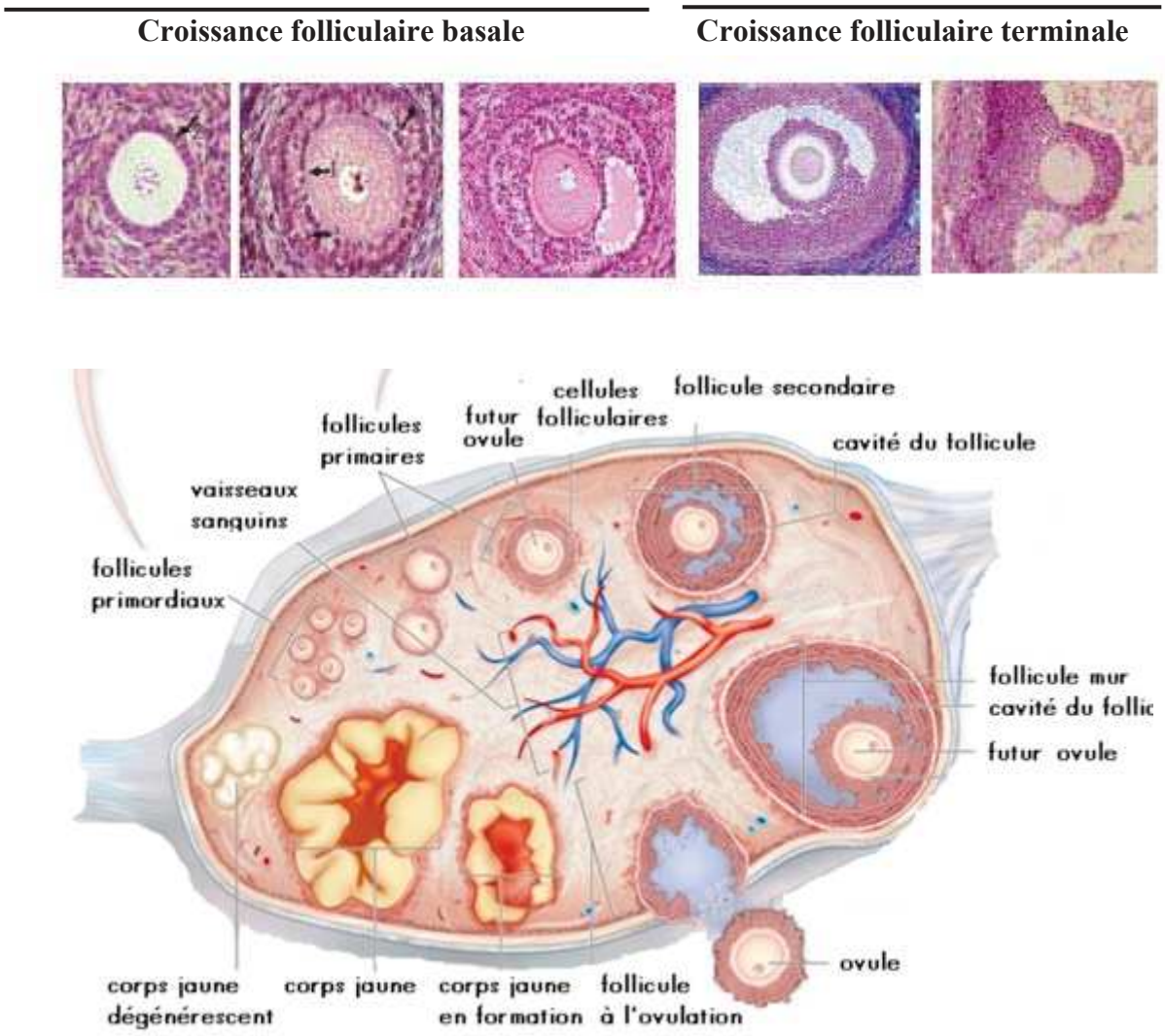


Figure 6: Croissance folliculaire basale et terminale
D'après Futura Science, 2006.

C1.1) Le recrutement initial

Chez la femelle, chaque jour des follicules dont le nombre varie selon l'espèce quitte la réserve ovarienne et débute son développement. Ce recrutement n'est pas influencé par les gonadotrophines (Dufour et al., 1979; Monniaux et al., 1997; Roche, 1996), mais par des facteurs locaux chez la femme sécrétés par l'ovocyte et/ou les cellules de la granulosa, tels que les facteurs de croissance et certaines hormones comme celle de la famille du TGFβ (transforming growth factor), l'AMH (anti müllerian hormone), GDF-9 (growth

differentiatyion factor-9), BMP-15 (bone morphogenetic protein 15) (Maruo et al., 1993); (Dong et al., 1996); (Galloway et al., 2000) ou le kit ligand (Parrott & Skinner, 1999).

Ce processus qui commence dès la vie fœtale a lieu jusqu'à l'épuisement de la réserve ovarienne. Il présente différents stades morphologiques. Tout d'abord, les ovocytes sont entourés d'une couche unique de cellules aplaties, les cellules de la pré-granulosa, d'origine épithéliale, ils sont dénommés **follicules primordiaux**. Ce processus est caractérisé par une croissance ovocytaire bloquée en prophase de méiose I et par une prolifération des cellules de la granulosa. Le follicule se développe et se transforme en **follicule primaire** contenant un ovocyte entouré de cellules cuboïdales. A ce stade, les synthèses d'ARN et de protéines sont très importantes et certaines seront utilisées immédiatement pour la mise en place de la zone pellucide autour de l'ovocyte (afin d'éviter la polyspermie). Ensuite, le **follicule secondaire** est caractérisé par une intense prolifération des cellules de la granulosa et une différenciation cellulaire. Ainsi, il y a formation du cumulus autour de l'ovocyte et des cellules de la thèque entourant les cellules de la granulosa. Ces différents types cellulaires échangent entre eux via des jonctions communicantes permettant un dialogue cellulaire indispensable au bon développement des follicules et à la maturation ovocytaire (Figure 7) (Levasseur et al., 2001).

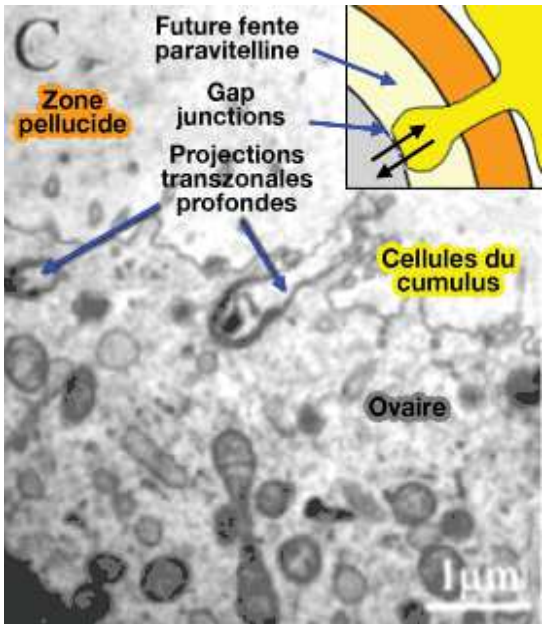


Figure 7: Jonctions communicantes entre cellules du cumulus et l'ovocyte
D'après (Chastant -Maillard et al., 2012)**.**

C1.2) La croissance folliculaire basale

La croissance folliculaire basale est définie comme étant la transformation des follicules basaux en **follicules pré-antraux** ou follicules tertiaires ou **follicules De Graaf** (Figure 8) (Gougeon, 1996).

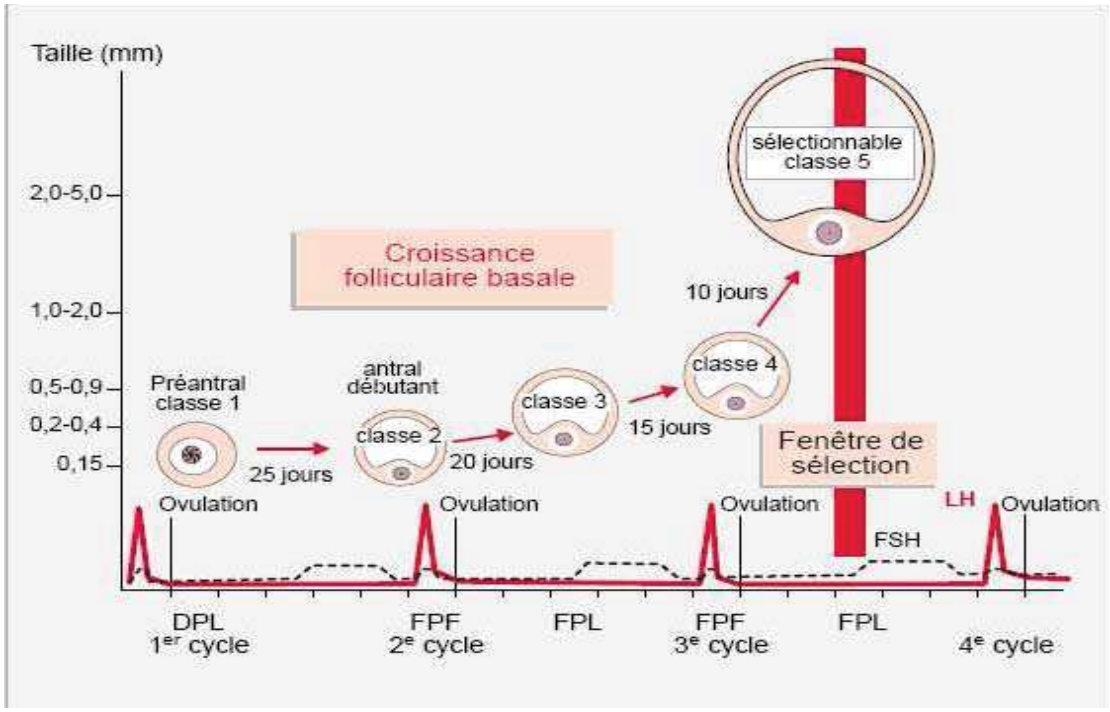


Figure 8: Chronologie du développement folliculaire de la cohorte d'où sera issu le follicule ovulatoire

(DPL : Début de Phase Lutéale ; FPF: Fin de Phase Folliculaire; FPL: Fin de Phase Lutéale; LH: luteinizing hormone; FSH: Follicle Stimulating Hormone), d'après (Gougeon, 1996).

Au cours de chaque cycle chez la femme, des cohortes de follicules se différencient en follicules pré-antraux mais une seule cohorte donnera un follicule mature prêt à ovuler. La croissance des follicules dure 65 jours et débute en **phase lutéale**. Par ailleurs, les cellules de la thèque se vascularisent en raison d'une forte production locale de facteurs angiogéniques (VEGF, bFGF, TGFα et β). Ces cellules subissent une différenciation morphologique interne (couche interne/couche externe) et fonctionnelle et se séparent des cellules de la granulosa par la lame basale. Après 25 jours de croissance, environ 70% des follicules présentent entre les cellules de la granulosa de petites cavités qui confluent pour créer une cavité unique appelée **antrum**. **L'antrum** contient le liquide folliculaire qui est riche en E2, de l'exsudat du plasma sanguin et des cellules folliculaires. Durant cette transformation, le nombre de récepteurs à la LH augmente au niveau des cellules de la thèque interne mais le nombre de récepteurs à la FSH reste constant (Yamoto et al., 1992).

C1.3) La croissance folliculaire terminale

Cette dernière phase de croissance est caractérisée par la dépendance des follicules aux gonadotrophines, elle dure deux semaines chez la femme et 4 à 5 jours chez la vache (Monniaux et al., 1997). Pour la majorité des espèces, la **croissance folliculaire** se déroule durant **la phase folliculaire** du cycle, excepté chez la vache et la jument où le phénomène est continu et génère des vagues folliculaires. La phase de croissance folliculaire terminale est caractérisée par 3 étapes successives ; **le recrutement, la sélection et le phénomène de dominance** (Ginther et al., 2001).

La FSH est le signal endocrinien du **recrutement** (Adams et al., 1992). C'est la phase d'entrée en croissance terminale des follicules gonado-dépendants (Driancourt, 2001). A partir de ce pool de follicules, des cohortes vont se former composées de petits follicules (2 à 5 mm chez la femme) avec un antrum. La dépendance aux gonadotrophines apparaît à une taille folliculaire précise. La FSH induit l'activité de la P450 aromatase, une enzyme impliquée dans la production d'E2. Elle stimule la production d'inhibine et de follistatine et inhibe l'expression d'IGFBP (Insulin-like growth factor binding protein), ce qui augmente la biodisponibilité des IGF. De plus, l'IGF-1 améliore la réponse des cellules de la granulosa à la FSH chez de nombreuses espèces (Adashi et al., 1988). Au cours de la sélection le follicule destiné à ovuler sera sélectionné, tous les autres dégénèrent par atrésie. Un seul follicule est sélectionné chez les espèces mono-ovulantes contre plusieurs chez les espèces poly-ovulantes.

Le mécanisme de **sélection** du follicule dominant n'est pas encore connu. Le follicule qui va devenir dominant pourrait être sélectionné sur un seuil de réponse à la FSH plus bas. En effet, l'inhibine et l'E2 exercent un rétrocontrôle négatif important sur la sécrétion de FSH (Ginther et al., 2000) et (Levasseur et al., 2001). Cette inhibition bloque le développement des follicules les plus demandeurs en FSH. A ce stade, les follicules produisent peu d'E2, mais les cellules de la thèque interne du follicule en voie de sélection contiennent un plus grand nombre de récepteurs à la LH d'où une plus grande production d'androstènedione. C'est au cours de cette phase de sélection que la dépendance des follicules aux gonadotrophines s'oriente d'une sensibilité de la FSH vers la LH.

Le phénomène de **dominance** est caractérisé par la maturation terminale (dépendante de la LH) du follicule malgré une diminution constante des taux de FSH contrôlée par l'E2 et l'inhibine. Il a été observé en parallèle un blocage de recrutement des follicules et une atrésie

des follicules de la cohorte. Chez le bovin, Yuan et al ont également observé une diminution des IGFBP dans le liquide folliculaire associée à une augmentation de l'ARNm de l'IGF-1 et de l'inhibine dans les cellules de la granulosa (Sartori et al., 2001). L'IGF-1 va avoir pour rôle de potentialiser d'une part, les effets de la LH sur les cellules de la thèque et d'autre part, les effets de la FSH sur les cellules de la granulosa. La FSH augmente l'activité de la P450 aromatase dans les cellules de la granulosa et donc la sécrétion d'E2 (Young., 1999). La thèque interne produit plus d'androgènes, et donc plus de substrats pour la synthèse d'E2 (Hillier et al., 1981). L'IGF-2 agit par un mécanisme autocrine en synergie avec la FSH (Giudice, 1992). Le follicule dominant présente une sensibilité à la FSH plus importante, cette caractéristique n'est pas due à un plus grand nombre de récepteurs mais probablement à une meilleure transduction du signal (Levasseur et al., 2001). Son volume va augmenter en raison de l'accumulation de liquide dans l'antrum. La dominance du follicule va être caractérisée par une augmentation de l'expression de VEGF (*vascular endothelial growth factor*) et de FGF2 (*fibroblast growthfactor 2*), ces deux acteurs sont impliqués dans le processus de vascularisation (Berisha et al., 2000). La régulation de la dominance est essentiellement contrôlée par les gonadotrophines mais certains facteurs locaux interviennent (Figure 9) (Knight & Glister, 2006). L'IGF-1 agit de manière autocrine sur les cellules de la granulosa et pourrait avoir une action paracrine sur les cellules de la thèque chez la rate et chez la poule (Hernandez, 1995). De plus, l'IGF-1 stabilise les transcrits de R-LH (récepteurs LH) dans les cellules de la granulosa de rate (Hirakawa et al., 1999) et stimule l'expression de R-FSH chez la souris (Zhou et al., 1997). Cette phase est caractérisée par l'apparition des récepteurs de la LH sur les cellules de la granulosa (Yamoto et al., 1992). Egalement, d'autres acteurs participent à cette régulation de la folliculogenèse terminale comme l'EGF (epidermal growth factor) chez le hamster (Roy, 1991) ou le bovin (Franchimont et al., 1986), le TGFβ chez le hamster (Roy, 1993), la follistatine chez le bovin (Glister et al., 2004) et certains stéroïdes chez la rate (Billig et al., 1993). L'achèvement de la croissance folliculaire terminale est favorisé par l'augmentation de l'AMPc qui est la conséquence de la liaison de la LH à son récepteur actif (Marion et al., 2002) et (Lécureuil et al., 2007).

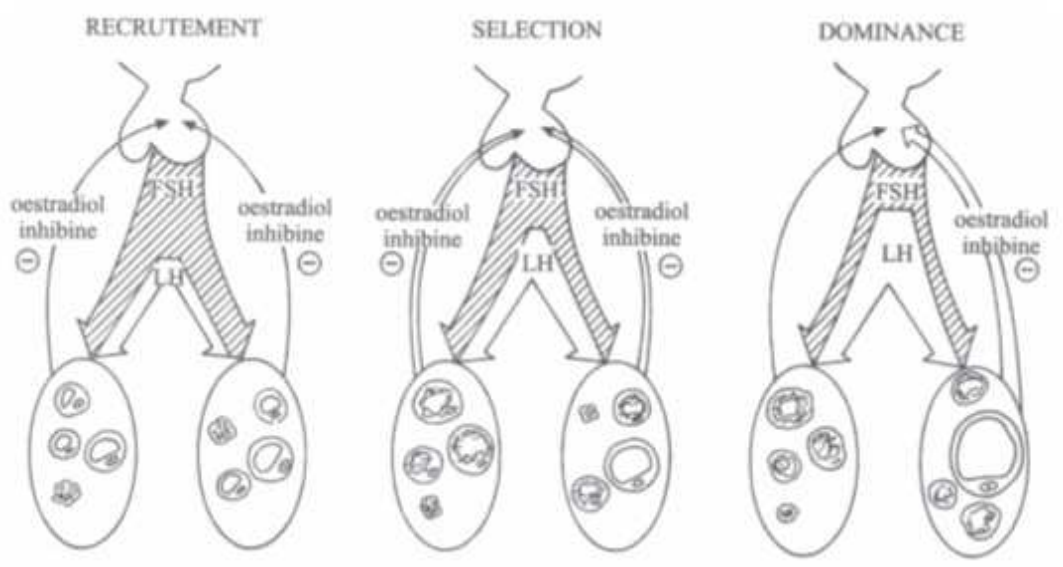


Figure 9: Chronologie du développement folliculaire de la cohorte d'où sera issu le follicule ovulatoire

L'épaisseur des flèches indique l'importance de chaque sécrétion. Figure tirée et adaptée de (Levasseur et al., 2001**).**

C2) L'ovulation

L'ovulation est l'étape ultime où le follicule dominant de la phase folliculaire du cycle œstral libère son ovocyte pour la fécondation. Cette libération est chez la plupart des espèces le début de la phase lutéale au cours de laquelle les sécrétions de stéroïdes sont modifiées. Elle se termine au premier jour des règles chez la femme et quelques dizaines de jours après chez d'autres espèces comme le chien (pseudogestation). Chez la femme, cet évènement a lieu autour du 14ème ou 15ème jour du cycle menstruel. Il est la conséquence de l'augmentation des taux circulants d'E2 en fin de croissance terminale qui induit la décharge gonadotrope ovulante et permet l'expulsion de l'ovocyte mûr : c'est l'ovulation. Cette rupture est l'aboutissement de remaniements morphologiques et cytologiques (Levasseur et al., 2001). Tout d'abord, le pic de LH, conséquence d'une rétroaction positive d'E2 au niveau hypothalamo-hypophysaire, indique le commencement de l'ovulation (Levasseur et al., 2001). Les pulses d'E2 provenant de l'ovaire vont provoquer l'augmentation de la synthèse des prostaglandines (PGE et PGF) qui sont responsables de la libération d'enzymes protéolytiques et favorisent l'ovulation (Levasseur et al., 2001). Le plasminogène contenu dans le liquide folliculaire est transformé en plasmine (protéase impliquée dans l'ovulation) par un activateur contenu dans les cellules de la granulosa. En parallèle, se produisent une dissociation du collagène et de l'albuginée, une rupture des jonctions entre les cellules de la granulosa et l'ovocyte, une expansion des cellules du cumulus oophorus due à une forte sécrétion d'acide

hyaluronique et enfin la disparition de la lame basale. De même, les cellules folliculaires situées à l'apex du follicule meurent par apoptose ou par nécrose, ce qui permet l'infiltration du liquide folliculaire. Devant cet affaiblissement des liaisons cellulaires et la pression croissante, le follicule se contracte permettant ainsi l'expulsion de l'ovocyte. La rupture de la membrane folliculaire résulte ainsi d'une réaction inflammatoire localisée. En effet, les macrophages et les lymphocytes sont présents dans l'ovaire autour des follicules pré-ovulatoires (Brannstrom et al., 1994). Ils sécrètent l'interleukine 1β (IL-1β) et le *tumor necrosis factor* α (TNFα) (Terranova & Rice, 1997). *In vitro*, l'IL-1β augmente le taux d'ovulation, chez la jument (Martoriati et al., 2003), et chez la rate (Brännström et al., 1993). Le TNF-α présente un rôle éventuel dans le mécanisme de la rupture folliculaire. Chez la rate, les concentrations ovariennes et sériques de TNF-α sont élevées à l'ovulation. De plus, des ovaires de rates perfusées libèrent du TNF-α dans l'effluent veineux pendant l'ovulation (Brännström et al., 1994) et l'ajout de TNF-α au liquide de perfusion améliore les taux d'ovulation en réponse à la LH (Brännström et al., 1995). Enfin, chez des brebis l'ovulation est bloquée après l'injection d'anticorps anti-TNF-α dans le liquide folliculaire (Murdoch et al., 1997).

C3) Le corps jaune

Juste après la rupture du follicule mature débute la **lutéogenèse**, il s'agit de la différenciation du follicule ovulé pour devenir une glande endocrine : le corps jaune = phénomène de lutéinisation (Smith et al., 1994). Durant la décharge gonadotrope de la LH, un réseau vasculaire provenant de la thèque interne se forme autour des cellules de la granulosa lutéinisées. Ces cellules sont alors capables de synthétiser de l'E2 et de la P4 chez l'humain et les rongeurs (Young, 1999). Le pic ovulatoire de la LH induit l'expression de nombreux gènes, comme celui du récepteur de la P4 (rat),(Park & Mayo, 1991) et celui de la cyclo-oxygénase 2 (cox-2) (rongeur) (Lim et al., 1997) ainsi que l'expression protéique de la P450 arom et de la P450-scc, impliquées dans la biosynthèse de l'E2 et de la P4 (Natraj & Richards, 1993). Les cellules de la granulosa et de la thèque, étant devenues des cellules lutéales, sécrètent de la P4 pour une éventuelle nidation et sortent du cycle cellulaire (arrêt en G0/G1), (Green et al., 2000). Si la fécondation n'a pas lieu, le corps jaune dégénère selon le processus de **lutéolyse** au bout de 14 à 16 jours formant ainsi le corpus albicans (Figure 10).

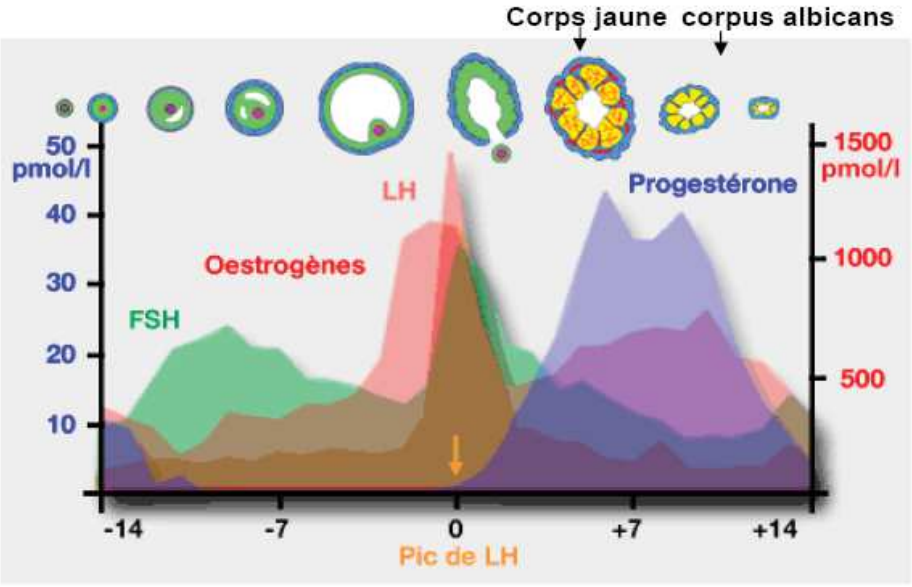


Figure 10: Chronologie du développement folliculaire et des sécrétions de stéroïdes et de gonadotrophines chez la femme
D'après (Zeleznik & Hillier, 1984)**.**

Cette régression est initiée par certains facteurs comme la prostaglandine 2α (Sugimoto et al., 1997). Chez la femme, la régression du corps jaune est provoquée par la diminution de la sécrétion de LH (Irianni & Hodgen, 1992) alors que l'expression de l'ARNm du récepteur de la LH augmente en phase lutéale dans le corps jaune avant de disparaître dès le début des règles (Nishimori et al., 1995).

C4) L'atrésie

L'atrésie correspond à la dégénérescence des follicules permettant la sélection du follicule ayant vraisemblablement le plus de chance de réussite de reproduction chez les mammifères. 99% des follicules qui entrent en croissance dégénèrent, les petits follicules dégénèrent à cause d'un signal apoptotique de l'ovocyte alors que dans les follicules à antrum ce sont les cellules de la granulosa qui enclenchent ce signal apoptotique et donc l'atrésie (Levasseur et al., 2001). L'étape déterminante dans la survie ou non des follicules est l'expression de récepteurs à la LH par les cellules de la granulosa lors de la transition follicule préantral/follicule ovulatoire. L'atrésie est marquée par des modifications morphologiques comme l'apparition de grains de pycnose (Abdennebi et al., 1999). Plusieurs facteurs sont impliqués dans ce processus ; l'IGF-1 amplifie l'action des gonadotrophines, des facteurs pro-apoptotiques comme Bax voient leur expression augmenter (Abdennebi et al., 1999), l'expression de la P450-arom dans les cellules folliculaires est abolie chez la rate, la truie et la

brebis (Jolly et al, 1997; Tilly et al., 1992; Uilenbroek et al., 1980). Egalement, l'expression protéique d'IGFBP-2, 4 chez la femme et -5 pour les bovins augmente durant l'atrésie dans le liquide folliculaire (Levasseur et al, 2001). Le choix du follicule qui va continuer son développement alors que les autres subissent l'atrésie semble aléatoire mais une fois sélectionné il est difficilement réversible.

C5) L'activité cyclique de l'ovaire

L'activité de l'ovaire fonctionne de manière cyclique chez la femme et la vache, elle débute par le **cycle oestrien** (comportement d'œstrus, chevauchement chez les bovins) et se poursuit par le **cycle menstruel ou utérin** (menstruation). La durée du cycle oestrien dépend de l'espèce (Tableau 1). Le cycle menstruel décrit précédemment est divisé en une **phase folliculaire** et une **phase lutéale**. Chez la femme, les cycles se mettent en place à la puberté et s'arrêtent à la ménopause (période de la vie où la réserve folliculaire est tarie). Chez la souris ou la rate, cet arrêt n'existe pas mais en fin de vie un dérèglement de l'axe gonadotrope apparaît et perturbe les cycles.

Espèce	Cycle (J)	Phase folliculaire (J)	Phase lutéale (J)	Œstrus (J)
Femme	28	14	14	Non déterminé
Vache	21	4	17	20
Rate	4-5	3	1-2	8-10

Tableau 1: Variabilité inter-espèce de la durée du cycle ovarien adapté de (Driancourt 2001) (Levasseur et al, 2001).

C6) L'ovogenèse

L'ovogenèse est un processus discontinu qui se produit en parallèle de la folliculogenèse et qui aboutit à la production d'ovocytes matures bloqués en métaphase II chez la femme. Ce phénomène trouve son origine dès la vie fœtale où les cellules germinales primordiales d'origine extra-gonadique migrent en direction des deux crêtes génitales et les colonisent pour former les ébauches gonadiques indifférenciées qui deviendront les ovaires. Ces cellules se différencient en **ovogonies**, s'entourent de cellules pour former les follicules primordiaux, entrent en méiose et se bloquent en prophase de première division méiotique (Stade diplotène). Ce sont des **ovocytes primaires de type I** (Eppig et al., 1996) qui vont rester au repos jusqu'à la puberté. Cependant, l'ovocyte va subir une croissance importante au sein du

follicule (Gosden et al., 1997). A la puberté, sous l'influence de la sécrétion de gonadotrophines les ovocytes I commencent leur maturation terminale afin d'assurer la réussite d'une fécondation et d'un développement embryonnaire précoce. La reprise de la méiose serait due à une levée d'inhibition, les ovocytes seraient bloqués au stade diplotène sous l'influence de substances appelées OMI (Oocytes Meiotic Inhibitor) sûrement sécrétées par la thèque interne et atteignant l'ovocyte par l'intermédiaire des cellules de la corona radiata (Tsafriri & Pomerantz, 1986). Un seul ovocyte atteint la deuxième division méiotique suivie de l'ovulation, les autres dégénèrent (Abdennebi et al., 1999).

C6.1) La croissance ovocytaire

Au cours de la croissance des follicules primordiaux, les ovocytes réalisent également leur croissance. Chez la femme, lorsque les follicules présentent un antrum l'ovocyte a atteint 80% de sa taille définitive. C'est à partir de ce moment que la croissance ovocytaire ralentit, le volume de l'ovocyte augmente par expansion de son cytoplasme et accumulation de réserves telles que les acides aminés, les métabolites glucidiques, et les acides nucléotidiques (Eppig, 1991; Gosden et al., 1997). Dans le cytoplasme de nombreuses modifications ont lieu : les mitochondries changent de morphologie, se multiplient et se rassemblent à proximité du réticulum endoplasmique, l'appareil de Golgi augmente son activité afin de produire la zone pellucide correspondant à une épaisse enveloppe glycoprotéique entourant l'ovocyte. De même, les granules corticaux sont en formation et augmentent en nombre, ils seront indispensables au moment de la fécondation afin d'éviter des problèmes de **polyspermie**. Durant cette phase de croissance, l'ovocyte va accumuler et stocker une grande quantité **d'ARN totaux** (10 ng chez certaines espèces) dont environ 10% sont des ARNm très stables. La transcription va être très intense en début de croissance afin de permettre la synthèse des protéines ZP (Zona pellucida) -1, -2, -3 qui constitueront la zone pellucide (Huntriss, 2002). Tous ces évènements sont réalisables si, et seulement si, il y a formation de jonctions entre l'ovocyte et les cellules folliculaires. Ces jonctions, qui au départ sont adhérentes ou intermédiaires vont devenir, des jonctions communicantes ou « gap junctions » (Buccione, 1990; Eppig, 1982), Elles sont indispensables pour le bon déroulement de la folliculogenèse (Mitchell & Burghardt, 1986). En effet, l'ovocyte est entouré par la corona radiata, ils sont en contact par des extensions cytoplasmiques traversant la zone pellucide (De Loos et al., 1991). Le nombre de ces « gap-junctions » augmente avec la croissance du follicule (Wert & Larsen, 1989). Les jonctions communicantes sont assemblées par des connexines. La connexine cx-37 intervient dans la liaison ovocyte/cellules de la granulosa alors que la connexine cx-43

intervient dans la liaison des cellules de la granulosa entre elles (Li et al., 2007). Des souris déficientes pour le gène codant la cx-37 sont viables cependant il y a une absence de jonctions communicantes, ainsi ces souris n'ovulent pas (Carabatsos et al, 2000; Simoni et al.,1997). De la même manière, les souris déficientes pour le gène cx-43 présentent peu de cellules germinales (Juneja, 1999). Leurs ovocytes sont morphologiquement anormaux, méiotiquement incompétents et non fertiles.

Au cours des dernières phases de maturation avant l'ovulation, l'ovocyte de mammifère va acquérir ce que l'on appelle sa **compétence au développement** (Mermillod & Marchal, 2012). Les compétences ovocytaires (méiotique et au développement) sont obtenues grâce à des interactions avec les cellules somatiques, il s'agit de reprendre la méiose et de donner un embryon viable, sain et capable de s'implanter. Il est aujourd'hui établi que ces modifications impliquent à la fois le compartiment nucléaire et le cytoplasme, mais on sait encore peu de choses sur ces phénomènes. Certains paramètres sont cruciaux pour ces processus comme le diamètre folliculaire (Royère, 2006). Chez la femme, au-dessus d'un diamètre folliculaire de 15 mm, un pourcentage d'ovocytes matures constant est obtenu. Le seuil critique se situe entre 12 et 14 mm où seulement 30 % des ovocytes arrivent en métaphase II, en dessous de 11 mm très peu d'ovocytes arrivent à maturation (<10%) (Royère, 2006). L'acquisition de la compétence au développement est également sensible à l'expansion des cellules du cumulus ou à la qualité et la taille ovocytaire (Blondin & Sirard, 1995; Crozet et al., 1995). La conformation et les sécrétions des cellules du cumulus jouent également un rôle dans l'élaboration de cette compétence. En effet, chez les bovins les ovocytes dénudés de leurs cellules du cumulus présentent des taux de maturation et de fécondation *in vitro* fortement réduits et ne produisent pas d'embryons sains (Fatehi et al., 2002; Zhang et al., 1995). Des résultats similaires ont été observés chez la souris et le porc (Chesnel et al., 1994; Maedomari et al., 2007). Egalement, la production de E2 et P4 par les cellules du cumulus est augmentée lors de la maturation ovocytaire (Liu et al., 2002) mais beaucoup d'autres acteurs sont supposés intervenir dans ce processus (Pic de LH, GAP-junctions, Meiosis Activating Sterols, PKA et les voies de signalisation des protéines kinases) (Royère, 2006). Enfin, la synthèse très intense en début de croissance des ARN totaux est un facteur déterminant de l'acquisition de la compétence au développement (Browder, 1985).

C6.2) La maturation ovocytaire

La maturation nucléaire a lieu au sein du follicule en fin de croissance où l'ovocyte reprend sa méiose si, et seulement si, il réalise sa croissance avec succès à l'intérieur d'un follicule de taille prédéterminée prêt pour l'ovulation. On parle de **compétence méiotique** pour décrire l'activation d'effecteurs moléculaires impliqués dans la reprise de la méiose (Mermillod & Marchal, 1999). On distingue deux grands évènements : **la reprise de la méiose** du stade VG au stade métaphase II et **la maturation cytoplasmique** qui est l'acquisition de l'aptitude à une fécondation normale et au démarrage du développement (Levasseur et al., 2001).

La maturation nucléaire de l'ovocyte

Pour être fécondable, l'ovocyte doit reprendre sa méiose, c'est-à-dire réduire de moitié le nombre de ses chromosomes. La méiose correspond à deux divisions nucléaires successives. La première est une mitose classique, (**méiose I**, division réductionnelle) cependant elle génère un ovocyte II et un premier globule polaire qui va être expulsé. Jusqu'à la fécondation la maturation de l'ovocyte est bloquée, c'est un signal calcique induit par le spermatozoïde qui va enclencher la méiose II correspondant à la deuxième division (division équationnelle) (Runft et al., 2002). Au cours de cette division les chromatides sœurs se séparent et cela donne lieu à l'expulsion d'un deuxième globule polaire.

La maturation ovocytaire débute au stade de prophase I ou stade vésicule germinale (VG), *in vivo* dans les heures qui suivent la décharge ovulante de LH (Hyttel et al., 1986). Le stade VG se caractérise par la présence d'une membrane nucléaire et par l'évolution de l'état de la chromatine, il s'en suit **la rupture de la membrane nucléaire ou GVBD** (Germinal Vesicle BreakDown). Le noyau disparait quelques heures après le pic de LH et correspond aux évènements de la reprise de méiose (Szollosi et al., 1972). La membrane nucléaire se plisse dû à la condensation des chromosomes qui sont attachés à l'enveloppe par une extrémité. Les pores nucléaires, puis l'enveloppe, se fragmentent et disparaissent, il ne reste alors que des saccules (résidus de la paroi) qui vont participer à la formation des enveloppes nucléaires des pro-noyaux après la fécondation (Levasseur et al., 2001). Seul le réseau de lamines, protéines qui ont été phosphorylées par l'action de kinases, subsistera jusqu'à la métaphase II (Murray et al., 1994). Les chromosomes poursuivent leur condensation et une fois libérés des fragments d'enveloppe nucléaire, ils se placent de part et d'autre de la plaque métaphasique

de la première division méiotique (**métaphase I**). En parallèle, les kinétochores (points d'ancrage des chromosomes aux microtubules) sont captés par les microtubules en cours d'élongation depuis les **MTOCs** (microtule organizing center). Les MTOCs forment les deux pôles d'un fuseau aplati, en forme de tonneau, ce qui va permettre aux chromosomes de migrer à chaque pôle de l'ovocyte ; c'est **l'anaphase I** suivie de la **télophase**. Ces deux phases sont rapides (migration des chromosomes) elles génèrent un ovocyte de type II (prophase II) pendant que le corps intermédiaire induit un étranglement qui conduit à l'expulsion du **premier globule polaire**. Après la télophase, les chromosomes se replacent sur la plaque métaphasique pendant la formation du second réseau méiotique. A ce stade l'ovocyte est en **métaphase II**, sa maturation est de nouveau bloquée jusqu'à la fécondation ou la parthénogenèse. Ce blocage méiotique est maintenu par un facteur cytostatique le **CSF** (cytostatic factor) (Tunquist & Maller, 2003).

Les mécanismes moléculaires impliqués dans la régulation de la maturation nucléaire

La reprise de la méiose signifie que l'ovocyte passe du stade G2 au stade M du cycle cellulaire. Cette transition repose sur une sérine/thréonine kinase le **MPF** (M-phase Promoting Factor) (Masui, 2001). Il s'agit d'un hétérodimère constitué d'une sous-unité catalytique, la protéine **CDK1** (cyclin dependent kinase I) une sérine-thréonine kinase de 34 kDa et d'une sous unité régulatrice, la **cycline B** de 65 kDa qui est présente en phase G2 du cycle cellulaire. Les phosphorylations/déphosphorylations de certains sites de CDK1, comme le résidu Tyr15 et Thr 161 reflètent les niveaux d'activité du MPF (Tunquist & Maller, 2003). Ainsi, l'activité du MPF est maintenue par le CSF au cours de l'arrêt en métaphase II (Madgwick & Jones, 2007). Les mécanismes d'activation du MPF sont variables selon les espèces et le stade méiotique. Le niveau d'activité de MPF chute à l'anaphase I suite à la dégradation des cyclines et remonte après la néo-synthèse de cycline pour le réarrangement des chromosomes en métaphase II (Levasseur et al., 2001). Chez la vache, la brebis ou la truie, la synthèse d'inhibiteurs protéiques est nécessaire pendant la maturation nucléaire alors que pour d'autres espèces comme la souris, l'entrée en méiose ne nécessite pas de néo-synthèse protéique puisque qu'un pré-MPF est présent dans l'ovocyte compétent dès le stade VG (Mermillod & Marchal, 1999). Ces divers mécanismes expliquent les différences observées des temps de latence entre les différentes espèces entre l'initiation de reprise de méiose et la GVBD. De même, la voie de signalisation MAPK-ERK1/2 est indispensable pour le maintien de l'arrêt méiotique en métaphase par le CSF (Lefebvre et al., 2002). Chez

les bovins, l'activation de la voie MAPK est nécessaire à l'activation du MPF (Fissore et al., 1996). Cette voie de signalisation intervient dans l'organisation des microtubules et dans l'assemblage du fuseau méiotique (Fan & Sun, 2004). *In vitro*, les MAPK-ERK1/2 sont indispensables lorsque la maturation a lieu en présence de FSH ou de LH, elle permet l'induction du gène cox-2 lors de l'expansion des cellules du cumulus (Su et al., 2003).

Les principaux facteurs de régulation de la maturation nucléaire

De nombreux facteurs sont impliqués dans la régulation de la maturation nucléaire, cependant ce mécanisme reste à ce jour mal connu. Ici nous nous concentrerons sur les facteurs étudiés au cours de la thèse.

Les gonadotrophines LH et FSH stimulent la maturation ovocytaire (Okazaki et al., 2003; Patsoula et al., 2003). La LH agit via plusieurs mécanismes, elle permet la rupture des jonctions perméables unissant l'ovocyte aux cellules avoisinantes et induit la levée passive du blocage méiotique (Levasseur et al., 2001). De plus, la LH induit la production de stérols activateurs de méiose (MAS) comme le 4,4-dimethyl-5R-cholesta-8,14,24-trien-3,-ol (FF-MAS) ou le 4,4-dimethyl-5R-cholesta-8,24-dien-3,-ol (T-MAS) par les cellules du cumulus chez l'humain et les murins.(Motola et al., 2008; Xu et al., 2002). Les gonadotrophines agissent via la voie de signalisation de l'AMPc ou la voie du calcium (Goren et al., 1990).

L'AMPc, provenant de la dégradation de l'ATP par l'adénylate cyclase joue un rôle important dans le blocage et la reprise de la méiose de nombreuses espèces (Downs et al., 1988). La voie de signalisation de la LH conduit à l'activation de l'adénylate cyclase, menant à l'augmentation du niveau d'AMPc qui provoque une activation de la voie PKA (protéine kinase dependante de l'AMPc) liée à la reprise ou non de la méiose (Parrish, Kim, & Bae, 1992). L'AMPc est produite au niveau de l'ovaire par l'ovocyte, les cellules de la granulosa et du cumulus. Notamment chez les rongeurs, des niveaux élevés d'AMPc prolongent le blocage méiotique *in vitro*, tout comme ces analogues perméables comme dibutyril-cAMP, le 8-bromo-39, le 59-cAMP, et la forskoline (activateur de l'adénylate cyclase) ou l'usage d'inhibiteurs des phosphodiestérases (isobutyl-méthyl xanthine, IBXM hypoxanthine). Ces molécules sont couramment utilisées *in vitro* pour anticiper à la reprise de la méiose, au cours de ce processus l'AMPc module l'activité des MAPK (Sun et al., 1999). A l'inverse, des niveaux faibles d'AMPc enclenchent la reprise de la méiose (Dekel et al., 1984).

Les stéroïdes jouent un rôle encore mal connu dans la maturation. Les œstrogènes bloquent la maturation ovocytaire *in vitro* chez l'humain (Vitek et al., 2013). L'inhibition de la synthèse de P4 par les cellules du cumulus ou le blocage du récepteur à la P4 provoque une diminution du développement embryonnaire chez le bovin (Aparicio et al., 2011). De même chez, le porc la sécrétion de P4 par les cellules du cumulus est positivement corrélée avec les taux de GVBD obtenus *in vitro* (Yamashita et al., 2003). Les facteurs IGF-1, IGF-2, insuline, et leurs récepteurs sont exprimés au cours du développement de l'ovocyte et favorisent la maturation chez les mammifères via des mécanismes paracrines. L'IGF-1 joue un rôle important dans la reproduction femelle car son invalidation chez la souris bloque l'ovulation et donc altère la fertilité des animaux. Egalement l'IGF-1 et l'insuline améliorent la maturation ovocytaire *in vitro*. Cependant, une étude montre que des souris déficientes en récepteurs à l'IGF et/ou à l'insuline dans l'ovocyte ne présentent aucun trouble des fonctions reproductives. Ces souris sont fertiles ce qui suggère que la signalisation insuline/IGF-1 n'est essentielle que pour la maturation ovocytaire (Pitetti et al., 2009). Cependant, une autre étude affirme que chez les souris déficientes pour IGF-1 le développement folliculaire est arrêté à la fin du stade pré-antral, le stade ou la croissance folliculaire est dépendante des gonadotrophines (Zhou et al., 2000).

L'expansion du cumulus

Les cellules du cumulus sont importantes pour le bon déroulement de la maturation cytoplasmique (Menck et al., 1998). En effet, des ovocytes dépourvus de cellules du cumulus arrivent à se développer jusqu'au stade de métaphase II mais la fécondation est anormale et ils ne peuvent former un embryon viable. Les cellules du cumulus vont subir une importante expansion (ou mucification) en fin de maturation ovocytaire car les espaces intracellulaires augmentent en raison à la formation d'une matrice viscoélastique composée essentiellement d'acide hyaluronique (Salustri et al., 1989). Cette matrice permet au complexe cumulus-ovocyte (COCs) de rester associé même après la ponction folliculaire. Les jonctions entre les cellules du cumulus et de la granulosa sont importantes pour la maturation ovocytaire mais en fin du processus d'expansion elles diminuent car l'ovocyte devient plus indépendant (Buccione, 1990; Eppig, 1982). Ainsi, la diminution de ces jonctions va faciliter l'expulsion de l'ovocyte au moment de l'ovulation (Zhuo & Kimata, 2001). Les gonadotrophines et l'IGF-1 stimulent l'expansion des cellules du cumulus oophorus via l'induction de la

production d'acide hyaluronique (Schoenfelder & Einspanier, 2003) et l'IGF-1 potentialise les effets de la FSH (Singh & Armstrong, 1997).

La maturation cytoplasmique

Le fait caractéristique de la maturation cytoplasmique est **la migration des granules corticaux** (d'origine golgienne). Les granules localisés dans le cytoplasme diffusent dans l'ovocyte immature mais dès la reprise de la méiose ils migrent vers la zone corticale de l'ovocyte en association avec le cytosquelette (filaments d'actine) (Ducibella et al., 1994). La fécondation provoque la libération du contenu des granules corticaux dans l'espace périvitellin entre l'ovocyte et la zone pellucide. Cette libération induit des modifications majeures de la zone pellucide (partie interne rendue impénétrable) en quelques secondes et bloque la pénétration de spermatozoïdes surnuméraires chez l'homme. Le processus de maturation cytoplasmique est dépendant de la qualité du follicule ; taille, aspect, diamètre de l'ovocyte (Blondin & Sirard, 1995; Pavlok et al., 1992). La maturation cytoplasmique conditionne la compétence au développement de l'œuf puisque c'est au cours de cette étape que l'ovocyte stocke les ARNm et les protéines nécessaires au bon développement embryonnaire des premiers jours (Eppig et al., 1996)

C7) La stéroïdogenèse

L'ovaire présente une activité cyclique maintenue en l'absence de fécondation ; les sécrétions hormonales varient en fonction de ces phases. Ce sont les cellules de la granulosa et de la thèque entourant l'ovocyte qui vont sécréter les stéroïdes sexuels. Les cellules de la granulosa sécrètent la P4 et l'E2 et les cellules de la thèque interne la P4, les androgènes et les oestrogènes. Les cellules de la thèque externe ne présentent pas de fonction sécrétoire. Tous les gènes codant pour les enzymes impliquées dans la production de stéroïdes ont été clonés et séquencés chez l'humain et leur expression au sein de l'ovaire décrite (Simpson et al., 1992).

C7.1) La biosynthèse des enzymes de la stéroïdogenèse

Le substrat de base des stéroïdes est le cholestérol. Il est converti en androgène dans les cellules de la thèque interne stimulées par la LH. Il existe 2 voies de production du cholestérol ; une voie minoritaire à partir de l'acétyl-CoA par la 3-hydroxy-3-méthylglutaryl-coenzyme A (HMGCoA) réductase et une voie majoritaire à partir des esters de cholestérols véhiculés par les lipoprotéines de basse densité (LDL). Le transport du cholestérol est assuré

par la protéine de transport StAR (steroidogenic acute regulatory protein) jusqu'à la membrane interne des mitochondries avant d'être clivé au niveau de la chaine latérale (C27) par l'enzyme P450-scc (P450 side chain cleavage) en prégnénologne (C21). Elle sera par la suite convertie en P4 (C19 ou C18) par la 3βHSD (3-β-hydroxysteroid-deshydrogenase) avant d'être transformée en E2 puis en androgènes (C19) (testostérone et androsténédione) par la P450C17α, dans les cellules de la thèque (Figure 11).

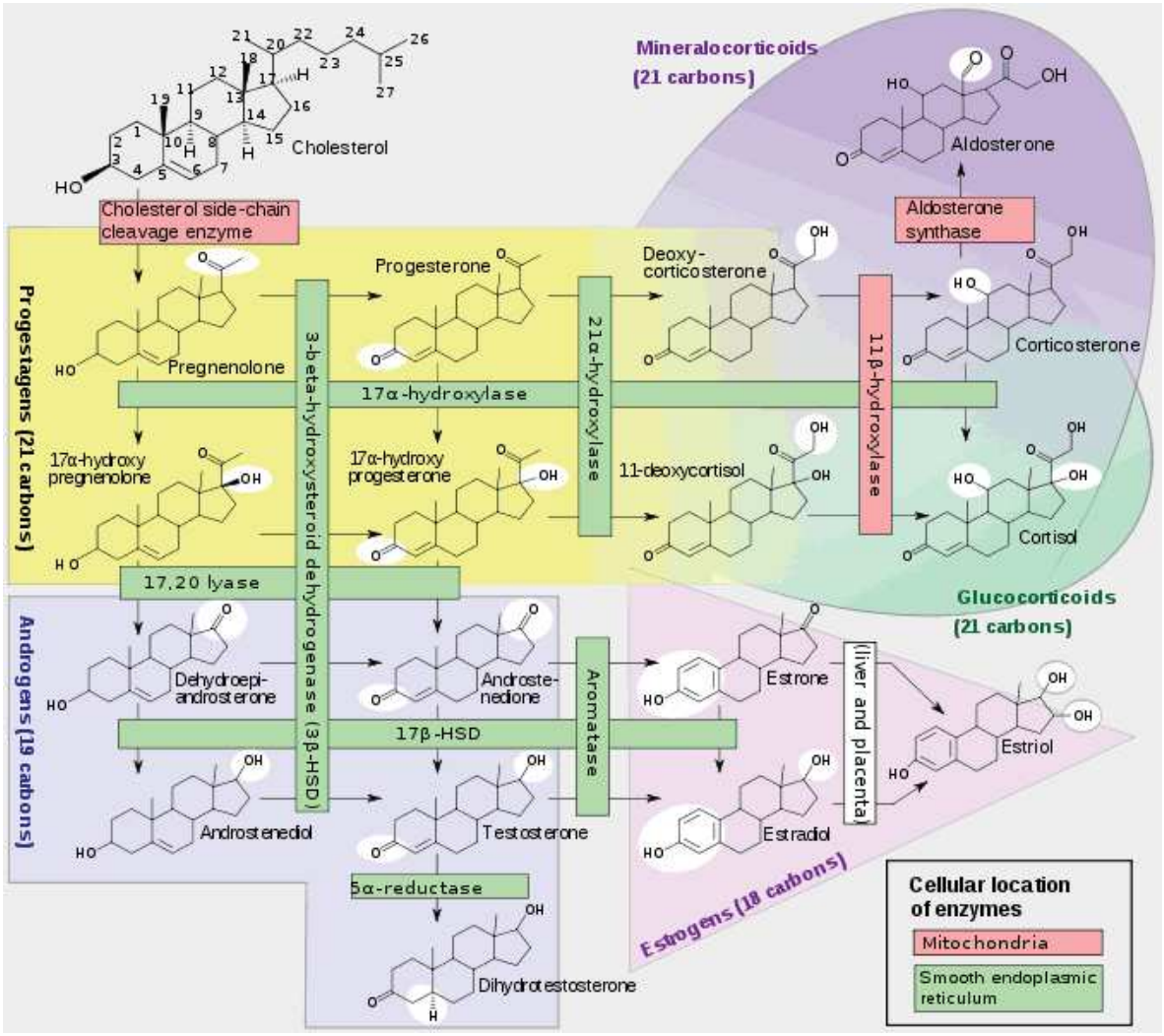


Figure 11: Voie de biosynthèse des stéroïdes sexuels chez la femme

D'après http://en.wikipedia.org/w/index.php?title=File:Steroidogenesis.svg License: GNU Free Documentation License Contributors: User:Mikael Häggström,User:Slashm

Cette enzyme possède une activité 17α-hydroxylase et 17-20 lyase et transforme la P4 en Δ4 androstènedione. La P4 sera également aromatisée en E2 dans les cellules de la granulosa par la p450 arom stimulées par la FSH (Sasano et al., 1989). Afin d'avoir une production suffisante d'E2 par le follicule, les cellules folliculaires (cellules de la granulosa et de la thèque) doivent dialoguer/collaborer ensemble (Young, 1999). Les cellules de la granulosa, en

produisant l'activine et l'inhibine, vont favoriser ou diminuer la sécrétion d'androstenédione respectivement par les cellules de la thèque (Young & McNeilly, 2012). L'augmentation de la production d' E2 va provoquer le pic de LH et permettre la mise en place du rétrocontrôle positif chez les agnelles (Clarke & Cummins, 1984). L'augmentation de la concentration de LH va induire la diminution de la sécrétion d'E2 et l'augmentation de la production de la P4 et de la 17 hydroxyP4. Cette concentration plus élevée en P4 est maintenue pendant la diminution de la LH et la phase lutéale. Elle va potentialiser les effets de l'E2 sur le rétrocontrôle positif chez l'ensemble des mammifères. Pendant cette phase, l'inhibine et l'E2 bloquent la sécrétion de la FSH et donc le déclenchement de la folliculogenèse. Après la lutéolyse et la transition lutéo-folliculaire, la sécrétion de FSH s'intensifie de manière à permettre le début d'un nouveau cycle marqué par le déclenchement des menstruations. La phase folliculaire est donc sous dominance **oestrogénique** alors que la phase lutéale est sous dominance **progestative**.

C7.2) La régulation de la stéroïdogenèse par les gonadotrophines

Les deux gonadotrophines LH et FSH induisent les sécrétions d'E2 et de P4 par les cellules ovariennes (Loucks & Heath, 1994) (Figure 12).

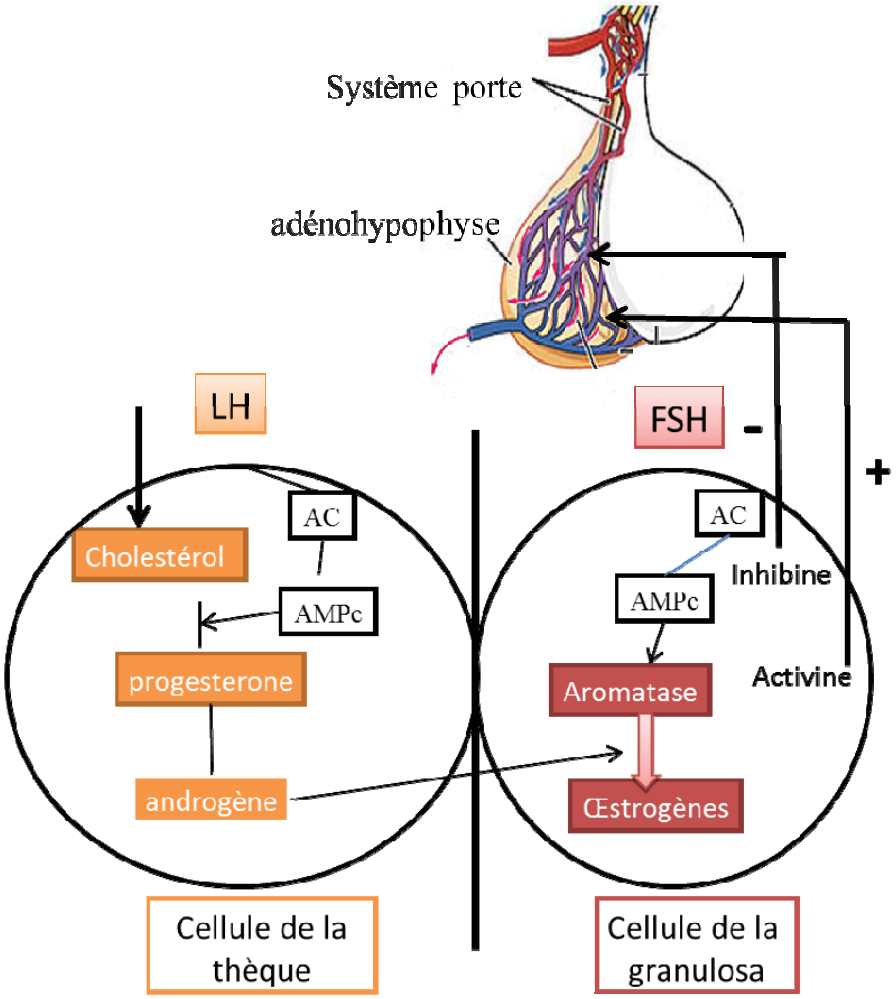


Figure 12: Régulation de la stéroïdogenèse par les gonadotrophines
AMPc Adénosine MonoPhosphate cyclique ; AC : Adénylate Cyclase**.**

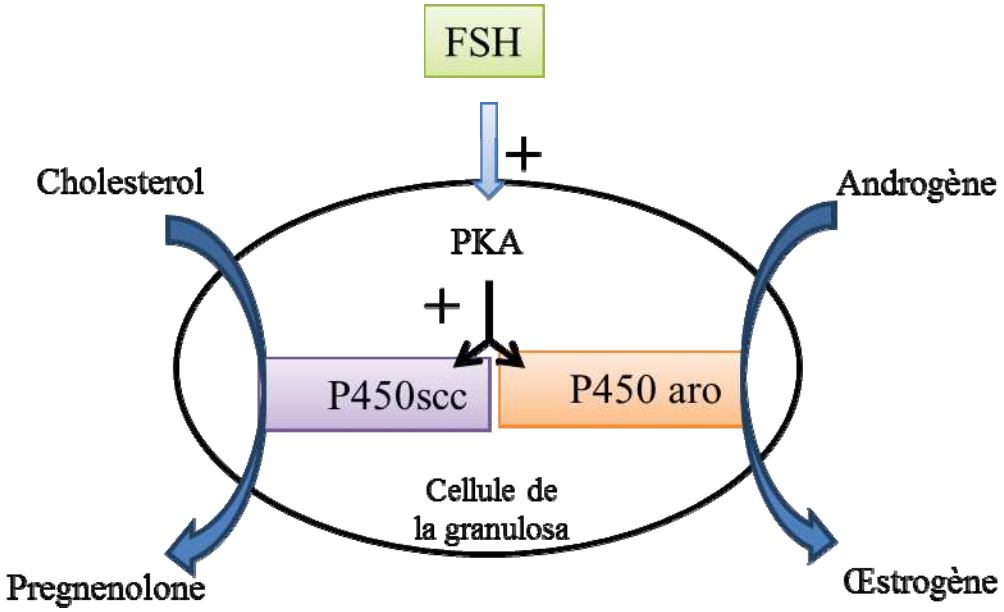


Figure 13: Effet de la FSH sur la sécrétion de stéroïdes dans les cellules de la granulosa chez l'humain et l'ovin

La FSH induit la production de stéroïdes via la voie PKA (Legault et al., 1999) qui active l'expression de la P450-scc et de la P450 arom (Pescador, 1997; Silva et al., 2006) (Figure 13). De plus chez le porc, la FSH stimule l'activité de la 3β-HSD (Dorrington & Armstrong, 1979; Pescador, 1997). Egalement, la sécrétion de stéroïdes notamment celle de l'E2 est modulée par la voie de signalisation MAPK-ERK1/2 qui régule négativement l'expression de la StAR et positivement celle de la P450 arom induite par la FSH dans des lignées de cellules de la granulosa de rate (Moore et al., 2001; Seger et al., 2001). La majorité des effets de la FSH sont transmis par l'AMPc et la PKA. La LH est connue pour inhiber les taux plasmatiques ovariens de l'E2 pendant la période pré-ovulatoire via une réduction de la disponibilité des androgènes ovariens et une inhibition de l'activité de la P450 arom chez la rate (Katz & Armstrong, 1976). Le pic de LH inhibe l'expression de P450C17α et de la P450-scc (sauf chez la femme) au moment de lutéinisation. Cependant, la LH induit la phosphorylation et la néosynthèse de la protéine StAR dans un délai de quelques minutes **en réponse à une augmentation de l'AMPc** (Levasseur et al., 2001). Par ailleurs, les récepteurs de la FSH et ceux de la LH induisent via leur protéine (Gαs) l'activation de l'AC et une augmentation de la concentration en AMPc intracellulaire et en conséquence l'activation de la PKA (Wood & Strauss, 2002). Cette voie de signalisation va augmenter l'expression et l'activité de la StAR. De plus, l'AMPc favorise la production des œstrogènes et des androgènes en modulant l'expression de la P450-scc chez l'humain dans les cellules de la

thèque (Havelock et al., 2004). La fixation de la LH et de la FSH sur leur récepteur peut aussi activer les protéines Gq et Gαi (Rajagopalan-Gupta et al., 1998). Ces protéines G activent la PLC générant la synthèse de l'inositol -1,4,5-triphosphate (IP) et du diacylglycérol (DAG). Ces derniers acteurs vont moduler la concentration calcique intracellulaire et l'activité de la PKC qui chez la souris peut réduire la stéroïdogenèse en inhibant l'AC (Leung & Steele, 1992).

C7.3) La régulation de la stéroïdogenèse par les facteurs locaux

In vitro l'**E2** agit en synergie avec la FSH, elle active l'expression du récepteur de la FSH et de la LH et de la P450 scc et de la P450 arom chez la rate (Silva et al., 2000). De plus, l'E2 stimule l'activité de la P450C17 et donc la sécrétion d'androgènes dans les cellules de thèques de rates et *in vivo* chez la femme, tandis qu'elle diminue la production de P4 chez le porc (Thanki & Channing, 1978). Cependant, l'E2 ne semble pas indispensable au développement folliculaire puisque l'invalidation du gène de ses récepteurs (α et β) ou l'utilisation d'un antagoniste n'empêche pas le développement jusqu'au stade préovulatoire (Nagy, 2000). L'**IGF-1** augmente *in vitro* la sécrétion de P4 en stimulant l'action de la P450scc dans les cellules de la granulosa issues de gros follicules ovins (Monniaux et al., 1994). L'IGF-1 stimule également la production d'E2 chez la souris (Demeestere et al., 2004). L'équilibre du ratio **IGF/IGFBPs** détermine la biodisponibilité des IGFs pour les cellules de la granulosa et régule leur réponse à la FSH (Levasseur et al., 2001). Dans les follicules à antrum, **l'inhibine** amplifie l'action stimulante de la LH sur la sécrétion d'androgènes par les cellules de la thèque interne chez l'humain contrairement à **l'activine** et inhibe la production de P4 dans les cellules tumorales humaines de la thèque, les HOTT (Sawetawan et al., 1996). Chez le bovin, la sécrétion d'E2 par les cellules de la granulosa en réponse à la FSH est inhibée par le TGFα et des activateurs des PKC qui régulent directement la PKA (Legault et al., 1999).

C8) La signalisation des récepteurs à la FSH et à l'IGF-1

Les cellules de la granulosa représentent le modèle cellulaire que nous avons le plus étudié au cours de cette thèse. Ainsi, nous avons choisi de détailler dans ce paragraphe les mécanismes d'action des récepteurs à la FSH et à l'IGF-1. En effet, comme décrit précédemment ces deux hormones sont fortement impliquées dans la régulation des fonctions des cellules de la granulosa.

La FSH se fixe sur un récepteur à 7 domaines transmembranaires couplé aux protéines hétérotrimériques Gs (Figure 14).

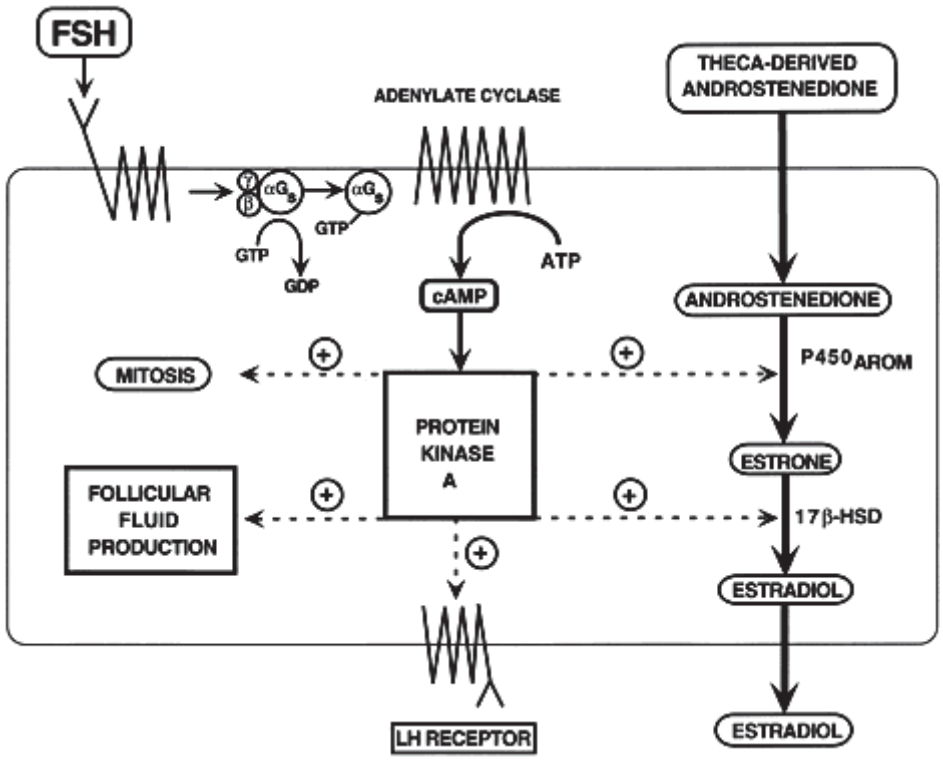


Figure 14: Diagramme du mécanisme d'action du contrôle de l'expression du récepteur FSH dans les cellules de la granulosa
D'après (Lobo, 1997)**.**

Cette fixation permet d'activer l'adénylate cyclase associée à la membrane plasmique (Leung & Steele, 1992; Simoni et al., 1997) ce qui induit une augmentation des niveaux d'AMPc intracellulaire qui a pour conséquences d'activer 2 voies. La voie majoritairement activée correspond à la PKA (*protéine kinase A*, dépendante de l'AMPc) qui a son tour va activer la voie des MAPK (*mitogen-activated protein kinase*), ERK1/2 (*extracellular signal-regulated kinase ½*) ou p38 qui sont connues avoir un impact sur la stéroïdogenèse (Conti, 2002). La phosphorylation de p38 est associée chez la femme avec l'augmentation de la synthèse d'androgènes dans les cellules de la thèque (Wood & Strauss, 2002). La PKA augmente l'activité transcriptionelle du promoteur de la StAR ((Wood & Strauss, 2002) et de SF-1 (Christenson et al., 1999). Egalement, la PKA régule l'expression de gènes, par exemple celui de la sous-unité α de l'inhibine ou celui de la P450 aromatase en activant des facteurs de trancription tel que CREB (*cAMP response element-binding protein*). La voie mineure est celle de la PI3K (phosphatidyl-inositol-3' kinase) / Akt (ou PKB) (Conti, 2002). La signalisation du récepteur à la FSH peut activer la PLC et la PKC ce qui chez la souris inhibe

la production d'AMPc et peut avoir un impact négatif sur la stéroïdogenèse (Leung & Steele, 1992). La voie MAPK-ERK1/2 est dépendante de l'AMPc dans les cellules de la granulosa de rate (García et al., 2012). Les modes d'activation de la voie MAPK ERKs varient en fonction du type cellulaire et des récepteurs impliqués et en fonction de l'espèce. Enfin, les MAPK ERK1/2 régulent directement la transcription des proto-oncogènes, comme c-fos et c-jun impliqués par exemple dans la prolifération cellulaire (Meredith et al., 1996).

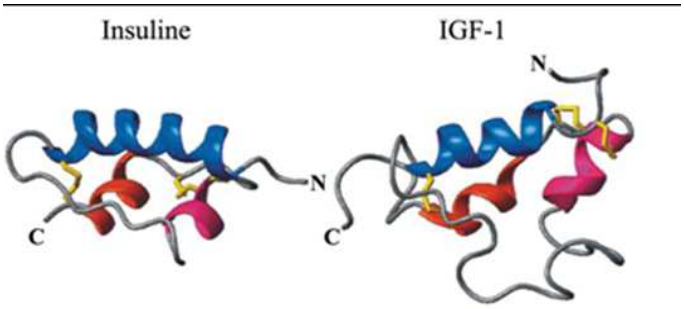


Figure 15: Structure en ruban de l'insuline et IGF-1.

Les structures en ruban de l'insuline et de l'IGF-1 sont illustrées. L'hélice du domaine B (bleu), les ponts disulfures (jaune) et les deux hélices du domaine A sont représentés (rose et rouge).

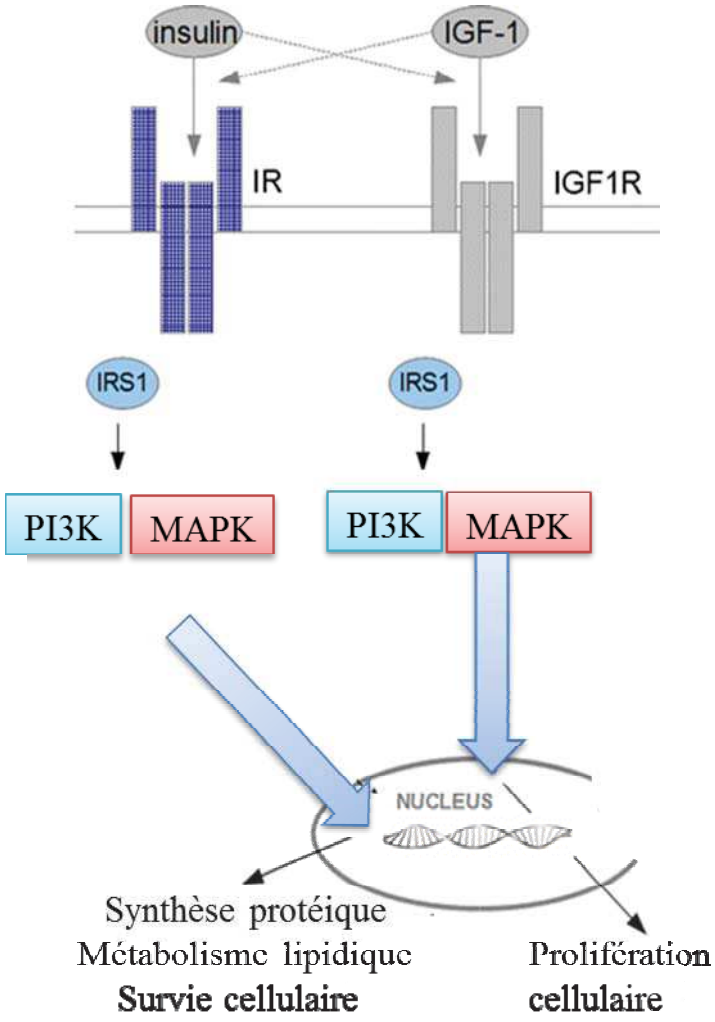


Figure 16: Voie de signalisation associée au récepteur de l'IGF-1 et de l'insuline
D'après (Trobec et al., 2011) Voie de signalisation associée au récepteur de l'IGF-1 et de l'insuline ; PI3K et MAPK d'après (Denley et al., 2005.).

L'IGF-1 présente une structure proche de celle de l'insuline (Figure 15). Il agit via son récepteur de type I à activité tyrosine kinase (Beitner-Johnson & LeRoith, 1995). Chez les mammifères non primates (rongeurs, porc, bovin), l'IGF-1 est le facteur le plus synthétisé par les cellules de la granulosa. Son récepteur (proche structuralement du récepteur de l'insuline) active les voies de signalisation PI3K et MAPK (Figure 16) (Dupont & LeRoith, 2001).

Les voies de signalisation de la FSH et d'IGF-1 s'entrecroisent (Gonzalez-Robayna et al., 2000). Ainsi, l'IGF-1 potentialise les effets de la FSH sur l'accumulation d'AMPc dans les cellules de la granulosa de rate en culture primaire (Adashi et al., 1986). L'IGF-1 et la FSH stimulent la prolifération des cellules de la granulosa de truie (Babu et al., 2000) et de brebis (Froment et al., 2003). De plus, l'IGF-1 influence la stéroïdogenèse et notamment la

production d'E2 chez différentes espèces (rongeurs, ovins, porcins), l'activation de son récepteur augmente l'activité de la P450 17α, P450 arom et P450-scc (Demeestere et al., 2004; LaVoie et al., 1999). Ces effets sont potentialisés par la FSH. (Demeestere et al., 2004; Monniaux et al., 1994). Enfin, l'IGF-1 active dans les cellules de la granulosa la voie MAPK-ERK½ qui est impliquée dans la production des stéroïdes et le bon développement folliculaire et ovocytaire (Wood & Strauss, 2002).

II) Métabolisme énergétique et reproduction : rôle du tissu adipeux et des adipocytokines

Il est reconnu aujourd'hui que la balance énergétique qu'elle soit négative ou positive influence le bon fonctionnement de l'axe reproductif hypothalamo-hypophyso-gonadique (HHG) (Clarke et Henry 1999). Ces observations sont valables chez le mâle comme chez la femelle. Ici nous explorerons ces effets seulement chez la femelle. Un déséquilibre négatif de la balance énergétique dû à une restriction alimentaire réduit les sécrétions de GnRH et de gonadotrophines chez l'animal et l'humain immatures et adultes (Tanaka et al., 2000). Un déficit de masse graisseuse peut entrainer un retard, voire une absence de puberté. A l'inverse, une masse adipeuse trop importante induit une puberté précoce (Styne, 2004). De même, chez les athlètes de haut niveau, la dépense d'énergie excessive et le stress physique amènent à des cas d'aménorrhée, d'oligoménorrhée et d'infertilité (Mastorakos et al.,2005.). On observe également une diminution du nombre d'ovulation chez les espèces poly-ovulantes en balance énergétique négative ce qui révèle que les fonctions ovariennes peuvent être directement ou indirectement affectées (Loucks & Heath, 1994). Ainsi, le maintien de l'équilibre de la balance énergétique est essentiel pour un bon fonctionnement de l'axe HHG (Clarke & Henry, 1999). Pour maintenir cette balance énergétique, l'organisme doit en permanence recueillir et interpréter des signaux hormonaux, métaboliques et nerveux émis par les tissus périphériques impliqués dans l'utilisation et le stockage de l'énergie (foie, muscle, tissu adipeux). Au cours de cette thèse nous nous sommes intéressés à certaines molécules exprimées et secrétées par le tissu adipeux, appelées adipocytokines. En effet, le tissu adipeux exerce une double fonction qui le place au cœur de l'homéostasie énergétique chez les mammifères. D'une part, c'est le seul tissu capable de stocker les réserves de l'organisme sous forme de triglycérides dans des cellules hautement spécialisées, les adipocytes. D'autre part, il secrète des molécules

biologiquement actives, collectivement appelées “ adipocytokines ”, qui sont impliquées dans la balance énergétique et le métabolisme glucido-lipidique. Les adipocytes eux-mêmes, mais également les cellules non-adipocytaires du tissu adipeux participent à cette fonction de sécrétion. Nous décrirons donc ci-dessous le tissu adipeux puis nous nous attarderons plus précisément sur certaines adipocytokines comme la leptine et l’adiponectine, les plus étudiées comme des liens potentiels entre les fonctions du métabolisme et de la reproduction. Nous présenterons aussi la chemerine, la visfatine et la résistine que nous avons étudiées au cours de ces trois dernières années.

A) Le tissu adipeux

Le tissu adipeux est une forme de tissu conjonctif lâche qui emmagasine les graisses (triglycérides) dans les cellules adipeuses. Ce tissu, longtemps considéré comme un organe de stockage inerte, se révèle depuis quelques années être un organe endocrine fortement impliqué dans le métabolisme énergétique. Il existe deux types de tissu adipeux ; le blanc et le brun. Le tissu adipeux brun présent en plus faible quantité, ne sera pas étudié ici, il joue un rôle essentiel dans le contrôle de la thermogenèse chez le nouveau-né, les petits mammifères, les rongeurs et les hibernants. Le tissu adipeux blanc ou « tissu graisseux » est essentiellement composé d’adipocytes isolés dans un tissu conjonctif bien vascularisé et innervé ou groupé en amas pour former le tissu adipeux. Les adipocytes ont pour fonction de synthétiser et de stocker des acides gras sous forme de triglycérides, puis de les libérer en fonction des besoins de l’organisme. Le tissu adipeux sécrète de nombreux facteurs dont **les adipocytokines** (cytokines) qui peuvent avoir des fonctions multiples via des actions endocrines, autocrines et/ou paracrines (Figure 17).

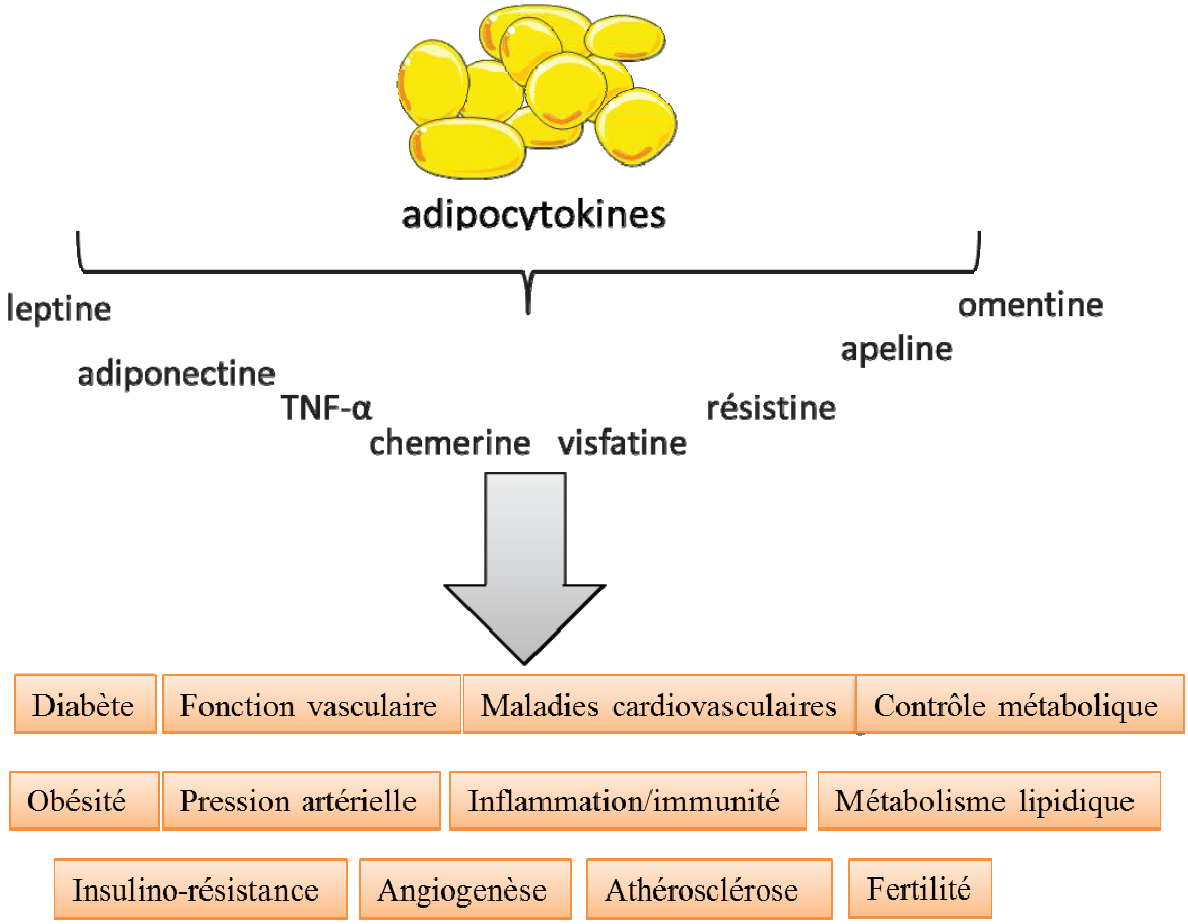


Figure 17: Exemple d'adipocytokines sécrétées par le tissu adipeux et leurs multiples fonctions paracrine et endocrine

Le tissu adipeux blanc représente 10 à 25 % de la masse totale de l'organisme humain (variable suivant le sexe), sa localisation est sous cutanée et péri-viscérale (Seale et al., 2009). Au sein du tissu adipeux, on retrouve également les fibroblastes, les cellules endothéliales, les leucocytes et macrophages qui ont aussi une activité sécrétoire (Zuk et al., 2001). L'adipocyte est une cellule qui peut varier de volume d'un facteur 1000. Le tissu adipeux va stocker l'énergie sous forme de triglycérides lors d'apport nutritionnel et va libérer ces molécules en cas de jeûn ou d'efforts physiques (lipolyse). La lipolyse (libération des lipides) est contrôlée par l'action de lipases présentent dans le cytoplasme qui sont activées par l'adrénaline et la noradrénaline et qui libèrent dans le sang des acides gras libres utilisables à des fin énergétiques par les autres cellules de l'organisme. Trois lipases interviennent, la monoglycéride lipase, la lipase hormonosensible (HSL) et l'Adipose triglyceride lipase (ATGL) (Fredrikson et al., 1986; Zimmermann et al., 2004). La LPL et la HSL provoquent la lipolyse des triglycérides en acides gras et en glycérol. La LPL une enzyme qui hydrolyse les triglycérides contenus dans les VLDL (Very Low Density Lipoproteins) et les chylomicrons

en deux acides gras libres et une molécule de monoacylglycérol. C'est la forme courte de HSL qui est exprimée dans le tissu adipeux, où elle hydrolyse les triglycérides. Son action doit être combinée avec la monoglycéride lipase pour obtenir une dégradation complète. Plusieurs hormones régulent les fonctions du tissu adipeux : par exemple l'insuline va stimuler la lipogenèse tandis que les catécholamines stimulent la lipolyse. Durant un repas, les taux d'insuline, de sucre et de lipides dans le sang augmentent ce qui conduit au stockage de l'énergie sous forme de triglycérides dans le tissu adipeux et le foie. A l'opposé, au cours d'un jeûne l'insulinémie et la glycémie diminuent ce qui induit la glycogénolyse et la lipolyse via l'activation du système nerveux sympathique et l'augmentation du glucagon, de l'épinéphrine et des glucocorticoïdes. Ces derniers maintiennent des apports de glucose suffisants pour le cerveau et les organes vitaux. Les acides gras vont être transformés par les muscles et le foie en corps cétoniques qui servent de carburant au cerveau et aux organes périphériques. **Le tissu adipeux est donc un véritable organe endocrine qui régule le métabolisme énergétique par ses propriétés anaboliques et cataboliques. Les différents acteurs sécrétés par le tissu adipeux ont des actions endocrines, paracrines ou autocrines. Ainsi, ce tissu n'est pas seulement un lieu de stockage de l'énergie mais bien un organe intervenant dans plusieurs grandes fonctions comme ; l'inflammation, l'homéostasie énergétique, le métabolisme mais aussi la reproduction.** (Waki & Tontonoz, 2007; Wozniak et al., 2009).

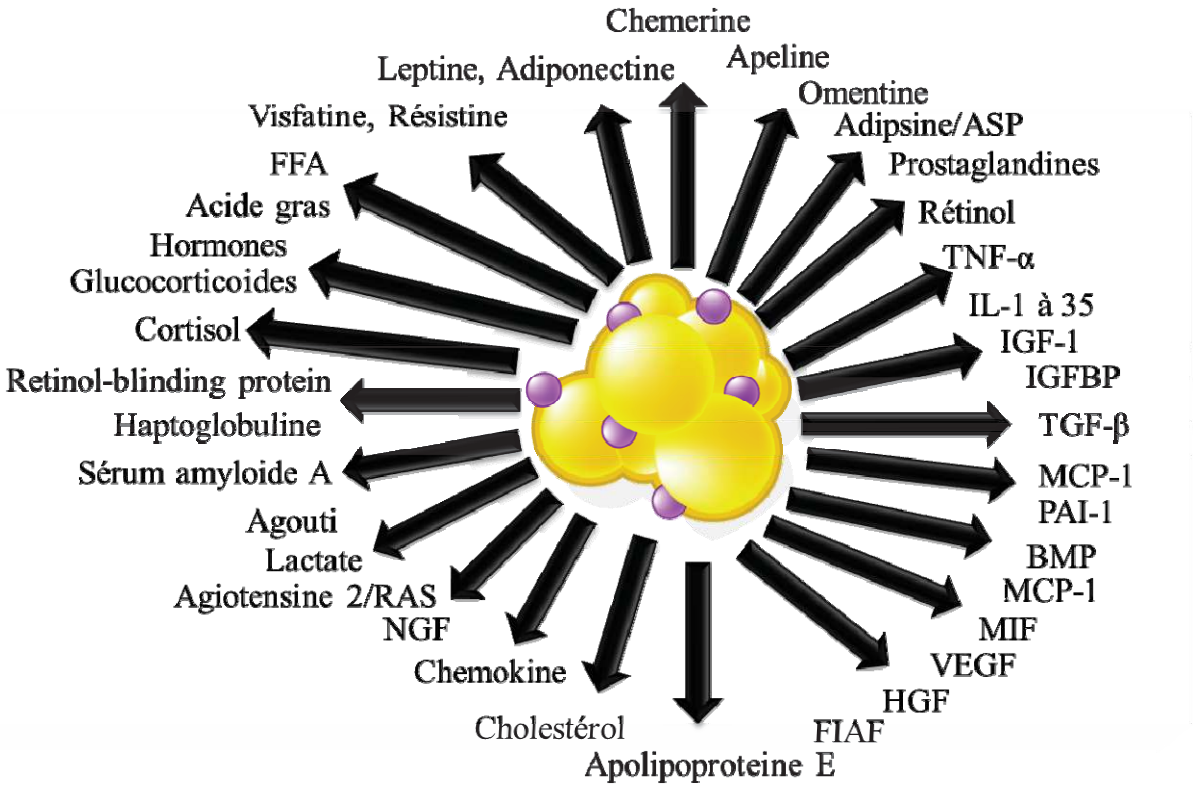
A1) L'adipogenèse

L'adipogenèse correspond à la formation d'adipocyte mature à partir d'une cellule progénitrice. Ce processus permet le maintien de l'expansion du tissu adipeux (TA) sous cutané en limitant l'accumulation ectopique de lipides impliqués dans les pathologies associées à l'obésité. Cette transformation nécessite deux grandes étapes ; **la détermination** et **la différenciation**. La détermination comprend une étape de sélection des cellules progénitrices à partir d'un précurseur mésenchymateux qui va emprunter la voie de détermination adipocytaire pour former un pré-adipocyte. L'existence de ce précurseur justifie que le tissu adipeux soit considéré comme une source de cellules souches multipotentes. Le tissu adipeux est à l'origine de cinq types cellulaires : myocyte, chondrocyte, ostéocyte, adipocyte et fibroblaste. Après une phase de croissance les pré-adipocytes commencent la seconde étape ; **la différenciation** qui consiste en la formation d'adipocytes matures et

fonctionnels. Cela commence par le remodelage du cytosquelette, de la matrice extracellulaire et l'expansion clonale (Bouloumié et al., 2001). Ces cellules qui arrêtent leur croissance, vont acquérir les propriétés lipogéniques et lipolytique, leur hormmono-sensibilité et leur capacité sécrétoire caractéristiques des adipocytes matures. De nombreuses études réalisées chez la souris ont permis de révéler les différents mécanismes et acteurs moléculaires fondamentaux impliqués dans l'adipogenèse. L'acquisition du phénotype adipocytaire mature dépend d'une programmation génétique précise, contrôlée par de nombreux facteurs de transcription. Il existe un équilibre entre les facteurs pro- et anti-adipogéniques. Les principaux facteurs de transcription qui participent dans l'adipogenèse sont PPARγ et la C/EBPα (CCAAT/enhancer binding protein-alpha). En effet, PPARγ a le potentiel d'induire l'adipogenèse dans des cellules non adipogéniques tels que les fibroblastes NIH-3T3. Des modèles murins ont permis de montrer *in vivo* l'implication de PPARγ dans l'adipogenèse (Rosen et al., 1999). Il existe deux isoformes de PPARγ nommées PPARγ_1 et PPARγ_2 qui diffèrent. D'après une étude de (Ren et al., 2002) c'est principalement l'isoforme γ_2 qui contrôle l'adipogenèse en régulant la transcription des adipocytes en cours de maturation (Fève et al. 2006; Koutnikova & Auwerx, 2001; Rosen et al., 2000). Cette isoforme est spécifiquement exprimée dans les adipocytes et plus particulièrement durant leurs différenciations. Son rôle semble indispensable puisque l'introduction de PPARγ dans des cellules dépourvues de C/EBPα restaure presque totalement le phénotype adipocytaire excepté la sensibilité à l'insuline. A l'inverse si l'activité de PPARγ est inhibée dans des cellules, la seule présence de C/EBPα n'est pas suffisante pour restaurer les fonctions adipocytaires (Rosen et al., 2002). PPARγ et C/EBPα orchestrent donc une voie de signalisation unique du développement et du maintien du phénotype adipocytaire. Cependant, les facteurs C/EBPβ et C/EBPδ induisent l'expression du PPARγ et du C/EBPα. Ces deux protéines induites très tôt influencent la maturation, elles organisent alors la pleine différenciation et le maintien du phénotype adipocytaire. C/EBPα peut augmenter l'expression de certains gènes qui induisent sa propre expression ainsi que celle de PPARγ. Les C/EBPs (β et δ) participent à l'action *in vitro* de PPARγ et leur activité combinée semble activer l'expression de C/EBPα. D'autres facteurs tel que ADD1 (adipocyte determination and differenciation factor 1) qui se fixe à un élément de régulation au stérol appelé SREBP-1 (Sterol regulatory element binding protein-1) (Yokoyama et al., 1993) semblent être impliqués dans l'adipogenèse et la lipogenèse en stimulant l'expression de gènes d'enzymes. ADD1 correspond à l'extrémité amino terminale de l'isoforme SREBP-1c qui prédomine dans les tissus animaux. L'expression des ARNm de ADD1/SREBP-1c augmente fortement dans des cultures de pré-adipocytes stimulés pour subir la différenciation en adipocytes

matures (Kim & Spiegelman, 1996). De plus, leur profil d'expression est similaire à celui de PPARγ. Une surexpression de ADD1/SREBP-1c dans des cultures cellulaires de pré-adipocytes 3T3-L1 en présence d'hormones inductrices de la différenciation conduit à une augmentation de l'expression des marqueurs des adipocytes et d'accumulation de lipides comparés à des cellules contrôles. De plus, ces deux facteurs pourraient être un relais de l'insuline (Foretz et al, 1999).

Il existe de nombreux autres facteurs pro-adipogéniques comme il existe un grand nombre de facteurs anti-adipogéniques. L'équilibre entre ces molécules, s'accompagne d'une adipogénèse adaptée. La voie de signalisation Wnt est l'exemple du contrôle négatif de la différenciation adipocytaire. Les facteurs Wnt sont des protéines sécrétées par de nombreuses cellules et se fixent sur des récepteurs de type Frizzled (Polakis, 2000). Ces facteurs favorisent les lignages musculaires et osseux et inhibent l'adipogénèse. Les BMPs qui appartiennent à la superfamille des TGF-β, notamment BMP-2 et BMP-4 sont impliqués dans l'adipogenèse, ils favorisent l'engagement des cellules dans la voie adipogénique (Huang et al., 2011). Les corégulateurs peuvent être cités : TRAP (Thyroid hormone receptor-associated protein), PGC-1, SRC-1, TIF-2, des coactivateurs ou NCoR, SMRT, des corépresseurs (Farmer, 2006). En s'associant aux facteurs de transcription tels que PPARγ ou C/EBP, ils modulent positivement ou négativement la différenciation (E D Rosen et al., 2000). Enfin, des facteurs extracellulaires et des voies de signalisation peuvent agir sur la différenciation adipocytaire. Ces effecteurs pro ou antiadipogéniques peuvent avoir une action endocrine ou paracrine (origine préadipocytaire ou adipocytaire).



ASP= Acylation-stimulating protein
FFA= Free fatty acid
FIAF= Fasting-induced adipose factor
HGF= Hepatocyte growth factor
IGF-1 = Insulin-like growth factor-1
IL= Interleukin
MCP-1= Monocyte chemoattractant protein-1
MIF= Macrophage migration inhibitory factor
NGF= Nerve growth factor
PAI-1= Plasminogen activator inhibitor-1
PGE_2= Prostaglandin E_2
$PGF_{2\alpha}$= 8-iso-prostaglandin $F_{2\alpha}$
RAS= Renin-angiotensin system
TGF-β= Transforming growth factor-β
TNF-α= Tumor necrosis factor-α
VEGF= Vascular endothelial growth factor
BMP= Bone morphogenetic protein

Figure 18 : Ensemble de molécules sécrétées par le tissu adipeux blanc

A.2) Un organe endocrine / les adipocytokines

Depuis une dizaine d'année le tissu adipeux est considéré comme un organe endocrine, la liste de peptides sécrétés par le tissu adipeux est en constante augmentation grâce à l'usage de nouvelles technologies comme la transcriptomique et la protéomique. Aujourd'hui plus de 600 protéines potentiellement sécrétées ont été identifiées (Lehr et al., 2012), cependant une étude suggère que seulement 10 % des protéines eucaryotes prédites ont été identifiées (Kussmann et al., 2008) (Figure 18). Le tissu adipeux sécrète des cytokines, des hormones, des facteurs de croissances, des protéines et des peptides (Celis et al., 2005). Les adipocytokines sont des cytokines produites et secrétées dans la circulation par le tissu adipeux. En 2007, 70 protéines sécrétées par le tissu adipeux ont été identifiées (Alvarez-Llamas et al., 2007). Elles peuvent être synthétisées par les adipocytes mais aussi pour certaines selon les espèces par les macrophages (Curat et al., 2006; Frühbeck et al., 2001;

Rajala & Scherer, 2003; Trayhurn & Beattie, 2001). Une étude sur l'expression des gènes a trouvé des différences majeures entre les adipocytes et les pré-adipocytes provenant du tissu adipeux sous-cutané ou viscéral (Gesta et al., 2006) suggérant des fonctions différentes entre les cellules du tissu adipeux viscéral et sous-cutané. Le tissu adipeux viscéral semble sécréter quantitativement plus de protéines que le tissu adipeux sous-cutané. Dans l'étude de Hockin et al, 59 % des protéines sécrétées proviennent du tissu adipeux viscéral contre 22 % à partir d'explant de tissu adipeux sous-cutané (Hocking et al ., 2010).

Les adipocytokines sont impliquées dans la régulation du métabolisme énergétique mais aussi dans d'autres fonctions comme l'inflammation et la reproduction. A ce jour, celles qui ont été le plus étudiées au niveau des fonctions du métabolisme et de la reproduction sont la leptine et l'adiponectine. Au cours de cette thèse nous nous sommes intéressés aux adipocytokines suivantes : résistine, visfatine et chemerine. Je décrirais donc principalement ces molécules en précisant brièvement leur rôle dans le métabolisme énergétique et surtout celui connu à ce jour dans les fonctions de la reproduction chez la femelle.

B) La leptine

B1) Structure et signalisation

La leptine identifiée par Samal et al en 1994 est une protéine de 16 KDa (167 aa) qui a été largement étudiée pour son rôle dans la régulation de l'homéostasie énergétique (Zhang et al., 1995). Vingt-et-un acides aminés constituent un peptide signal, qui, une fois clivé donne naissance à une protéine mature de 146 acides aminés (Zhang et al., 1994). 67 % de la séquence peptidique de la leptine a été conservée au cours de l'évolution, preuve du rôle primordial joué par cette hormone pour la survie des espèces (Masuzaki et al., 1995) (Figure 19). Il existe 83% d'homologie entre les leptines de rat et de souris contre 84% d'homologie entre les leptines murine et humaine (Masuzaki et al., 1997).

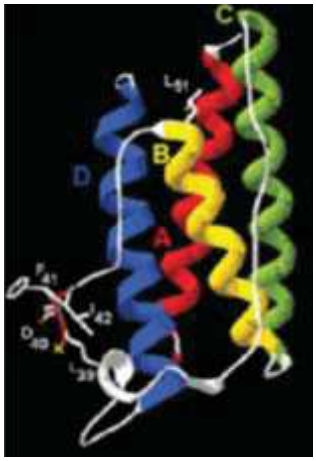


Figure 19: Modélisation de la structure de la leptine
D'après (Niv-Spector et al., 2005).

La synthèse de leptine a initialement été attribuée au seul tissu adipeux, majoritairement par les adipocytes blancs (Green et al., 1995; Zhang et al., 1994) et en moindre partie par le tissu adipeux brun (Cinti et al., 1997). Cette production étant proportionnelle à la quantité de tissu adipeux (Maffei et al., 1995), les niveaux plasmatiques de leptine sont donc le reflet du statut énergétique d'un individu. Depuis, une synthèse ectopique de l'hormone par d'autres tissus a été mise en évidence. Ainsi, l'estomac (Bado et al., 1998; Sobhani et al., 2000), l'épithélium intestinal (Sitaraman et al., 2004), le foie (Taouis et al., 1998), le muscle squelettique (Wang et al., 1998), la peau (Glasow et al., 2001) et les gonades (Cioffi et al., 1997; Ryan et al., 2002).

Le récepteur de la leptine fait partie de la famille de récepteurs de l'interleukine-6 (IL-6) puisqu'il contient un domaine de liaison extracellulaire, un domaine membranaire ainsi qu'un domaine cytosolique (Tartaglia et al, 1995). L'épissage alternatif du gène codant pour le récepteur à la leptine a donné naissance à au moins six différentes isoformes de ce type de récepteur (Figure 20).

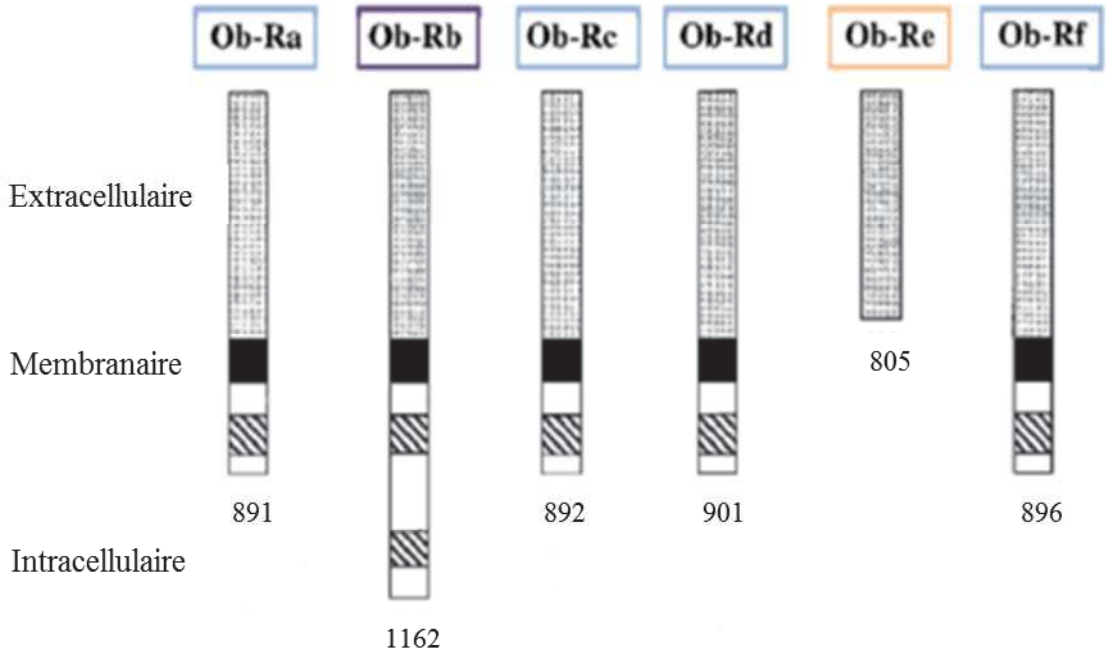


Figure20: Les différentes isoformes du récepteur de la leptine
D'après (Ahima & Flier, 2000).

Ces isoformes ont en commun un domaine extracellulaire de 800 acides aminés, un domaine transmembranaire de 34 acides aminés ainsi qu'un domaine intracellulaire variable. La plus longue isoforme du récepteur à la leptine (OB-Rb) est la seule capable de transduire du signal car, seul son domaine intracellulaire d'environ 300 résidus possède les motifs requis afin d'interagir avec les protéines nécessaires et transmettre un signal (Tartaglia et al., 1995). Quant au domaine extracellulaire d'OB-Rb, il a deux motifs similaires aux récepteurs des cytokines ainsi que quatre domaines fibronectine de type III (Tartaglia, 1997). L'isoforme la plus courte du récepteur à la leptine (OB-Ra) et OB-Rb ont en commun le domaine transmembranaire et extracellulaire ainsi que les 29 premiers résidus du domaine intracellulaire. Cette isoforme ne participe qu'à la clearance de la leptine et/ou au transport de celle-ci à travers la barrière hémato-encéphalique (Zhang et al., 2005). La leptine se retrouve dans la circulation sous forme libre ou encore liée. La signalisation intracellulaire de la leptine est médiée via son association non covalente avec une tyrosine kinase de la famille Janus Kinase (JAK), acteur principal de la voie de signalisation JAK/STAT. Toutefois, il faut noter que le récepteur OB-Rb peut transmettre son signal via plusieurs voies de signalisation tel que SHC/GRB2, IRS-2, PI-3 Kinase, MAPK, la voie AMPK ainsi que l'activation ERK1/2 qui est dépendante de SHP-2 (Otero et al., 2006).

B2) Rôle au niveau du métabolisme énergétique

Le rôle principal de la leptine est de contrôler la prise alimentaire ainsi que la dépense energétique via son interaction avec différents neuropeptides tels que le Neuropeptide Y (NPY) ou encore l'agouti related peptide (AgRP). Chez les souris obèses (ob/ob) déficientes en leptine biologiquement active, on observe une augmentation de la prise alimentaire, ces animaux ne connaissent pas la notion de satiété, présentent des troubles de la régulation thermique et un hypogonadisme (Zhang et al., 1994). Une injection de leptine recombinante provoque une réduction de la prise alimentaire et une augmentation des dépenses énergétiques (Halaas et al., 1995). Ces souris sont insulino-résistantes et diabétiques, chez l'humain les effets sont moins marqués cependant en cas de déficience du gène de la leptine (rare) on observe des patients obèses, hyperphagiques et une puberté retardée. En dehors du métabolisme énergétique, la leptine a démontrée des effets pléiotropiques dans plusieurs tissus périphériques (cf tableau ci-dessous).

Tissu ou organe	**Effets biologiques**	**Références**
Foie	Stimule la néoglucogenèse Inhibe la formation des triglycérides	Kamohara et al., 1997 ; Rosetti et al., 1997 ; Flier et al., 1997
Pancréas	Stimule la production d'insuline	Fehmann et al., 1997 ; Seufert et al., 1999 ; Poitout et al., 1998
Muscle squelettique	Stimule le transport du glucose Stimule l'expression d'UCP2 UCP3	Muojo et al., 1997 ; Gong et al., 1997 ; Liu et al., 1998
Tissu adipeux	Inhibe l'accumulation des triglycérides Stimule la lipolyse Stimule la thermmogenèse	Cusin et al., 1998 ; Gong et al., 1997 ; Commis et al., 1999 ; Scarpace et al., 1998
Tractus gastro-intestinal	Favorise l'absorption des nutriments	Guilmeau et al., 2004

Tableau 2: Action de la leptine au niveau des organes périphériques
D'après (Margetic et al., 2002)

Notamment, la leptine régule l'hématopoïèse, la pression sanguine, la fonction des lymphocytes T, la masse osseuse mais aussi la reproduction (Sirotkin & Grossmann, 2007; Zhang et al., 2005). Au niveau des fonctions reproductrices, elle agit à la fois au niveau des gonades et de l'axe hypothalamo-hypophysaire.

B3) Rôle au niveau de l'ovaire

Plusieurs études montrent que la leptine est importante au niveau du fonctionnement ovarien (Brann et al., 2002). Ob-R a été retrouvé dans plusieurs cellules ovariennes chez différentes espèces : cellules de la granulosa et de la thèque chez l'humain, la rate, la brebis et la truie (Batista et al., 2013; Löffler et al., 2001; Smolinska et al., 2013). La leptine réduit la production d'E2 par les cellules de la granulosa humaine, diminue la synthèse d'androgènes induite par la LH dans les cellules de thèques bovines. De plus, la présence d'Ob-R au niveau messager et protéique a été détectée dans l'ovocyte de plusieurs espèces ainsi que la protéine de la leptine cependant son ARNm n'a pas été identifié ce qui suggère qu'il doit être produit ailleurs et transporté. Chez la souris, la leptine améliore le taux de reprise de méiose des ovocytes pré-ovulatoires en agissant de manière indirecte sur les cellules de la thèque (Ryan et al., 2002). La leptine facilite la maturation ovocytaire via la voie de signalisation MAP kinase chez la truie (Craig et al., 2004). La leptine est présente à la fois dans le sérum et dans le liquide folliculaire à une concentration similaire. Via toutes ces actions, la leptine paraît être un signal hormonal informant l'axe reproducteur sur l'état nutritionnel de l'organisme.

B4) Rôle au niveau de l'axe hypothalamo-hypophysaire

La leptine tient une place importante dans la régulation centrale de la reproduction. L'expression du récepteur de la leptine (OB-R) a été montrée par RT-PCR dans l'hypophyse antérieure et l'hypothalamus. (Cai & Hyde, 1998; Dieterich & Lehnert, 1998; Shimon et al. 1998; Yu et al., 1997). Dans l'hypothalamus, elle régule positivement la sécrétion pulsatile du GnRH. Dans l'hypophyse elle contrôle les sécrétions de LH et de FSH. Plus précisément, plusieurs études *in vitro* ont montré que la leptine améliore les sécrétions de gonadotrophines par les cellules hypophysaires de différentes espèces.

C) L'adiponectine

C1) Structure et signalisation

L'adiponectine est une protéine de 247 acides aminés chez l'homme et de 244 acides aminés chez la souris. Sa séquence est très bien conservée chez les mammifères (80% d'identité avec la souris). Le monomère de l'adiponectine comprend 4 domaines : au niveau de l'extrémité N-terminale, une séquence signal puis une séquence hypervariable, un domaine collagène-like et au niveau de l'extrémité C-terminale, un domaine globulaire (tête) (Figure 21). Cette protéine peut exister sous forme entière ou sous forme globulaire de plus petite taille.

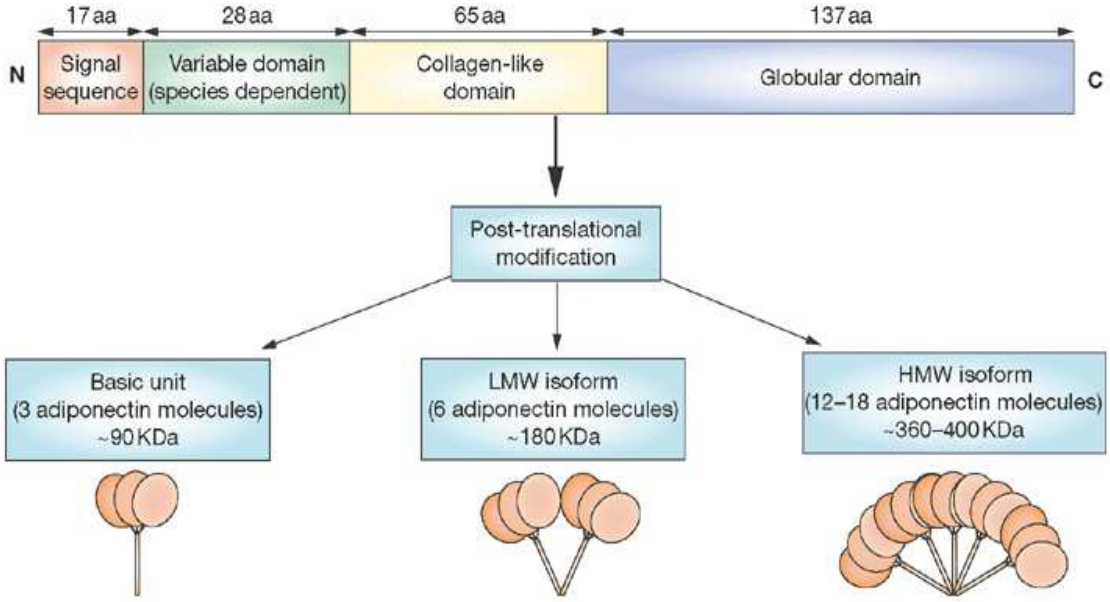


Figure 21: Les diverses formes moléculaires de l'adiponectine sécrétées par le tissu adipeux blanc.
LMW : Low Mollecular Weight, Middle Mollecular Weight, HMW : High Mollecular Weight
D'après (Goldstein et al., 2009)**.**

L'adiponectine a la capacité de s'oligomériser, puisque sur gel, la protéine existe en quatre formes, trois de ces formes n'existant qu'en conditions non dénaturantes. En conditions dénaturantes, la protéine a un poids moléculaire de 28 kilodaltons (kDa). Les formes oligomériques ont des poids moléculaires de 70 kDa, 150 kDa et de 440 kDa, correspondants respectivement au trimère, à l'hexamère et à la forme de haut poids moléculaire (HMW) (Nishimori et al., 1995). La formation des oligomères est possible grâce à des ponts disulfures

qui sont nécessaires à la forme HMW et à l'hexamère mais non essentiels pour le trimère bien qu'ils soient présents (Tsao et al., 2003). Chez un homme sain, l'adiponectinémie est de l'ordre de 5 à 30 mg/L ce qui représente 0,01 % des protéines plasmatiques totales. L'adiponectine est majoritairement produite par le tissu adipeux blanc mais elle est aussi retrouvée dans d'autres tissus comme l'os et le muscle (Berner et al., 2004; Lord et al., 2005). Il existe un rythme circadien de sécrétion avec une concentration maximale le matin vers 8h00, une diminution nocturne et une concentration minimale vers 4h00 du matin (Gavrila et al., 2003). Il existe aussi un dimorphisme sexuel. Chez la femme, les concentrations sont plus élevées que chez l'homme. Cette différence apparaît au moment de la puberté (Böttner et al., 2004). Ce dimorphisme peut être expliqué par une adiposité plus marquée chez la femme que chez l'homme. Les androgènes et plus particulièrement la testostérone pourrait jouer un rôle inhibiteur (Combs et al., 2003; Nishizawa et al., 2002).

Pour agir au niveau cellulaire, l'adiponectine se fixe principalement à deux récepteurs, AdipoR1 et AdipoR2, préférentiellement exprimés dans le muscle et le foie, respectivement. Les AdipoR sont des récepteurs à sept domaines transmembranaires, mais présentent une topologie inversée par rapport aux récepteurs de même type associés aux protéines G (Yamauchi et al., 2003) (Figure 22). AdipoR1 et AdipoR2 peuvent se dimériser de façon homologue ou hétérologue (Kadowaki & Yamauchi, 2005). L'analyse de ces protéines suggère que l'adipoR1 a une affinité plus élevée pour la forme globulaire de l'adiponectine alors que l'affinité de l'adipoR2 est supérieure pour la forme pleine longueur de celle-ci (Yamauchi et al., 2003).

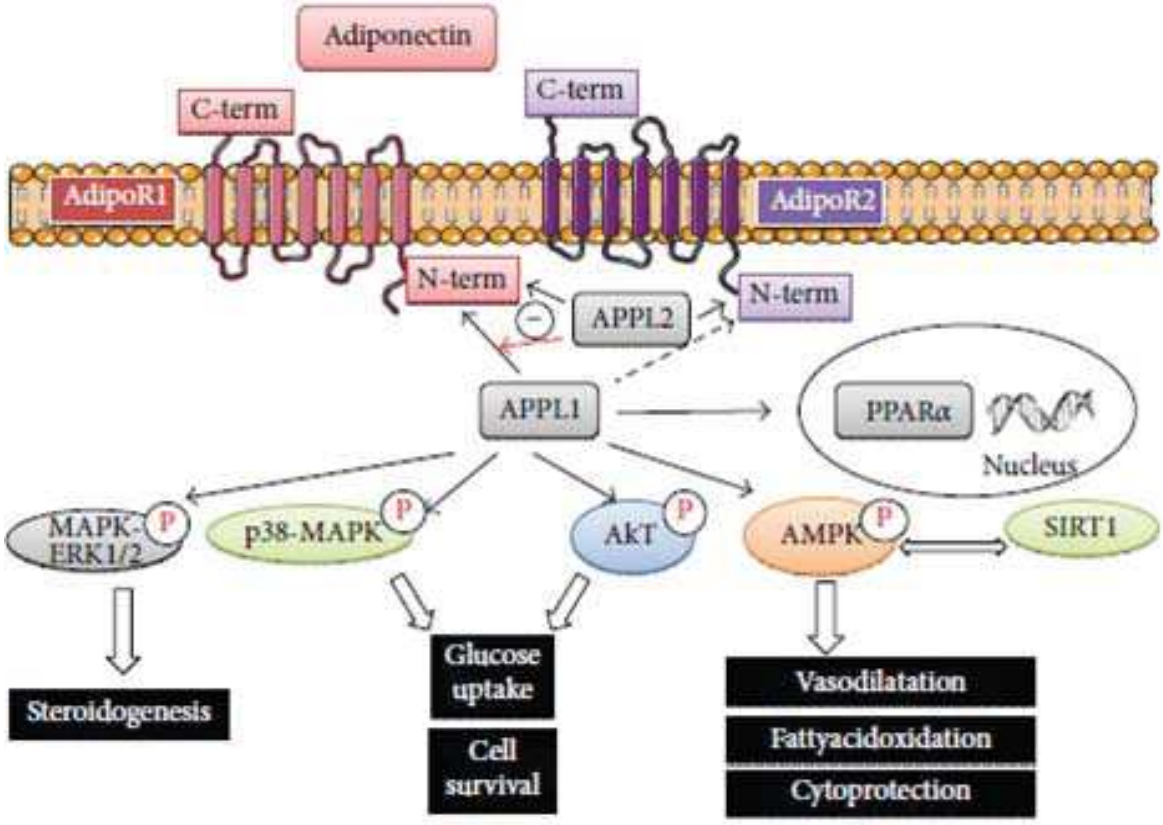


Figure 22: Structure et signalisation des récepteurs adiponectine AdipoR 1 et AdipoR2
D'après (Reverchon et al., 2013).

Un troisième récepteur, plus controversé, a été identifié pour l'adiponectine, T-cadhérine aussi connu comme H-cadhérine (Hug et al., 2004). Cette protéine aurait la capacité de lier la forme hexamèrique et HMW de l'adiponectine (Hug et al., 2004) mais l'absence d'une queue cytoplasmique pose un problème pour la signalisation.

Une fois activés, les AdipoR lient une protéine adaptatrice, APPL présente sous deux isoformes APPL1 et APPL2 qui relayent l'activation d'autres kinases comme les MAPK ERK1/2, p38, Akt et l'AMPK (Adenosine Monophosphate activated Kinase) (Tosca et al., 2008). Lorsque le récepteur AdipoR1 est inactif, APPL2 se lie et inhibe APPL1. APPL1 peut aussi se lier à une petite hydrolase du GTP (GTPase), Rab5. Celle-ci active la translocation du transporteur de glucose Glut4 qui active la capture du glucose dans les muscles (Mao et al., 2006). L'APPL1 active deux autres enzymes qui par la translocation de Glut4 à la membrane vont augmenter la capture du glucose : la protéine kinase activée par les mitogènes (MAPK) (Kadowaki et al., 2006) et l'adénosine monophosphate (AMP) kinase (AMPK) (Zhou et al., 2009). La phosphorylation de l'AMPK entraine aussi la phosphorylation de l'acétyle-

coenzyme A carboxylase (ACC), inhibant ainsi l'enzyme et activant l'oxydation des acides gras dans les muscles et le foie (Lafontan & Viguerie, 2006).

C2) Rôle au niveau du métabolisme énergétique

L'adiponectine est impliquée dans les métabolismes lipidiques et glucidiques et joue un rôle primordial dans la physiopathologie de l'obésité, du diabète de type 2. L'adiponectine limite la prise de poids chez des rongeurs recevant un régime riche en graisses et en sucres (Milan et al., 2002). La suppression de l'expression d'AdipoR1 et d'AdipoR2 s'accompagne d'une diminution de l'activité de PPARalpha ***(Peroxisome Proliferator-Activated Receptor*** alpha***)***, de l'oxydation des acides gras et de l'utilisation du glucose (Yamauchi et al., 2007). Chez la souris, l'adiponectine globulaire augmente l'oxydation des acides gras dans les muscles ce qui entraîne une perte de poids chez l'animal (Fruebis et al., 2001) (Figure 23).

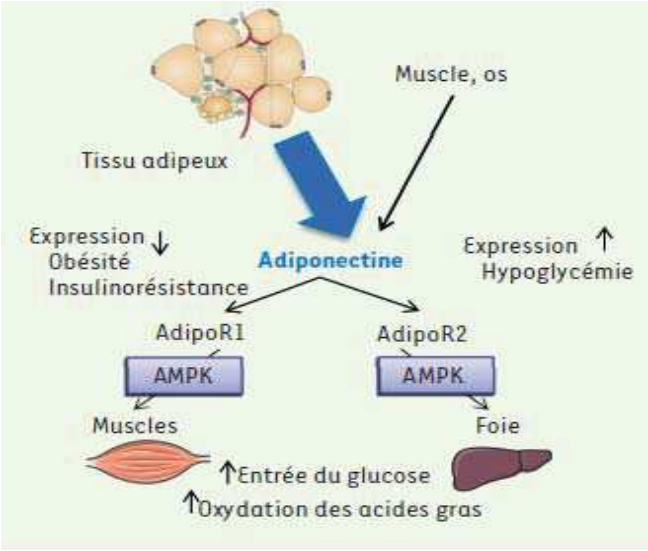


Figure 23: Lieu de production, exemples de cibles et rôles de l'adiponectine dans les tissus périphériques
D'après (Reverchon et al., 2013).

La forme pleine longueur et la forme globulaire de l'adiponectine augmentent l'utilisation du glucose et l'oxydation des acides gras dans le muscle par un mécanisme impliquant l'AMPK (Yamauchi et al., 2002) (Figure 22). Dans certaines situations, les effets de l'adiponectine forme longue et de sa forme globulaire se distinguent entre autre à cause de leur affinité différente pour les récepteurs de l'adiponectine (Palanivel et al., 2007). L'adiponectine globulaire augmente aussi l'oxydation des acides gras dans le cœur par le même intermédiaire, l'AMPK (Ding et al., 2007). En plus des effets sur l'utilisation du

glucose, l'adiponectine inhibe dans le foie la synthèse endogène de glucose en réprimant l'expression d'enzymes gluconéogéniques (Combs et al., 2001). Par ailleurs, l'adiponectine, en activant le système nerveux central, agit sur le métabolisme énergétique systémique en causant l'augmentation de l'absorption de la nourriture et la baisse de dépense énergétique (Kubota et al., 2007). L'adiponectine augmente les effets de l'insuline (Berg et al., 2001) et elle corrige partiellement la résistance à l'insuline due à l'obésité (Yamauchi et al., 2001). Ainsi, l'adiponectine offre une protection contre le diabète de type 2 (Maeda et al., 2002) et sa forme globulaire protège les souris ob/ob contre le diabète (Yamauchi et al., 2003). Des animaux génétiquement modifiés n'exprimant pas l'adiponectine présentent plus d'insulino-résistance (Kubota et al., 2002) et les agonistes de PPARγ y ont une moins bonne capacité d'amélioration de la tolérance au glucose (Nawrocki et al., 2006). En plus de diminuer la résistance à l'insuline, l'adiponectine globulaire augmente la sécrétion d'insuline par les cellules β du pancréas en situation d'hyperglycémie par une voie de signalisation impliquant l'AMPK (Gu et al., 2006). De plus, Civitarese et coll. ont observé que tout comme l'adiponectine, l'expression des deux récepteurs est diminuée chez les sujets ayant des antécédents familiaux de diabète de type 2 (Civitarese et al., 2004).

C3) Rôle au niveau de la reproduction femelle

C3.1) Rôle au niveau de l'ovaire

Après la leptine, l'adiponectine est l'adipocytokine qui a été le plus étudiée au niveau des cellules ovariennes. Dans l'ovaire, l'adiponectine a été identifiée dans le liquide folliculaire (femme et truie) (Chabrolle et al., 2009; Ledoux et al., 2006) et est exprimée dans différentes cellules ovariennes tels que l'ovocyte (rate), les cellules de la thèque (poule et rate) (Chabrolle et al., 2007; Chabrolle et al., 2007) et les cellules du corps jaune. Les récepteurs AdipoR1 et AdipoR2 ont été identifiés au niveau des différents types cellulaires du follicule (ovocyte, *cumulus oophorus*, cellules de la granulosa et de la thèque) et dans le corps jaune chez différentes espèces (rate, vache, truie, poisson, poule) y compris chez la femme. Le rôle de l'adiponectine a été étudié *in vitro* dans la stéroïdogenèse des cellules de la granulosa et de thèque et dans la maturation ovocytaire chez plusieurs espèces. Chez la rate et la femme, l'adiponectine augmente la sécrétion de la P4 et celle de l'E2 en réponse à l'IGF-1 (Insulin Like Growth Factor 1) (Chabrolle et al., 2009). Chez la rate, cette augmentation s'explique par une amélioration de la signalisation du récepteur de l'IGF-1 et chez la femme par une augmentation de l'expression de la protéine aromatase (Gutman et al., 2009). Chez la vache,

l'adiponectine diminue *in vitro* la production des stéroïdes par les cellules de la thèque en réduisant l'expression des récepteurs LH et des enzymes CYP11a1 (cytochrome P450, family 11, subfamily A, polypeptide 1) et CYP17a1 (cytochrome P450, family 17, subfamily A, polypeptide 1) (Lagaly et al., 2008). Lors de protocole de fécondation *in vitro* chez la femme et la souris, l'adiponectine améliore la maturation ovocytaire et le développement de l'embryon (Richards et al., 2012). Chez le porc, l'adiponectine améliore la maturation ovocytaire via la voie de signalisation des MAPK-P38 (Chappaz et al., 2008). Enfin dans cette précédente étude, l'adiponectine dans le milieu de culture augmente le nombre d'embryons se développant jusqu'au stade blastocyste.

C3.2) Rôle au niveau de l'endomètre et le placenta

Chez la femme, une forte expression des récepteurs a été observée dans l'endomètre pendant la période de l'implantation de l'embryon. Une étude récente indique que l'expression d'AdipoR1 et AdipoR2 est diminuée (60 %) dans l'endomètre des femmes en échec d'implantation après une procédure de FIV (fécondation *in vitro*) pour infertilité comparée à l'expression dans l'endomètre de femmes fertiles (Dos Santos et al., 2012). D'autre part, la concentration plasmatique d'adiponectine est fortement diminuée chez des patientes atteintes d'endométriose ou de cancer de l'endomètre (Michalakis & Segars, 2010). Ceci suggère que l'adiponectine et/ou ses récepteurs pourrait être impliqués dans la réceptivité endométriale (Dos Santos et al., 2012; Kim et al., 2011). L'adiponectine et ses récepteurs sont aussi présents dans le placenta humain et de rat. Dans le placenta de rate, l'ARN messager de l'adiponectine augmente au cours de la gestation puis diminue lors d'une restriction alimentaire (Caminos et al., 2005). Enfin, une dérégulation de l'expression du système adiponectine/AdipoR pourrait survenir dans certaines conditions pathologiques associées à des fausses couches ou à des pathologies de l'implantation (Kim et al., 2011).

C3.3) Rôle au niveau de l'axe hypothalamo-hypophysaire

L'adiponectine, AdipoR1 et AdipoR2 sont présents dans l'hypothalamus et l'hypophyse chez l'homme et les rongeurs. Au niveau hypothalamique, des études *in vitro* sur une lignée neuronale hypothalamique GT1-7 montrent que l'adiponectine en activant l'AMPK réduit la sécrétion *in vitro* de GnRH (Wen et al., 2008). Au niveau hypophysaire, Lu et al ont montré que dans la lignée LβT2 (cellules gonadotropes immortalisées de souris) l'adiponectine réduit

la sécrétion de LH en absence ou en présence de GnRH *via* la phosphorylation de l'AMPK (Lu et al., 2008). Egalement, ils montrent qu'une injection intraveineuse d'un adénovirus exprimant l'adiponectine chez les souris mâles réduit les niveaux de LH sériques sans modifier les taux de FSH. De plus, l'expression de l'adiponectine dans l'hypophyse est inhibée par le GnRH dans des cultures primaires d'hypophyse de rats et dans la lignée gonadotrope LβT2 (Kim et al., 2013).

La leptine et l'adiponectine représentent les deux adipocytokines les plus étudiées à ce jour, cependant le tissu adipeux sécrète bien d'autres molécules. Dans cette thèse, nous nous sommes intéressés aux effets des adipocytokines chemerine, visfatine et résistine sur les fonctions ovariennes.

D) La chemerine

D1) Structure et signalisation

La chemerine ou RARRES2 (Retinoic Acid Receptor Responder Protein 2) ou TIG2 (Tazarotene Induced Gene 2 Protein) est une autre adipocytokine de 163 aa avec un poids moléculaire de 14 kDa secrétée sous forme de précurseur inactif, la pro-chemerine (Meder et al., 2003) (Figure 24).

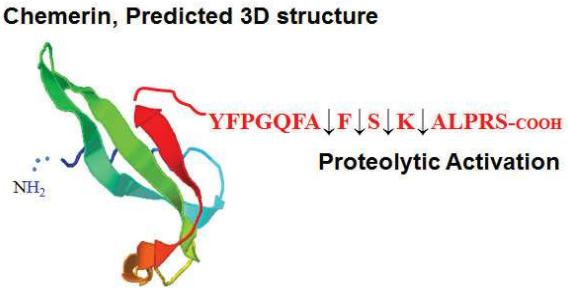


Figure 24: Structure predictive en 3D de la chemerine
D'après the Zabel laboratory at the veterans affairs Palo Alto Healthcare system, California.

La prochemerine subit alors un clivage de sa partie C-Terminale (6 aa) par une sérine protéase. La chemerine participe à la différenciation des adipocytes, au développement du tissu adipeux et à la régulation du métabolisme glucidique (Goralski et al., 2007). Elle est également impliquée dans la lipogenèse, l'adipogenèse, l'angiogenèse et elle améliore le

chimiotactisme des cellules immunitaires sur les sites d'inflammation (Bozaoglu et al., 2010; Goralski et al., 2007; Wittamer et al., 2003). La chemerine peut fixer trois récepteurs avec 7 domaines transmembranaires couplés aux protéines G appelés CMKLR1, GPR1 et CCRL2 (Figure 25).

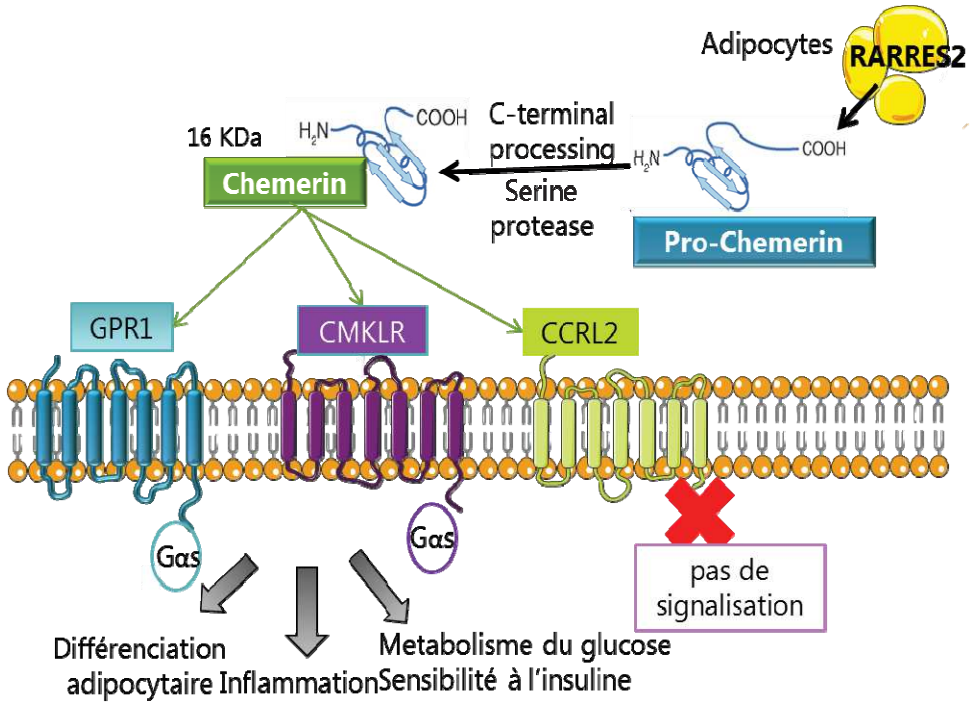


Figure 25: Activation de la pro-chemerine après clivage de sa partie C-terminale en chemerine active, fixation sur ses trois récepteurs CMKLR1, GPR1 et CCRL2 et ses principales fonctions endocrines

D'après (Reverchon et al., 2012)**.**

CMKLR1 (chemokine like receptor 1 aussi connu sous les noms chemR23 ou GPCR-DEZ) (Wittamer et al., 2003) est le plus étudié. Il est majoritairement exprimé par les cellules dendritiques plasmacytoïdes, les monocytes/macrophages et les cellules lymphocytaires NK (encore appelées « natural killers ») (Parolini et al., 2007). Le récepteur GPR1 présente une forte affinité pour la chemerine au niveau d'un peptide sur sa partie C-terminale. La fixation de la chemerine sur son récepteur GPR1 augmente la concentration calcique intracellulaire dans des cellules transfectées avec le récepteur GPR1 (Barnea et al., 2008). GPR1 est principalement exprimé dans le foie, le rein, les intestins et le tissu adipeux. Enfin, CCRL2 (Chemokine (C-C motif) (Zabel et al., 2008) est un récepteur qui n'induit pas de signalisation intracellulaire connue à ce jour (Yoshimura & Oppenheim, 2011). Cependant, ce récepteur pourrait réguler la biodisponibilité de la chemerine pour les deux autres récepteurs. CCRL2

présente une forte expression dans les cellules endothéliales du poumon et une plus faible dans le foie (Monnier et al., 2012). Chez l'humain, la chemerine est retrouvée dans le tissu adipeux notamment les adipocytes, le foie (Krautbauer et al., 2013), le pancréas, (Chamberland et al., 2013), le poumon (Ntikoudi et al., 2014) et l'ovaire (Reverchon et al., 2012).

D2) Rôle au niveau métabolisme énergétique

La concentration plasmatique de la chemerine est augmentée dans des modèles de souris obèses et avec l'augmentation de la masse graisseuse chez l'humain (Bozaoglu et al., 2007; Parlee et al, 2010; Weigert et al., 2010). Cependant son expression dans le tissu adipeux humain est controversée. Certaines études montrent une augmentation de l'expression de la chemerine alors que d'autres la trouvent inchangée en cas d'obésité (Bozaoglu et al., 2007; Ernst & Sinal, 2010; Parlee et al., 2010).

Les niveaux circulants de chemerine sont positivement corrélés avec l'indice de masse corporelle, le taux de triglycérides et les niveaux plasmatiques de cytokines inflammatoires (Bozaoglu et al., 2007; Lehrke et al., 2009). Chez l'homme et la souris, la synthèse de chemerine par les adipocytes est induite par les cytokines pro-inflammatoires comme IL-1β et TNF-α (Cawthorn & Sethi, 2008; Kralisch et al., 2009). En effet, Parlee et al ont observé qu'un traitement des 3T3-L1 adipocytes avec du TNF-α augmente la concentration de chemerine bioactive dans le milieu de culture (Parlee et al., 2010). Chez la vache, les niveaux d'expression de l'ARNm de la chemerine et de son récepteur CMKLR1 sont modifiés durant la différenciation des adipocytes et sont régulés par le TNF-α, l'adiponectine et un analogue de la chemerine (Suzuki et al., 2012).

Des explants de tissu adipeux de donneurs obèses sécrètent plus de chemerine que des explants de donneurs témoins et la concentration de chemerine dans le surnageant est négativement corrélée avec la sensibilité à l'insuline de ces cellules (Sell et al., 2009). Ainsi, les niveaux circulants de chemerine plus élevés en cas d'obésité seraient induits par les cytokines inflammatoires et produits par les adipocytes. *In vitro*, la chemerine recombinante inhibe l'absorption de glucose par les adipocytes induite par l'insuline (Kralisch et al., 2009) alors que l'injection de chemerine recombinante *in vivo* durant un test de tolérance au glucose n'a pas d'effet sur l'absorption du glucose par le tissu adipeux chez la souris (Ernst & Sinal,

2010). Chez l'humain, l'insuline augmente *in vitro* l'expression de la chemerine dans des explants de tissus adipeux et *in vivo* la concentration de chemerine augmente suite à une hyper-insulinémie prolongée chez des individus sains (Tan et al., 2009). Ceci suggère que cette induction de chemerine serait un mécanisme d'action pour diminuer l'activité de l'insuline.

La chemerine et son récepteur CMKLR1 joue un rôle important dans l'adipogenèse et le métabolisme énergétique. En effet, une invalidation du gène de la chemerine ou de celui de son récepteur CMKLR1 dans des pré-adipocytes ou des adipocytes matures réduit l'expression de gènes impliqués dans l'homéostasie du glucose et des lipides comme GLUT4 (Glucose Transporter-4) ou HSL (Hormone Sensitive Lipase) (Goralski et al., 2007). Dans des adipocytes matures un traitement à la chemerine bloque l'effet potentialisateur de l'isoproterenol sur la lipolyse de manière indépendante à CMKLR1 tout en améliorant l'absorption de glucose stimulée par l'insuline en activant la phosphorylation de la tyrosine d'IRS1, un substrat du récepteur de l'insuline (Insulin Receptor Substrate 1 (Goralski et al., 2007; Takahashi et al., 2008).

Dans des cultures d'adipocytes appauvris en cholestérol, la synthèse de chemerine au niveau messager et protéique est augmentée en parallèle du facteur de transcription SREBP2 alors que l'expression de SREBP1c est diminuée. Chez l'humain et la souris, le promoteur de la chemerine présente des sites de liaison pour SREBP (Zabel et al., 2014). Chez des sujets obèses l'expression de SREBP1c est également diminuée donc l'augmentation de la synthèse de chemerine semble être due à SREBP2. La plus grande quantité protéique de chemerine pourrait être expliquée par des mécanismes transcriptionnels. De plus, des pré-adipocytes 3T3-L1 traités avec un siRNA contre SREBP2 présentent une forte diminution en cholestérol, triglycérides et chemerine. Un faible taux de cholestérol est directement lié à une augmentation de la chemerine plasmatique (Bauer et al., 2011). De plus, chez la vache, Muruganandan et son équipe ont montré que la chemerine possède sur sa région promotrice des éléments de réponse à PPAR-γ (PPRE), ce qui concorde avec les données de Suzuki et al qui ont observé une augmentation simultanée de l'expression de PPAR-γ2 et de la chemerine durant la différenciation des adipocytes (Muruganandan et al., 2010; Suzuki et al., 2012).

D3) Rôle au niveau de la reproduction femelle

D3.1) Rôle au niveau ovarien

La chemerine a tout d'abord été identifiée dans l'ovaire humain par (Nagpal et al., 1997). Elle est particulièrement abondante dans le liquide d'ascite de patientes atteintes du cancer de l'ovaire (Wittamer et al., 2003). Plusieurs études montrent un rôle de la chemerine dans les fonctions des cellules ovariennes (stéroïdogenèse, apoptose et prolifération cellulaire). Chez des rates traitées au 5α-dihydrotestosterone la chemerine recombinante diminue la sécrétion d'E2 par les cellules de la granulosa et induit l'apoptose de ces cellules (Kim et al., 2013). Egalement, la chemerine bloque l'expression du facteur dérivé de l'ovocyte, GFD9 qui favorise la prolifération cellulaire et la différenciation des follicules pré-antraux en follicules antraux (Kim et al., 2013). La chemerine supprime aussi l'effet inducteur de la FSH sur la sécrétion de P4 et d'E2 dans des cultures de follicules pré-antraux et de cellules de la granulosa (Wang et al., 2012). La chemerine inhibe également la sur-expression des récepteurs nucléaires NR5a1 et NR5a2 qui activent l'expression d'enzymes de la stéroidogenèse, P450-scc et P450arom. La chemerine apparaît donc comme un important régulateur intra-ovarien et pourrait contribuer à la dérégulation du développement folliculaire.

Il est connu que les concentrations sériques de plusieurs adipocytokines comme la leptine, la visfatine la résistine et le TNF-α sont plus élevées au dernier stade de la gestation alors que les niveaux circulant d'adiponectine sont diminués (D'Ippolito et al., 2011; Mazaki-Tovi et al., 2007). Les niveaux de chemerine sérique sont faibles durant le début de la grossesse et sont élevés en fin de gestation, ces fluctuations sont inversement corrélées avec les niveaux d'adiponectine (Garces et al., 2013) chez la femme. La chemerine est également localisée dans le placenta humain et sa forte concentration en fin de grossesse suggère que la chemerine joue un rôle dans le transport de nutriments et la croissance embryonnaire (Kasher-Meron et al., 2014).

D3.2) Rôle au niveau de l'axe hypothalamo-hypohysaire

A ce jour il existe aucune donnée bibliographique qui relate un rôle de la chemerine dans les fonctions reproductrices au niveau de l'axe hypothalamo-hypophysaire. Seuls quelques travaux sur le rôle de la chemerine au niveau de la prise alimentaire ont été décrits. Cependant, l'effet de la chemerine au niveau central sur la prise alimentaire est controversé. En effet, en 2011 Brunetti et al ont montré que la chemerine n'avait pas d'effet sur la prise

alimentaire chez des rats Wistar adultes. Puis très récemment, ils ont observé qu'une administration de chemerine (8 et 16 µg/kg) par voie intrapéritonéale pendant 17 jours diminue la prise alimentaire et la masse corporelle comparé à des rats témoins. Ceci pourrait être associé à une augmentation significative de la synthèse et de la libération de sérotonine dans l'hypothalamus (Brunetti et al., 2014). Chez l'humain, il a été montré que les astrocytes expriment au niveau messager le récepteur CMKLR1 en absence de toute stimulation laissant penser un potentiel rôle de la chemerine dans les fonctions centrales (Croitoru-Lamoury et al., 2003).

E) La visfatine

E1) Structure et signalisation

La visfatine également appelée Nicotinamide phosphoribosyltransférase (Nampt) ou pre-B cell colony-enhancing factor (PBEF) est codée par le gène PBEF1 chez l'humain puis transcrit et traduit en une protéine de 491 aa avec un poids moléculaire de 52 kDa (Figure 26).

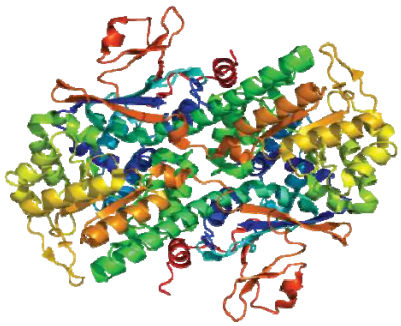


Figure 26: Structure de la protéine de la visfatine humaine
D'après (Wang et al., 2006)**.**

Initialement, la visfatine a été identifiée comme un facteur de croissance pour les cellules B à un stade précoce et comme une cytokine améliorant les effets de l'interleukine 7 (Samal et al., 1994), puis il a été raporté que la visfatine était plus fortement exprimée dans le tissu adipeux viscéral et que sa concentration plasmatique était grandement corrélée avec la masse de graisse viscérale chez la souris (Berndt et al., 2005). Cependant, elle est retrouvée dans de nombreux autres tissus tels que le tissu adipeux sous-cutané, la moelle osseuse, les lymphocytes activés, le foie, le cœur, les membranes fœtales ou les muscles (Fukuhara et al., 2005). La visfatine est reconnue comme une protéine multifonctionnelle ayant au moins trois

propriétés :1. -c'est une enzyme impliquée dans la synthèse du NAD 2. –c'est une adipocytokine secrétée 3. –c'est une cytokine pro-inflammatoire.

La visfatine possède une activité nicotinamide phosphoribosyltransférase, pour cela elle doit s'homodimériser afin de former son site catalytique pour permettre la réaction chimique suivante:

nicotinamide D-ribonucleotide + diphosphate ⇌ nicotinamide + 5-phospho-alpha-D-ribose 1-diphosphate

Les deux substrats de la visfatine sont le nicotinamide D-ribonucleotide et le diphosphate alors que ces deux produits sont nicotinamide et 5-phospho-alpha-D-ribose 1 –diphosphate. Une augmentation de la concentration de la visfatine via le NAD+ stimule l'activité d'une d'une histone déacétylase, la sirtuine 1 (Sirt 1) impliquée dans la régulation du métabolisme énergétique et plus récemment dans la stéroidogenèse des cellules ovariennes (Morita et al., 2012). Si la visfatine a été identifiée dans de nombreuses espèces comme l'humain, le chien, les rongeurs, le porc et le poulet (Chen et al., 2006; Klöting & Klöting, 2005; McGlothlin et al., 2005; Ocón-Grove et al., 2010; Samal et al., 1994), elle n'a cependant pas encore de récepteur connu à ce jour. Quelques fonctions de la visfatine sont résumées ci-dessous (Figure 27).

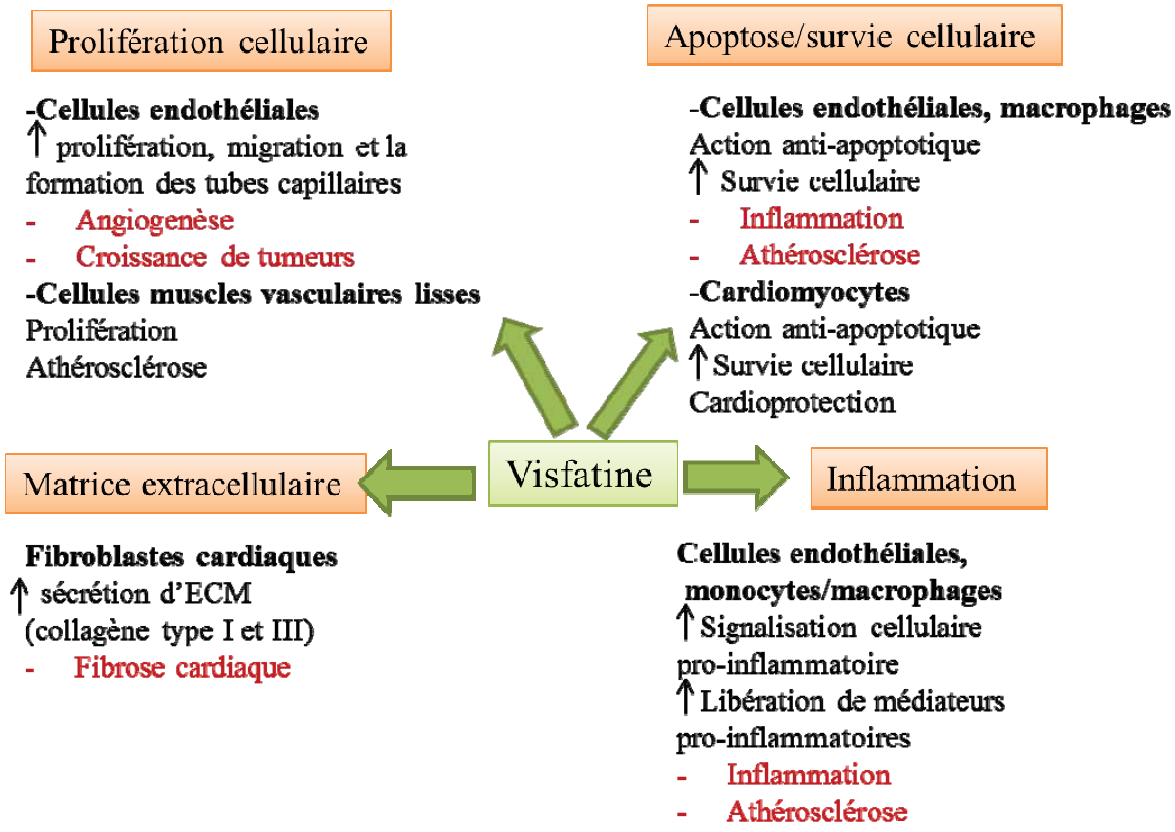


Figure 27 : Principales fonctions endocrines de la visfatine
D'après (Peiró et al;, 2010).

E2) Rôle au niveau du métabolisme énergétique

La visfatine a surtout été remarquée par son effet mimétique de l'insuline, en effet, elle participe à la diminution de l'insulino-résistance en voyant sa sécrétion augmentée en cas de glycémie élevée (fréquemment observé dans le cas d'obésité et de diabète de type 2 (Berndt et al., 2005; Chen et al., 2006). En 2005, Fukuhara et al, reportait qu'elle pourrait se lier de manière non spécifique au récepteur de l'insuline, fait démenti aujourd'hui. Cependant dans les cellules beta de pancréas de souris la visfatine active le récepteur de l'insuline ainsi que la voie de signalisation MAPK-ERK1/2 (Revollo et al., 2007). De plus, dans des ostéoclastes humains primaires la visfatine induit la phosphorylation des tyrosines du récepteur de l'insuline (IRS-1 et IRS-2) (Xie et al., 2007).

Les niveaux plasmatiques de visfatine sont positivement corrélés avec la pression artérielle systolique, le poids corporel, la glycémie à jeun, l'insulino-résistance, les taux plasmatiques de MCP-1 et l'albuminurie (Berndt et al., 2005; Kang et al., 2010). La

concentration de visfatine est pourtant plus élevée chez les patients atteints du diabète de type 2 que chez des personnes témoins (Chen et al., 2006). A l'inverse, le niveau plasmatique de visfatine est diminué lors d'un diabète gestationnel chez la femme (Chan et al., 2006; Haider et al., 2007). Dans des cultures d'adipocytes, une forte concentration en glucose et en angiotensine II diminue nettement la synthèse de visfatine. Un traitement des adipocytes *in vitro* avec de la visfatine exogène augmente l'activité transcriptionnelle de NF-kB et les molécules pro-inflammatoires. Ceci suggère que la synthèse de visfatine par le tissu adipeux est favorisée dans un contexte d'hyperinsulinémie, qu'elle induit l'activation de NF-kB et conduit à l'activation de cytokines pro-inflammatoires (Kang et al., 2010). **Ainsi, la question se pose de savoir si la visfatine serait un marqueur de la sensibilité à l'insuline ou si elle correspondrait plutôt à un nouveau marqueur de l'inflammation.**

Il a également été montré qu'un traitement à la rosiglitazone (un sensibilisateur à l'insuline) chez des patients atteints du diabète de type 2 réduit de manière significative les niveaux de visfatine circulante (McGee et al., 2011). De plus, ces mêmes auteurs ont montré que l'insuline augmente l'expression protéique de la visfatine et que cet effet est atténué par un traitement à la rosiglitazone. Ils ont aussi ont observé une meilleure réponse de la visfatine avec des doses plus faibles d'insuline suggérant ainsi une boucle de régulation négative de l'insuline sur la visfatine dans le tissu adipeux. Egalement, dans ce tissu des études *in vivo* et *in vitro* ont révélé que la visfatine est régulée par NF-kB et c-Jun Kinase (JNK). Lors d'une analyse du gène de la visfatine, des éléments de réponse au NF-kB ont été identifiés sur le gène de la visfatine. De plus, NF-kB régule positivement l'expression de la visfatine dans les cellules épithéliales humaines (Romacho et al., 2013). Ces découvertes suggèrent que les voies de signalisations JNK et NF-kB sont impliquées dans la régulation de l'expression de la visfatine dans les adipocytes humains.

Chez la souris, le lien entre la visfatine et l'obésité/insulino-résistance apparaît plus clairement que chez l'humain. Afin d'explorer l'effet de la visfatine, Fukuhara et son équipe ont généré des souris déficientes en visfatine (-/-) qui malheureusement meurent dans les stades précoces de l'embryogenèse. Ainsi, ils ont étudié des souris hétérozygotes déficientes en visfatine (+/-), ces souris sont viables et présentent 2/3 de niveaux plasmatiques de visfatine par rapport aux souris sauvages (Fukuhara et al., 2005). Ces souris hétérozygotes ne présentent pas de modification de la masse corporelle totale, ni de la nourriture ingérée ou de leur taux de croissance. Cependant, les souris hétérozygotes visfatine (+/-) ont un niveau de glucose plus élevé suggérant que la visfatine aurait un rôle physiologique dans la diminution

du niveau de glucose plasmatique chez cette espèce. A l'inverse, une étude de Klöting comparant l'expression du gène de la visfatine dans les adipocytes de rats WOKW (Wistar Ottawa Karlsburg) un modèle animal pour le syndrome métabolique avec des rats témoins DA (Dark Agouti) ne trouve aucune différence. Ainsi, ce résultat de l'expression relative du gène de la visfatine montre qu'il n'y a pas de relation entre l'expression du gène de la visfatine dans les adipocytes et le syndrome métabolique des rats WOKW (Klöting & Klöting, 2005).

En conclusion, les mécanismes d'action de la visfatine sur l'obésité, l'inflammation et l'homéostasie glucidique sont loin d'être totalement élucidés.

E3) Rôle au niveau de la reproduction femelle

E3.1) Rôle au niveau ovarien

La visfatine a tout d'abord était localisée dans les cellules de la granulosa humaine par Shen et son équipe en 2010. Egalement, la visfatine est présente dans le fluide folliculaire, Shen et al, ont montré que la concentration de visfatine dans le liquide folliculaire est corrélée positivement avec le nombre d'ovocytes recueillis chez l'humain (Shen et al., 2010). Une étude plus récente vient de décrire la présence de la visfatine chez la souris dans les cellules de la granulosa, du cumulus, les cellules stromales et endothéliales et moins abondamment dans l'ovocyte (Choi et al., 2012). Dans cette étude, un traitement des souris avec de la PMSG (Pregnant mare serum gonadotropin) augmente significativement le niveau de visfatine au niveau messager et protéique dans l'ovaire. Le rôle de ces molécules dans l'ovocyte n'est toujours pas compris.

Shen et al ont montré que l'expression de la visfatine dans des cultures primaires de cellules de la granulosa est augmentée par des traitements à l'hCG (Hormone Chorionique Gonadotrophique) et la prostaglandine 2 (Shen et al., 2010). Chez l'humain d'autres études sont nécessaires afin de définir le rôle précis de la visfatine dans la reproduction. Chez les rongeurs, une administration de visfatine durant la superovulation améliore la compétence de développement des ovocytes et le potentiel de fertilité chez la souris âgée (Choi et al., 2012).

E3.2) Rôle au niveau de l'axe hypothalamo-hypophysaire

Comme pour la chemerine, il n'existe à ce jour aucune donnée sur les effets de la visfatine au niveau des sécrétions de LH, FSH ou encore de la sécrétion ou expression du GnRH ou kisspeptine.

La visfatine est retrouvée dans le fluide cérébrospinal à une concentration d'environ 10% de celle du plasma (Hallschmid et al., 2009). De plus, une injection de visfatine intracérébro-ventriculaire diminue la prise alimentaire 8h après l'injection et induit une diminution du poids chez la souris (Park et al., 2011). Egalement, la visfatine diminue plus fortement la perte de poids par rapport à des peptides identifiés comme amincissants. Ceci suggère que l'un des rôles principaux de la visfatine au niveau central est impliqué dans le processus de consommation de l'énergie. Byong seo Park a observé que la visfatine augmente la synthèse d'α-MSH, un neuropeptide anorexigène. La visfatine pourrait induire un effet anorexique en agissant sur la voie de signalisation centrale des mélanocortine qui joue un rôle important dans la médiation de l'anorexie et de la cachexie (Laviano et al., 2008). De plus, Park et al., (2011) ont trouvé qu'en bloquant le récepteur des mélanocortines avec SHU9119, un antagoniste MC3/4R, ils atténuent l'effet anorexigène de la visfatine. Ainsi la voie de signalisation des mélanocortines est un potentiel médiateur de l'effet de la visfatine sur le comportement alimentaire. A l'inverse chez le poulet une injection intracérébro-ventriculaire de visfatine augmente la prise alimentaire (Cline et al, 2008). Ainsi, la visfatine semble avoir des effets spécifiques suivant l'espèce.

F) La résistine

F1) Structure et signalisation

La résistine a été identifiée en 2001 par deux études indépendantes montrant que cette protéine constitue un lien potentiel entre l'obésité, l'inflammation et l'insulino-résistance. C'est une protéine de 108 acides aminés chez l'homme (12,5 kDa) et de 114 acides aminés chez la souris appartenant à la famille des « resistin–like molecules » ou « FIZZ » (*found in inflammatory zone*) (Schwartz & Lazar, 2011). Elle est constituée par des homodimères reliés par des ponts disulfures (Figure 28). La résistine est retrouvée dans le sang sous forme de faible poids moléculaire (trimère) qui apparaît biologiquement active, et sous une forme hexamérique.

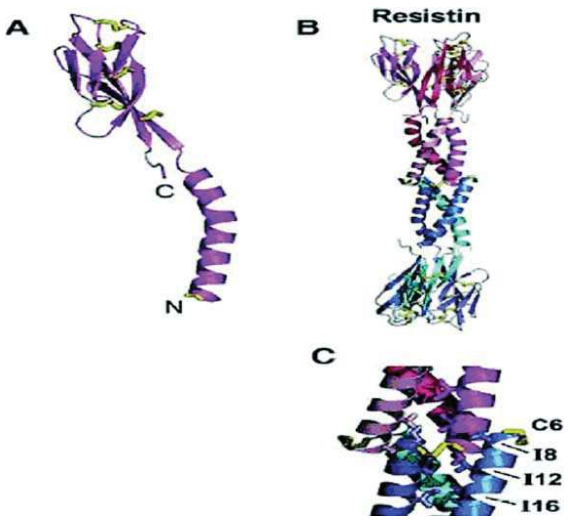


Figure 28: Structure de la résistine humaine
(A) Structure monomérique de la résistine, alors que l'image (B) montre sa forme hexamèrique comprenant deux trimères liés par des ponts disulfures. (C) Liens disulfures inter-chaîne présents dans la forme hexamèrique de la résistine. D'après (Patel et al ., 2004)**.**

Bien que la résistine ait d'abord été proposée comme jouant un rôle dans l'homéstasie du glucose, elle est également impliquée dans d'autres fonctions comme la régulation de la réponse immunitaire. Les différentes cibles de la résistine sont décrites ci-dessous :

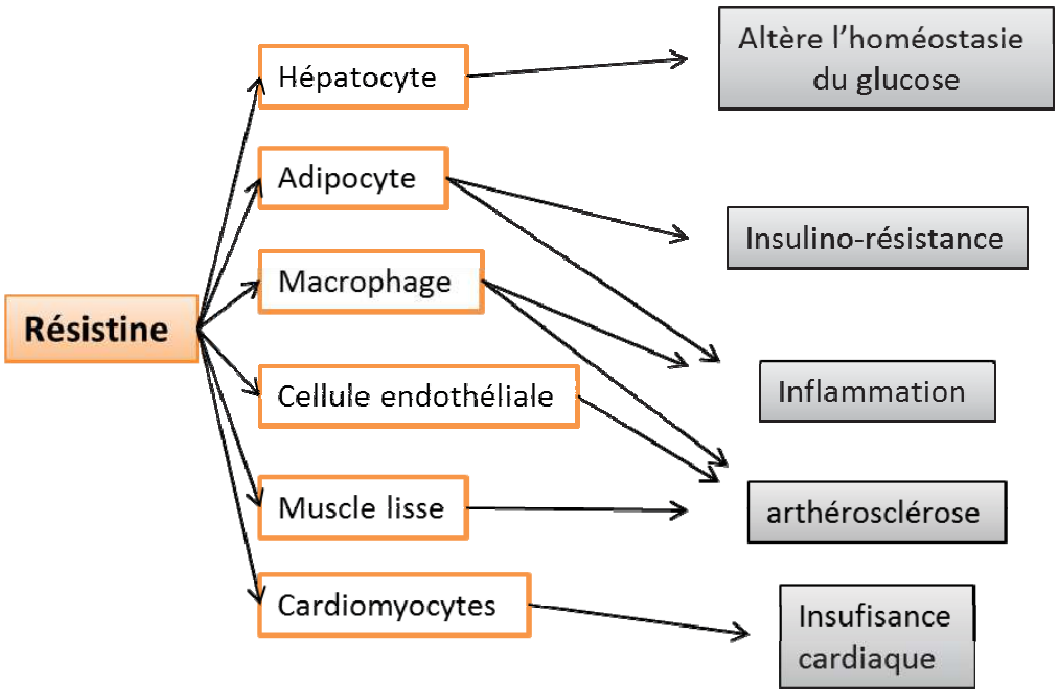


Figure 29: Principales cibles de la résistine humaine
D'après (Schwartz & Lazar, 2011)**.**

Chez la souris, la résistine est directement produite par les adipocytes alors que chez l'homme, elle est exprimée par la moelle osseuse et les macrophages qui la transportent vers les adipocytes (Kaser et al., 2003;Patel et al., 2003). Le gène de la résistine localisé sur le chromosome 19 chez l'humain a été mis en évidence lors de recherche sur les nouvelles cibles des TZD (traitement contre l'insulino-résistance et le diabète de type II (Steppan et al., 2001). La résistine est une adipocytokine corrélée au degré d'insulino-résistance chez la souris alors que les données chez l'homme sont plus contradictoires. Chez la souris, son taux diminue sous l'influence des thiazolidinediones (sensibilisateurs à l'insuline); inversement, il est augmenté en cas d'obésité. Très peu d'informations sont connues à ce jour quant au mode d'action de la résistine. Aucun récepteur n'a été clairement identifié, alors que la voie de signalisation utilisée demeure tout aussi obscure. Des études récentes suggèrent que la résistine pourrait se lier au récepteur tyrosine kinase appelé ROR1 (receptor tyrosine kinase-like orphan receptor) dans la lignée murine de pré-adipocytes 3T3-L1 (Sánchez-Solana et al., 2012) ou encore aux récepteurs TLR4 (Toll-like receptor 4) dans l'hypothalamus de souris (Benomar et al., 2013). Une isoforme de decorine a également été identifiée comme un potentiel récepteur de la résistine impliqué dans l'expansion du tissu adipeux (Daquinag et al., 2011).

F2) Rôle au niveau du métabolisme énergétique

Contrairement à l'adiponectine, le rôle de la résistine dans le métabolisme est moins connu chez l'homme. Chez la souris obèse, les concentrations sériques de résistine sont augmentées (Silha et al., 2006). Chez ces animaux, l'administration d'un anticorps anti-résistine corrige les niveaux de glucose circulant et améliore la sensibilité à l'insuline. Cependant, l'administration de résistine recombinante n'entraîne qu'une intolérance au glucose. Chez l'homme, le concept de «résistine» en tant qu'hormone principalement inductrice d'insulino-résistance, reste à confirmer. Il faut noter que le gène de la résistine possède une région capable de lier la protéine C/EBPα qui appartient à la famille des facteurs de transcription et qui, comme les PPARs, sont importants dans la régulation de la différenciation adipocytaire. Certaines hormones semblent contrôler l'expression du gène de la résistine dans le tissu adipeux. Cependant les données sont contradictoire, chez les rongeurs obèses (souris db/db et ob/ob), Way et al montre que les agonistes du récepteur PPARγ (thiazolidinédione, rosiglitazone, MCC-555 et GW_{1929}) augmentent l'expression de la résistine

alors que moore et al et Haugen et al observe que la rosiglitazone (TZD) inhibe son expression dans les adipocytes 3T3-L1 en culture et chez les souris db/db *in vivo* (Haugen et al., 2001; Moore et al., 2001; Way et al., 2001). La régulation de l'expression de la résistine reste controversée dans les adipocytes 3T3-L1, l'insuline diminue l'expression de la résistine tandis qu'*in vivo,* chez le rat obèse Zucker et chez les souris diabétiques (induit par la streptozotocine) l'insuline a un effet stimulant sur son expression (Kim et al.,2001).

F3) Rôle au niveau de la reproduction femelle

F3.1) Rôle au niveau ovarien

La résistine est présente dans le liquide folliculaire, l'ovocyte, les cellules de la thèque et le corps jaune chez la rate et la vache (Maillard et al., 2011). Contrairement à la rate, elle est retrouvée dans les cellules de la granulosa chez la femme et la vache. Dans les cellules de thèques humaines en culture, la résistine augmente l'expression de la 17 alpha hydroxylase en présence de forskoline et/ou de forskoline +/- insuline suggérant un rôle de la résistine dans la production d'androgènes (Munir et al., 2005). Chez la vache et la rate, la résistine module différemment la stéroïdogenèse et la prolifération des cellules de la granulosa en réponse à l'IGF-1 suggérant un effet spécifique de l'espèce (Maillard et al., 2011).

F3.2) Rôle au niveau de l'axe hypothalamo-hypophysaire

La résistine est également présente au niveau central dans l'hypophyse et l'hypothalamus (Morash et al., 2002). Au niveau de l'hypophyse, l'expression de la résistine est faible à la naissance chez la rate et augmente jusqu'à l'âge de 28 jours. Son expression augmente au sevrage chez la femelle et à la puberté chez le mâle. De plus, sa concentration hypophysaire est deux à trois fois plus importante chez les mâles que chez les femelles. La sur- ou la sous-expression de cette molécule ne semble pas avoir de conséquence sur la reproduction chez le rat (Juan et al., 2001; Pravenec et al., 2003). D'autre part, la résistine diminue *in vitro* l'expression des récepteurs AdipoR1 et AdipoR2 dans les cellules hypophysaires de rat en culture (Rodríguez-Pacheco et al., 2009). Chez cette espèce, elle co-localise avec les neurones contrôlant le comportement alimentaire au niveau hypothalamique (Mitchell et al., 2005). Le rôle de la résistine au niveau hypothalamique et hypophysaire dans les fonctions de la reproduction reste à déterminer.

D'autres adipocytokines non présentées ici telles que le TNFα sont connus affecter les fonctions ovariennes. D'autre part, récemment dans le laboratoire nous avons montré que l'omentine encore appelée intelectine est une autre adipocytokine impliquée dans la régulation de la stéroïdogenèse des cellules de la granulosa humaine (Cloix et al., 2014).

III. Exemple d'infertilités liées à un déséquilibre de la balance énergétique chez la femelle

A. Le SOPK chez la femme

Le syndrome des ovaires polykystiques est une pathologie affectant 6 à 10 % des femmes en âge de procréer. Selon la conférence de consensus de Rotterdam en 2003, une femme est considérée atteinte du SOPK lorsqu'elle répond positivement à 2 des 3 critères suivants : une hyper-androgénie clinique et/ou biologique, des cycles inférieur ou supérieur à 28-34 jours et plus de 12 follicules de 2 à 9 mm de diamètre par ovaire ou volume ovarien. Ce syndrome est associé dans 50% des cas avec une insulino-résistance (hyperinsulinémie et/ou dislipidémie) (De Leo et al., 2003). Ainsi 40% des patientes sont hypofertiles et le taux de fausse couche spontanée est doublé par rapport à la moyenne. Cette pathologie induit une dérégulation de la stéroïdogenèse des cellules de la thèque et une hypersensibilité de l'ovaire à l'action de l'insuline et des gonadotrophines. L'implication des adipocytokines dans le SOPK n'est pas claire. De nombreux résultats contradictoires sont présentés dans la littérature. Des agents sensibilisateurs tels que la metformine et les thiazolidinediones (TZD) sont utilisés pour traiter certaines de ces patientes atteintes du SOPK. Ces agents améliorent la fertilité et réduisent le taux d'androgènes (De Leo et al., 2003). Un traitement à la metformine durant 6 mois diminue de manière significative la concentration de chemerine sérique et en parallèle diminue l'insulino-résistance. Ainsi le niveau élevé de chemerine chez les patientes atteintes du SOPK pourrait être un mécanisme compensatoire à l'insulino-résistance. De plus, la metformine inhibe l'activité ovarienne de la p450 aromatase chez la femme atteinte du SOPK (La Marca et al., 2002), ainsi la question d'une action directe de la metformine sur les cellules ovariennes se pose.

B. Bilan énergétique négatif après vêlage et infertilité chez la vache laitière

Chez la vache laitière, une forte mobilisation des réserves adipeuses après le vêlage conduit à un déficit énergétique qui peut selon son importance engendrer un allongement de l'anoestrus post-partum à savoir un retard de la reprise de cyclicité (Levasseur et al., 2001). Ce déficit énergétique se manifeste au niveau métabolique par une diminution des taux plasmatiques d'IGF-1, d'insuline, de glucose et une augmentation des concentrations de la GH et des acides gras libres (Beam & Butler, 1999; Rutter et al., 1989). Il peut également conduire à une diminution des pulses de LH (Grimard et al., 1995). Une balance énergétique trop négative est également associée à une diminution de la taille des follicules (Jolly et al., 1995). Dans ce cas-là, Beam et Bulter ont constaté que le follicule dominant ovule dans seulement 46% des cas.

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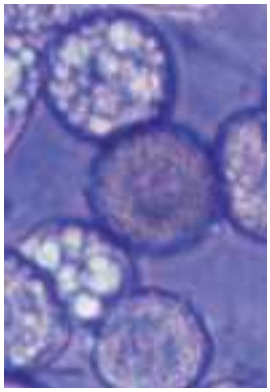
Adiponectine et résistine

Un rôle dans les fonctions de reproduction ?

Maxime Reverchon, Virginie Maillard, Pascal Froment, Christelle Ramé, Joëlle Dupont

> Les adipocytokines sont des hormones principalement produites par le tissu adipeux blanc, un organe endocrine impliqué dans l'homéostasie énergétique. Elles jouent un rôle important dans le contrôle, non seulement du métabolisme lipidique et glucidique, mais aussi du fonctionnement des gonades et de l'axe hypothalamo-hypophysaire. En effet, certaines adipocytokines, comme l'adiponectine et la résistine, régulent la stéroïdogenèse, la maturation des cellules germinales et la sécrétion des hormones gonadotropes chez différentes espèces. Ainsi, elles pourraient faire le lien entre la reproduction et le métabolisme énergétique, et être à l'origine des infertilités rencontrées dans certains troubles métaboliques, comme l'obésité ou le syndrome des ovaires polykystiques. <



Vignette (Photo © Inserm - Philippe Valet).

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L'adiponectine et la résistine sont deux adipocytokines, hormones sécrétées par le tissu adipeux. Elles induisent des effets opposés sur la régulation de la sensibilité à l'insuline. L'adiponectine améliore la sensibilité à l'insuline alors, qu'à l'inverse, la résistine induit un état d'insulinorésistance.

• L'adiponectine est une protéine de 244 acides aminés majoritairement produite par le tissu adipeux blanc, mais elle est aussi retrouvée dans d'autres tissus comme l'os et le muscle [1, 2] *(Figure 1)*. Chez l'homme, le niveau d'expression de son ARN messager varie selon la localisation, puisqu'il est plus faible dans le tissu adipeux viscéral que dans le tissu adipeux sous-cutané, que les sujets soient minces ou obèses. L'adiponectine est impliquée dans le métabolisme lipidique et glucidique, et semble jouer un rôle primordial dans la physiopathologie de l'obésité, du diabète de type 2 et de la maladie coronarienne. Chez un homme sain, l'adiponectinémie est de l'ordre de 5 à 30 mg/L, ce qui représente 0,01 % des protéines plasmatiques totales. Une adiponectinémie basse est prédictive d'une insulinorésistance ou d'un diabète de type 2. L'adiponectine plasmatique existe sous diverses formes moléculaires : forme monomérique et multimérique (monomères d'adiponectine organisés en une structure plus complexe grâce à des ponts disulfures), que l'on peut classifier en haut poids moléculaire (HMW), en poids moléculaire moyen (MMW), et en bas poids moléculaire (LMW). Pour agir au niveau cellulaire, l'adiponectine se fixe principalement sur deux récepteurs, AdipoR1 et AdipoR2, préférentiellement exprimés dans le muscle et le foie, respectivement. Les AdipoR sont des récepteurs à sept domaines transmembranaires, mais présentent une topologie inversée par rapport aux récepteurs de même type associés aux protéines G : la partie amino-terminale est intracellulaire et la partie carboxy-terminale extracellulaire [3]. Une fois activés, les AdipoR lient une protéine adaptatrice, APPL, qui relaye l'activation d'une *adenosine monophosphate activated kinase* (AMPK) [4]. L'AMPK est un composant majeur de la voie de signalisation de l'adiponectine impliquée dans ses effets métaboliques.

• La résistine est une protéine de 108 acides aminés chez l'homme et de 114 acides aminés chez la souris, qui appartient à la famille des *resistin-like molecules* ou FIZZ (*found in inflammatory zone*) [5]. Elle est constituée d'homodimères reliés par des ponts disulfures. Chez la souris, la résistine est directement produite par les adipocytes alors que chez l'homme, elle est exprimée dans les cellules de la moelle osseuse et les macrophages qui la transportent jusqu'aux adipocytes *(Figure 1)* [6, 7]. La résistine est corrélée au degré d'insulinorésistance chez la souris, alors que les données chez l'homme sont plus contradictoires. Chez la souris, son taux diminue sous l'influence des thiazolidinediones (qui améliorent la sensibilité à l'insuline) ; inversement, il est augmenté en cas d'obésité. Très peu d'informations

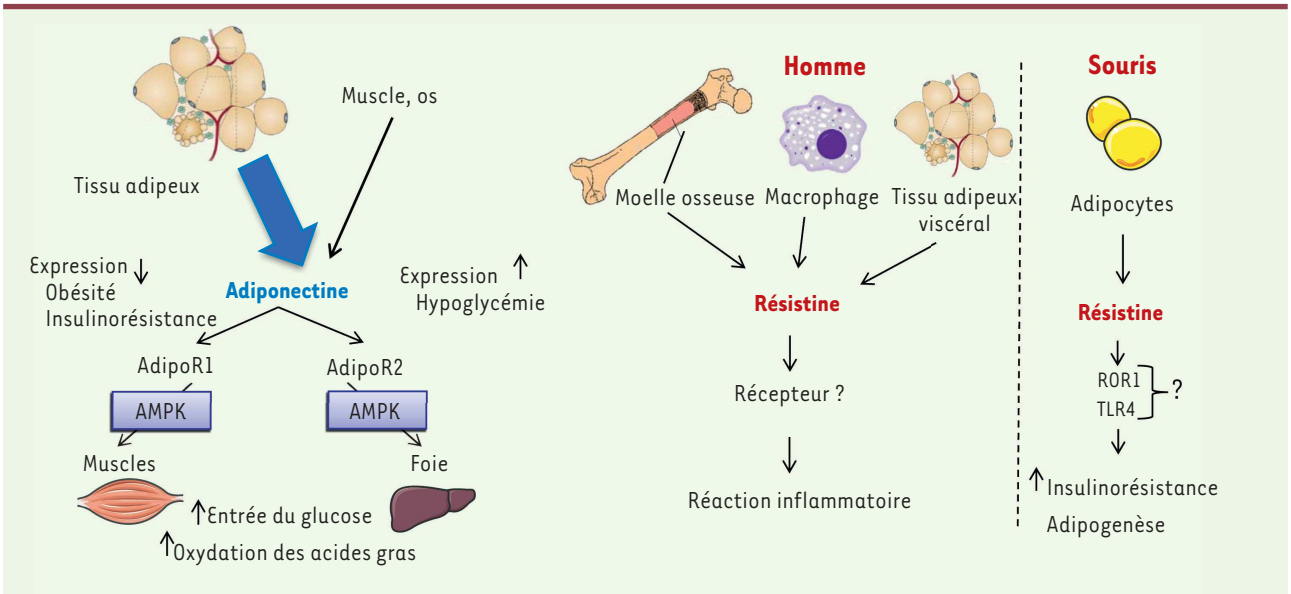

Figure 1. ***Lieu de production, exemples de cibles et rôles de l'adiponectine et de la résistine dans les tissus périphériques.*** L'adiponectine majoritairement produite par le tissu adipeux est aussi trouvée dans d'autres organes comme le muscle et l'os. L'adiponectine présente des propriétés de sensibilisation à l'insuline, et sa concentration est diminuée en cas d'obésité souvent associée à une insulinorésistance. Elle agit *via* ses récepteurs AdipoR1 et AdipoR2 et améliore l'entrée cellulaire du glucose et l'oxydation des acides gras au niveau des muscles et du foie. La résistine est majoritairement produite par les macrophages et la moelle osseuse chez l'homme, et les adipocytes chez la souris. Chez cette dernière, elle est fortement impliquée dans les phénomènes d'insulinorésistance et d'adipogenèse, alors que chez l'homme son rôle est moins clair. ROR1 : *receptor tyrosine kinase-like orphan receptor 1* ; TLR4 : *Toll-like receptor 4*.

sont connues à ce jour quant au mode d'action de la résistine. Aucun récepteur n'a été clairement identifié, et la voie de signalisation utilisée demeure tout aussi obscure. Des études récentes suggèrent que la résistine pourrait se lier au récepteur tyrosine kinase ROR1 (*receptor tyrosine kinase-like orphan receptor 1*) dans la lignée murine de préadipocytes 3T3-L1 [8], ou encore au récepteur TLR4 (*Toll-like receptor 4*) dans l'hypothalamus de souris [9].

Dans cette synthèse, nous rapporterons brièvement les rôles connus des adipocytokines adiponectine et résistine dans le métabolisme énergétique et la reproduction chez la femelle et le mâle. Puis nous décrirons le lien qu'elles pourraient établir entre ces deux fonctions, ainsi que leur possible implication dans le syndrome des ovaires polykystiques (SOPK) chez la femme et les infertilités liées à l'obésité.

Rôle de l'adiponectine et de la résistine dans la régulation du métabolisme énergétique

L'adiponectine limite la prise de poids chez des rongeurs recevant un régime riche en graisses et en sucres, et il existe une relation inverse entre la résistance à l'insuline et les concentrations plasmatiques d'adiponectine [10]. La suppression de l'expression d'AdipoR1 et d'AdipoR2 s'accompagne d'une diminution de l'activité de PPARα (*peroxisome proliferator-activated receptor* α), de l'oxydation des acides gras et de l'utilisation du glucose [11]. De plus, Civitarese *et al.* [12] ont observé que l'expression des deux récepteurs est diminuée chez les sujets ayant des antécédents familiaux de diabète de type 2. Ces données confèrent ainsi à l'adiponectine un rôle protecteur contre le développement du diabète de type 2. En fait, l'adiponectine peut être considérée comme un agent antidiabétique qui augmente la sensibilité à l'insuline en activant l'AMPK. Dans le foie, l'adiponectine augmente la phosphorylation du récepteur à l'insuline, l'oxydation des acides gras, et diminue la néoglucogenèse [13, 14]. Dans le muscle, l'activation de l'AMPK par l'adiponectine conduit à l'inactivation de l'acétylCoA, ce qui augmente l'oxydation des acides gras [14, 15]. Dans ce tissu, l'adiponectine favorise également l'utilisation du glucose en facilitant la translocation des transporteurs GLUT4 sensibles à l'insuline [16].

Chez l'homme, le rôle de la résistine dans le métabolisme est moins connu que celui de l'adiponectine. Chez la souris obèse, la concentration sérique de résistine est augmentée [17]. Chez ces animaux, l'administration d'un anticorps antirésistine corrige le taux de glucose circulant et améliore la sensibilité à l'insuline, mais l'administration de résistine recombinante n'entraîne qu'une intolérance au glucose. Chez l'homme, l'hypothèse selon laquelle la résistine serait une hormone principalement inductrice d'insulinorésistance reste à confirmer.

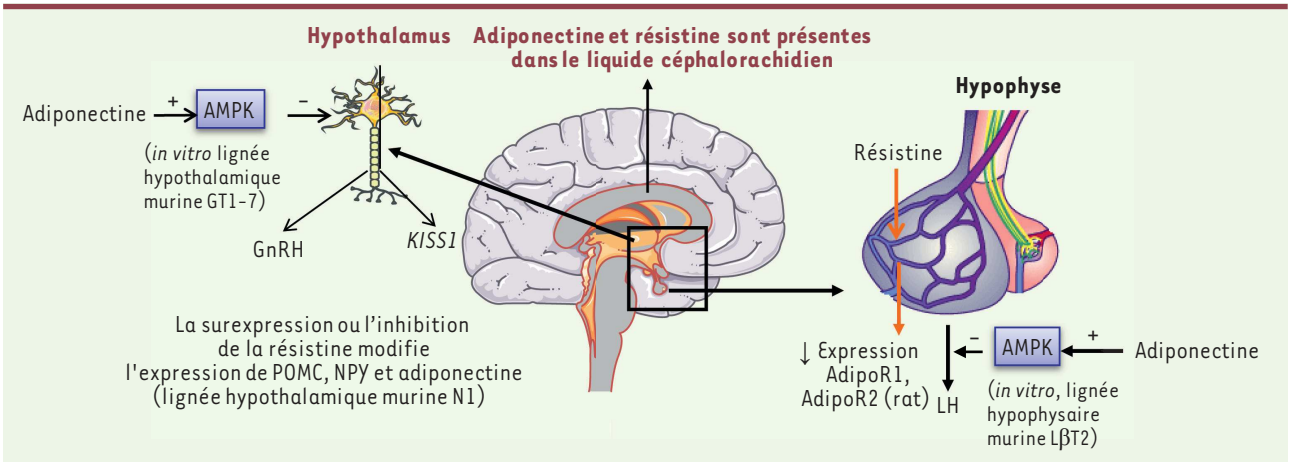

Figure 2. Schéma résumant les effets de l'adiponectine et de la résistine au niveau de l'axe hypothalamo-hypophysaire. L'adiponectine et la résistine sont présentes dans le liquide céphalorachidien, ainsi qu'au niveau des cellules hypothalamiques et hypophysaires où sont aussi exprimés les récepteurs AdipoR1 et AdipoR2. L'adiponectine réduit, *via* l'AMPK, les secrétions hypothalamiques de GnRH et la transcription du gène *KISS1 in vitro* dans les cellules GT1-7. Dans la lignée hypothalamique murine N1, une surexpression ou une inhibition de la résistine modifie l'expression des neuropeptides POMC et NPY, et aussi de l'adiponectine. Enfin, la résistine pourrait diminuer l'expression des récepteurs de l'adiponectine dans les cellules hypophysaires de rat. LH : *luteinizing hormone* ; NPY : neuropeptide Y ; AMPK : *adenosine monophosphate activated kinase* ; POMC : proopiomélanocortine ; GnRH : *gonadotropin releasing hormone*.

Rôle de l'adiponectine et de la résistine dans les fonctions centrales de la reproduction

Pour contrôler les fonctions de la reproduction, l'adiponectine et la résistine pourraient également agir sur l'axe hypothalamo-hypophysaire (*Figure 2*). En effet, l'adiponectine, AdipoR1 et AdipoR2 et la résistine sont présentes dans l'hypothalamus et l'hypophyse chez l'homme et les rongeurs (pour revue voir [18]). Des études *in vitro* montrent que l'adiponectine, en activant l'AMPK, réduit la sécrétion *in vitro* de GnRH (*gonadotropin releasing hormone*) par les cellules hypothalamiques GT1-7. Ces effets pourraient s'expliquer par une inhibition de l'expression du gène *KISS1* qui joue un rôle central dans la régulation de l'axe gonadotrope [19] (→). De plus, l'adiponectine inhibe également la libération de LH (*luteinizing hormone*) qu'elle soit basale ou induite par le GnRH dans la lignée murine gonadotrope LβT2 [20]. Au niveau de l'hypophyse, l'expression de la résistine est faible à la naissance chez la rate et augmente jusqu'à l'âge de 28 jours. Elle augmente au sevrage chez la femelle et à la puberté chez le mâle. De plus, sa concentration hypophysaire est deux à trois fois plus importante chez les mâles que chez les femelles. D'autre part, la résistine diminue *in vitro* l'expression des récepteurs AdipoR1 et AdipoR2 dans les cellules hypophysaires de rat en culture [21]. Le rôle de la résistine au niveau hypothalamique et hypophysaire reste à déterminer.

(→) Voir *m/s* n° 3, vol. 29, mars 2013, page 242

Rôle de l'adiponectine et de la résistine au niveau des gonades

L'ovaire

L'adiponectine est, avec la leptine, l'adipocytokine qui a été la plus étudiée au niveau des cellules ovariennes. Dans l'ovaire (*Figure 3*), l'adiponectine a été identifiée dans le liquide folliculaire (femme et truie) [22, 23]. Elle est exprimée dans différents compartiments : l'ovocyte (rate) [24], le corps jaune [24], les cellules de la thèque (poule et rate) [24, 25], et très faiblement dans les cellules de la granulosa de poule (à tous les stades du développement folliculaire) et de rate. Les récepteurs AdipoR1 et AdipoR2 ont été identifiés dans différents types cellulaires du follicule (ovocyte, *cumulus oophorus*, cellules de la granulosa et de la thèque) et dans le corps jaune chez différentes espèces (rate, vache, truie, poisson, poule) y compris chez la femme. Le rôle de l'adiponectine a été étudié *in vitro* dans la stéroïdogenèse des cellules de la granulosa et de thèque et dans la maturation ovocytaire chez plusieurs espèces. Chez la rate et la femme, l'adiponectine augmente la sécrétion de la progestérone et celle de l'œstradiol en réponse à l'IGF-1 (*insulin like growth factor-1*) [23]. Chez la rate, cette augmentation s'explique par une augmentation de la signalisation du récepteur de l'IGF-1, et, chez la femme, par une augmentation de l'expression de l'enzyme aromatase responsable de la biosynthèse d'œstrogènes. Dans la lignée de granulosa humaine KGN, l'inactivation spécifique d'AdipoR1 et d'AdipoR2 montre qu'AdipoR1 est impliqué dans la survie cellulaire, alors qu'AdipoR2 est préférentiellement impliqué dans la stéroïdogenèse [26]. En 2008, Gutman *et al.* [27] ont montré une augmentation de la concentration de l'adiponectine dans le liquide folliculaire en réponse aux gonadotropines ovariennes après administration de LH recombinante. Chez la vache, l'adiponectine diminue *in vitro* la

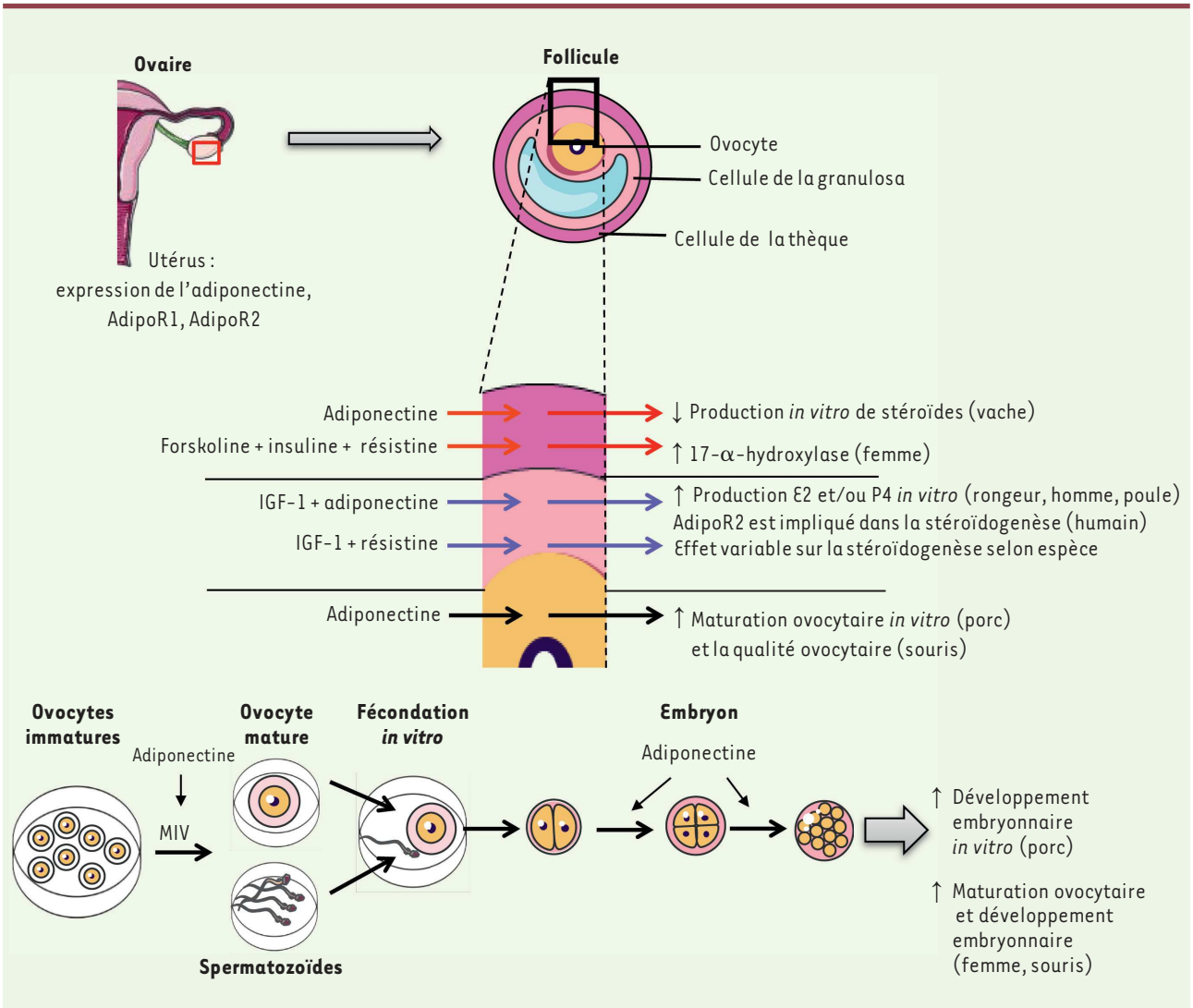

Figure 3. ***Schéma illustrant les effets de l'adiponectine et de la résistine au niveau de l'ovaire.*** Au niveau ovarien, l'adiponectine et ses récepteurs ont été identifiés dans différent types cellulaires, tels que l'ovocyte, les cellules de la granulosa et les cellules de la thèque. Le système adiponectine est aussi présent au niveau de l'utérus. Au niveau des cellules de la thèque, il a été montré que, chez la vache, l'adiponectine diminue la sécrétion des stéroïdes. Chez la femme, la résistine, en présence ou non de forskoline et d'insuline, augmente l'activité de la 17-α-hydroxylase. D'autre part, il a été montré dans des études *in vitro* sur des cellules de la granulosa que l'adiponectine augmente, en réponse à l'IGF-1, la sécrétion de stéroïdes chez la poule, la rate et la femme. Quant à la résistine, elle exerce, en réponse à l'IGF-1, des effets variables sur la sécrétion de stéroïdes suivant les espèces. Enfin, l'adiponectine améliore la maturation ovocytaire (femme, souris), ainsi que le développement embryonnaire (femme, souris, porc). E2 : œstradiol ; P4 : progestérone; IGF-1 : *insulin like growth factor* ; MIV maturation *in vitro*.

production des stéroïdes par les cellules de la thèque en réduisant l'expression des récepteurs de la LH et des enzymes CYP11a1 (cytochrome P450, famille 11, sous-famille a, polypeptide 1) et CYP17a1 (cytochrome P450, famille 17, sous-famille a, polypeptide 1) [28]. Dans les cellules de granulosa de porc cultivées *in vitro*, l'adiponectine augmente l'expression de molécules impliquées dans l'ovulation, telles que Cox2 (cyclooxygénase 2), PGE2 (prostaglandine E2) et EGF (*epidermal growth factor*) [22]. Lors de protocoles de fécondation *in vitro* chez la femme, la souris et le porc, l'adiponectine améliore la maturation ovocytaire et le développement de l'embryon [29, 30]. Chez cette espèce, plusieurs polymorphismes des gènes codant pour l'adiponectine et ses deux récepteurs ont été identifiés. Certains variants et haplotypes identifiés sont associés à des tailles de portées plus grandes, un plus petit nombre de porcelets mort-nés et momifiés, et un intervalle sevrage-œstrus plus court [31].

La résistine est présente dans le liquide folliculaire, l'ovocyte, les cellules de la thèque et le corps jaune chez la rate et la vache [32]. Contrairement à la rate, elle est retrouvée dans les cellules de la granulosa

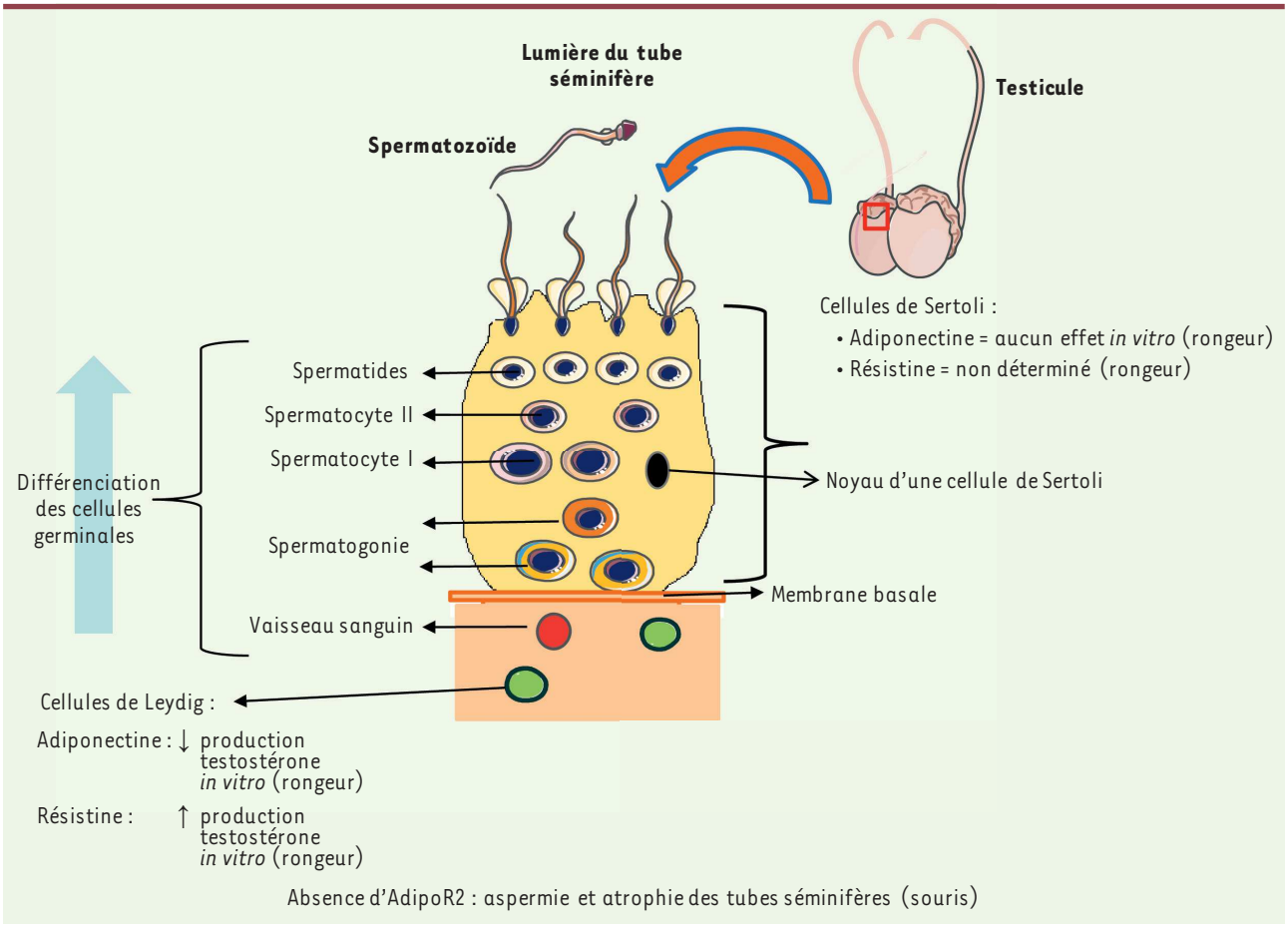

Figure 4. ***Schéma illustrant les effets de l'adiponectine et de la résistine au niveau du testicule.*** L'adiponectine, l'AdipoR1, l'AdipoR2 et la résistine sont retrouvés au niveau des cellules testiculaires. L'adiponectine est localisée au niveau de cellules de Leydig, alors que ces récepteurs sont présents dans les tubes séminifères. L'adiponectine diminue la production de testostérone *in vitro*. L'absence d'AdipoR2 chez la souris conduit à une aspermie et une atrophie des tubes séminifères. La résistine est détectée dans les cellules de Leydig et à l'intérieur des tubes séminifères dans les cellules de Sertoli. Son rôle reste encore à déterminer.

chez la femme et la vache. Dans les cellules de thèques humaines en culture, la résistine augmente l'expression de la 17-α-hydroxylase en présence de forskoline et/ou de forskoline + insuline, suggérant un rôle de la résistine dans la production d'androgènes [33]. Chez la vache et la rate, la résistine module différemment la steroïdogenèse et la prolifération des cellules de la granulosa en réponse à l'IGF-1, suggérant un effet dépendant de l'espèce [32].

Le testicule

Même si la plupart des études sur les adipocytokines se sont principalement focalisées sur l'ovaire, des travaux montrent la présence de l'adiponectine, d'AdipoR1, d'AdipoR2 et de la résistine au niveau des cellules testiculaires. Dans le testicule de rat, l'adiponectine est surtout présente dans les cellules interstitielles de Leydig, alors que ses récepteurs (et principalement AdipoR1) sont présents dans les tubes séminifères [34]. L'adiponectine diminue *in vitro* la production de testostérone en présence ou non d'hCG (hormone chorionique gonadotrope), alors qu'elle n'a aucun effet sur l'expression des gènes codant pour l'AMH (hormone anti-mullérienne) et le SCF (*stem cell factor*), spécifiques de la cellule de Sertoli [34]. Chez la souris, la déficience en récepteur AdipoR2 conduit à une atrophie des tubes séminifères et à une aspermie sans modification de la concentration de testostérone [35]. Chez le poulet, des travaux montrent une augmentation des récepteurs de l'adiponectine au cours de la maturation sexuelle, et suggèrent un rôle de l'adiponectine dans la stéroïdogenèse, la spermatogenèse, la fonction des cellules de Sertoli et la motilité des spermatozoïdes [36]. Dans le testicule de rat, la résistine est localisée dans les cellules de Leydig et les cellules de Sertoli à l'intérieur des tubes séminifères. Son expression est régulée par le jeûne, les gonadotrophines et la leptine. De plus, elle varie au cours du développement postnatal atteignant un maximum à l'âge adulte [37]. Ainsi, l'adiponectine et la résistine produites localement ou par voie endocrine sont des

senseurs énergétiques capables de réguler les fonctions gonadiques femelles et mâles *(Figures 3* et *4)*.

Rôle de l'adiponectine et de la résistine au cours de l'implantation de l'embryon et de la formation du placenta

Implantation de l'embryon

L'adiponectine et ses récepteurs sont exprimés dans l'utérus de différentes espèces (lapin, porc, homme et rongeurs). Chez la femme, une forte expression des récepteurs a été observée dans l'endomètre pendant la période de l'implantation de l'embryon. Une étude récente indique que l'expression d'AdipoR1 et AdipoR2 est diminuée (60 %) dans l'endomètre des femmes en échec d'implantation après une procédure de FIV (fécondation *in vitro*) pour infertilité comparée à l'expression dans l'endomètre de femmes fertiles [38]. D'autre part, les taux plasmatiques d'adiponectine sont fortement diminués chez des patientes atteintes de diabète gestationnel, du syndrome des ovaires polykystiques (SOPK), mais aussi d'endométriose et de cancer de l'endomètre (voir [18]). Ces études suggèrent qu'une modification de l'expression de l'adiponectine et/ou de ses récepteurs pourrait être impliquée dans la réceptivité endométriale [38, 39]. De plus, une dérégulation de l'expression de ce système pourrait survenir dans certaines conditions pathologiques associées à des fausses couches ou à des pathologies de l'implantation [39]. Contrairement à l'adiponectine, peu de données sont disponibles à ce jour sur un rôle potentiel de la résistine lors de l'implantation de l'embryon.

Placenta

L'adiponectine, AdipoR1 et AdipoR2, ainsi que la résistine ont été identifiés dans le placenta chez la femme et la rate [40]. Dans le placenta de femme, la résistine et l'adiponectine sont principalement exprimées par les cellules trophoblastiques. La résistine est aussi présente dans la membrane amniotique. Dans le placenta de rate, l'expression de l'ARN messager de l'adiponectine augmente au cours de la gestation puis diminue lors d'une restriction alimentaire. En revanche, celle d'AdipoR2 diminue et n'est pas affectée par la restriction alimentaire [40]. L'adiponectine diminue la prolifération *in vitro* des lignées trophoblastiques JEG-3 et BeWo [41], stimule la différenciation du trophoblaste villeux en syncytiotrophoblaste et favorise la sécrétion d'hormones placentaires, de l'hCG (hormone chorionique gonadotrope), et de leptine. Le profil d'expression de la résistine placentaire n'est pas encore connu. Cependant, une étude montre que la résistine est capable de moduler le transport du glucose dans les cellules trophoblastiques humaines.

Adiponectine et résistine : quelle implication dans les infertilités liées à l'obésité, à l'insulinorésistance et au syndrome des ovaires polykystiques ?

De nombreuses études épidémiologiques sur de grandes cohortes de femmes enceintes ont parfaitement démontré le lien entre l'indice de masse corporelle au moment de la conception et les chances de grossesses. Le risque de mettre plus d'un an à concevoir est augmenté de 27 % en cas de surpoids et de 78 % en cas d'obésité [42]. Les patientes obèses, enceintes après un traitement d'infertilité, ont un taux significativement plus élevé de fausses couches spontanées, taux qui est corrélé à l'importance de l'obésité. L'obésité peut affecter le tissu reproducteur à différents niveaux chez la femme : ovocyte, embryon, placenta et environnement utérin. Chez l'homme, une étude récente réalisée en France chez près de 2 000 individus, présentée à l'*European society of human reproduction* (ESHRE) en juillet 2011, indique que le surpoids entraîne une modification des paramètres du sperme, du fait probablement de désordres hormonaux ; les déficits en nombre, en mobilité et en vitalité des spermatozoïdes qui sont observés entraînent une réduction de la fécondité [43]. Il est difficile, pour l'instant, de décrire les mécanismes par lesquels l'obésité ou le surpoids affecte la qualité des gamètes mâles et femelles. Il peut s'agir de l'altération des concentrations plasmatiques des hormones de la reproduction et du métabolisme. En effet, le tissu adipeux est un site de production d'hormones stéroïdiennes, mais aussi d'adipocytokines dont les profils d'expression tissulaire et/ou plasmatique sont modifiés par l'obésité. L'excès de graisse favorise le métabolisme des œstrogènes en deux types d'hydroxyœstrogènes moins actifs, le stockage des hormones stéroïdes dans le tissu adipeux et modifie l'insulinosensibilité et la sécrétion de la SHBG (*sex hormone-binding globulin*), une protéine de liaison aux hormones sexuelles (testostérone et œstradiol) (pour revue voir [44]). Comme nous l'avons décrit ci-dessus, l'adiponectine et ses récepteurs, et la résistine sont exprimés par les tissus reproducteurs. Ainsi, ces hormones pourraient agir localement par voie paracrine ou autocrine, ou par voie endocrine sur les cellules de l'axe gonadotrope. Une obésité est souvent observée chez les patientes atteintes du SOPK (syndrome des ovaires polykystiques). Ce syndrome comporte à la fois des troubles de la fertilité et des troubles du métabolisme. Les patientes, 5-10 % des femmes en âge de procréer, présentent une hyperandrogénie, leurs cycles sont irréguliers (témoin de l'oligo-anovulation) et leurs ovaires micropolykystiques à l'échographie. Dans le SOPK, la concentration de l'adiponectine est diminuée indépendamment de l'indice de masse corporelle, mais est associée à l'obésité abdominale et à l'hyperandrogénie [45]. L'adiponectinémie est également diminuée chez un modèle murin de SOPK [46]. De plus, dans ce syndrome, un traitement par la metformine, antidiabétique de la famille des biguanides qui améliore la sensibilité à l'insuline et active l'AMPK, augmente dans

certaines études le taux d'ovulation [47], et aussi l'adiponectinémie [48]. Quelques travaux ont également relié les taux plasmatiques de la résistine avec le SOPK. Ainsi, l'adiponectine et la résistine, comme d'autres adipocytokines, pourraient être un lien physiopathologique entre les anomalies métaboliques (insulinorésistance et obésité) et les troubles de la fertilité constatés chez les patientes souffrant d'un SOPK.

Conclusions et perspectives

Plusieurs travaux ont montré la présence de l'adiponectine, de ses récepteurs et de la résistine au niveau de l'axe gonadotrope. Une hypothétique régulation centrale de la fonction de reproduction par ces deux adipocytokines semble possible, mais reste à explorer. Au niveau des gonades, l'adiponectine et la résistine régulent *in vitro* la stéroïdogenèse, ainsi que la maturation ovocytaire et le développement embryonnaire. Chez la femme, l'adiponectine pourrait jouer un rôle dans le développement embryonnaire pré-implantatoire. D'autre part, l'adiponectine émerge comme un nouveau régulateur positif de la fonction trophoblastique qui favorise la formation d'un placenta fonctionnel. Ainsi, comme dans le cas du syndrome métabolique, l'adiponectine ou ses analogues (adiponectine recombinante, agoniste des récepteurs de l'adiponectine) pourraient être utilisés dans le traitement de certaines infertilités. Enfin, il convient de s'interroger sur le rôle des autres adipocytokines (visfatine, chémérine, omentine, etc.) dans les pathologies du système reproducteur liées à l'insulinorésistance et à l'obésité. ◊

SUMMARY

Adiponectin and resistin: a role in the reproductive functions?

Adipokines are hormones mainly produced by the white adipose tissue, an endocrine organ involved in energy homeostasis. They play an important role in the regulation of lipid and glucose metabolisms, in inflammation and immune disorders. New roles for adipokines have recently emerged in the field of fertility and reproduction. Indeed, adipokines such as adiponectin and resistin are able to regulate the functions of male and female gonads and of the hypothalamic-pituitary axis. For example, they modulate steroidogenesis of gonadic somatic cells, germ cell maturation and secretion of gonadotrope hormones in various species. The reproductive system is tightly coupled with energy balance, and thereby metabolic abnormalities can lead to the development of physiopathological situations such as the polycystic ovary syndrome (PCOS). Obesity and overweight are significantly involved in the declining natural fertility and decrease the effectiveness of treatments. Women with obesity and/or PCOS have abnormal plasma adiponectin and resistin profiles. Thus, these adipokines could be a link between reproduction and energy metabolism and could partly explain some infertility related to obesity or PCOS. ◊

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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TIRÉS À PART

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Review Article

Adipokines and the Female Reproductive Tract

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It is well known that adipose tissue can influence puberty, sexual maturation, and fertility in different species. Adipose tissue secretes molecules called adipokines which most likely have an endocrine effect on reproductive function. It has been revealed over the last few years that adipokines are functionally implicated at all levels of the reproductive axis including the gonad and hypothalamic-pituitary axis. Many studies have shown the presence and the role of the adipokines and their receptors in the female reproductive tract of different species. These adipokines regulate ovarian steroidogenesis, oocyte maturation, and embryo development. They are also present in the uterus and placenta where they could create a favorable environment for embryonic implantation and play a key role in maternal-fetal metabolism communication and gestation. Reproductive functions are strongly dependent on energy balance, and thereby metabolic abnormalities can lead to the development of some pathophysiologies such as polycystic ovary syndrome (PCOS). Adipokines could be a link between reproduction and energy metabolism and could partly explain some infertility related to obesity or PCOS.

1. Introduction

It is now recognized that the white adipose tissue is a multifunctional organ. In addition to its key role of lipid storage, it has a crucial endocrine function secreting many hormones called adipokines [1]. These molecules are cytokines produced in the main by adipocytes or adipose stromal cells. Adipokines are implicated in adipocyte differentiation, energy metabolism, insulin resistance, inflammation, immunity, cancer, and angiogenesis [2–5]. It is well known that an excess or deficiency of white adipose tissue affects puberty, sexual maturation, and fertility in different species [6]. Furthermore, variations of white adipose tissue quantities modulate the expression level and serum concentrations of adipokines.

Obesity and excess weight are significantly involved in the decline in the natural fertility of mammals. New roles of adipokines have recently emerged in the field of fertility and reproduction [7]. Indeed, adipokines such as leptin, adiponectin, and resistin are able to regulate the functions of gonads and the hypothalamic-pituitary axis [8, 9]. Furthermore, the reproductive tract is tightly coupled with energy balance, and thereby metabolic abnormalities can lead to the development of some pathophysiologies such as polycystic ovary syndrome (PCOS) [10]. PCOS is the commonest endocrine disorder in women, affecting 5–10% of females of reproductive age. In this review, we focus on the localization and the role of some adipokines (in particular, adiponectin, resistin, visfatin, and chemerin) and their receptors on the female reproductive tract including ovary, placenta, and uterus. Finally, we will discuss their potential as actors involved in PCOS.

1.1. Adiponectin. Adiponectin was discovered in 1995 after leptin. It is a protein of 244 amino acids (30 kDa) produced mainly by white adipose tissue but is also found in other

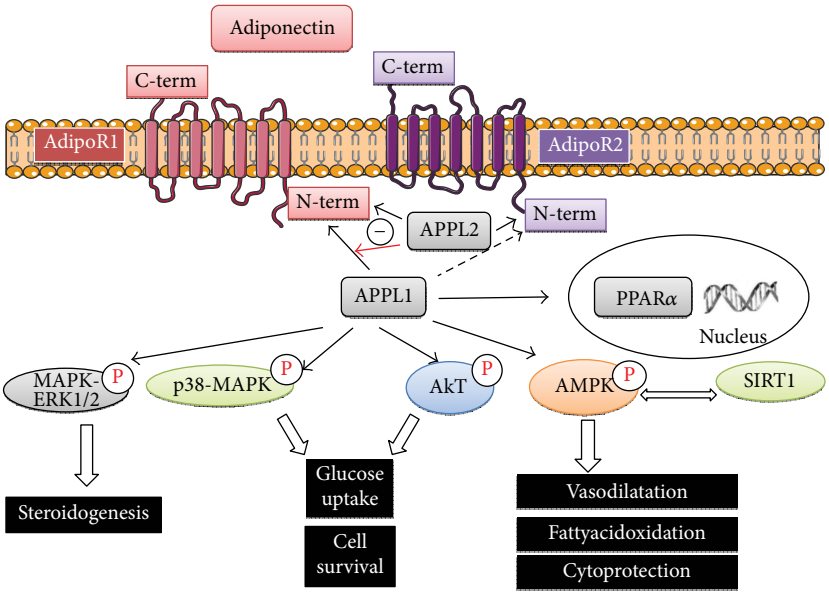

Figure 1: Signalling transduction via adiponectin receptors (AdipoR1 and AdipoR2) activation. The binding of the different forms of adiponectin to the two known adiponectin receptors, AdipoR1 and AdipoR2, can lead to stimulation of various signaling pathways. Indeed, interacting directly with the N-terminal of at least AdipoR1 and possibly AdipoR2, APPL1 elicits signalling through not only PPARα, AMPK, and AMPK/SIRT1 but also p38-MAPK, ERK1/2-MAPK, and Akt. APPL2 binds to AdipoR1 and AdipoR2. Unlike APPL1, APPL2 inhibits AdipoR1 dependent signaling. According to the tissue, activation of both receptors results in modulation of different biological effects such as steroidogenesis, glucose uptake, cell survival, fatty acid oxidation, vasodilatation, and cytoprotection. APPL1/2: adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1; PPARα: peroxisome proliferator-activated receptor α; SIRT1: sirtuin 1 (a NAD-dependent deacetylase); AMPK: 5′ adenosine monophosphate-activated protein kinase; MAPK: Mitogen-activated protein kinase; ERK1/2: extracellular signal-regulated kinases 1/2 (−: inhibition).

tissues such as muscle and bone [11, 12]. Adiponectin is also known as Acpr30 (adipocyte complement-related protein 30 kDa), apM1 (adipose most abundant gene transcript-1), or GBP 28 (gelatin-binding protein) [13]. Although adiponectin is secreted mainly by the adipose tissue, more recent studies have indicated that it is more widely expressed in various species [14, 15]. In humans, the expression level of adiponectin mRNA varies depending on its location since expression is lower in visceral adipose tissue as opposed to subcutaneous adipose tissue [16]. Unlike other adipocyte-derived hormones, adiponectin gene expression and blood concentrations are inversely associated with body mass index [17]. In serum, adiponectin assembles into several oligomeric multimers including trimers, known as low molecular weight (LMW); hexamers, known as medium molecular weight (MMW) and higher molecular weight (HMW) multimeric complexes [18]. HMW are considered the most biologically active isoforms [13, 18]. Adiponectin structurally belongs to the complement 1q family and is found at high concentrations (>0.01% of the total protein) in serum of healthy individuals [13]. It is well known for its effect in improving insulin sensitivity [19] and regulating various processes including lipid synthesis, energy homeostasis, vasodilatation, and atherogenic activity [13, 19]. Adiponectin acts mainly through two G-coupled receptors named AdipoR1 and AdipoR2 (Figure 1). Interestingly the intracellular/extracellular orientation of the N-terminus and C-terminus is the opposite of classical G-coupled receptors. In addition, T-cadherin has also been reported to serve as a receptor for high-order multimers of adiponectin [20]. In HEK293 cells, transcriptional down regulation of T-cadherin largely improves adiponectin-mediated ERK1/2 activation suggesting that T-cadherin either competes with AdipoR1/R2 for adiponectin binding or interferes with the coupling of adiponectin-bound AdipoR1/R2 to downstream effectors [21]. However, a more detailed analysis of the adiponectin/T-cadherin function remains to be determined.

Downstream of AdipoR1 and AdipoR2: the biological effects of adiponectin are mediated by different signaling pathways involving the following molecules: AMPK, PPARγ ERK, AKT, and P38 [13, 22]. Besides these signaling pathways, APPL1 and APPL2 [23], Ca2+, and SIRT1 [24] are emerging downstream effectors of the AdipoRs [25]. AdipoR signaling can be modulated by the interaction with two adaptor proteins: adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1 (APPL1 and APPL2) (Figure 1). Following adiponectin-AdipoR1 binding, APPL1 mediates a number of downstream signaling events associated with adiponectin function [26]. When the receptor is inactive APPL2 binds and inhibits APPL1 function, but APPL2 binding is displaced upon activation of AdipoR1 [27]. Thus, adiponectin, which is an abundant circulating protein synthesized mainly in adipose tissue, appears to be a major modulator of insulin action.

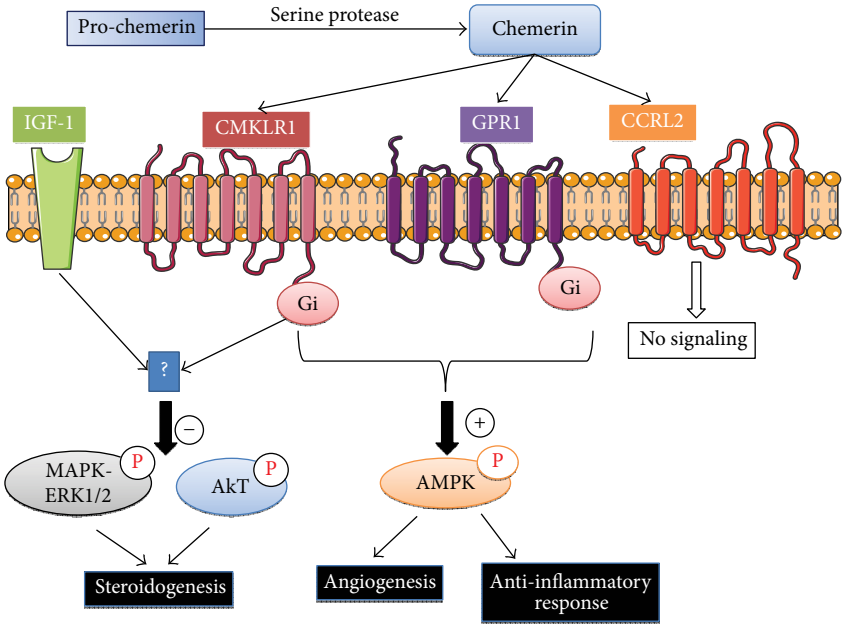

Figure 2: Chemerin receptors, CMKLR1, GPR1, and CCRL2 signaling pathways. Chemerin is able to bind three different G protein-coupled receptors: CMKLR1 (chemokine-like receptor 1), GPR1 (G protein-coupled receptor 1), and CCRL2 (Chemokine (CC motif) receptor-like 2). This latter receptor does not appear to be a signaling receptor. Once activated, CMKLR1 and GPR1 stimulate or inhibit different signaling pathways including MAPK ERK1/2, Akt, and AMPK to regulate different biological processes such as angiogenesis, inflammation, and steroidogenesis. In particular, our group showed that chemerin decreases IGF-1-induced steroid production through MAPK ERK1/2 phosphorylation in human granulosa cells (−: inhibition, +: stimulation).

1.2. Resistin. Resistin was identified in 2001 by Steppan et al. [28]. It is a circulating cysteine rich protein of 12 kDa that belongs to the family of "resistin-like molecules" or "FIZZ" (found in inflammatory zone) [29]. It consists of homodimers connected by disulfide bridges. Resistin is strongly involved in insulin resistance and obesity in rodents but its role in humans is still unclear. In humans, resistin is mainly produced by monocytes and macrophages and less in pancreatic β-cells [30] in lung [31], and placental tissue [32], whereas in rodents it is more expressed in adipose tissue [33]. Resistin injection in rodent causes insulin resistance whereas with antibodies against resistin increased the insulin sensitivity in obese mice [28]. These data suggest that resistin induced insulin resistance and an increase in resistin circulating level contributes to decreased insulin sensitivity in obesity. However, in healthy humans, resistin gene expression is very low. Therefore the involvement of resistin in insulin resistance needs to be confirmed. Furthermore, after much research on resistin's mechanism of action its receptor and the signaling pathway involved are still unknown. Recently, Sánchez-Solana et al. suggest that resistin can bind the receptor tyrosine-kinase-like orphan receptor (ROR1) in murine pre 3T3-L1 adipocytes [34]. Benomar et al. suggest also that resistin can bind the Toll-like receptor 4 (TLR4) in the mouse hypothalamus [35]. Thus, in rodents, resistin has opposite effects on the regulation of insulin sensitivity as compared to adiponectin.

1.3. Visfatin. Visfatin also known as PBEF (pre-B-cell colony enhancing factor) or Nampt (nicotinamide phosphoribosyltransferase) was discovered in 2005 by Fukuhara et al. [36]. This protein was initially characterized as a growth factor for early-stage B cells [37] and is a 52 kDa protein of 491 amino acids expressed in several tissues including muscle, bone marrow, liver, lymphocytes, and fetal membranes [36] but predominantly in visceral adipose tissue [38]. Fukuhara et al. showed a correlation between visfatin levels in mice and humans and the proportion to the amount of visceral fat [36]. Visfatin presents insulin-mimetic effects, stimulates glucose uptake in adipocytes and muscle cells, and suppresses glucose release from hepatocytes [36, 39]. Visfatin is a rate limiting enzyme involved in NAD biosynthesis from nicotinamide. Like for resistin, visfatin's receptor is still unknown and also the signaling pathway involved. Fukuhara et al. suggested that visfatin can bind to the insulin receptor but at a different site. However this suggestion was later retracted [36].

1.4. Chemerin. Chemerin or RARRES2 (retinoic acid responder protein 2) or TIG2 (tazarotene induced gene 2 protein) is a new adipokine of 163 amino acids and a molecular weight of 14 kDa [40]. Chemerin is secreted as a precursor named prochemerin that is cleaved at the C-terminus by a serine protease to become active [40]. Chemerin can bind three G-coupled receptors: CMKLR1, GPR1, and CCRL2 (Figure 2). CMKLR1 (chemokine-like receptor 1) is predominantly expressed by plasmacytoid dendritic cells, monocytes/macrophages, and natural killer [41] whereas GPR1 is mainly expressed in the liver, intestine, kidney, and adipose tissue [42]. CCRL2 is another GPCR ((chemokine (C-C motif)) that presents high expression in lung endothelial

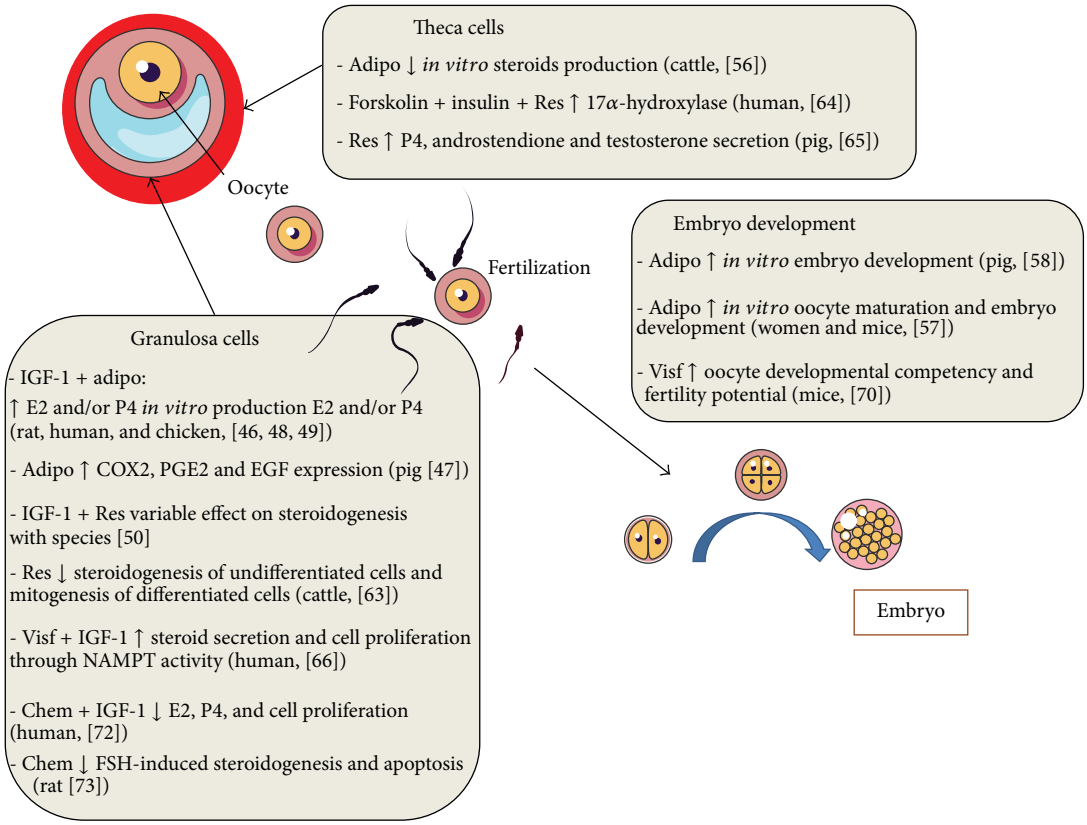

Figure 3: Adiponectin, resistin, visfatin, and chemerin effects on ovarian cells in various species. Adipo: adiponectin, Res: resistin, Visf: visfatin, Chem: chemerin, E2: oestradiol, P4: progesterone, and IGF-1: insulin-like growth factor-1.

cells and less in liver endothelium [43, 44] but it does not induce chemerin signaling [45]. It is suggested that CCRL2 can regulate the bioavailability of chemerin to other chemerin receptors [44].

2. Adipokines and Ovary (Figure 3)

2.1. Adiponectin. In the ovary, adiponectin has been identified in follicular fluid (woman and sow [46, 47]) and is expressed in different compartments such as the oocyte (rat [48]), the corpus luteum (rat [48]), and the theca cells (chicken [49] and rat [48]). Interestingly, adiponectin was almost undetectable in rat, chicken, and human granulosa cells [46, 48, 49]. AdipoR1 and AdipoR2 receptors have been identified in different cell types of the follicle (oocyte cumulus, granulosa cells, and theca cells) in different species (rat, cow, pig, fish, and chicken) including women [46–51]. However their expression level differs; mRNA AdipoR1 level was higher than that of AdipoR2 in granulosa and theca cells from large follicles, whereas an opposite expression pattern was observed in oocytes from large bovine follicles [52]. In chicken ovarian adiponectin, AdipoR1 and AdipoR2 were expressed in whole ovary [48]. In this species adiponectin mRNA expression was twofold lower in F4 theca cells than in F1 theca cells and opposite results were obtained in granulosa cells. And the expression of adiponectin in theca cells was 10- to 30-fold higher than in granulosa cells [48]. These results show that the expression of adiponectin and its receptors is differentially modulated with cell type and cell maturity.

The role of adiponectin has been studied *in vitro* on steroidogenesis of granulosa and theca cells and oocyte maturation in several species. In primary rat and human granulosa cells, adiponectin increases progesterone and estradiol secretions in response to IGF-1 (insulin-like growth factor 1) [46]. In rats, this increase is due to an activation of IGF-1 receptor signaling and an increase in the protein expression of aromatase [49]. In the human granulosa cell line, KGN, the specific AdipoR1 and AdipoR2 inactivations showed that AdipoR1 is involved in cell survival whereas AdipoR2 is preferentially involved in steroidogenesis [53]. In 2008, Gutman et al. have shown an increase in the concentration of adiponectin in follicular fluid in response to gonadotropin in human ovarian after treatment with recombinant LH (luteinizing hormone) [54]. In cultured bovine theca cells, adiponectin suppresses *in vitro* androstenedione production and gene expression of the LH receptor and key enzymes in the androgen synthesis pathway (CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) and CYP17A1 (cytochrome P450, family 17, subfamily A, polypeptide 1)) [55, 56]. Moreover in these cells, knockdown of genes for AdipoR1 and AdipoR2 was associated with increased androstenedione secretion [55]. In pigs, adiponectin increases *in vitro* granulosa cell expression

of molecules involved in ovulation (COX2 (cyclooxygenase 2), PGE2 (prostaglandin E2), and EGF (epidermal growth factor)) and improves *in vitro* embryo development [47]. During IVF protocol in women and mice, adiponectin improves oocyte maturation and embryo development [57]. Similar results were observed for the *in vitro* development of embryos in pig [58]. In this species, several polymorphisms of adiponectin and its receptors (AdipoR1 and AdipoR2) have been identified. Certain variants and haplotypes identified are associated with larger litters, a smaller number of stillborn and mummified piglets, and shorter weaning-estrus intervals [59].

2.2. Resistin. Resistin is expressed in bovine, rat, and human ovarian cells [60–62]. Moreover, resistin modulates granulosa cell function, such as steroidogenesis and proliferation, in basal state or in response to IGF-I *in vitro* [60]. Furthermore, Spicer et al. showed that resistin inhibits steroidogenesis of undifferentiated (small follicles) granulosa cells and inhibits mitogenesis of differentiated (large follicles) granulosa cells collected from cattle [63]. In human cultured theca cells, recombinant resistin triggered 17α-hydroxylase activity, a marker of ovarian hyperandrogenism in women with PCOS [64]. Furthermore, recently in pig, resistin increased progesterone, androstenedione, and testosterone secretion by upregulating the steady state levels of CYP11A1, 3betaHSD, CYP17A1, and 17beta HSD. In the latter study, recombinant resistin had no effects on oestradiol secretion and CYP19A expression in ovarian follicles [65]. All these data suggest that resistin could affect *in vivo* ovarian function.

2.3. Visfatin. The presence of visfatin in human ovarian follicles has been shown in oocytes, cumulus cells, granulosa, and theca cells [66]. In primary human granulosa cells and KGN cells, visfatin expression (mRNA and protein) is regulated by metformin, an antidiabetic agent through AMPK activation and SIRT1 activity [66]. Furthermore, recombinant human visfatin increases IGF-1-induced steroid secretion and cell proliferation through NAMPT activity in primary human granulosa and KGN cells [66]. Shen et al. have also showed that visfatin expression in primary human granulosa cells is increased by hCG and prostaglandin E2 treatments [67]. In humans, the precise reproductive role of resistin remains controversial. Indeed, Seow et al. showed that resistin was not a major determining factor in the growth and maturation of oocytes during ovarian stimulation [68], whereas Chen et al. (2007) demonstrated a negative correlation between serum resistin levels and the number of oocytes retrieved during IVF [69]. In rodents, a recent study showed that administration of visfatin during superovulation improves the developmental competency of oocytes and fertility potential in old female mice suggesting a role of this adipokine in ovarian function and oocyte quality in older mammals [70].

2.4. Chemerin. Chemerin and CMKLR1 are expressed in the mouse, bovine, and human ovary [71, 72]. In humans, our group showed that chemerin and CMKLR1 are present in granulosa and theca cells and follicular fluid [72]. Chemerin levels are significantly higher in follicular fluid than in plasma [72]. *In vitro*, we have shown that rhChem inhibited IGF-1-induced progesterone, E2 secretion, and cell proliferation in human granulosa cells and this was associated with a reduction in the levels of p450 aromatase and a decrease in the tyrosine phosphorylation of IGF-1Rβ subunit and phosphorylation of Akt and MAPK ERK1/2 [72]. In rodents, ovarian and circulating chemerin levels are elevated in a chronically androgenised rat model and chemerin suppresses FSH-induced steroidogenesis [73] and induces apoptosis in granulosa cells, thereby suppressing follicle growth [74]. Furthermore in rat, chemerin suppresses the expression of the oocyte-derived factor, GDF9, that promotes granulosa cell proliferation and preantral/early antral follicle growth. It also suppresses GDF9-induced follicular growth *in vitro*. Thus, chemerin appears as an important intraovarian regulator and could contribute to the dysregulation of follicular development.

3. Adipokines in Uterus and Endometrium

In pig, AdipoR1 and AdipoR2 are also highly expressed in the endometrium [12] and both were localized in the endometrial and glandular human epithelium [75]. Interestingly, AdipoR1 and AdipoR2 transcript levels are higher during the midluteal phase suggesting that adiponectin may affect implantation [75]. Adiponectin, AdipoR1, and AdipoR2 are expressed in the uterus in different species (rabbit [76], pig [12], human [75], and rodents ([77]). In human, a strong expression of the receptors was observed in the endometrium during the implantation of the embryo. A recent study indicates that the expression of AdipoR1 and AdipoR2 is decreased (<60%) in the endometrium of women who experience implantation failure compared to fertile women with embryo implantation [78]. In contrast, plasma adiponectin levels are greatly reduced in patients with not only gestational diabetes and polycystic ovary syndrome (PCOS), but also endometriosis and endometrial cancer (cited in [79]). These studies suggest that a change in the expression of adiponectin and/or its receptors may be involved in endometrial receptivity [77, 78]. In addition, deregulation of the expression of this system could occur in certain pathological conditions associated with miscarriage or bad implantation [77]. Chemerin is also expressed and produced by the human uterus [80]. It is differentially expressed by decidual cells during early pregnancy, being present at high levels in stromal cells and extravillous trophoblast cells but not in decidual endothelial cells. Chemerin production in the uterus is upregulated during decidualization suggesting an important role in vascular remodeling during early pregnancy [80]. Visfatin is expressed in pig uterus [81]. However, few data are available to date on the potential role of not only visfatin but also resistin during embryo implantation.

4. Adipokines and Placenta

There is evidence indicating that several adipocytokines play an important role in placental function. The expression

of adiponectin in the human placenta is contradictory in the literature [82–85]. However, AdipoR1 and AdipoR2 are expressed in placental trophoblasts at the mRNA level [82], but only AdipoR2 protein is reported in human [86] and mouse trophoblast plasma membranes [87]. McDonald and Wolfe demonstrated that globular adiponectin attenuates mRNA expression and/or production of placental lactogen, chorion gonadotropin, and progesterone in trophoblast cells [85]. Adiponectin has been reported to promote syncytialization in BeWo cells and in primary human trophoblastic cells (PHT) isolated from early first trimester placentas [88] but inhibit syncytialization in PHT isolated later in gestation [85, 88]. Adiponectin decreases the *in vitro* proliferation of BeWo and JEG-3 trophoblastic lines, stimulates the differentiation of trophoblast in villous syncytiotrophoblast, and promotes secretion of placental hormones (hCG (human chorionic gonadotropin) and leptin) [89]. Like adiponectin receptors, resistin is present in human placenta especially in trophoblastic cells [32]. In humans, resistin expression increases during the gestation. Indeed, placenta resistin expression is significantly higher at term than in the first trimester [32] and resistin has been reported to play a role in pregnancy. It induces BeWo cell invasiveness and could contribute to the control of placental vascular development [90]. Resistin is also able to modulate glucose transport in human trophoblast cells [91]. Chemerin was detected in human placenta in the third trimester in the cytotrophoblast [92]. It is known that the third trimester of gestation in human is characterized by an anti-inflammatory response, thus chemerin could play a role to induce an anti-inflammatory environment. In the rat, chemerin expression is higher at day 16 and then decreases significantly towards the end of pregnancy [92]. Moreover, in this latter study, rat serum chemerin levels were decreased as gestation progressed [92]. Preeclampsia is a pathology characterized by high blood pressure and significant amounts of protein in the urine of a pregnant woman. As with resistin, maternal chemerin concentrations are significantly higher in preeclampsia patients compared to control patients [93, 94]. In humans, visfatin is also expressed in the placenta [95] where it activates proinflammatory cytokine release and phospholipid metabolism via activation of the NF-κB pathway [84]. In preeclampsia, plasma levels of visfatin and adiponectin do not change even with the severity of the disease [96]. All these data suggest that the adipokines are involved during embryonic implantation and gestation. They could play an important role in the maternal-fetal metabolism and metabolic homeostasis during pregnancy.

5. Adipokines: Implications in Infertility Associated with Obesity/Insulin Resistance and Polycystic Ovary Syndrome (PCOS)

The effects of obesity on female reproduction have also been extensively investigated. Several epidemiological studies using large cohorts of pregnant women have demonstrated a link between body mass index and the chances of pregnancy. The risk of taking more than a year to conceive is increased by 27% for overweight women and 78% for obese women [97]. Obese patients, pregnant after infertility treatment, have a significantly higher rate of spontaneous abortion that is correlated to the extent of obesity. Obesity can affect reproductive tract at different levels: oocyte, embryo, placenta, and uterine environment. It is difficult to describe the mechanisms by which obesity or excess weight affects the quality of female gametes. Obesity can affect the quality of gametes by altering the plasma concentrations of reproductive hormones and metabolism. Indeed, the adipose tissue is a site of production of not only steroid hormones but also adipocytokines. Obesity modifies the tissue and/or plasma expression profiles. Obesity is often observed in patients with polycystic ovary syndrome (PCOS) (about 50% of PCOS patients are obese). This syndrome presents both fertility and metabolic disorders. It is one of the most common causes of female infertility, which affects 5–10% of women of reproductive age. It is a heterogeneous syndrome with the characteristics of hirsutism, acne, anovulation, hyperandrogenemia, polycystic ovaries, and infertility [98]. Data regarding the levels of adipokines including adiponectin, resistin, and visfatin in PCOS patients are still controversial (references cited in [10]). Serum adipokines concentrations are reduced in PCOS patients compared with controls in some studies whereas in other reports serum adipokines did not differ between PCOS and control (references cited in [10]). Several meta-analyses have reported the PCOS association with polymorphisms of the adiponectin and resistin genes [99–101]. Although the profile of most adipokines such as adiponectin, resistin, and visfatin is still unknown in PCOS due to the conflicting data, the dysregulated adipokine levels in PCOS patients suggest that adipokines contribute to the pathology of PCOS.

6. Concluding Remarks

White adipose tissue can influence and communicate not only with other peripheral organs but also with reproductive tissues through the production of adipokines such as adiponectin, resistin, visfatin, and chemerin. These adipokines are also expressed in the reproductive tract suggesting not only endocrine but also autocrine or paracrine effects of these molecules. It would be important to inhibit each adipokine and/or its receptor in a cell specific manner in order to determine the role of these adipokines in different cells of the reproductive tract. Some adipokines seem to be involved in gestational pathologies like gestational diabetes mellitus and preeclampsia by their impact on insulin sensitivity and energy homeostasis. In our review we focus on female reproductive cells but adipokines and their receptors are also expressed in male gonad and consequently could affect spermatogenesis. The reproductive tract is regulated by hormones produced by the pituitary-hypothalamus axis where some adipokines are also present. Thus, adipokines could influence the central regulation of reproductive function by modulating the LH and FSH secretion. The secretion of adipokines and its influence on PCOS are very controversial. Further investigation is warranted to better understand the relationship between the adipokines and reproductive function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hypothèses de travail

et objectifs de la thèse

Hypothèses de travail et objectifs de la thèse

De nombreux travaux révèlent l'importance du lien entre le métabolisme énergétique et la reproduction. Comme nous l'avons décrit dans l'introduction, le tissu adipeux est un organe endocrine sécrétant de nombreuses molécules dont les adipocytokines pouvant intervenir sur différentes fonctions de l'organisme. Les adipocytokines interviennent dans les processus d'insulino-résistance, de diabète de type 2, d'obésité et d'inflammation. De plus, leur expression et leur concentration varient en fonction de l'état nutritionnel de l'individu et de sa masse graisseuse (Koerner et al., 2005). De nombreux travaux sur la leptine ou l'adiponectine montrent que les adipocytokines sont non seulement impliquées dans la régulation des fonctions métaboliques mais également dans celles de la reproduction (Campos et al., 2008). Les adipocytokines récemment identifiées telles que la chemerine, la visfatine et la résistine ont été très peu étudiées au niveau des fonctions gonadiques.

Ainsi, les objectifs de cette thèse ont consisté à :

1. **Rechercher la présence des adipocytokines (messager et protéine) chemerine, visfatine et résistine au niveau de l'ovaire chez la femme et la vache**. Nous avons choisi le modèle bovin car depuis années les vaches laitières principalement de race Prim Holstein voient leur fertilité diminuer due en partie à une forte sélection pour leur production laitière. En effet, plus une vache va produire plus elle va mobiliser ses réserves adipeuses après vêlage pour répondre à ses besoins pour la production de lait. Cette forte lipomobilisation n'est pas sans conséquences pour la fertilité de la vache. Notre hypothèse est que les adipocytokines plasmatiques ou tissulaires pourraient être des indicateurs de fertilité.

2. **Etudier l'effet des adipocytokines sur les différentes fonctions ovariennes et plus particulièrement au niveau des cellules de la granulosa humaine ou bovine (stéroïdogenèse, prolifération, activation de voies de signalisation) puis au niveau de l'ovocyte chez le bovin**.

3. **Etudier la concentration plasmatique de la résistine et le rôle de cette adipocytokine au niveau du tissu adipeux chez la vache.**

Enfin, afin de mieux comprendre le rôle de ces adipocytokines dans les fonctions de la reproduction, nous avons mené une étude préliminaire sur **l'effet de la chemerine recombinante humaine sur les sécrétions de LH et de FSH à la fois dans les cellules primaires hypophysaires bovines et dans la lignée gonadotrope murine, LβT2**.

Méthodologie

Méthodologie

A) Patientes et animaux

Au cours de cette thèse nous avons utilisé majoritairement deux modèles : la femme et la vache. Le modèle de la femme a été étudié car l'impact des adipocytokines au niveau de l'axe reproducteur (hypothalamo-hypophyso-ovarien) est peu connu. De plus, ces molécules pourraient être impliquées dans plusieurs problèmes de fertilité, notamment la première cause d'infertilité des pays développés ; le syndrome des ovaires polykystiques (SOPK) qui peut être associé à l'obésité (Kousta et al., 2005). Le modèle bovin a été utilisé dans un intérêt agronomique comme présenté précédemment.

A.1) Les patientes

Pour chaque étude, nous avons utilisé des patientes suivies dans le cadre d'un protocole de fécondation *in vitro* au sein du service de Biologie de la Reproduction du Professeur Royère, du CHRU Bretonneau à Tours. Pour chacune de ces patientes les causes d'infertilité du couple ont été mécaniques ou d'origine masculine sans endocrinopathie connue (hypo ou hyperthyroïdie). Les patientes ont donné leur consentement éclairé de manière écrite et n'ont reçu aucune compensation monétaire pour participer à l'étude. Pour chacune des patientes un seul cycle a été inclus dans l'étude.

A.2) La vache

Nous avons étudié au cours de leur lactation des vaches laitières (Prim Holstein) de l'unité expérimentale UEPAO. Pour les cultures primaires des cellules de la granulosa et de l'hypophyse, nous avons collecté les organes (ovaires et hypophyse) sur des vaches issues d'abattoirs (Vendôme ou Sablé sur Sarthe). Ainsi, pour ces animaux, il nous est impossible de connaître le statut nutritionnel, le stade au niveau du cycle ni l'historique des animaux. Les organes une fois prélevés sont déposés dans une solution saline (1% NaCl) à 37°C pour le transport.

B) Prélèvements et cultures cellulaires

B.1) Culture primaire :

B.1a) Chez la vache :

- L'hypophyse

Les hypophyses de vaches sont récupérées directement en abattoir à l'aide d'un boucher qui ouvre le crâne de l'animal pour permettre le prélèvement. Une fois collectées, les hypophyses situées au niveau de la selle turcique sont transportées dans une solution saline (1% NaCl) jusqu'à la salle de culture. Les hypophyses sont nettoyées (les membranes entourant les hypophyses sont ôtées) dans du Ham's F12 (Sigma) et la post-hypophyse est enlevée. Elles sont ensuite coupées en morceaux de 1 à 2 mm^3. Ces morceaux sont rincés trois fois dans du milieu Ham's F12 et incubés 20 minutes à 37°C dans du Ham 's F12 contenant de la collagénase A 0,4% (40 mg de collagénase pour 100 ml soit 0,4%, Roche Diagnostic) et de la DNase (12,5 mg dans 10 ml, DN25 Sigma). Les morceaux d'hypophyses sont lavés avec du Ham's F12 et passés dans un seringue de 20 ml une dizaine de fois puis ensuite successivement avec des aiguilles avec un diamètre de plus en plus petit (aiguilles blanches (18G (1,2 mm) x 40 mm), roses 19G (1,1 mm) x 40 mm puis vertes 21G (0,8 mm) x 40 mm (dissociation mécanique). Après un lavage les morceaux sont à nouveau incubés 45 à 60 minutes à 37°C dans 20 ml de milieu Ham's/collagénase A comme décrit précédemment sous forte agitation. Si des morceaux n'ont pas été digérés complètement ils sont remis à incuber avec de la collagénase A à 37°C pendant 15 minutes. Le tout est centrifugé 5 minutes à 1800 rpm et les cellules hypophysaires sont lavées puis resuspendues dans du milieu Mc Coy sans rouge de Phénol supplémenté avec 10% de sérum fœtal bovin (FBS, PAA laboratoires, les Mureaux, France), 20 mmol/Hepes pH 7,4, pénicilline (100 U/ml), streptomycine (100 mg/l).

- Les cellules de la granulosa

Arrivés au laboratoire les ovaires sont lavés puis les petits follicules (3 à 5 mm de diamètre) disséqués puis sont grattés dans un peu de milieu Mc Coy afin d'expulser les cellules de la granulosa. Le tout est centrifugé cinq minutes puis le culot est soit lavé une fois avec 2 ml de milieu Mc Coy afin d'éliminer un maximum d'hématies soit déposé sur un gradient de percoll (Sigma) de 40% et 60%, et dilué avec du milieu Ham's F-12 avec L-glutamine (PAA laboratories). Une centrifugation de 20 minutes à 1800 rpm permet de

séparer les hématies (au niveau du gradient à 60 %), des cellules de la granulosa (au sommet du gradient à 40 %). Les cellules de la granulosa en surface sont récupérées, puis lavées dans du milieu Ham's F- 12 avec L-glutamine puis centrifugées 8 minutes à 1800 rpm. Le culot est resuspendu dans du milieu Mc Coy5A modifié (20 mmol/Hepes pH 7,4, pénicilline (100 U/ml), streptomycine (100 mg/l), L-glutamine (3 mmol/l), 0.1% BSA, 5 mg/l transferrine, 20 mg/l selenium, and 10% sérum bovin fœtal (FBS, PAA laboratories, les Mureaux, France) puis mis en culture selon les conditions souhaitées pour l'expérimentation. Après 24 h de culture les cellules sont sevrées pendant une nuit avec du milieu McCoy's 5A contenant 1% de FBS avant d'être stimulées durant un temps souhaité avec ou sans réactif. Toutes les cultures cellulaires sont réalisées dans une étuve à 37 °C, 95 % O_2 / 5 % CO_2.

B.1.b) Chez la femme

Au laboratoire de PMA (Procréation Médicalement Assistée) du CHRU de Bretonneau les complexes cumulo-ovocytaires (COC) une fois ponctionnés avec le liquide folliculaire sont déposés dans une boite de pétri et isolés par un technicien. Nous récupérons la boîte de pétri contenant du liquide folliculaire, les cellules de la granulosa et les hématies. L'ensemble est centrifugé puis le culot est passé sur un gradient de percoll comme décrit dans le paragraphe précédent. Les cellules sont ensemencées dans le milieu Coy5A modifié contenant pénicilline (100 U/ml), streptomycine (100 mg/l) et 10% de FBS puis mises en culture selon les conditions souhaitées pour l'expérimentation.

B.2) Culture de lignée cellulaire de la granulosa humaine, KGN

La lignée cellulaire KGN (human granulosa like tumor cell line) correspond à des cellules de la granulosa humaine issues d'un carcinome ovarien. Ces cellules ont été obtenues des Drs Masatoshi Nomura et Hajime Nawata, Kyushu University, Japan (Nishi et al., 2001). Les cellules KGN conservent les caractéristiques physiologiques des cellules ovariennes incluant l'expression du récepteur FSH fonctionnel et l'expression de l'aromatase. Elles sont cultivées dans un milieu Dulbecco's minimal essential (DMEM) et Ham's F12 (Sigma, St. Louis, MO, USA) supplémenté avec 10% de FBS et des antibiotiques (100 IU/ml pénicilline et100 mg/ml streptomycine obtenus de Sigma) dans une étuve à 37 °C, 95 % O_2 / 5 % CO_2.

B.3) Culture de lignée cellulaire gonadotrope murine, LbetaT2

Les cellules LBetaT2 ont été fournies par P. Mellon et al. (2001) Les cellules sont cultivées dans du milieu DMEM supplémenté avec 10% de sérum de veau fœtal et des antibiotiques (penicilline 100 unités/ml et streptomycine 100 mg/ml) dans une étuve à 37 °C, 95 % O_2 / 5 % CO_2.

B.4) Inhibition de l'expression de protéines par infection avec un lentivirus dans les cellules de la granulosa bovine

Les cellules de la granulosa bovine ont été infectées par deux lentivirus recombinants bloquant l'activité de la visfatine (shNAMPT) d'après la littérature (Yang et al., 2007). Le premier est ***shNAMPT1 (5-GAGTGTTACTGGCTTAACAA-3)*** et le second se nomme ***shNAMPT2 (5-GAGTGTTACGGGGTTCCAG-3).*** Les séquences de shRNA ont été modifiées en mutant des bases critiques sur le shRNA contre NAMPT2. Pour cloner les shRNA, nous avons utilisé le vecteur lentiviral pSicoR (NAMPT-pSicoR lentiviral vector), dans ce vecteur le promoteur U6 dirige l'expression le shRNA et un autre promoteur précoce contrôle la cassette d'une protéine fluorescente verte (GFP). La VSV-G (une protéine G du virus de la stomatite vésiculaire), les vecteurs lentiviraux pseudotypés shRNA ont été produits. Les cellules ont été incubées avec le vecteur lentiviral pendant 48 heures pour que toutes les cellules soient infectées et surtout que l'expression de la visfatine soit fortement diminuée avant d'être soumises aux traitements souhaités**.**

B.5) Maturation ovocytaire bovine in vitro

B.5.a) Récolte, sélection et mise en culture des ovocytes

Une fois au laboratoire les ovaires sont stockés à 34°C et sont soumis à une ponction, les petits follicules sont aspirés comme décrit précédemment (Pennetier et al., 2006). Le liquide de ponction est mis à décanter, le culot est ensuite déposé sous loupe binoculaire puis les ovocytes sont triés et sélectionnés. Seuls les ovocytes sains c'est-à-dire entourés de plus de trois couches de cellules du cumulus présentant un aspect uniforme et non expansés sont conservés pour l'étude. Les ovocytes sont lavés dans une solution de TCM 199 (Sigma M 4530) supplémenté avec BSA 4mg/L et mis en culture dans du milieu Hepes 199 (Sigma M 7528) avec de la BSA (0,4g/L et de la gentamycine (2,5ml/L) avec ou sans les réactifs

d'intérêt pendant 10h ou 22h., 22 h correspondant au temps de maturation d'un ovocyte bovin. La culture est réalisée à 39°C dans une atmosphère saturée en eau avec 95% O_2 / 5 % CO_2.

B.5.b) Montage sur lame et coloration au Hoescht

Après stimulation, les ovocytes sont décoronisés c'est-à-dire séparés des cellules du cumulus. Pour cela, ils sont placés dans une goutte de 100 µl de TCM 199 supplémenté par 20 µl de hyaluronidase (40mg/ml) puis agités dans un cône par des mouvements de va et vient. Ensuite les ovocytes sont lavés par transfert de goutte en goutte de milieu TCM 199 avant d'être déposés sur une lame pendant 24h. Puis les lames sont déposées pendant une nuit dans l'alcool 95%, elles sont retirées, une fois l'alcool évaporé les ovocytes sont colorés avec une solution de citrate de sodium 0,1 M additionnée de Hoescht 0,1 µg/ml afin de marquer les noyaux cellulaires. Les lames sont recouvertes par 50 µl de vectashield (molecular probes, P36930) et montées sous lamelle qui sera scellée grâce à du vernis à ongles. Les lames sont conservées à 4°C à l'abri de la lumière avant d'être lues au microscope à fluorescence.

C) Mise en évidence des transcrits

C.1) Extraction des ARN

- Préparation des échantillons

Les ARN totaux sont extraits à partir de tissus broyés (cœur, foie poumon, muscle, rate, rein, tissu adipeux viscéral et sous-cutané, hypophyse) ou de cellules cultivées (cellules de la granulosa, cellules KGN, cellules hypophysaires bovines) puis lysées. Les tissus ou cellules sont stockés à -80°C avant l'extraction.

- Protocoles d'extraction

Les tissus ou les cellules (100 mg) sont resuspendus dans 1 ml de Trizol (mélange d'isothiocyanate de guanidine et de phénol, Sigma-Aldrich) puis broyés avec un ultra turax. Deux cents µl de chloroforme 10 % (Biomedical) sont ajoutés, après une forte agitation le tout est centrifugé pendant 20 minutes à 4°C à 15000g. Cela permet l'apparition de 3 phases, la phase aqueuse contenant les acides nucléiques est récupérée. Une fois la phase aqueuse prélevée la même quantité d'isopropanol pur (biomedical) est ajoutée de manière à précipiter les ARN totaux. Les tubes sont agités fortement puis placés sur glace pendant 10 minutes,

avant une nouvelle centrifugation à 15000g pendant 20 minutes à 4°C. Le culot d'ARN obtenu est lavé avec 750 µl d'éthanol 70 %, puis centrifugé pendant 5 minutes à 7500g. L'éthanol est éliminé et le culot d'acides nucléiques est remis en suspension dans 15 µl d'eau déminéralisé stérile. Les ARN totaux sont quantifiés par mesure de leur absorbance à 260 nm au spectrophotomètre et conservés à -80°C.

C.2) Caractérisation des ARNm extraits par RT-PCR

La RT-PCR a permis de mettre en évidence l'expression des adipocytokines et de leurs récepteurs au sein des cellules et tissus d'intérêt. Un microgramme d'ARN totaux est dénaturé puis rétrotranscrit avec la réverse transcriptase (RT) de type MMLV (Moloney Murine Leukemia Virus reverse transcriptase) dans un mélange réactionnel de 20 µl contenant 50 mM de Tris HCl (pH 8,3), 75 mM de KCL, 3 mM de $MgCl_2$, 200 µM de chaque désoxynuléotide triphosphate (Amersham), 50 pmol d'oligo (dT) 15, 5U d'inhibiteur de ribonucléases et 15 U de MMLV (Moloney murine leukemia virus reverse transcriptase). Le tout est incubé pendant 1 heure à 37°C. L'ADNc produit est amplifié par Polymerase Chain Reaction (PCR) dans un mélange réactionnel (2 µl de produit de RT, 10 mM de Tris-HCl (pH 9), 50 mM de KCl, 1,5 mM de $MgCl_2$, 200 mM de chaque désoxynucléotide triphosphate, 10 pmol de chaque amorce et 1 U de Taq polymerase). Les amorces utilisées sont décrites dans le tableau 3. Une première dénaturation à 94°C est effectuée pendant 5 minutes puis 35/40 cycles incluant pour chacun d'entre eux une étape de dénaturation (1 minute à 95°C), une étape d'hybridation (1 minute à 58°C) et une étape d'élongation (1 minute à 72°C. La PCR se termine par une élongation terminale (10 minutes à 72°C). Du tampon de charge (bleu de bromophénol 0,25 %; xylène cyanol 0,25 % ; glycérol 30 %) est ajouté aux produits de PCR puis le tout est déposé sur un gel d'agarose 1,5 % coloré au bromure d'éthidium. La migration s'effectue dans du tampon TBE 0,5X (0,045 mM Tris pH 8 ; 0,045 mM acide borique ; 0,025 mM EDTA) à 100 V pendant 30 minutes. Les ADNc amplifiés sont visualisés sous rayonnement UV et extraits, séquencés et comparés dans les banques de données.

Gene	Taille en paire de bases	Amorce sens	Amorce anti-sens
Amorce humaine			
visfatine	228 pb	5'AAGAGACTGCTGGCATAGGA3'	5'AAGAGACTGCTGGCATAGGA3'
Chemerine	251 pb	5'AGACAAGCTGCCGGAAGAGG3'	5'TGGAGAAGGCGAACTGTCCA3'
Résistine	229 pb	5'TGGTGTCTAGCAAGACCCTG3'	5'GCAGTGACATGTGGTCTCGG3'
Actine	188 pb	5'GTCCCAGTCTTCAACTATAC3'	5'ACGGAACCACAGTTATCAT3'
Cyclophiline A	217 pb	5'GCATACAGGTCCTGGCATCT3'	5'TGTCCACAGTCAGCAATGGT3'
RPL19	156 pb	5'AATCGCCAATGCCAACTC3'	5'CCCTTTCGCTTACCTATACC3'
Amorce Bovine			
visfatine	190 pb	5'AAGAGACTGCTGGCATAGGA3'	5'ACCACAGATACAGGCACTGA3'
chemerine	266 pb	5'GAGGAGTTCCACAAGCATC3'	5'ACCTGAGTCTGTATGGGACA3'
GPR1	571 pb	5'CTGTCATTTGGTTCACAGGA3'	5'AACAACCTGAGGTCCACATC3'
CCRL2	131 pb	5'CGTCATGATCACGTGCAAGA3'	5'GCAGGAAGTTGCTGATCTTG3'
CMKLR1	221 pb	5'CGGCCATGTGCAAGATCAGC3'	5'CAGGCTGAAGTTGTTAAAGC3'

Tableau 3: Amorces utilisées pour amplifier les fragments d'ADNc d'intérêt

C.3) Etude de l'expression des ARNm par PCR en temps réel (QPCR)

La PCR en temps réel est une technique permettant de visualiser au cours des cycles de PCR la quantité d'ADNc amplifiée. Pour cela, après la rétro-transcription les ADNc des cellules de la granulosa humaine ou bovine sont dilués au 1/5. Un pool des ADNc est dilué en série au 1/3 jusqu'à 1/3645 afin de déterminer l'efficacité des amorces. La réaction de qPCR se fait dans un volume final de 20 µl comprenant 5 µl de solution d'ADNc et 15 µl de mix master. Le mix master contient 10 µl iQ SYBR Green supermix (Bio-Rad), 0,25 µl d'amorce sens (10 µM), 0,25 µl d'amorce antisens (10 µM) et 4,5 µL d'eau. Le protocole de PCR suivant est utilisé sur le système MyiQ Cycler (Bio-Rad), les échantillons sont tout d'abord dénaturés (5 minutes à 95°C), puis amplifiés durant 40 cycles (dénaturation pendant 30 secondes à 95°C, hybridation pendant 30 secondes à 60°C et élongation pendant 30 secondes à 72°C) avant une élongation finale pendant 8 minutes à 72°C. Nous avons utilisés trois gènes de référence, l'actine F (5'-ACGGAACCACAGTTTATCATC-3' et R 5'GTCCCAGTCTTCAACTATACC3'), RPL19 (F 5'-AATCGCCAATCCAACTC-3' et R 5'-CCCTTTCGCTTACCTATACC-3'), et la cyclophiline A (F 5'-GCATACAGGTCCTGGCATCT-3' et R 5'-TGTCCACAGTCAGCAATGGT-3'). Le Ct

(cycle threshold) est déterminé pour chaque échantillon, il correspond au nombre de cycles minimal nécessaire pour que la fluorescence émise par le SYBR green dépasse le seuil de détection (supérieur au bruit de fond). Ce seuil va permettre d'effectuer la quantification relative de matériel de chaque échantillon. Pour cela, il est nécessaire de connaître l'efficacité(E) des amorces, elle doit être comprise entre 1,75 et 2 pour être considérée comme optimale selon i-Cycler, Bio-Rad. L'efficacité des couples d'amorces est testée en faisant une qPCR avec un mix d'ADNc, représentant le pool des échantillons dilués comme cité ci-dessus. Les logarithmes des Ct obtenus sont calculés et l'équation de la régression linéaire passant par ces points permet de calculer l'efficacité de chaque couple d'amorces. ($E=10^{-1/k}-1$, ou K= coefficient directeur de la droite). Un premier ratio est réalisé entre les valeurs de Ct est le Ct de la plus grande dilution (1/3645). La valeur de Ct de chaque échantillon obtenu est comparée à un gène ubiquiste, ici Cyclophiline A, RPL 19 (Ribosomal Protein L 19) et l'actine. Ainsi, le ratio calculé permet de connaître l'expression relative de nos adipocytokines et de leurs récepteurs par rapport aux gènes de référence. (R = (puissance de l'eff du gène étudié – Ctgène étudié)/ (puissance de l'eff du gène de référence – Ct gène de référence).

D) Mise en évidence des protéines

D.1) Etude quantitative des protéines par Western Blot

D.1.1) Extraction des protéines

Les cellules ou tissus sont lysés à froid dans le tampon suivant : (10 mM Tris (pH 7,4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0,5% Igepal) contenant des inhibiteurs de protéases (2 mM PMSF, 10 µg/ml leupeptin) et de phosphatases (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Les lysats cellulaires sont ensuite centrifugés à 16 000g pendant 20 minutes à 4°C et leur concentration protéique est déterminée par dosage colorimétrique (dosage BCA Interchim, Montluçon, France).

D.1.2) Protocole du western blot

Les extraits protéiques (30 à 50 µg) sont dénaturés dans du tampon Laemmli 5X (Tableau 4) avec du β-mercaptoéthanol et chauffés pendant 5 minutes à 95°C. Les échantillons sont ensuite soumis à une électrophorèse, sur un gel de SDS-polyacrylamide à 12 % en condition dénaturante, pendant 3 à 4 heures à 70 V puis 90 V, dans du tampon de migration (Tableau 5). Les protéines sont transférées pendant une heure et demie à 80 V sur

une membrane de nitrocellulose dans du tampon de transfert (Tableau 6). Un passage des membranes dans le rouge de Ponceau permet de vérifier la présence des protéines et la qualité du transfert. Les membranes sont ensuite incubées dans du TBS-Tween (Tableau 6) contenant 5 % de lait écrémé, permettant ainsi de bloquer les sites de liaisons non spécifiques. Ensuite, les membranes sont incubées une nuit à 4°C avec l'anticorps primaire approprié (dilution 1/1000) pendant 16 heures à 4°C, et sont lavées dans du TBS-Tween-0,5 %. Enfin, les membranes sont incubées 1 heure 30 min à température ambiante avec l'anticorps secondaire anti-lapin ou souris couplé à la peroxydase (BioRad) (dilution finale 1/5000). La révélation se fait selon le principe de chemiluminescence, le substrat de la peroxydase, le réactif ECL (Enhanced CheLuminescence, Perkin Elmer, Courtaboeuf, France) est déposé sur la membrane. Le signal émis est détecté grâce à une G :Box de SynGene (Ozyme) en utilisant le logiciel GenSnap (Release 7.09.17). La quantification a été réalisée avec le logiciel GeneTools (Release 4.01.02).

Tampon Laemmli 5X	
Glycérol	50%
SDS	350 mM
Hepes 1M pH 7,6	20 %
2β-Mercapthétanol	25 %

Tableau 4: Composition du Tampon de Laemmli 5X

Tampon de migration	
Tris Base (ultra pure)	20 mM
Glycine	200 mM
Méthanol	20 %
SDS 10 %	10 %

Tableau 5: Composition du Tampon de migration

Tampon TBS-Tween	
Tris HCL pH 7,6	2 mM
NaCl	15 mM
Tween 20	0,1%

Tableau 6: Composition du Tampon TBS-Tween

D.2) Localisation tissulaire par immunohistochimie

Les lames commerciales d'ovaires humains ou celles d'ovaires bovins réalisées par le laboratoire sont déparaffinées et sont réhydratées à l'aide de bains successifs : 2 fois 5 minutes dans du toluène ; 2 fois 3 minutes dans de l'éthanol (EtOH) 100 % ; 1 fois 2 minutes dans de l'EtOH 95 % ; 1 fois 2 minutes dans de l'EtOH 90 % et 1 fois 2 minutes dans de l'EtOH 70 %. Ensuite pour bloquer l'activité peroxydase endogène, les coupes sont baignées pendant 20 minutes dans une solution composée de 10 % de méthanol et 0,3 % de peroxyde d'hydrogène (H_2O_2). Les lames sont rincées au PBS 1X (Phosphate Buffered Saline) pendant 5 minutes, 2 fois. Les antigènes sont alors démasqués par un tampon composé de citrate (1/100, Vector Antigen Unmasking Solution, AbCys, Paris, France) pendant 10 minutes à température ambiante puis les lames sont rincées 2 fois 5 minutes dans du PBS 1X et les liaisons non spécifiques sont bloquées avec du sérum d'agneau à 5 % pendant 30 minutes. Puis l'anticorps primaire dirigé contre la protéine d'intérêt ou des immunoglobulines G de lapin, servant de témoin négatif, sont déposés sur les lames, le tout est incubé une nuit à 4°C. Les coupes sont de nouveau lavées au PBS 1X, 2 fois 5 minutes. L'anticorps secondaire anti-lapin ou anti-souris prêts à être utilisé, couplé à la HRP, est incubé 30 minutes à température ambiante puis les lames sont rincées au PBS 1X. La révélation est réalisée avec la DAB (DiAmino-3,3'Benzidine tetrachlohydrate, kit DakoCytomation, Envision®+ System-HRP, Dako, Trappes, France) et la réaction est stoppée par immersion dans du PBS 1X. Afin de mettre en évidence les structures cellulaires du tissu, une contre-coloration à l'hématoxyline est effectuée pendant environ 5 minutes. Les coupes sont enfin déshydratées par bains successifs de 2 minutes d'EtOH 70 %, 90 %, 95 % et 2 fois 100 %. Deux bains de 10 minutes de toluène permettent le montage final au DePex des lamelles sur les coupes. Des clichés sont pris à l'aide du logiciel Spot Camera (version 4.0.1.1). Nous avons utilisé des anticorps de lapin anti-IgG comme témoin négatif.

E) Dosage des hormones

E.1) Dosage radio-immunologique (RIA) de la P4 *et de l'*E2-*17β*

Les concentrations de P4 et d'E2-17β ont été mesurées dans les milieux de cultures des cellules de la granulosa humaine ou bovine. Les cellules de la granulosa des patientes ou des vaches provenant d'abattoir (environ 300 000 cellules/puits) sont ensemencées sur des

plaques 48 puits avec 300 µL de milieu McCoy's + BSA (5%). Après 24 h de sevrage les cellules sont stimulées pendant 48 h. Le milieu de culture est prélevé et dilué au 1/10 dans du milieu McCoy's + BSA (5%). Puis 50 µL du milieu de culture + 50 µL d'anticorps anti-P4 (diluée 1/72000) + 50 µL de P4* tritiée (10 000 cpm) + 350 µL de tampon phosphate (6,08g/l de sodium phosphate monosodique, 21,85g/l de sodium phosphate disodique, 9g/l de sodium chlorure, 1g/l de sodium azide, 1g/l de gélatine) sont incubés une nuit à 4°C. Le principe est de créer une compétition, représentée schématiquement (Figure 30), entre de la P4 marquée radioactive (P4 chaude) et la P4 non marquée (dite froide) pour la liaison à l'anticorps. L'ajout d'une solution de charbon et de Dextran permet de précipiter la P4 liée à l'anticorps. Une centrifugation à 3200 tours / minutes pendant 30 minutes sépare la P4 liée de la P4 libre contenue dans le surnageant qui est récupéré et dosé. Une émulsion scintillante (Perkin Elmer) est ajoutée au surnageant contenant le stéroïde marqué et la radioactivité est mesurée dans un « β-photomultiplier » à scintillation liquide pendant 2 minutes par fiole. Les résultats sont exprimés comme la concentration de stéroïdes (ng/ml)/concentration protéique/puits. La limite de détection de la P4 est de 12 pg/tube (60 pg/puits) et les coefficients de variation intra- et inter-dosages sont inférieurs à 10% et 11%, respectivement. La limite de détection pour l'E2 est de 1,5 pg/tube (7,5 pg/puit) et les coefficients de variation intra- et interdosage sont de 7% et 9%, respectivement. Les résultats sont exprimés en quantité d'hormone secrétée pour 100 µg de protéines.

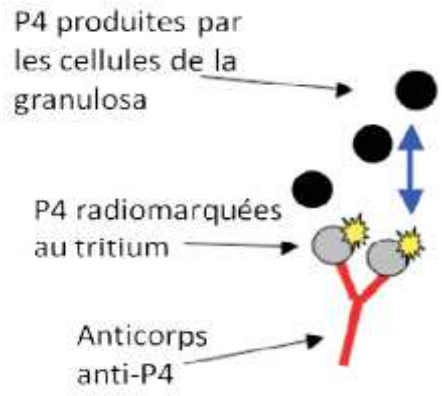

Figure 30: Principe du dosage radio-immunologique de la P4 et de l'E2

E.2) Dosage de la chemerine, la visfatine, la résistine dans le sérum et le liquide folliculaire humain et bovin

Les dosages des adipocytokines dans le plasma et le liquide folliculaire des patientes et des vaches ont été réalisés en utilisant des kits ELISA (Biovendor, Heidelberg, Germany) spécifiques des molécules à doser et de l'espèce (Humain, bovin). Ces kits ELISA comme

décrit dans chacun des articles qui seront présentés dans cette thèse ne distinguent pas forcément les différentes isoformes des adipocytokines dosées.

F) Prolifération cellulaire

F.1) Incorporation de thymidine tritiée

La mesure de la prolifération cellulaire est réalisée via l'incorporation de thymidine tritiée lors de la réplication de l'ADN. Une fois les cellules confluentes (environ $2x10^5$ cellules viables / 500µl) et sevrées, elles sont incubées avec les différents traitements puis diluées dans du milieu de culture contenant 1 µCi/µl de 3H-thymidine (AmershamLife Science) pendant 24 h. Les cellules sont rincées 2 fois dans du PBS 1X puis fixées grâce à une solution de 50 % d'acide trichloroacétique (TCA) et laissées sur glace pendant 15 minutes. Une solution de 0,5 M de NaOH (500 µL) permet la lyse des cellules, enfin les lysats sont placés dans des fioles de comptage (Packard Bioscence) avec 3,2 ml d'émulsion scintillante (Perkin Elmer, Courtaboeuf, France). La mesure de la radioactivité est déterminée par un « β-photomultiplier ».

F.2) Viabilité cellulaire : coloration bleu trypan

Les cellules sont décollées de leur support avec une solution de trypsine-EDTA (Sigma), centrifugées, colorées au bleu trypan et dénombrées dans une cellule de comptage (grille de Thomas) sous microscope.

G) Analyse statistique

Les résultats des différentes expériences sont exprimés en moyenne ± SEM et sont représentés sous la forme d'un histogramme. Elles résultent d'au moins trois séries indépendantes. Pour tester la significativité des variations de divers facteurs, un test ANOVA factoriel est utilisé et complété par un test de Fisher quand le test ANOVA est significatif ($P<0,05$).

Résultats

Résultats

Identification et rôle des adipocytokines (chemerine, visfatine, résistine) dans les cellules ovariennes humaines

Nous avons choisi d'étudier le rôle des adipocytokines, chemerine, visfatine et résistine dans la fonction de reproduction femelle chez la femme et plus particulièrement au niveau des cellules de la granulosa. Tout d'abord, nous avons recherché la présence des adipocytokines au sein de l'ovaire par RT-PCR et western blot mais également par immunohistochimie. Nous avons observé leurs effets sur la prolifération des cellules de la granulosa ainsi que sur la sécrétion de stéroïdes en absence ou en présence d'IGF-1 et de FSH. Egalement, nous avons analysé l'effet d'insulino-sensibilisateur sur l'expression des adipocytokines, étant donné leur relation étroite avec le diabète et l'insulino-résistance. Enfin nous avons étudié quelles voies de signalisation étaient activées par les adipocytokines dans les cellules ovariennes en effectuant des cinétiques courte et longue. Nous avons souhaité caractériser ces systèmes chez la femme car l'effet de la chemerine, de la visfatine et de la résistine au sein de l'ovaire humain sont peu connus à ce jour. Cette étude devrait permettre de mieux comprendre les liens entre le tissu adipeux et les fonctions ovariennes. Les différents points suivant seront abordés :

Caractérisation des systèmes chemerine, visfatine et résistine dans l'ovaire humain et rôle *in vitro* dans les cellules de la granulosa humaine

A) Localisation des adipocytokines dans l'ovaire humain (**Articles 1,2 et 3**).

B) Dosage folliculaire et plasmatique des adipocytokines (**Articles 2 et 3**).

C) Impact des adipocytokines sur la prolifération des cellules de la granulosa humaine (**Articles 1, 2 et 3**).

D) Effets des adipocytokines sur la stéroïdogenèse (**Articles 1, 2 et 3**).

E) Influence d'insulino-sensibilisateurs sur l'expression des adipocytokines (**Article 2**).

F) Voies de signalisation activées par les adipocytokines dans les cellules de la granulosa humaine (**Articles 1, 2 et 3**).

Caractérisation des systèmes chemerine, visfatine et résistine dans l'ovaire humain et rôle in vitro dans les cellules de la granulosa humaine

A) Identification des adipocytokines dans l'ovaire humain (Articles 1,2 et 3).

Chez l'humain les adipocytokines sont exprimées dans de nombreux tissus. Nous avons montré la présence des adipocytokines chemerine et son récepteur CMKLR1, visfatine et résistine dans des cultures primaires de cellules de la granulosa humaine et dans la lignée cellulaire de granulosa humaine, KGN. Nous avons amplifié par RT-PCR : des fragments de 251 pb et 400 pb correspondant à la chemerine et son récepteur, respectivement (**Fig. 1A, article 1**) un fragment de 228 pb correspondant à la visfatine (**Fig 1A, article 2**) et un fragment d'ADNc de 300 pb représentant la résistine (**Fig 2A, article 3**). Les tissus adipeux viscéral et sous-cutanés ont été utilisés comme témoins positifs. Par western blot nous avons obtenu des résultats similaires au niveau protéique (**Fig 1B, article 1 ; Fig 1B, article 2 et Fig 2A, article 3**). Par immunohistochimie sur des coupes commerciales d'ovaire humain nous avons mis en évidence la présence de la chemerine et de son récepteur CMKLR1 et de la visfatine dans les cellules de la granulosa et moins abondamment dans les cellules de la thèque (**Fig 1C, article 1 et Fig 1C, article 2**). De plus, la visfatine a été localisée dans les cellules du cumulus et l'ovocyte (**Fig 1C, article 2**). La résistine a été observée dans l'ensemble des cellules citées précédemment. Elle est présente en quantité égale dans les cellules des thèques des gros follicules et dans les cellules de la granulosa à tous les stades (**Fig 2C, article 3**).

Ainsi, dans l'ovaire de femme les adipocytokines, chemerine et son récepteur CMKLR1, visfatine et résistine sont exprimés dans les follicules ovariens humains. La chemerine, son récepteur et la visfatine sont plus exprimés dans les cellules de la granulosa et la résistine est présente à tous les stades de développement du follicule ainsi que dans les cellules de la thèque des gros follicules. L'expression des adipocytokines semble varier suivant le stade de développement des follicules.

B) Dosage folliculaire et plasmatique des adipocytokines (Articles 2 et 3).

Nous avons mesuré la concentration en chemerine et en résistine dans le liquide folliculaire et le plasma de patientes suivies dans le cadre d'un protocole de fécondation *in vitro* au CHRU de Bretonneau. La concentration de chemerine est deux fois plus élevée dans le liquide folliculaire que dans le plasma chez 8 des 10 patientes étudiées (**Fig 1D, article 1**). A l'inverse, les niveaux de résistine sont significativement plus élevés dans le plasma que dans le liquide folliculaire, malgré un petit nombre de patientes (n=9, $P < 0,05$) (**Fig 1, article 3**).

C) Impact des adipocytokines sur la prolifération et la viabilité des cellules de la granulosa humaine (Articles 1, 2 et 3).

Les cellules primaires de la granulosa ou les cellules KGN traitées pendant 24h avec IGF-1 (10^{-8}M) augmentent significativement leur incorporation de [^{3}H]-thymidine tritiée (Pierre et al., 2009). Les trois adipocytokines étudiées présentent des effets différents sur la prolifération cellulaire. Cependant elles n'affectent pas toutes les trois la prolifération basale ou induite par FSH. Un traitement à la chemerine recombinante humaine (10ng/ml ou 100 ng/ml) pendant 24 h diminue l'incorporation de thymidine induite par IGF-1 dans les cellules de la granulosa humaine et KGN (**Fig 4, article 1**). A l'inverse, la visfatine recombinante humaine (10ng/ml) augmente la prolifération cellulaire induite par IGF-1 de 1,3 fois dans les cellules de la granulosa humaine et dans les cellules KGN (**Fig 8A, article 2**). Nous avons confirmé cet effet en utilisant un inhibiteur spécifique de l'activité de la visfatine, le **FK 866** (**Fig 8A, article 2**). Un traitement à la résistine recombinante humaine (10 ng/ml) n'a pas d'effet sur l'incorporation de thymidine par les cellules de la granulosa et les cellules KGN (résultats non montrés). Par ailleurs, aucune de ces adipocytokines n'affecte la viabilité cellulaire (données non présentées).

Ainsi, la chemerine recombinante (10 ng/ml, 24h) diminue l'incorporation de thymidine tritiée induite par IGF-1 *in vitro* dans les cellules de la granulosa humaine (primaires et KGN) tandis que la visfatine recombinante (10 ng/ml) l'augmente et la résistine recombinante (10 ng/ml) n'a aucun effet significatif.

D) Effets des adipocytokines sur la stéroïdogenèse (Articles 1, 2 et 3).

- Impact sur la sécrétion de stéroïdes (P4, E2)

Les cellules primaires de la granulosa humaine ou les cellules KGN ont été incubées pendant 48h avec chacune des adipocytokines recombinantes humaines étudiées en présence ou en absence de FSH (10^{-8} M) et/ou IGF-1 (10^{-8} M). Comme attendu, les traitements avec FSH et/ou IGF-1 seuls augmentent les sécrétions de P4 et d'E2 dans les cellules KGN et CGH (**Fig. 2, article 1**). Cependant, dans ces dernières cellules, les adipocytokines chemerine, visfatine et résistine n'affectent pas la sécrétion de stéroïdes en absence ou en présence de FSH. En revanche, la chemerine recombinante humaine (rh chemerine) (10 et 100 ng/ml) et la résistine recombinante humaine (rh résistine) (10 ng/ml) inhibent l'effet de l'IGF-1 sur la sécrétion de stéroïdes dans les cellules primaires de la granulosa humaine et les KGN (**Fig 2, article 1 et Fig 3 A et B, article 3**). En effet, la sécrétion de stéroïdes induite par IGF-1 est réduite de trois fois par la rh chemerine (10 ng/ml) et de 2,2 fois par la rh résistine ($P<0,001$). Pour la résistine, ce résultat a été confirmé par l'utilisation d'un anticorps monoclonal 1H7 (10 µg/ml) dirigé contre la sous-unité beta de l'IGF-1R décrit comme inhibiteur de l'activité biologique d'IGF-1 (**Fig. supplémentaire 1, disponible en ligne**). A l'inverse, la visfatine augmente la sécrétion de stéroïdes. En effet, la visfatine recombinante humaine (rh visfatine) (10 ng/ml) augmente la sécrétion de E2 et P4 basale dans les cellules KGN ($P<0.001$) mais pas dans les cellules primaires (**Fig 6, article 2**). De plus, la rh visfatine augmente de 2 fois l'effet de l'IGF-1 sur la sécrétion de stéroïdes ($P<0.001$) dans les CGH et les KGN (**Fig 6, article 2**).

Ainsi les adipocytokines, chemerine et résistine ont un effet inhibiteur sur la sécrétion de stéroïdes induite par IGF-1 tandis que la visfatine a un effet stimulateur dans les cellules primaires de la granulosa humaine et les KGN.

- Impact des adipocytokines sur les enzymes de la stéroïdogenèse

Etant donné l'effet des adipocytokines sur la sécrétion de stéroïdes, nous avons déterminé si elles affectent l'expression des enzymes de la stéroïdogenèse (P450 scc, P450 aromatase et 3β-HSD) ainsi que celle de la protéine de transport du cholestérol (StAR). Un traitement de 48 h des cellules primaires de la granulosa humaine avec la rh chemerine n'a pas d'effet sur les niveaux protéiques de la StAR, la P450scc et la 3β-HSD à l'état basal ou en réponse à l'IGF-1 ou à la FSH (**Fig 3, article 1**). Cependant, la rh chemerine diminue la

quantité de la P450 aromatase en présence d'IGF-1 ($P<0.05$) **(Fig 3D, article 1**) mais n'a pas d'effet en son absence. De la même manière, la rh résistine diminue le niveau protéique de la P450 aromatase mais aussi de la P450scc en présence d'IGF-1 et ne présente aucun effet significatif à l'état basal ou en présence de FSH (**Fig 3C et D, article 3**). La rh visfatine n'affecte pas dans nos conditions le niveau d'expression des enzymes de la stéroïdogenèse et de la StAR.

Ainsi, les effets inhibiteurs de la rh chemerine et la rh résistine sur la production de stéroides *in vitro* par les cellules de la granulosa en réponse à l'IGF-1 pourraient en partie s'expliquer par une diminution de l'expression des enzymes de la stéroïdogenèse (P450 aromatase et p450scc).

E) Influence d'insulino-sensibilisateurs (rosiglitazone et metformine) sur l'expression des adipocytokines (Article 2).

Connaissant le lien étroit entre les adipocytokines et le diabète de type 2/obésité, nous avons testé l'effet de deux insulino-sensibilisateurs utilisés lors de protocole de fécondation *in vitro* sur l'expression de la visfatine dans des cultures primaires de cellules de la granulosa humaine et les cellules KGN. Par PCR en temps réel, après 24 h de stimulation nous avons observé que la metformine (0, 0,1, 1 et 10 mM) augmente l'expression de la visfatine de manière dose-dépendante alors que la rosiglitazone (0; 0,1; 1 et 10 μM) l'augmente seulement à la concentration de 10 μM (**Fig 2 A, article 2**). Nous obtenons des effets opposés après 48 h d'incubation. Suite à ces résultats nous avons confirmé au niveau protéique l'effet de ces insulino-sensibilisateurs. Dans les cellules KGN, la metformine (10 mM) après 24 h de traitement augmente le niveau protéique de la visfatine de deux fois alors qu'il est diminué de moitié après 48 h (**Fig 2B, article 2**). La rosiglitazone ne présente aucun effet significatif. Des résultats similaires ont été obtenus avec les cellules primaires de la granulosa humaine. Nous avons ensuite étudié par quels mécanismes moléculaires la metformine influence l'expression messager et protéique de la visfatine.

- Implication de la voie de signalisation AMPK dans les effets de la metformine sur l'expression de la visfatine dans les cellules primaires de la granulosa humaine

Richardson et al ont déjà montré qu'un traitement à la metformine active la voie de signalisation AMPK dans plusieurs types cellulaires (Richardson et al., 2009). Ici, nous montrons que la metformine (10 mM) dans les cellules KGN et les cellules primaires de la

granulosa humaine augmente la phosphorylation de l'AMPK sur le résidu Thr 172 après 1 et 2 h de stimulation (**Fig 3A, article 2**). A l'inverse, après 48h de stimulation la phosphorylation de l'AMPK est diminuée (**Fig 3 B, article 2**). De plus, afin de déterminer de manière indirecte l'activité de l'AMPK nous avons mesuré la phosphorylation de sa cible, l'acétyl CoA carboxylase (ACC). La phosphorylation de l'ACC s'avère être parallèle à la Thr 172 de l'AMPK (données non montrées). Afin de vérifier l'implication de l'AMPK dans la régulation de l'expression de la visfatine par la metformine, nous avons utilisés le « composant C » un inhibiteur spécifique de l'AMPK. Le « composant C » (10 µM) inhibe totalement les effets de la metformine sur l'expression de la visfatine et la phosphorylation de l'AMPK après 24 et 48 h de traitement (**Fig 3D, article 2**). Egalement, pour s'assurer du rôle de l'AMPK dans la régulation de l'expression de la visfatine dans les CGH, nous avons utilisé l'AICAR un activateur de l'AMPK. L'AICAR (10 µM) augmente la phosphorylation du résidu Thr 172 de l'AMPK après 30, 60 et 120 minutes de stimulation (**Fig 4 A, article 2**). De plus, l'AICAR augmente l'expression du messager de la visfatine de 3 fois après 24 h de traitement et la réduit de 3 fois après 48 h (**Fig 4 B et C, article 2**). Le « composant C » inhibe totalement l'effet de l'AICAR (**Fig 4 B et C, article 2**). Des résultats similaires ont été observés au niveau protéique (**Fig 4 D et E, article 2**).

- Implication de SIRT1 dans les effets de la metformine sur l'expression de la visfatine dans les cellules primaires de la granulosa humaine

De la même manière que précédemment, il est connu que la metformine active la protéine déacétylase SIRT1 (Caton et al., 2010) et plusieurs études ont montré que l'activation de l'AMPK pouvait mener à l'augmentation de l'activité des SIRT1 (Cantó & Auwerx, 2009; Cantó et al., 2009; Fulco et al., 2008). Nous avons observé que la metformine augmente l'activité des SIRT1 après 24h de traitement dans les cellules KGN et CGH (**Fig 5A, article 2**). Afin de vérifier ce résultat nous avons utilisé le Sirtinol, un inhibiteur spécifique de SIRT1, ce traitement réduit l'expression de la visfatine en réponse à 24h de traitement à la metformine (**Fig 5 A, article 2**). Nous obtenons des résultats similaires au niveau protéique (**Fig 5 B, article 2**). La viabilité des cellules n'est pas affectée par un traitement au « Composant C » ou au Sirtinol (Données non montrées).

Ainsi la metformine induit l'expression de la visfatine via les voies de signalisation AMPK et SIRT1.

F) Voies de signalisations activées par les adipocytokines dans les cellules de la granulosa humaine (Articles 1, 2 et 3).

Des études précédentes menées au laboratoire ont montré que les adipocytokines comme l'adiponectine et la résistine peuvent moduler la phosphorylation des voies de signalisation MAPK -Erk1/2 et p38, AMPK et Akt. Ainsi, nous avons choisi d'étudier ces voies de signalisation dans les cellules de la granulosa humaine. De plus, constatant les effets des adipocytokines associés à l'IGF-1 sur la prolifération et la sécrétion de stéroïdes nous avons analysé certains composants de la signalisation du récepteur à IGF-1.

Les cellules primaires de la granulosa ont été stimulées pendant 48h avec une adipocytokine recombinante humaine en présence ou en absence d'IGF-1 (10^{-8}M) ou FSH (10^{-8}M) (condition identique à celle utilisée pour la stéroïdogenèse). Le traitement avec IGF-1 seul (10^{-8}M, 48h) augmente la phosphorylation de la sous-unité beta du IGF-1R (**Fig 5A, article 1**), de la voie MAPK-ERK1/2 (**Fig 5 B, article 1**) et de la voie Akt (**Fig 5 C, article 1**). Un traitement par la FSH augmente seulement la phosphorylation de la voie MAPK-ERK ½ (**Fig 5 A-C, article 1**).

Après 48h d'incubation avec la chemerine ou la résistine (10 ng/ml) les effets d'IGF-1 sur ces voies de signalisation sont totalement abolis dans les cellules primaires de la granulosa alors que les effets de la FSH ne sont pas affectés. De plus, ces deux adipocytokines inhibent la phosphorylation de la sous-unité beta du IGF-1R induite par IGF-1 (**Fig 5, article 1 et Fig 4, article 3**). Ce résultat a été confirmé pour la résistine par l'utilisation d'un anticorps monoclonal (1H7, 10 µg/ml)) dirigé contre la sous-unité beta de l'IGF-1R (**Fig supplémentaire 2, disponible en ligne**).

Les trois adipocytokines étudiées chemerine, visfatine et résistine activent rapidement (1-5 minutes ($P< 0,05$) les voies de signalisation MAPK-Erk ½ et P38, et Akt (**Fig 6, article 1 ; Fig 8, article 2 et Fig 4 article 3**). Cependant, la chemerine active ces voies de signalisation de manière transitoire alors qu'elle est maintenue après stimulation avec la visfatine. La résistine active de manière transitoire les voies de signalisation MAPK-Erk ½ et P38 alors que la phosphorylation de la voie Akt est maintenue durant les 60 minutes de stimulation.

La chemerine active plus tardivement la voie de signalisation AMPK (après 30 minutes de stimulation, **Fig 6 D, article 1**) alors que la visfatine et la résistine n'affectent pas cette voie de signalisation (données non montrées).

En résumé, ces trois adipocytokines activent de manière rapide les voies de signalisation MAPK-ERK ½ et P38, et Akt. Seule la chemerine active plus tardivement la voie de signalisation AMPK.

Conclusions et discussion

L'objectif de ces études était dans un premier temps d'identifier les trois adipocytokines, chemerine, visfatine et résistine au niveau des cellules ovariennes humaines puis dans un second temps d'observer leurs effets sur la prolifération, la stéroïdogenèse ainsi que les voies de signalisation mises en jeu dans les cellules de la granulosa.

Nous avons mis en évidence pour la première fois la présence des adipocytokines dans l'ovaire humain. En effet, nous avons confirmé la présence de la chemerine de la visfatine et de la résistine au niveau du messager et de la protéine dans les différentes cellules ovariennes. Ces résultats sont en accord avec de précédentes études où TIG2 (autre appellation pour la chemerine) a été détecté dans l'ovaire humain par Northern blot (Nagpal et al., 1997). De plus, la visfatine et la résistine ont été observées dans l'ovocyte comme d'autres adipocytokines telles que la leptine et l'adiponectine qui ont été retrouvées dans l'ovocyte de plusieurs espèces (Chabrolle et al., 2007). La résistine avait déjà été localisée dans l'ovocyte de vache par notre équipe (Maillard et al., 2011) mais elle est absente dans les cellules de la granulosa de rate, ce qui laisse penser que les adipocytokines ont une expression tissu spécifique suivant l'espèce. D'ailleurs, la localisation cellulaire de la résistine dans le tissu adipeux humain et murin est différente (Steppan et al., 2001).

Egalement, la chemerine, la visfatine et la résistine ont été identifiées dans le fluide folliculaire. Lors du dosage de la chemerine et de la résistine dans le plasma et le liquide folliculaire un jour avant la ponction ovocytaire, nous avons obtenu des résultats inverses avec ces deux adipocytokines. La concentration de chemerine est plus importante dans le liquide folliculaire que dans le plasma comme l'adiponectine (Chabrolle et al., 2009) à l'inverse de la résistine. Nos résultats sont en accord avec une précédente étude qui raporte des niveaux de résistine plus bas dans le liquide folliculaire que dans le plasma chez des patientes contrôles et atteinte du SOPK (Seow et al., 2005). Ces résultats sont limités par le fait que les kits ELISA utilisés pour le dosage ne détectent pas toutes les isoformes des adipocytokines. Dans notre étude et celle de Seow et al (2005) la concentration de résistine dans le liquide folliculaire est beaucoup plus faible que celle du plasma suggérant que la résistine n'a pas ou très peu d'effet paracrine/autocrine dans le processus de développement du follicule chez les femmes suivies pour un protocole de FIV. A l'inverse de la chemerine

qui pourrait avoir un effet paracrine/autocrine sur la maturation ovocytaire. De plus, il a été montré que d'autres adipocytokines telles que la leptine et le TNF-α (Tumor necrosis factor alpha) affectent la fertilité et le développement embryonnaire (Brännström et al., 1993). Ainsi, l'état corporel de l'individu modifie l'expression et les concentrations des adipocytokines qui pourraient affecter les fonctions de reproduction.

Dans l'étude de la visfatine nous avons analysé l'effet de deux insulino-sensibilisateurs, metformine et rosiglitazone, sur l'expression de la visfatine dans les cellules primaires de la granulosa humaine. La rosiglitazone modifie l'expression de la visfatine seulement à sa plus forte concentration (10 μM) contrairement à la metformine où nous observons un effet dose-réponse. La rosiglitazone est connue augmenter l'expression de la visfatine dans le tissu adipeux viscéral de rat et dans les macrophages humains (Choi et al., 2005; Mayi et al., 2010). La metformine affecte la visfatine au niveau protéique, elle augmente l'expression de la visfatine après 12 et 24h de stimulation et la réduit après 48h. D'autres études ont déjà décrit l'effet de la metformine sur la visfatine dans d'autres types cellulaires (Caton et al., 2010). Ici nous avons analysé le mécanisme moléculaire impliqué dans la régulation de l'expression de la visfatine. L'AMPK est une cible potentielle de la metformine (Tosca et al., 2008), nous avons confirmé leurs interactions dans les cellules primaires de la granulosa humaine. De plus, l'utilisation d'un inhibiteur spécifique de l'AMPK, le composant C, inhibe totalement l'effet de la metformine sur l'expression de la visfatine. Nous avons également montré l'implication de SIRT1, une histone déacétylase qui comme l'AMPK est présente dans l'ovaire et régule l'homéostasie énergétique (Morita et al., 2012).

Nous montrons que la metformine active SIRT1 dans les cellules primaires de la granulosa humaine et un inhibiteur de SIRT1 (le sirtinol) abolit les effets de la metformine sur l'expression de la visfatine. Ainsi, l'AMPK mais aussi SIRT1 sont impliqués dans la régulation de l'expression de la visfatine. Des études antérieures ont montré que l'AMPK active SIRT1 dans le muscle squelettique et les cultures de myocytes (Cantó & Auwerx, 2009; Fulco et al., 2008). Ainsi, l'expression de la visfatine est régulée par la metformine dans les cellules primaires de la granulosa humaine via les voies de signalisation AMPK/SIRT1. Maintenant, il reste à déterminer si l'effet de la metformine sur l'expression de la visfatine via l'AMPK est dépendant ou non de SIRT1 et quel est l'impact sur les cellules de la granulosa.

Dans les trois articles nous avons remarqué un effet des adipocytokines sur la sécrétion de stéroïdes induites par IGF-1 dans les cultures de cellules de la granulosa humaine

et les cellules KGN. La chemerine (rh chemerine) et la résistine (rh résistine) recombinantes humaine diminuent la sécrétion de P4 et E2 induite par IGF-1 alors que la visfatine l'augmente. Des études précédentes ont déjà montré que la rh résistine altère la sécrétion de stéroïdes *in vitro* dans les cellules de la granulosa de rate et de vache (Maillard et al., 2011). La rh chemerine et la rh résistine affectent la sécrétion de stéroïdes en diminuant la phosphorylation de la sous-unité β du récepteur IGF-1 et la phosphorylation de la voie MAPK-ERK1/2, connue pour réguler positivement la stéroïdogenèse dans des cultures de cellules de la granulosa de rate et de femme (Seto-Young et al., 2003; Tosca et al., 2005). Nous ne savons pas si les effets de la chemerine sur le récepteur IGF-1 sont dus à l'activation de CMKLR1 par la chemerine ou à un effet direct de la chemerine sur IGF-1R. De plus, ces adipocytokines réduisent les niveaux protéiques de certaines enzymes de la stéroïdogenèse. La rh chemerine et la rh résistine diminuent la quantité de P450 aromatase induite par IGF-1 et la rh résistine réduit aussi le niveau de la P450 aromatase mais aussi de la P450 scc. Ceci pourrait expliquer l'effet de ces adipocytokines sur la stéroïdogenèse. La rh visfatine, active également la voie de signalisation MAPK-ERK1/2, cependant elle potentialise les effets d'IGF-1 dans les CGH et les cellules KGN. Au laboratoire, il a été observé que l'adiponectine augmente également la sécrétion de stéroïdes induite par IGF-1 dans les cellules de la granulosa. Tout comme la visfatine, l'adiponectine active la voie de signalisation MAPK-ERK1/2 qui semble pouvoir influencer de manière positive et négative la sécrétion de stéroïdes (Chabrolle et al., 2009). Les effets de la rh visfatine sont abolis par l'utilisation d'un inhibiteur spécifique de l'activité NAMPT de la visfatine, le FK866. Maintenant, il reste à étudier si la rh visfatine peut phosphoryler ou moduler la phosphorylation d'IGF-1R. Ces trois adipocytokines ne modifient pas la sécrétion de stéroïdes induites par la FSH, alors que la FSH seule augmente la sécrétion de P4 et E2.

Dans cette étude nous avons constaté que la chemerine et la résistine réduisent l'incorporation de la thymidine tritiée induite par IGF-1 dans les cellules KGN et les cellules primaires de la granulosa humaine à l'inverse de la visfatine qui l'augmente. Ceci pourrait être expliqué par le fait que ces trois adipocytokines activent la voie de signalisation Akt, connue pour être impliquée dans la prolifération cellulaire. Nous avons analysé plusieurs voies de signalisations et nous avons observé que la chemerine, la visfatine et la résistine activent les voies MAPK-ERK1/2 et P38 et Akt dans les cellules primaires de la granulosa humaine et seule la chemerine active la voie de signalisation AMPK.

Ainsi, nos travaux montrent que les adipocytokines, chemerine, visfatine et résistine sont présentes au sein de l'ovaire humain et plus particulièrement dans les cellules de la granulosa. De plus, ces adipocytokines influencent la stéroïdogenèse en réponse à l'IGF-1 et la prolifération des cellules primaires de la granulosa humaine. Ceci pourrait être expliqué par l'effet des adipocytokines sur l'expression de certaines enzymes de la stéroïdogenèse et/ou la voie de signalisation MAPK-ERK1/2. Egalement, l'expression de la visfatine est régulée par la metformine via les voies de signalisation AMPK/SIRT1. Ces découvertes améliorent notre compréhension du rôle des adipocytokines dans l'ovaire, cependant d'autres études sont nécessaires pour comprendre leur effet dans d'autres types cellulaires ovariens (cellules de la thèque par exemple) et leurs potentielles implications dans le syndrome des ovaires polykystiques.

Article 1

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ORIGINAL ARTICLE *Reproductive biology*

Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells

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BACKGROUND: Chemerin is a novel adipokine involved in the regulation of adipocyte development, inflammation and metabolic functions. To date, no role of this adipokine in reproductive functions has been described. In the present study, we identified chemerin and its receptor, CMKLR1 (chemokine-like receptor 1), in primary human granulosa cells (hGCs) and in a human ovarian granulosa-like tumour cell line (KGN). We also investigated the effects of recombinant human chemerin (rhChem) on steroid production and on various signalling pathways.

METHODS AND RESULTS: By RT–PCR immunoblotting and immunohistochemistry, we showed that chemerin and CMKLR1 are expressed in hGCs and KGN cells. By ELISA, we also found chemerin in human follicular fluid and we observed that in 8 of 10 women the chemerin level was at least 2-fold higher in follicular fluid than in plasma. rhChem (10 or 100 ng/ml) significantly decreased insulin-like growth factor-1 (IGF-1) (10^{-8} M)-induced secretion of progesterone and estradiol (as determined by radioimmunoassay) but did not affect basal-or FSH (10^{-8} M)-induced steroid secretion in hGCs and KGN cells. In parallel, it also decreased IGF-1-induced p450 aromatase protein levels without affecting the protein levels of other factors involved in steroidogenesis (steroidogenic acute regulatory protein, 3-beta-hydroxysteroid dehydrogenase and p450 side-chain cleavage enzyme) in hGCs cells. All these changes were associated with a decrease in the IGF-1-induced tyrosine phosphorylation of IGF-1 receptor beta subunit and phosphorylation of mitogen-activated protein kinase extracellular signal-regulated kinases 1/2 (MAPK ERK1/2) and Akt. In hGCs and KGN cells, rhChem also decreased IGF-1-induced thymidine incorporation. Finally, we showed that rhChem rapidly activates MAPK ERK1/2, MAPK P38 and Akt phosphorylation and more slowly AMP-activated protein kinase phosphorylation under basal conditions (no IGF-1 or FSH) in primary hGC cells.

CONCLUSIONS: Taken together, chemerin and its receptor (CMKLR1) are present and active in hGCs. Chemerin reduces IGF-1-induced steroidogenesis and cell proliferation through a decrease in the activation of IGF-1R signalling pathways in primary hGCs.

Key words: adipokine / ovary / steroidogenesis / growth factors / signalling pathways

Introduction

Adipose tissue (AT) is not only an energy storage organ but it is also able to secrete a number of hormone-like compounds. Among the main endocrine products of the AT are the proteins: leptin, adiponectin, resistin, visfatin and chemerin. Chemerin, also known as retinoic acid receptor responder protein 2 (RARRES2), is a novel adipokine that regulates adipocyte development and metabolic function (Roh *et al.*, 2007). It is a 16 kDa protein secreted in an inactive form as prochemerin and is activated through cleavage of the C-terminus by inflammatory and coagulation serine proteases (Zabel *et al.*, 2005). In humans, the plasma chemerin levels (~100–200 ng/ml, Bozaoglu *et al.*, 2010; Hu and Feng, 2011) are correlated with body fat, glucose and lipid metabolism, and inflammation. For example, plasma chemerin levels in patients who had undergone bariatric surgery for the purpose of weight loss were significantly reduced after surgery, and correlated with BMI and fat mass (Ress *et al.*, 2010; Sell *et al.*, 2010). A majority of human data indicate that plasma chemerin is elevated in obesity/diabetes (Bozaoglu *et al.*, 2007) and in inflammatory states (Pasceri and Yeh, 1999; Wittamer *et al.*, 2003). Recent animal studies reported the parallel findings that obese and diabetic mice have elevated circulating levels of

chemerin (Ernst *et al.*, 2010; Parlee *et al.*, 2010). Chemerin acts through its receptor, the G protein-coupled receptor, CMKLR1 (chemokine-like receptor 1), (Gantz *et al.*, 1996). Activation of CMKLR1 with its ligands results in intracellular calcium release, inhibition of cAMP accumulation and phosphorylation of mitogen-activated protein kinase extracellular signal-regulated kinases 1/2 (MAPK ERK1/2), through the Gi class of heterotrimeric G proteins (Yoshimura and Oppenheim, 2011). CMKLR1 is also able to activate the phosphatidylinositol 3-kinase/Akt signalling pathway (Yoshimura and Oppenheim, 2011).

During the last few years, new roles of adipokines have been emerging in the field of fertility and reproduction. Indeed, leptin, first described over a decade ago, has been thoroughly studied regarding reproduction and at several levels of the reproductive axis, from the pituitary to gonads (Moschos *et al.*, 2002). Adiponectin and adiponectin receptors (AdipoR1 and AdipoR2) are expressed in many reproductive tissues, including the central nervous system, ovaries, oviduct, endometrium and testes (Mitchell *et al.*, 2005). They influence gonadotrophin release in pituitary cells, steroid secretion in ovarian cells, normal pregnancy and assisted reproduction outcomes. In humans, high levels of adiponectin are associated with an improved menstrual function and better outcomes in assisted reproductive cycles (Michalakis and Segars, 2010). We previously identified resistin in rat and bovine ovary and showed that it can modulate *in vitro* granulosa cell steroidogenesis and proliferation in a species-specific manner (Maillard *et al.*, 2011). In humans, the precise reproductive role of resistin remains controversial. Indeed, Seow *et al.* (2005) showed that resistin was not a major determining factor in the growth and maturation of oocytes during ovarian stimulation, whereas Chen *et al.* (2007) demonstrated a negative correlation between serum resistin levels and the number of oocytes retrieved during IVF. Chemerin and CMKLR1 have been shown to be expressed in mouse ovary and placenta and in human placenta (Goralski *et al.*, 2007).

However, to date, no studies have described the presence of chemerin and CMKLR1 in the human ovary. In the present study, we identified chemerin and CMKLR1 in human ovarian follicles. We also investigated the effects of human recombinant chemerin on steroid production and on the activation of various signalling pathways in granulosa cells from women undergoing IVF and in KGN cells.

Materials and Methods

Patients

Twenty-two infertile women (36 ± 4 years old) attending the IVF unit of the department for ICSI cycles were recruited in 2011. The aetiology of infertility was mechanical, unexplained or male factor infertility without any known endocrinopathy [polycystic ovarian syndrome (PCOS), hyperprolactinemia, hypo-and-hyperthyroidism]. Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. From each patient, only one cycle was included in the study.

KGN cell culture

The human ovarian granulosa-like tumour cell line, KGN, was obtained from Drs Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan (Nishi *et al.*, 2001). KGN cells are undifferentiated and maintain physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of aromatase. They were cultured in Dulbecco's minimal essential medium/F12 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin obtained from Sigma) in a 5% CO_2 atmosphere at 37°C.

Hormones and reagents

Purified ovine FSH-20 (oFSH; lot no. AFP-7028D, 4453 IU/mg, FSH activity = 175 times the activity of oFSH-S1) used for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA. Recombinant human insulin-like growth factor-I (IGF-I) was from Sigma. Recombinant human chemerin (rhChem) was obtained from R&D (Lille, France). Human ovary sections embedded in paraffin were obtained from Euromedex (Souffelweyersheim, France).

Antibodies

Mouse monoclonal antibody to human chemerin for western blot was obtained from R&D system. Rabbit polyclonal antibodies to chemerin for immunohistochemistry were purchased from Phoenix France SAS (Strasbourg, France). Rabbit polyclonal antibodies to chemerin receptor (CMKLR1), phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser 473) and phospho-AMP-activated protein kinase (AMPK) alpha Thr172 were purchased from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to ERK2 (C14) and p38 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to AMPKα1 were obtained from Upstate Biotechnology Inc (Lake, Placid, NY, USA). Mouse monoclonal antibodies to Vinculin and p450 aromatase were obtained from Sigma and Serotec (Varilhes, France), respectively. Rabbit polyclonal antibodies against p450 side-chain cleavage enzyme (p450scc), steroidogenic acute regulatory (StAR) and 3-beta-hydroxysteroid dehydrogenase (3-β-HSD) were generously provided by Dr Dale Buchanan Hales (University of Illinois, Chicago, IL, USA) and Dr Van Luu-The (CHUL Research Center and Laval University, Canada), respectively. All antibodies were used at 1/1000 dilution in western blotting.

Isolation and culture of human granulosa cells

Granulosa cells were collected from pre-ovulatory follicles during oocyte retrieval for IVF. The ovarian stimulation protocol and IVF and ICSI procedures used have already been reported (Guerif *et al.*, 2004). After isolation of cumulus–oocyte complexes (used for IVF), follicular fluids were pooled and centrifuged (400*g*, 10 min). To remove most of the red blood cells, the pellet was centrifuged (400*g*, 20 min) on a two layer, discontinuous Percoll gradient (40%, 60% in Ham's medium, Gibco-BRL; Life Technologies, Cergy Pontoise, France). The 40% fraction was collected and treated with a haemolytic medium (NH_4Cl 10 mmol/l in Tris–HCl, 10 mmol/l, pH 7.5; Sigma, Isles d'Abeau, France) to remove as many as possible of the remaining red blood cells. Following centrifugation, the pellet was washed with a fresh medium (Hams F12); cells were counted in a haemacytometer and cell viability was determined using Trypan Blue dye exclusion. Cells were cultured in McCoy's 5A medium supplemented with 20 mmol/l HEPES, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine [3 mmol/l, 0.1% bovine serum albumin (BSA), 0.1 μmol/l androstenedione, 5 mg/l transferrin, 20 μg/l selenium] and 5% fetal bovine serum (FBS). The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO_2 at 37°C. We performed four cultures (one per week). Each culture was

performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in quadruplicate or duplicate, as indicated in the legend of figures.

RNA isolation and RT–PCR

Total RNA was extracted from human granulosa cells (hGCs), human ovarian granulosa tumour-derived cell line cells (KGN) and from human visceral or subcutaneous adipose tissue (Vis AT, Sc, AT), using Trizol reagent according to the manufacturer's procedure (Invitrogen). RT–PCR was performed to assay the expression of chemerin and CMKLR1 in hGCs and KGN cells and in Vis and Sc AT. RT of total RNA (1 μg) was carried out in a 20 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ, USA), 50 pmol of oligo(dT)15, 5 U of ribonuclease inhibitor and 15 U of Moloney murine leukemia virus reverse transcriptase. RT reaction was performed at 37°C for 1 h. The sets of primers for chemerin and CMKLR1 were: forward chemerin 'agacaagctgccggaagagg', reverse chemerin 'tggagaaggcgaactgtcca', forward CMKLR1 'acttcctgttcaacgtcttc' and reverse CMKLR1 'cagcttgtaga caatggtga'. PCRs were carried out using 2 μl of the RT reaction mixture in a volume of 50 μl containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U of Taq polymerase. The samples were processed for 35 PCR cycles (95°C, 1 min; 58°C, 1 min; 72°C, 1 min), with a final extension step at 72°C for 10 min. PCR products were visualized in an agarose gel (1.5%) stained with ethidium bromide, and the DNAs were extracted from the agarose using the gel extraction kit QIAEX II (Qiagen, Hilden, Germany) and sequenced by the Genome Express company (Meylan, France). PCR amplifications with RNA were performed in parallel as negative controls. RT–PCR consumables were purchased from Sigma (Isles d'Abeau), except that Moloney Murine Leukemia Virus reverse transcriptase and RNase inhibitor (RNasin) were from Promega (Madison, WI, USA).

Immunoprecipitation and immunoblotting

KGN cells, freshly collected hGCs purified on Percoll gradient and *in vitro* cultured hGCs and adipose tissues (Sc and Vis AT) were homogenized as previously described (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). Lysates were incubated on ice for 30 min and then centrifuged at 12 000*g* for 20 min at 4°C. The protein concentration in the resulting supernatants was then determined using the bicinchoninic acid protein assay.

To measure IGF-1Rβ tyrosine phosphorylation, lysates containing 100 μg of protein were incubated with IGF-1Rβ antibodies (1:1000 dilution) for 16 h at 4°C. Immune complexes were precipitated by incubation with protein G-agarose for 1 h at 4°C as described previously (Dupont *et al.*, 2000). Immunoprecipitates were subjected to SDS–PAGE and transferred to nitrocellulose membranes. Blots were blocked by incubation for 1 h at room temperature with 5% BSA dissolved in Tris-buffered saline supplemented with 0.1% Tween-20 and then probed with PY20 antibodies (1:1000 dilution). After extensive washing, immune complexes were detected using horse-radish peroxidise (HRP)-conjugated secondary antibodies (Amersham Biosciences) and an enhanced chemiluminescence detection system. Blots were then stripped and re-probed with IGF-1Rβ antibodies. The radiographs were scanned, and the optical density of each band was measured using the software Scion Image (4.0.2 version). In order to determine MAPK ERK1/2, Akt, AMPK phosphorylation levels and the amount of P450 aromatase, CMKRL1 and chemerin, the lysates (50 μg of protein) were then directly subjected to electrophoresis on 10% (w:v) SDS–PAGE under reducing conditions and transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France). Membranes were incubated overnight at 4°C with appropriate antibodies (final dilution 1:1000). The results are expressed as the intensity signal in arbitrary units after normalization allowed by the presence of MAPK ERK2, Akt, AMPK total (for ERK1/2, Akt and AMPK phosphorylation, respectively) and vinculin (for p450 aromatase) as an internal standard. The effect of rhChem was analysed on three or four different primary hGC cultures. Each primary culture was derived from a different patient.

Immunohistochemistry

Human ovary sections were deparaffinized, hydrated and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France), and then allowed to cool to room temperature. After washing in a phosphate-buffered saline (PBS) bath for 5 min, sections were immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). After two washes in a PBS bath for 5 min, non-specific background was eliminated by blocking with 5% lamb serum in PBS for 20 min, followed by incubation overnight at 4°C with PBS containing rabbit primary antibody raised against either chemerin (1:100) or CMKLR1 (1:100). Sections were washed twice for 5 min each time in a PBS bath and were incubated for 30 min at room temperature with a 'ready to use' labelled polymer-HRP anti-rabbit (DakoCytomation Envision Plus HRP system, Dako, Ely, UK). The sections were then washed twice in PBS and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System, DakoCytomation). Negative controls involved replacing primary antibodies with rabbit immunoglobulin G (IgG).

Thymidine incorporation into granulosa cells

Primary hGCs (2×10^5 viable cells/500 μl) were cultured in McCoy's 5A medium and 10% FBS during 48 h and were then serum-starved for 24 h followed by the addition of 1 μCi/μl of [^{3}H]-thymidine (Amersham Life Science, Arlington Heights, IL, USA) in the presence or absence of chemerin and/or IGF-1 (10^{-8} M). After 24 h of culture, the excess of thymidine was removed by washing the cells twice with PBS and then they were fixed with cold trichloroacetic acid 50% for 15 min and lysed by NaOH 0.5 N. The radioactivity was determined by scintillation fluid (Packard Bioscience) counting in a β-photomultiplier.

Progesterone and estradiol radioimmunoassay

The concentration of progesterone and estradiol (E_2) in the culture medium of KGN and hGCs cells was measured after 48 h of culture by a radioimmunoassay protocol as previously described (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). The limit of detection of progesterone was 12 pg/tube (60 pg/well), and the intra- and inter-assay coefficients of variation were <10 and 11%, respectively. The limit of detection of E_2 was 1.5 pg/tube (7.5 pg/well), and the intra-and inter-assay coefficients of variation were <7 and 9%, respectively. Results were expressed as the concentration of steroids/cell protein concentration/well. Results are means ± SEM of data obtained from KGN cells (at four different passages) and primary hGCs (four cultures, one per week). Each primary hGCs culture was performed pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in quadruplicate.

Chemerin plasma and follicular fluid levels

Chemerin plasma and follicular fluid concentrations were measured using a human chemerin ELISA kit (BioVendor, Heidelberg, Germany). This ELISA

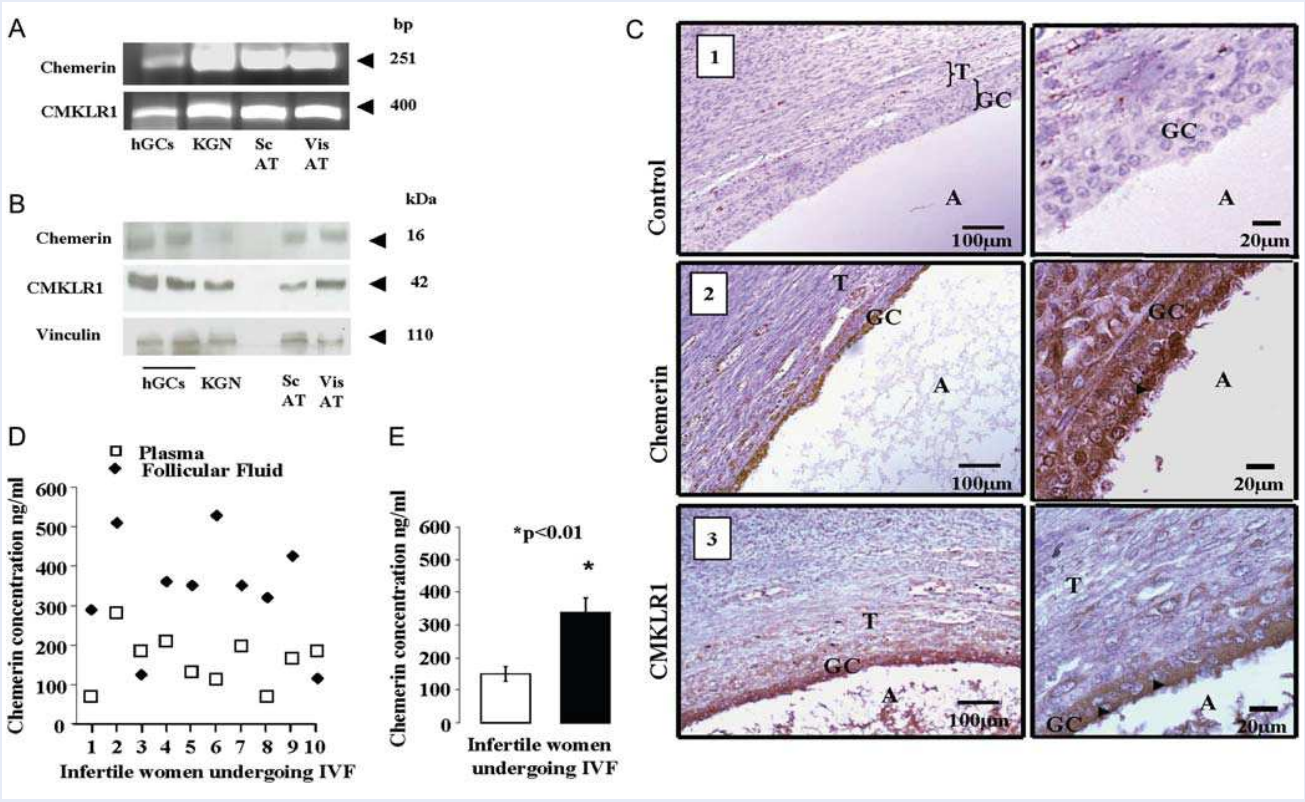

Figure 1 Chemerin and CMKLR1 expression in hGCs and measurement of the chemerin concentration in plasma and follicular fluid of women treated with IVF. (**A**) Total RNA from fresh hGCs obtained from women undergoing IVF (hGCs) and human ovarian granulosa tumour-derived cell line (KGN) was extracted as described in 'Materials and Methods' section. RT–PCR was performed by using primers designed to amplify fragments of chemerin (251 pb) and CMKLR1 (400 pb). Human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues were used as a positive control for chemerin and CMKLR1 expression [mRNA (A) and protein (B, see below)]. Tissues or cells from four different patients were used and two replications within patient were performed. (**B**) Protein extracts (50 µg) were resolved by SDS–PAGE, transferred to a nitrocellulose membrane and incubated with specific antibodies against chemerin and CMKLR1. Equal protein loading was verified by reprobing membrane with an anti-vinculin antibody. Tissues or cells from four different patients were used and two replications within patient were performed. (**C**) Localization of chemerin and CMKLR1 in human ovarian follicle by immunohistochemistry. The three panels on the right-hand side are higher magnifications of part of the panels on the left. DAB-immunoperoxidase staining was performed on paraffin-embedded human ovary using antibodies against chemerin (2), CMKLR1 (3) or no primary antibodies but rabbit IgG (1). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. Chemerin and CMKLR1 are detected in granulosa cells (GCs) and theca cells (T) of ovarian follicle. A, antrum. Bars = 100 or 20 µm. Immunohistochemistry was performed on two different human ovary slides from each of four patients (**D** and **E**). Plasma (□) and follicular fluid (◆) levels of chemerin after collecting blood and follicular fluid of 10 infertile women undergoing *in vitro* fertilization, on the day of oocyte retrieval. Each concentration was determined by ELISA method as described in 'Materials and Methods' section. The individual data (D) or the mean of these data (E) are represented. *$P < 0.05$.

kit does not distinguish between active chemerin and inactive prochemerin.

Statistical analysis

All experimental data are presented as the mean ± SEM. A *t*-test was used for statistical comparison of the means between two groups. A one-way analysis of variance (ANOVA) was used for multiple comparisons involving more than two treatment groups. The Student–Newman–Keuls test was used for *post hoc* comparison of the significant ANOVA. A $P < 0.05$ was considered significant (in each experiment, '*n*' is taken as the number of cultures).

Results

Chemerin and CMKLR1 expression in hGCs

We determined the expression of chemerin and its receptor CMKLR1 in fresh hGCs and in the hGC line KGN. As shown in Fig. 1A, we amplified by RT–PCR two cDNAs corresponding to the fragments of chemerin (251 pb) and CMKLR1 (400 pb). Vis AT was used as a positive control for chemerin and CMKLR1 expression. The transcripts of chemerin and CMKLR1 were largely expressed in granulosa cells and as expected in ATs (Fig. 1A). Immunoblotting protein extracts revealed one band corresponding to chemerin (16 kDa) and another band corresponding to CMKLR1 (42 kDa), showing

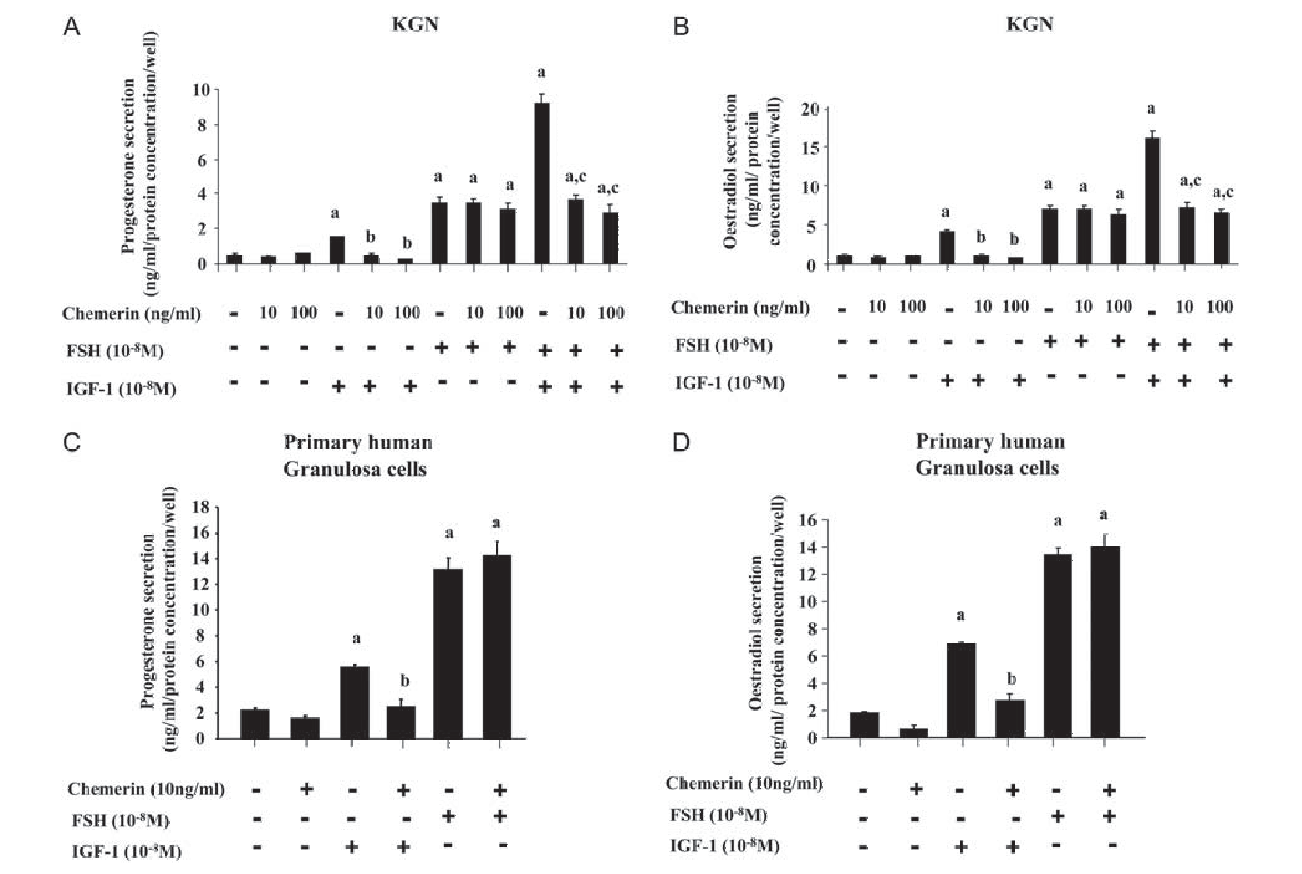

Figure 2 Effect of rhChem on basal and FSH and/or IGF-1-stimulated progesterone and E_2 secretions by KGN (**A**, **B**) and primary hGCs (**C**, **D**) hGCs (KGN and primary hGCs) were cultured in medium with serum and then in serum-free medium in the absence or in the presence of chemerin (10 and/or 100 ng/ml) ± FSH (10^{-8} M) and/or IGF-1 (10^{-8} M) for 48 h as described in 'Materials and Methods' section. The culture medium was collected, and progesterone (A, C) and E_2 (B, D) production was measured by radioimmunoassay and the data are represented as the progesterone or E_2 concentration (ng/ml)/cellular protein concentration/well. Results are means ± SEM of the four cultures of primary hGCs and four independent experiments (at four different passages) for KGN cells. For primary hGCs, each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in quadruplicate. $^{a}P < 0.05$ compared with the untreated control, $^{b}P < 0.05$ compared with the IGF-treated, $^{c}P < 0.05$ compared with the IGF/FSH-treated samples.

that chemerin and its receptor are produced in human ovary, and, in particular, in fresh granulosa cells (Fig. 1B). Immunohistochemistry with human ovarian sections confirmed the presence of chemerin and CMKLR1 in human follicles (Fig. 1C). CMKLR1 and its ligand were detected in granulosa cells and less abundantly in theca cells (Fig. 1C). At the cellular level, the staining for chemerin was mostly cytoplasmic (Fig. 1C2). For CMKLR1, the staining was cytoplasmic and also at the membrane level (see arrow Fig. 1C3). Thus, chemerin and its receptor (CMKLR1) are expressed in human ovary and more particularly in granulosa cells.

Chemerin plasma and follicular fluid levels in women

We measured the concentration of chemerin in plasma and follicular fluid of 10 infertile women. As shown in Fig. 1D, chemerin level was at least 2-fold higher in follicular fluid than in plasma in 8 of 10 subjects by pooling cells (Fig. 1D, $P < 0.05$).

Effect of human recombinant chemerin on basal and FSH- or IGF-1-stimulated progesterone and E_2 production in KGN and human primary granulosa cells

We next investigated the effects of chemerin treatment on steroidogenesis in KGN and primary hGCs. Cells were incubated in serum-free medium with human recombinant chemerin (10 or 100 ng/ml) for 48 h in the presence or absence of FSH (10^{-8} M) and/or IGF-1 (10^{-8} M). As expected, FSH and IGF-1 treatment alone increased progesterone and E_2 secretion in KGN cells (Fig. 2A and B, Pierre *et al.*, 2009) and primary hGCs cells (Fig. 2C and D, Chabrolle *et al.*, 2009). In KGN cells, this effect was enhanced when FSH and IGF-1 were combined (Fig. 2A and B, $P < 0.001$). In these latter cells, the IGF-1-induced secretion of progesterone (Fig. 2A, $P < 0.001$) and E_2 (Fig. 2B, $P < 0.001$) in the absence or in the presence of FSH was decreased by 3-fold with 10 and 100 ng/ml chemerin treatments, whereas in the absence of IGF-1 chemerin (10

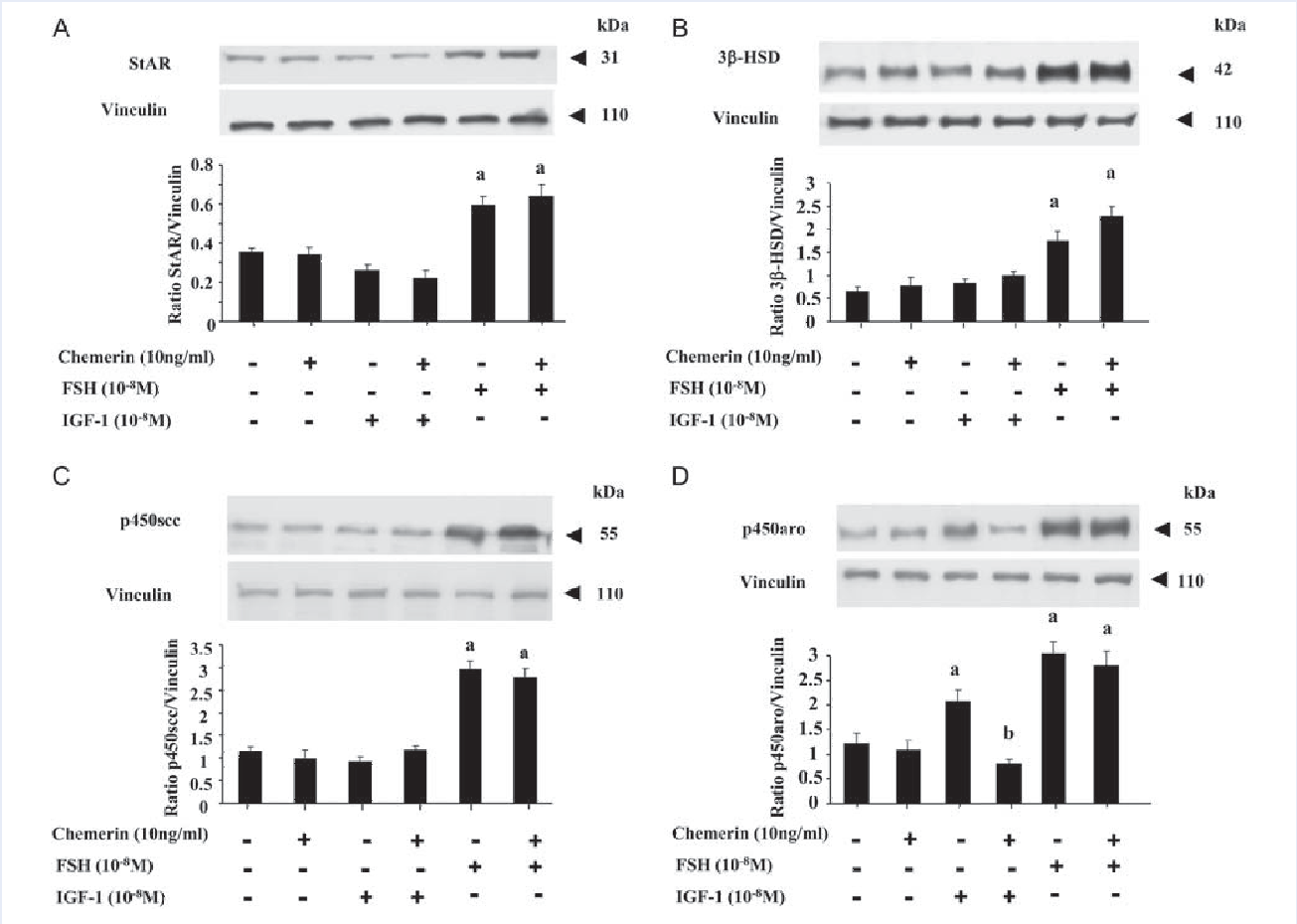

Figure 3 The effect of human recombinant chemerin on the amount of StAR, 3-β-HSD, p450scc and p450 aromatase proteins in primary hGCs. Protein extracts from primary hGCs, cultured for 48 h in the absence or in the presence of chemerin (10 ng/ml) ± FSH (10^{-8} M) or IGF-1 (10^{-8} M), were submitted to SDS–PAGE as described in 'Materials and Methods section. The membranes were probed with antibodies against StAR, 3-β-HSD, p450scc or p450 aromatase. Equal protein loading was verified by reprobing membranes with an anti-vinculin antibody. Blots were quantified and the StAR, 3-β-HSD, p450scc and p450 aromatase/vinculin ratios are shown. Results are representative of at least four cultures of granulosa cells. Each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in duplicate. The results are expressed as mean ± SEM. $^{a}P < 0.05$ compared with the untreated control, $^{b}P < 0.05$ compared with the IGF-treated.

and 100 ng/ml) had no effect on steroid production (Fig. 2A and B). In primary hGCs, similar results were observed with chemerin 10 ng/ml in response to IGF-1 (Fig. 2C and D). We confirmed these data with chemerin 100 ng/ml (data not shown). Thus, chemerin decreased IGF-1-stimulated progesterone and E_2 production in hGCs.

Then, we investigated the effects of human recombinant chemerin on the amount of the StAR protein, an important cholesterol carrier, and on the protein amount of three key enzymes of steroidogenesis, p450scc, 3-β-HSD and p450 aromatase in the primary hGCs cells. Chemerin treatment (10 ng/ml) for 48 h did not affect the protein level of StAR, p450scc and 3-β-HSD in basal state or in response to IGF-1 or FSH (Fig. 3A–C). By contrast, chemerin decreased the amount of the p450 aromatase in the presence of IGF-1 ($P < 0.05$, Fig. 3D), but had no significant effect in its absence.

Effects of human recombinant chemerin on hGC proliferation and viability in response to IGF-1

We also studied the effect of human recombinant chemerin on the number of KGN and primary hGCs in response to IGF-1. We determined [^{3}H]-thymidine incorporation and cell viability of primary hGCs treated with 10 or 100 ng/ml chemerin for 24 h in the presence or in the absence of IGF-1 (10^{-8} M). As expected, IGF-1 treatment significantly increased [^{3}H]-thymidine incorporation in KGN (Fig. 4A) (Pierre *et al.*, 2009) and primary granulosa cells (Fig. 4B) (Chabrolle *et al.*, 2009). In KGN cells, chemerin treatment (10 and 100 ng/ml) significantly reduced IGF-1-induced thymidine incorporation ($P < 0.05$, Fig. 4A). Similar results were observed with chemerin (10 ng/ml) in human primary granulosa cells (Fig. 4B). As revealed by the staining with trypan blue, chemerin (10 or 100 ng/ml) had no effect

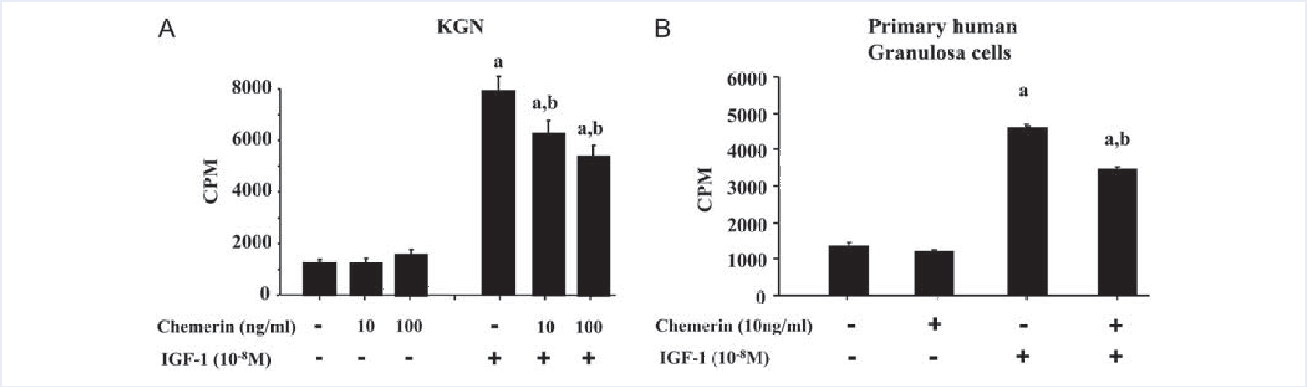

Figure 4 The effect of human recombinant chemerin on hGCs thymidine incorporation. Thymidine incorporation was determined in KGN (**A**) and primary hGCs (**B**) cultured for 24 h in the presence or absence of human recombinant chemerin (10 or 100 ng/ml) ± IGF-1 (10^{-8} M) as described in 'Materials and Methods' section. Results are representative of at least four cultures of granulosa cells. For primary hGCs, each culture was performed by pooling cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in quadruplicate. The results are expressed as mean ± SEM. $^{a}P < 0.05$ compared with the untreated control, $^{b}P < 0.05$ compared with the IGF-treated.

on the cell viability in the absence or in the presence IGF-1 in both KGN and primary hGCs cells (data not shown). Thus, human recombinant chemerin decreased IGF-1-induced thymidine incorporation without affecting the viability of hGCs.

Effect of human recombinant chemerin on signalling pathways in primary hGCs

Since chemerin treatment (10 ng/ml) significantly decreased IGF-1-induced steroidogenesis and proliferation in primary hGCs. We next investigated whether some components of IGF-1R signalling could be affected by human recombinant chemerin in these cells. Cells were incubated in serum-free medium with human recombinant chemerin (10 ng/ml) for 48 h in presence or absence of IGF-1 (10^{-8} M) or FSH (10^{-8} M) (the same conditions than those used to determine the effect of chemerin on progesterone and E_2 production). IGF-1 treatment (10^{-8} M, 48h) significantly increased IGF-1 receptor beta subunit (Fig. 5A), MAPK ERK1/2 (Fig. 5B) and Akt (Fig. 5C) phosphorylation levels when compared with the basal state (no IGF-1 or FSH), whereas FSH treatment (10^{-8} M, 48 h) significantly increased only MAPK ERK1/2 phosphorylation (Fig. 5A–C). As shown in Fig. 5A–C chemerin treatment (10 ng/ml, 48 h) totally abolished the IGF-1 effects on these signalling components, whereas it did not affect those stimulated by FSH. Thus, the negative effect of chemerin on IGF-1-induced steroidogenesis is probably due primarily to a reduction in the amount of IGF-1 receptor and consequently an inhibition of the activation of the downstream elements of IGF-1R.

Several adipokines including adiponectin, resistin and leptin have been shown to activate or inhibit AMPK, MAPK and Akt in various cell types ((Hegyi *et al.*, 2004; Chabrolle *et al.*, 2009; Pierre *et al.*, 2009; Maillard *et al.*, 2011). Thus, we examined which signalling pathways could be activated by chemerin (10 ng/ml) in primary hGCs. As shown in Fig. 6A–C, human recombinant chemerin (10 ng/ml) rapidly (after 1 min of stimulation), but transiently significantly increased, phosphorylation of MAPK ERK1/2 (Fig. 6A), MAPK P38 (Fig. 6B) and Akt (Fig. 6C), whereas it significantly increased more slowly phosphorylation of AMPK (after 30 min of stimulation, Fig. 6D). Thus, human recombinant chemerin activates MAPK (ERK1/2 and P38), Akt and AMPK signalling pathways in hGCs.

Discussion

In the present study, we report for the first time the presence of chemerin and CMKLR1 in human ovarian follicles and in the human ovarian granulosa-like tumour cell line, KGN. In primary hGCs and KGN cells, rhChem decreased IGF-1-induced thymidine incorporation and progesterone production without any variation of StAR, p450scc and 3-β-HSD-protein levels. Chemerin also decreased IGF-1-induced E_2 secretion and p450 aromatase protein level. All these data were associated with an inhibition of the IGF-1-induced tyrosine phosphorylation of IGF-1R beta subunit and phosphorylation of MAPK ERK1/2 and Akt.

We found chemerin in human ovarian follicles. This result is in good agreement with data from Nagpal *et al.* (1997) that detected TIG2 (another name of chemerin) by northern blot in human ovary. We have found chemerin in human follicular fluid. Moreover, in 8 out of 10 patients, we observed a higher concentration in human follicular fluid than in plasma on the day before the oocyte pick-up. As a limitation, it is recognized that the ELISA assay used in our study to measure plasma and follicular fluid levels of chemerin does not distinguish between active chemerin and inactive prochemerin. Thus, total measured chemerin concentrations might not equate to the actual amount of active chemerin. Follicular fluid is in part an exudate of serum and in part the product of ovary activity. Chemerin has been found in haemofiltrate and it is particularly abundant in ascites fluid from ovarian cancer patients (1.8–7 nM) (Wittamer *et al.*, 2003). We have showed that the messenger RNA and the protein of chemerin are expressed in human granulosa and theca cells. Thus, we can hypothesize that ovarian cells are able to produce chemerin. Other adipokines including leptin, adiponectin and resistin have already been detected in follicular fluid. Indeed, Gurbuz *et al.* (2005)

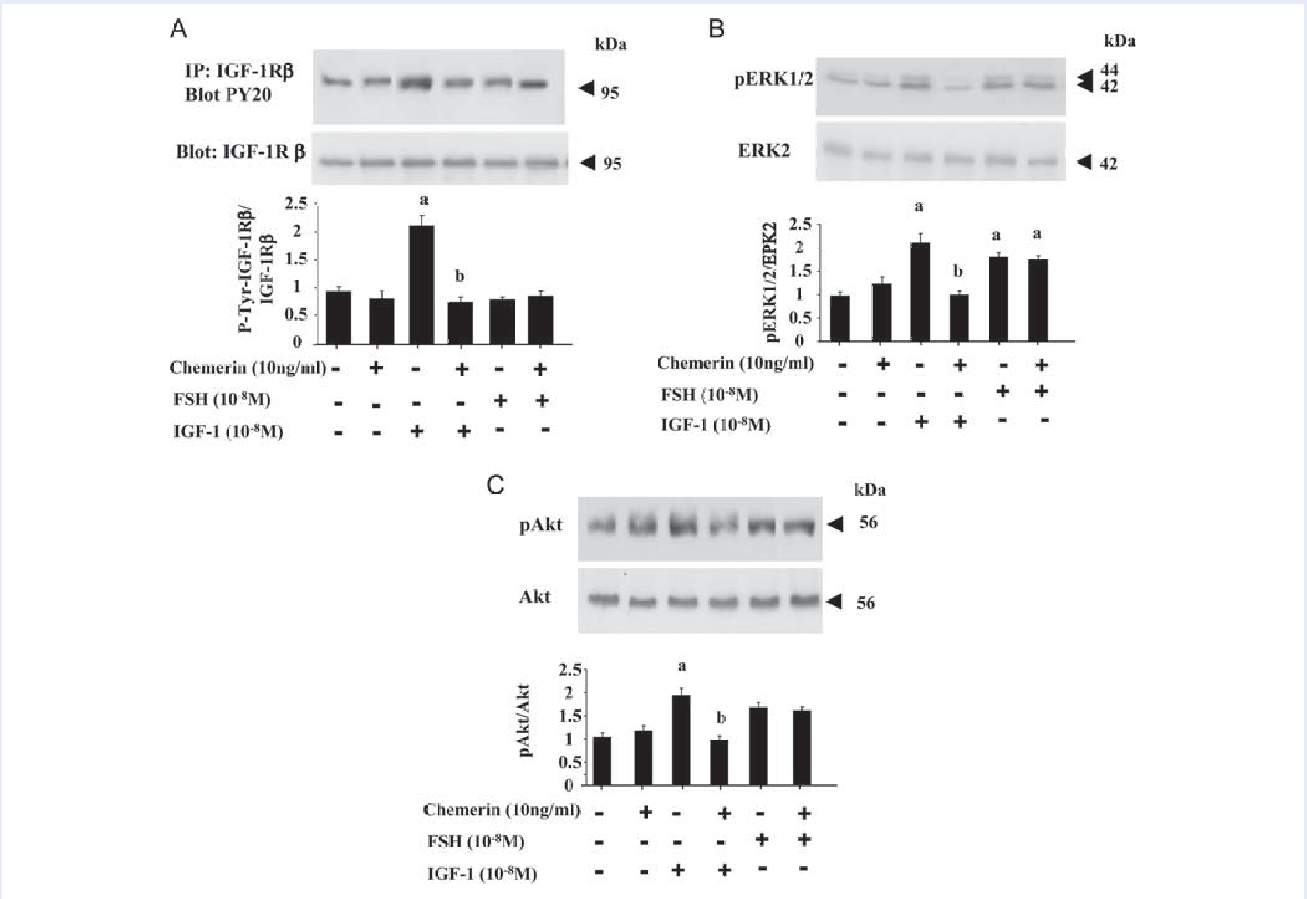

Figure 5 The effect of human recombinant chemerin on phosphorylation of IGF-1R β subunit, MAPK ERK1/2 and Akt in response to IGF1 or FSH in primary hGCs. Primary hGCs were cultured in a medium with serum and then in a serum-free medium in the absence or in the presence of chemerin (10 ng/ml) ± FSH (10^{-8} M) or IGF-1 (10^{-8} M) for 48 h (conditions used to measure progesterone and E_2 production). Cells were lysed, and tyrosine phosphorylation of IGF-IRβ was determined. IGF-1Rβ (**A**) was immunoprecipitated from whole cell lysates. Samples were then subjected to western blotting with antibodies recognizing phosphotyrosine (PY20). IGF-1Rβ levels were evaluated by reprobing the membranes with IGF-1Rβ antibodies. To determine MAPK ERK1/2 and Akt phosphorylation, cell lysates were directly subjected to immunoblotting with anti-phospho-MAPK ERK1/2 (**B**) or phospho-Akt (**C**) antibodies and then with anti-ERK2 or Akt protein antibodies. Representative blots from four different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in duplicate. Blots were quantified and the phosphorylated protein/total protein ratio is shown. The results are represented as means ± SEM. (i) $P < 0.05$ compared with the untreated control, (ii) $P < 0.05$ compared with the IGF-treated.

have observed similar levels of leptin in serum and in follicular fluid, whereas Chen *et al.* (2004) found higher follicular than serum leptin levels on the day of ovum pick up in women receiving IVF. Seow *et al.* (2005) have shown that the follicular fluid resistin levels were significantly lower than serum resistin levels in both the control and PCOS groups. In contrast, we have previously observed in six patients a higher concentration of adiponectin in human follicular fluid than in plasma on the day before the oocyte pick-up (Chabrolle *et al.*, 2009). The growth of the ovarian follicle and the oocyte maturation involve various autocrine and paracrine factors (Richards *et al.*, 2002). The fact that we observed a higher concentration of chemerin in human follicular fluid than in plasma on the day before the oocyte pick-up in 8 out of 10 patients may indicate that this hormone has a paracrine effect in the process of oocyte development in women receiving IVF. Indeed, it has been shown for other cytokines including leptin, tumour necrosis factor-α and interleukin-6 that their follicular fluid levels affect the success of fertilization and early embryonic development (Brannstrom and Norman, 1993; Adashi, 1994), including in women with PCOS (Mantzoros *et al.*, 2000).

By immunohistochemistry, we found that chemerin and CMKLR1 are expressed in both granulosa and theca cells and also in KGN cells. We have previously shown that adiponectin was almost undetectable in fresh hGCs and KGN cells, whereas AdipoR1 and AdipoR2 were present (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). Karamouti *et al.* (2003) did not find leptin mRNA transcripts in primary hGCs, whereas Abir *et al.* (2005) detected them as well as its receptors. Thus, we can speculate endocrine but also autocrine and/or paracrine effects for adipokines, in hGCs. In the present study, we showed that rhChem (10 ng/ml) treatment rapidly increased MAPK ERK1/2, p38, Akt and later AMPK phosphorylation,

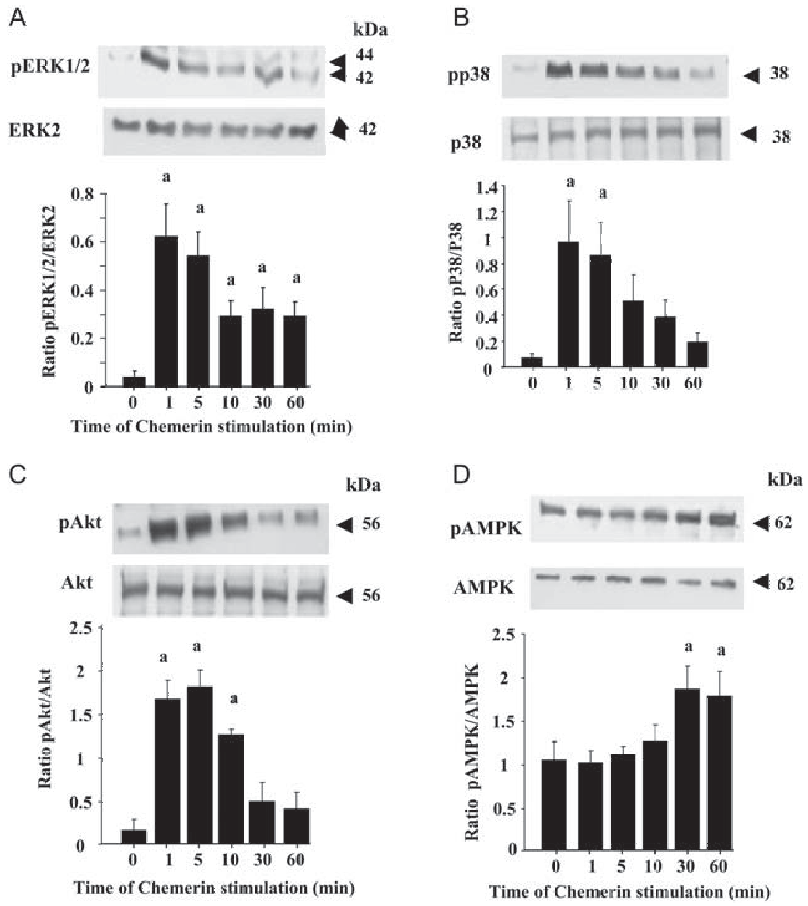

Figure 6 Effect of human recombinant chemerin on phosphorylation of MAPKs (ERK1/2 and P38), Akt and AMPK in primary hGCs Cell lysates were prepared from primary hGCs incubated with chemerin 10 ng/ml for various times: 0, 1, 5, 10, 30 or 60 min. Lysates (50 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-MAPK ERK1/2 (**A**), anti anti-phospho-P38 (**B**), anti-phospho-Akt (**C**) or anti-phospho-AMPK (**D**) and then with anti-ERK2, P38, Akt or AMPK protein antibodies. Representative blots from three different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (chemerin in the presence or in the absence of IGF-1 or FSH) was applied in duplicate. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as means ± SEM. $^{a}P < 0.05$ compared with the untreated control.

suggesting that CMKLR1 is functional in primary hGCs. Similar signalling pathways have already been activated in response to adiponectin, resistin or leptin in granulosa cells of various species (Chabrolle *et al.*, 2009; Maillard *et al.*, 2011).

As previously reported, we have shown that IGF-1 and FSH increased progesterone and E_2 production by hGCs and this was improved when both IGF-1 and FSH were combined (Bergh *et al.*, 1991; Chabrolle *et al.*, 2009). Furthermore, we observed here that rhChem inhibited IGF-1-induced progesterone and E_2 secretion in the absence or in the presence of FSH. In the absence of FSH, this was associated with a decrease in the IGF-1-induced tyrosine phosphorylation of IGF-1R beta subunit, and phosphorylation of MAPK ERK1/2 and Akt. From our results, we do not know whether the effects of chemerin on IGF-1R-mediated responses are due to chemerin activation of CMKLR1 or chemerin antagonism of the IGF-1R. In humans, it has been reported that higher chemerin release is associated with insulin resistance at the level of lipogenesis and insulin-induced antilipolysis in adipocytes (Sell *et al.*, 2009). Furthermore in this latter study, chemerin-induced insulin resistance in human skeletal muscle cells at the level of insulin receptor substrate 1, Akt and glycogen synthase kinase 3 phosphorylation and glucose uptake (Sell *et al.*, 2009). Thus, chemerin could antagonize the action of IGF-1, which plays a key role in the development of ovarian follicles (Adashi, 1998). Several reports indicate that the MAPK ERK1/2 signalling pathway positively regulates progesterone and E_2 production in cultured rat or hGCs (Seto-Young *et al.*, 2003; Tosca *et al.*, 2005). Thus, chemerin could decrease IGF-1-induced steroidogenesis by inhibiting MAPK ERK1/2 phosphorylation. In the present study, we observed that chemerin reduced IGF-1-induced thymidine incorporation in KGN and human primary granulosa cells. This could be due to the inhibition of the Akt signalling pathway in response to IGF-1. Indeed, this signalling pathway is involved in the proliferation of hGCs (Goto *et al.*, 2009). We have shown that chemerin reduces IGF-1-induced progesterone secretion without affecting

the protein levels of the cholesterol carrier, StAR and of p450scc and 3-β-HSD, two steroidogenesis enzymes. However, this could be due to an inhibition of the p450scc and 3-β-HSD enzyme activities that we did not measure. In contrast, we observed that chemerin decreased IGF-1-induced p450 aromatase protein level, which could explain the inhibitory effect of chemerin on IGF-1-induced E_2 secretion.

It was reported that circulating and AT chemerin levels increased in PCOS patients versus control (Tan *et al.*, 2009). The PCOS syndrome is the most common cause of anovulation and infertility, affecting 5–10% of women of reproductive age. It is characterized by hyperandrogenism, chronic anovulation, and, occasionally, obesity. In our present study, we showed that chemerin and CMKLR1 are expressed in hGCs and less abundantly in theca cells. It will be interesting to determine the expression and the functionality of chemerin and CMKLR1 in granulosa cells from PCOS patients in order to better understand whether and how chemerin contributes to PCOS. Serum levels of chemerin are also increased in another female population at risk for metabolic and vascular disease, i.e. women with pre-eclampsia during and 6 months after pregnancy (Stepan *et al.*, 2011).

In conclusion, we demonstrated for the first time the presence *in vivo* of chemerin and CMKLR1 in the human ovarian follicle and more particularly in granulosa and theca cells and follicular fluid. Chemerin levels are significantly higher in follicular fluid than in plasma. *In vitro,* we have shown that rhChem inhibited IGF-1-induced progesterone and E_2 secretion and cell proliferation in hGCs and KGN cells. This was associated with a reduction in the levels of p450 aromatase and a decrease in the tyrosine phosphorylation of IGF-1Rβ subunit and phosphorylation of Akt and MAPK ERK1/2 in hGCs. These findings significantly increase our understanding of the role of chemerin in hGCs. However, further investigations are necessary to understand the effects of chemerin on other human ovarian cells including theca cells and oocyte and also its potential implication in the PCOS.

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Authors' roles

M.R. participated together with J.D. in the design of the study. The experiments were carried out by M.R., M.C., C.R., F.G., D.R. and J.D. Data analysis were performed by M.R., M.C., F.G., L.T. and J.D. The manuscript was written by M.R. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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Article 2

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MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Visfatin is expressed in human granulosa cells: regulation by metformin through AMPK/SIRT1 pathways and its role in steroidogenesis

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ABSTRACT: Visfatin is a cytokine hormone and an enzyme involved in metabolic (obesity, type II diabetes) and immune disorders. Some data suggest a role of visfatin in ovarian function. Here, we identified visfatin in human follicles and investigated the molecular mechanisms involved in the regulation of its expression in response to insulin sensitizers, metformin (MetF) and rosiglitazone, in primary human granulosa cells (hGCs) and in a human ovarian granulosa-like tumour cell line (KGN). We also studied the effects of human recombinant visfatin (RhVisf) on steroid production and on the activation of various signalling pathways. By RT–PCR, immunoblotting and immunohistochemistry, we showed that visfatin is expressed not only in hGCs and KGN cells, but also in human cumulus cells and oocytes. In hGCs and KGN cells, MetF increased visfatin mRNA in a dose-dependent manner (0.1, 1 and 10 mM), and rosiglitazone increased visfatin mRNA expression (only at 10 μM) after treatments for 24 h, whereas both reduced it after 48 h of incubation. This regulation was confirmed at the protein level for the MetF treatment only. Using the compound C and Aicar, inhibitor and activator of AMP-activated protein kinase (AMPK), respectively, and Sirtinol, an inhibitor of sirtuin-1 (SIRT1), we observed that these MetF effects on visfatin expression were mediated through the AMPK/SIRT1 signalling pathways. RhVisf (10 ng/ml) significantly increased insulin-like growth factor-1 (IGF-1) (10 nM)- but not FSH (10 nM)-induced secretion of progesterone and estradiol as determined by radioimmunoassay and IGF-1-induced thymidine incorporation in hGCs and KGN cells. Finally, rhVisf rapidly activates the mitogen-activated protein kinase pathway via ERK1/2, P38 and Akt phosphorylation under basal conditions in primary hGC cells. In conclusion, visfatin is present in ovarian human follicles, and in hGCs and KGN cells, visfatin increases IGF-1-induced steroidogenesis and cell proliferation and MetF regulates visfatin expression through the AMPK/SIRT1 signalling pathway.

Key words: adipokine / ovary / insulin-like growth factors / signalling pathways

Introduction

Visfatin, also known as nicotinamide phosphoribosyltranferase (NAMPT) and pre-B cell colony-enhancing factor (PBEF), is a cytokine hormone and an enzyme involved in metabolic (obesity, type II diabetes) and immune disorders (Rongvaux *et al.*, 2002; Revollo *et al.*, 2004; Luk *et al.*, 2008). It is the most recently identified adipokine that regulates numerous processes, including glucose homeostasis, lipid metabolism, inflammation and angiogenesis (Stofkova, 2010). Visfatin is also the rate-limiting enzyme in the NAD biosynthesis pathway from nicotinamide. It exists in two molecular forms, and both the extracellular (cytokine like) and intracellular (enzymatic) forms seem to be responsible for its biological effects. Some evidence has shown that visfatin-mediated NAD biosynthesis regulates the activity of sirtuin 1 (SIRT1), a NAD-dependent deacetylase involved in the pathogenesis of age-associated complications including type 2 diabetes (Revollo *et al.*, 2004). Visfatin expression is regulated by anti-diabetic thiazolidinediones such as rosiglitazone in rat-isolated adipocytes (Kloting and Kloting, 2005), and visfatin secretion is improved by insulin and glucose in cultured human subcutaneous adipocytes (Haider *et al.*, 2006). Furthermore, Xie *et al.* have suggested that visfatin can activate insulin receptor and downstream signalling pathways (Xie *et al.*, 2007).

Some data suggest that visfatin, like other adipokines (Tersigni et al., 2011), could regulate reproductive functions. In the male, visfatin is expressed in chicken testis and particularly in Sertoli and Leydig cells and in germinal cells (Ocon-Grove et al., 2010). Furthermore, in this species, sexual maturation is associated with changes in testicular visfatin expression. In the female, a recent study showed that administration of visfatin during ovulation induction improves developmental competency of oocytes and fertility potential in old female mice (Choi et al., 2012), suggesting a role of this adipokine in ovarian function. Visfatin is expressed by human fetal membranes during pregnancy and is present throughout gestation in the amniotic epithelium and mesenchymal cells (Ognjanovic and Bryant-Greenwood, 2002). It may play an important role in placentation for normal pregnancy (Fasshauer et al., 2008; Mazaki-Tovi et al., 2010; Zulfikaroglu et al., 2010). In humans, Shen et al. reported a positive correlation between follicular fluid visfatin concentrations of women undergoing controlled ovarian stimulation and the number of oocytes retrieved (Shen et al., 2010).

It has recently been shown that visfatin is expressed in human granulosa cells (hGCs), and its expression *in vitro* is increased by hCG and prostaglandin E2 treatments (Shen et al., 2010). However, the role of visfatin in hGCs steroidogenesis is unknown. A previous study showed that women with polycystic ovary syndrome exhibit higher plasma visfatin levels than control subjects of similar body mass index, suggesting that plasma visfatin concentrations might affect ovarian functions (Chan et al., 2007; Kowalska et al., 2007). Furthermore, Ozkaya et al. found that metformin (MetF) treatment significantly reduced circulating plasma visfatin concentrations in PCOS patients (Ozkaya et al., 2010). In addition to its reproductive consequences (infertility, anovulation, polycystic ovaries and hyperandrogenism), PCOS is a metabolic disorder associated with insulin resistance and hyperinsulinemia (Dunaif, 1997). Peripheral effects of MetF, dependent or not on its insulin-sensitizing action, have been observed in several studies (Palomba et al., 2006; Diamanti-Kandarakis et al., 2010). Several reports have suggested a specific effect of MetF on ovaries, showing that PCOS patients ovulating under treatment have an improved ovarian artery blood flow and better dominant follicle and corpus luteum vascularization (Palomba et al., 2006). MetF is also able to exert direct effects on the ovary, by inhibiting, for example, *in vitro* steroid production by GCs in various species, including man (Mansfield et al., 2003; Tosca et al., 2006). Furthermore, *in vivo* MetF treatment reduces the stimulated activity of several steroidogenic enzymes without a reduction in circulating insulin in women with PCOS (Vrbikova et al., 2001). However, although MetF has been used in clinical practice for several years, its mechanism of action remains unclear. One hypothesis is that MetF treatment could affect visfatin expression in ovarian cells.

The objectives of this study was to explore the expression of visfatin in the human follicle and to study its regulation in hGCs (luteal GCs from women undergoing *in vitro* fertilization and KGN cells) in response to two insulin sensitizers, MetF and rosiglitazone. We also investigated the effects of human recombinant visfatin on steroid production and on the activation of various signalling pathways in these cells.

Materials and Methods

Patients

Twenty infertile women (35 ± 4 years old) were recruited at the Service de médecine et Biologie de la Reproduction, CHRU de Tours, in 2011 for mechanical, unexplained or male factor infertility without any known endocrinopathy (polycystic ovarian syndrome, hyperprolactinemia and hypo- and hypertrophism). Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. Only one cycle was studied from each patient.

KGN cell culture

The human ovarian granulosa-like tumour cell line, KGN, was cultured in Dulbecco's minimal essential medium/F12 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin obtained from Sigma) in a 5% CO_2 atmosphere at 37°C. KGN cells were obtained from Drs Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan (Nishi et al., 2001). They are undifferentiated and maintain the physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of aromatase.

Hormones and reagents

Purified ovine FSH-20 (oFSH; lot no.AFP-7028D, 4453 IU/mg, FSH activity = 175 times the activity of oFSH-S1) was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA. Recombinant human insulin-like growth factor-1 (IGF-1) used for culture treatment was from Sigma (St Louis, MO, USA). Recombinant human visfatin was from R&D (Lille, France). Human ovary sections embedded in paraffin were obtained from Euromedex (Souffelweyersheim, France). The visfatin inhibitor, FK866, the AMP-activated protein kinase (AMPK) inhibitor, compound C, the SIRT1 inhibitor, Sirtinol and the SIRT1 activity assay were obtained from Sigma (St Louis, MO, USA).

Antibodies

Affinity-purified rabbit anti-human PBEF (BL2122) polyclonal antibody and blocking peptides for the polyclonal antibody PBEF (BP2122) were purchased from Bethyl Laboratories Inc. (Montgomery, USA). Rabbit polyclonal antibodies to phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser 473) and phospho-AMPK alpha Thr172 were obtained from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to AMPKalpha were purchased from Upstate Biotechnology Inc. (Lake, Placid, NY, USA). Mouse monoclonal antibodies to Vinculin (VLC) were obtained from Sigma (St Louis, MO, USA). Rabbit polyclonal antibodies to ERK2 (C14) and p38 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies were diluted at 1/1000 in western blotting.

Isolation and culture of hGCs

The hGCs were collected from pre-ovulatory follicles during oocyte retrieval for IVF. The ovarian stimulation protocol and IVF and ICSI procedures used have already been reported (Guerif et al., 2004; Reverchon et al., 2012). After isolation of cumulus oocyte complexes (used for IVF), follicular fluids were pooled and then centrifuged (400*g*, 10 min). To remove most of the red blood cells, the pellet was centrifuged (400*g*, 20 min) on a two-layer discontinuous Percoll gradient (40, 60% in Ham's medium, Gibco-BRL; Life Technologies, Cergy Pontoise,

France). The 40% fraction was collected and treated with haemolytic medium (NH_4Cl 10 mmol/l in Tris–HCl pH 7.5; Sigma, Isles d'Abeau, France) to remove the remaining red blood cells as many as possible. Following centrifugation, the pellet was washed with fresh medium (Hams F12); cells were counted in a haemocytometer and cell viability was determined using Trypan Blue dye exclusion. Cell were cultured in McCoy's 5A medium supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l, 0.1% BSA, 0.1 μmol/l androstenedione, 5 mg/l transferrin and 20 μg/l selenium) and 5% fetal bovine serum (FBS). The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures were kept under a water-saturated atmosphere of 95% air/5% CO_2 at 37°C. We made four cultures (one per week). Each culture was made by pooling cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or absence of IGF-1) was applied in quadruplicate or duplicate as indicated in the figure legends.

RNA extraction and RT–PCR

Total RNA from hGCs, KGN cells and human visceral or subcutaneous adipose tissue (Vis AT, Sc AT) was extracted with Trizol reagent according to the manufacturer's procedure (Invitrogen). RT–PCR was used to detect visfatin expression in hGCs, KGN cells and in Vis and Sc AT. Reverse transcription of total RNA (1 μg) was denatured and retrotranscripted with the reverse transcriptase Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (15 U) in a 20 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 200 μM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ, USA), 50 pmol of oligo(dT)15 and 5 U of ribonuclease inhibitor. All was incubated at 37°C for 1 h. cDNAs were amplified in 50 μl reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U of Taq polymerase and 2 μl of the RT mix. PCR was performed with specific primer pairs for visfatin (forward: 5′-AAGAGACTGCTGGCATAGGA-3′ and reverse: 5′-ACCACAGATACAGGCACTGA-3′) and β-actin (forward: 5′-ACGGAACCACAGTTTATCATC-3′ and reverse 5′-GTCC CAGTCTTCAACTATACC-3′). First, the samples were denatured at 94°C for 5 min, then 35 PCR cycles were processed (95°C, 1 min; 58°C, 1 min; 72°C, 1 min), with a final extension step at 72°C for 10 min. PCR products were migrated on 1, 5% agarose gel stained with ethidium bromide and sequenced by the genome express company (Meylan, France). MMLV reverse transcriptase and RNase inhibitor (RNasin) were purchased from Promega (Promega, Madison, WI, USA), and RT–PCR consumables were purchased from Sigma (l'Isle d'Abeau Chesnes, France).

Protein extraction and western blot

Freshly collected hGCs purified on Percoll gradient, KGN cells, *in vitro*-cultured hGCs and adipose tissues (Sc and Vis AT) were homogenized as previously described (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). Lysates were incubated on ice for 30 min and then centrifuged at 12 000*g* for 20 min at 4°C. The protein concentration in the resulting supernatants was then determined using the BCA protein assay. After denaturation, the samples were subjected to electrophoresis on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France). The membrane was blocked for 30 min in TBS-Tween-milk 5% and incubated for 16 h with appropriate primary antibodies at a 1/1000 final dilution. Finally, the blots were incubated for 1 h 30 min at room temperature with a HRP-conjugated anti-rabbit or anti-mouse IgG (dilution 1/5000). Proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using a G:Box SynGene (Ozyme) with the GeneSnap software (release 7.09.17). Signals detected were quantified with the GeneTools software (release 4.01.02). The results were expressed as the intensity signal in arbitrary units after normalization, allowed by the presence of ERK2, p38, Akt, AMPK total (for ERK1/2, p38, Akt and AMPK phosphorylation, respectively) and VLC (for visfatin) as an internal standard. The effect of recombinant human visfatin was analysed on four different primary human granulosa cell cultures. Each primary culture was derived from a different patient.

SIRT1 activity assay

A SIRT1 activity assay was performed using the SIRT1 Fluorimetric Drug Discovery Kit (Biomol International). Briefly, total protein (25 μg) and acetylated substrate (Fluor-de-Lys-Sirt1 substrate, 100 μM) were incubated at 37°C in the presence of NAD (100 μM) for 30 min. After incubation, de-acetylated substrates were detected on a TECAN M200pro fluorometric plate reader at 'Physiologie de la Reproduction et des Comportements' (Nouzilly, France).

Immunohistochemistry

Human ovary sections were deparaffinized, hydrated and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France) and then allowed to cool to room temperature. After washing in a phosphate-buffered saline (PBS) bath for 5 min, sections were immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). After two washes in a PBS bath for 5 min, non-specific background was eliminated by blocking with 5% lamb serum in PBS for 20 min, followed by incubation overnight at 4°C with PBS containing rabbit primary antibody raised against visfatin (1:100). Sections were washed twice for 5 min each time in a PBS bath and were incubated for 30 min at room temperature with a 'ready to use' labelled Polymer-HRP anti-rabbit antibody (DakoCytomation Envision Plus HRP system, Dako, Ely, UK). The sections were then washed twice in PBS, and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System, DakoCytomation). Negative controls involved replacing primary antibodies with rabbit IgG.

Progesterone and estradiol radioimmunoassay

The concentration of progesterone and estradiol in the serum-free culture medium of KGN and hGCs cells was measured after 48 h of incubation with or without IGF-1, FSH or FK866 by a radioimmunoassay protocol as previously described (Chabrolle *et al.*, 2009; Pierre *et al.*, 2009). The limit of detection of progesterone was 12 pg/tube (60 pg/well), and the intra- and inter-assay coefficients of variation were less than 10 and 11%, respectively. The limit of detection of estradiol was 1.5 pg/tube (7.5 pg/well), and the intra-and inter-assay coefficients of variation were less than 7 and 9%, respectively. Results were expressed as the concentration of steroids/cell protein concentration/well. Results are mean ± SEM of data obtained from KGN cells (at four different passages) and primary hGCs (four cultures, one per week). Each primary hGCs culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment (in the presence or absence of IGF-1, FSH or FK866) was applied in quadruplicate.

Thymidine incorporation into GCs

hGCs (2×10^5 viable cells/500 μl) were cultured in McCoy's 5A medium and 10% FBS for 48 h and were then serum starved for 24 h, followed by

the addition of 1 μCi/μl of [3H] thymidine (Amersham Life Science, Arlington Heights, IL, USA) in the presence or absence of visfatin (10 ng/ml) and/or IGF-1 (10 nM). After 24 h of culture, excess thymidine was removed by washing the cells twice with PBS, and then they were fixed with cold trichloroacetic acid 50% for 15 min and lyzed by NaOH 0.5 N. The radioactivity was determined by scintillation fluid (Packard Bioscience) counting in a beta-photomultiplier.

Real-time quantitative PCR

Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers [visfatin (forward: 5′-AAGAGACTGCTGGCATAGGA-3′ and reverse: 5′-ACCACAGATACAGGCACTGA-3′), β-actin (forward: 5′-ACGGAA CCACAGTTTATCATC-3′ and reverse: 5′-GTCCCAGTCTTCAACTA TACC3′), ribosomal protein L19 (RPL 19) (forward: 5′-AATCGCCAATG CCAACTC-3′ and reverse: 5′-CCCTTTCGCTTACCTATACC-3′) and cyclophylin A (forward: 5′-GCATACAGGTCCTGGCATCT-3′ and reverse: 5′-TGTCCACAGTCAGCAATGGT-3′)] in total volume of 20 μl in a MyiQ Cycle device (Bio-Rad). Samples were tested in duplicate on the same plate, and PCR amplification with water, instead of cDNA, was done systematically as a negative control. After incubation for 2 min at 50°C and a denaturation step of 10 min at 95°C, samples were subjected to 40 cycles (30 s at 95°C, 30 s 60°C and 30 s at 72°C), followed by the acquisition of the melting curve. Primers' efficiency (E) was performed from serial dilutions of a pool of obtained cDNA and ranged from 1.8 to 2. Three reference genes were used: cyclophilin A, RPL19 and β-actin. For each gene, expression was calculated according to primer efficiency and Cq: expression $= E^{-Cq}$. Then, relative

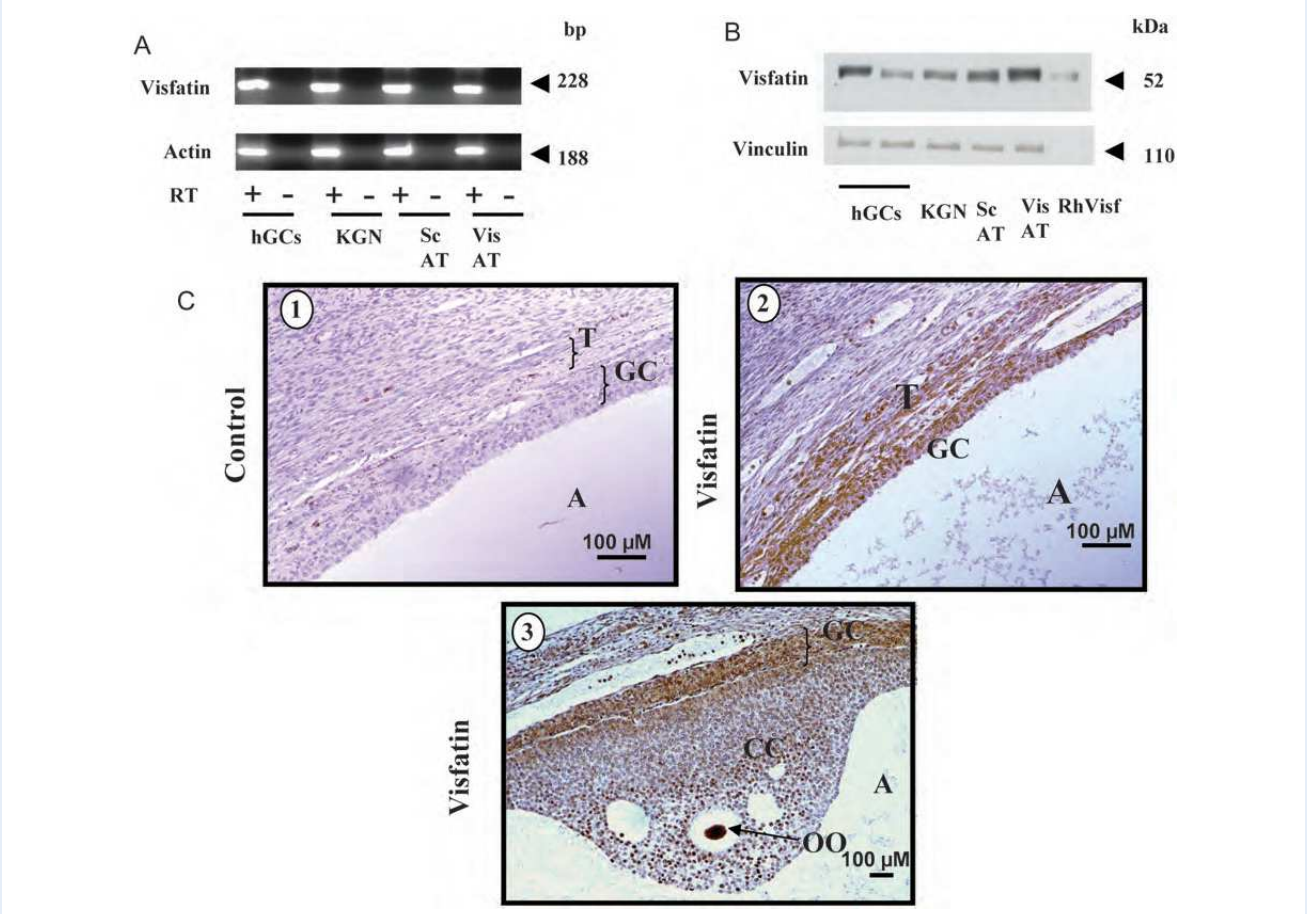

Figure 1 Expression of visfatin in hGCs and ovarian follicle. (**A**) RT–PCR from total RNA of hCGs, ovarian granulosa cell lines (KGN), human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues was performed with primers designed to amplify one fragment of visfatin (228 pb) and one fragment of actin (188 pb) as a housekeeping gene. Human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues were used as a positive control for visfatin expression [mRNA (A) and protein (**B**, see below)]. The expected sizes of the different amplified products by RT–PCR are indicated on the right. RT-corresponds to an RT–PCR carried out without reverse transcriptase. (B) Protein extracts (50 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with specific antibodies against visfatin. Equal protein loading was verified by a reprobing membrane with an anti-VLC antibody. hCGs from four different patients were used. The data presented are from one representative experiment with only one sample of hCGs from two different patients. Recombinant human visfatin was used as positive control. (**C**) Visfatin localization in human ovarian follicle by immunochemistry. DAB-immunoperoxidase staining was performed on paraffin-embedded human ovary using antibodies against visfatin (2, 3) or no primary antibodies but rabbit IgG (1). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. Visfatin is detected in GCs, CC, theca cells (T) and oocytes (OO) of ovarian follicles. A, antrum. Bars = 100 μm or 20 μm. Immunohistochemistry was performed on two different human ovary slides from each of four patients.

expression of visfatin/reference gene was analysed. Only one reference gene is represented in the figures.

Statistical analysis

All experimental results are expressed as the mean ± SEM. Statistical analyses were carried out using a *t*-test or one-way analysis of variance (ANOVA) (for comparison of various means), and if ANOVA revealed significant effects, it was supplemented with Fisher's test. A $P < 0.05$ was considered significant.

Results

Visfatin expression in hGCs

We determined the expression of visfatin in fresh hCGs and in the human granulosa tumour cell line, KGN. As shown in Fig. 1A, we amplified by RT–PCR one cDNA fragment of 228 pb, corresponding to visfatin. The specificity of the amplified products was assessed by sequencing. Visceral and subcutaneous adipose tissues (Vis AT, Sc AT) were used as a positive control. We found that the transcript of visfatin is strongly expressed in GCs and as expected in the adipose tissues (Fig. 1A). Immunoblotting of protein extracts revealed the presence of visfatin (52 kDa) in fresh hCGs and in KGN cells (Fig. 1B). Immunohistochemistry with human ovarian follicle sections confirmed the results obtained by immunoblotting. Visfatin was detected in not only GCs but also in cumulus cells (CC) and oocyte and less abundantly in theca cells (Fig. 1C). Thus, visfatin is expressed in human ovarian follicles and more particularly in GCs.

Effect of MetF and rosiglitazone on visfatin mRNA and protein expression in hGCs

We next investigated the effect of two insulin sensitizers, MetF and rosiglitazone, on visfatin expression in KGN (Fig. 2) and primary hGCs (data not shown). Overnight starved cells were incubated for different times (24 and 48 h) with different concentrations of MetF

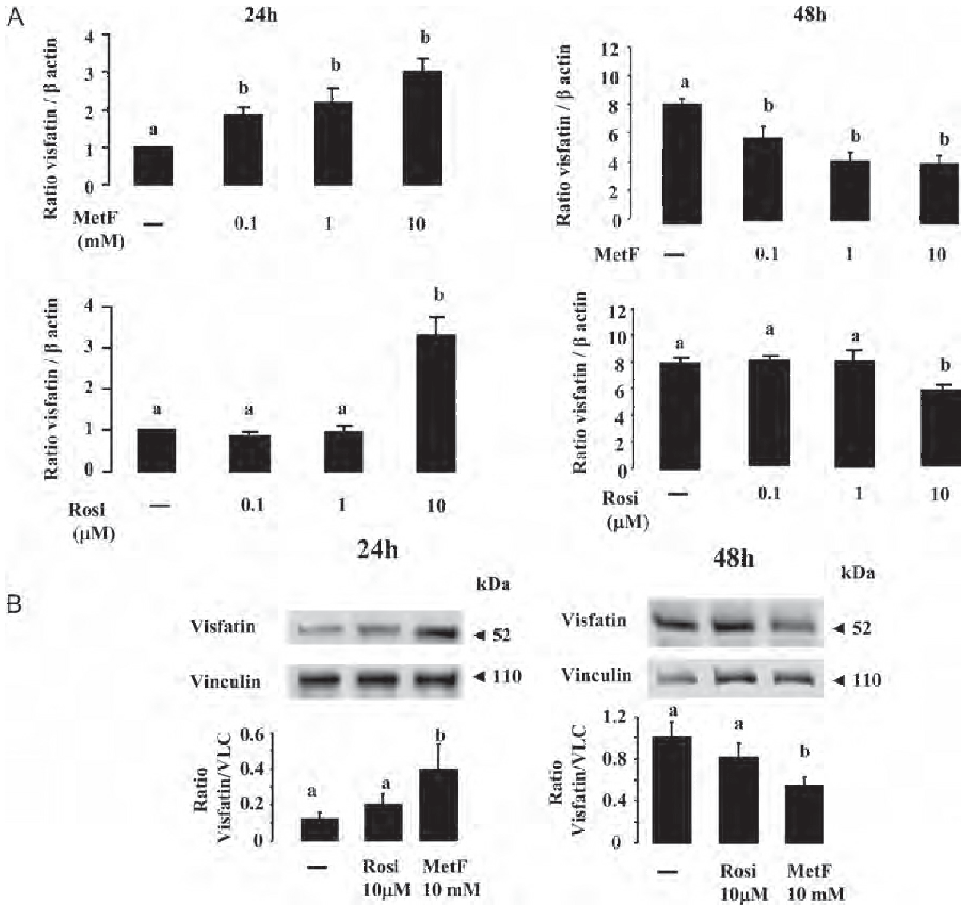

Figure 2 Effect of MetF and rosiglitzone (Rosi) on visfatin expression at the mRNA (**A**) and protein (**B**) level in KGN cells. (A) Visfatin gene expression was measured by quantitative real-time-PCR in KGN cells after 24 and 48 h of stimulation with or without different doses of rosiglitazone (Rosi, 0.1,1 and 10 μM) or MetF (0.1, 1 and 10 mM). β-actin was used as a reference gene. Similar results were obtained using two other reference genes, RPL19 and cyclophilin A. (B) Protein levels of visfatin were analysed after 24 and 48 h of stimulation with or without rosiglitazone (Rosi, 10 μM) or MetF (10 mM). VLC was used as a loading control. Results are representative of at least eight cultures of KGN cells obtained at different passages. In each culture, each treatment (in the presence or in the absence of MetF or rosiglitazone) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

(0, 0.1, 1 and 10 mM) or rosiglitazone (0, 0.1, 1 and 10 μM). By real-time quantitative PCR, we showed that after 24 h of stimulation, MetF increased visfatin mRNA expression in a dose-dependent manner, whereas rosiglitazone increased it only at the 10 μM concentration (Fig. 2A, left panel). As shown in Fig. 2A, right panel, we observed opposite effects after 48 h of incubation. Similar results were obtained using two other reference genes (RPL19 and Cyclophilin A). We next examined whether these effects of MetF and rosiglitazone on visfatin mRNA were also observed at the protein level. As shown in Fig. 2B, MetF treatment (10 mM) for 24 h increased the level of visfatin protein by more than 2-fold, whereas it halved it after 48 h in KGN cells (Fig. 2B). Rosiglitazone treatment (10 μM) did not affect visfatin protein expression at any time points that were studied (Fig. 2B). Similar results were observed in hGCs (data not shown). We next investigated the molecular mechanisms involved in the regulation of visfatin expression in response to MetF.

Involvement of AMPK in the MetF effects on visfatin expression in hGCs

It is well known that MetF treatment activates AMPK in various cell types, including hGCs (Richardson *et al.*, 2009). In KGN cells (Fig. 3A) and in hGCs (data not shown), we showed that MetF treatment (10 mM) increased AMPK phosphorylation on Thr172 residue after 60 and 120 min of incubation. Similar results were observed after 12 or 24 h of stimulation, whereas MetF treatment for 48 h significantly inhibited AMPK phosphorylation (Fig. 3B). We also indirectly assessed the AMPK activity by measuring the phosphorylation of its downstream target, Acetyl CoA carboxylase (ACC) (data not shown). ACC phosphorylation paralleled that of Thr172 AMPK in response to MetF (data not shown). To determine whether AMPK phosphorylation is involved in the regulation of visfatin expression in response to MetF in KGN cells, we investigated the effects of Comp

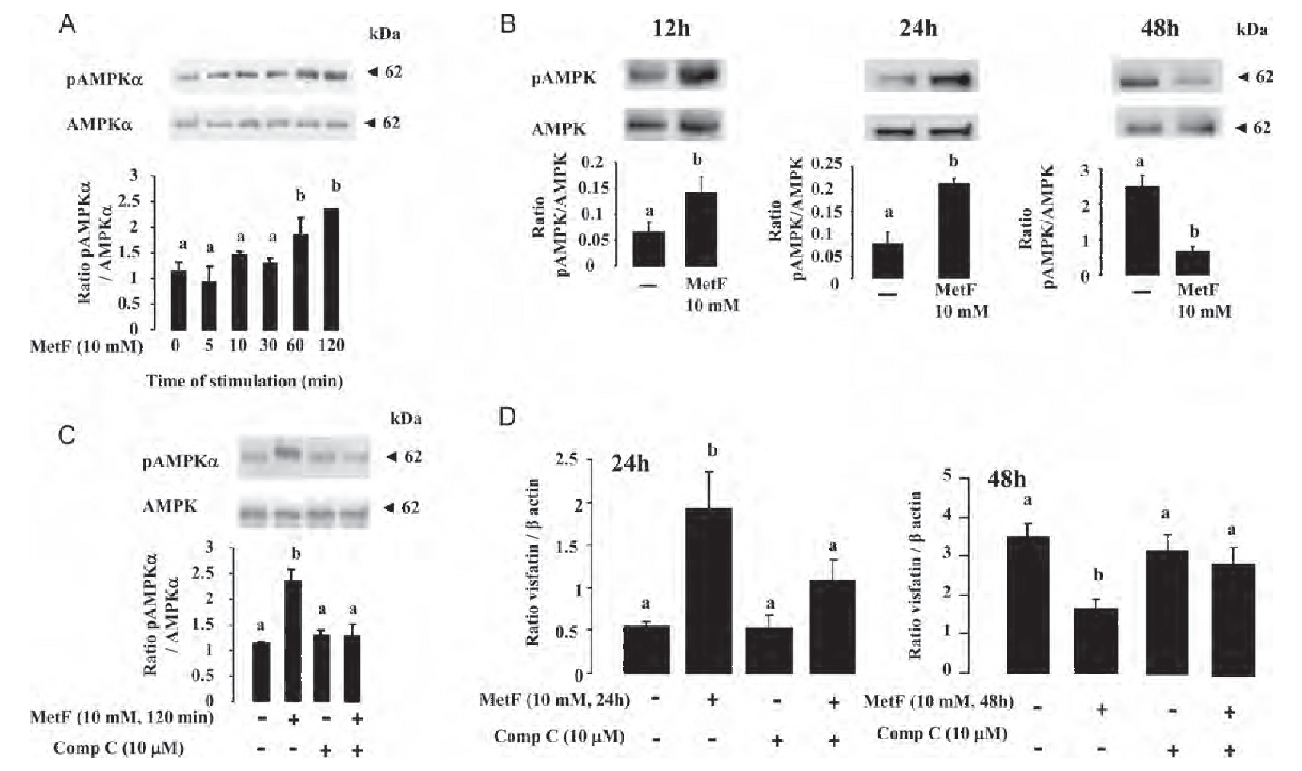

Figure 3 Effect of MetF on AMPK phosphorylation (**A**, **B**) and effect of compound C on visfatin expression in response to MetF in KGN cells (**C**, **D**). (A and B) Cell lysates were prepared from cells incubated in serum-free medium in the presence of MetF (10 mM) for various times: 0, 5, 10, 30, 60 or 120 min (A) or for 12, 24 or 48 h (B). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-phospho-AMPK and anti-AMPK (A and B). Results are representative of at least four cultures of KGN cells obtained at different passages. In each culture, each treatment (visfatin in the presence or absence of MetF) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$. (C) KGN cells were pre-incubated in the serum-free medium in the presence or absence of Comp C (10 μM) for 60 min and then stimulated or not with 10 mM MetF for 120 min in serum-free medium. Cells were then lyzed and subjected to western blotting with antibodies against phospho-AMPK (Thr 172) and AMPK. Representative blots for the three experiments are shown. Blots were quantified, and the phosphorylated protein:total protein ratio is shown. The results are mean ± SEM. Different letters indicate significant differences with $P < 0.05$. (D) KGN cells were pre-incubated in the serum-free medium in the presence or absence of Comp C (10 μM) for 60 min and then incubated or not with 10 mM MetF for 24 h or 48 h in serum-free medium. Visfatin gene expression was measured by quantitative real-time-PCR as indicated in Fig. 2. Results are representative of at least eight cultures of KGN cells obtained at different passages. In each culture, each treatment (in the presence or in the absence of Comp C and/or MetF for 24 or 48 h) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

C, a well-known inhibitor of AMPK. After 2 h of MetF stimulation in serum-free medium, immunoblot analysis confirmed that Comp C treatment (10 μM) significantly decreased AMPK phosphorylation when compared with the control treated with dimethyl sulphoxide (Fig. 3C). Comp C treatment (10 μM) totally eliminated the effect of MetF on visfatin expression after 24 and 48 h of incubation (Fig. 3D). Similar results were observed on the visfatin protein levels (data not shown). The viability of KGN cells as determined by trypan blue staining was not affected by Comp C treatment (data not shown). Thus, Comp C inhibited AMPK phosphorylation and eliminated the effects of MetF on visfatin expression. To confirm that AMPK is involved in the regulation of visfatin expression in hGCs, we incubated cells with Aicar, an activator of AMPK in the absence or presence of Comp C treatment, and investigated visfatin mRNA and protein expression. As shown in Fig. 4A, Aicar (1 mM) increased AMPK phosphorylation on Thr172 residue after 30, 60 and 120 min of incubation. Aicar treatment (10 μM) increased visfatin mRNA expression by about 3-fold after 24 h of incubation (Fig. 4B), whereas it reduced it by about 3-fold after 48 h of incubation (Fig. 4C). Comp C treatment (10 μM) totally abolished the effect of Aicar on visfatin expression after 24 and 48 h of incubation (Fig. 4B and C). Similar results were observed on the visfatin protein levels (Fig. 4D and E).

Involvement of SIRT1 in MetF-induced visfatin expression in hGCs

MetF is also known to activate the NAD+-dependent histone/protein deacetylase SIRT1 (Caton *et al.*, 2010). Furthermore,

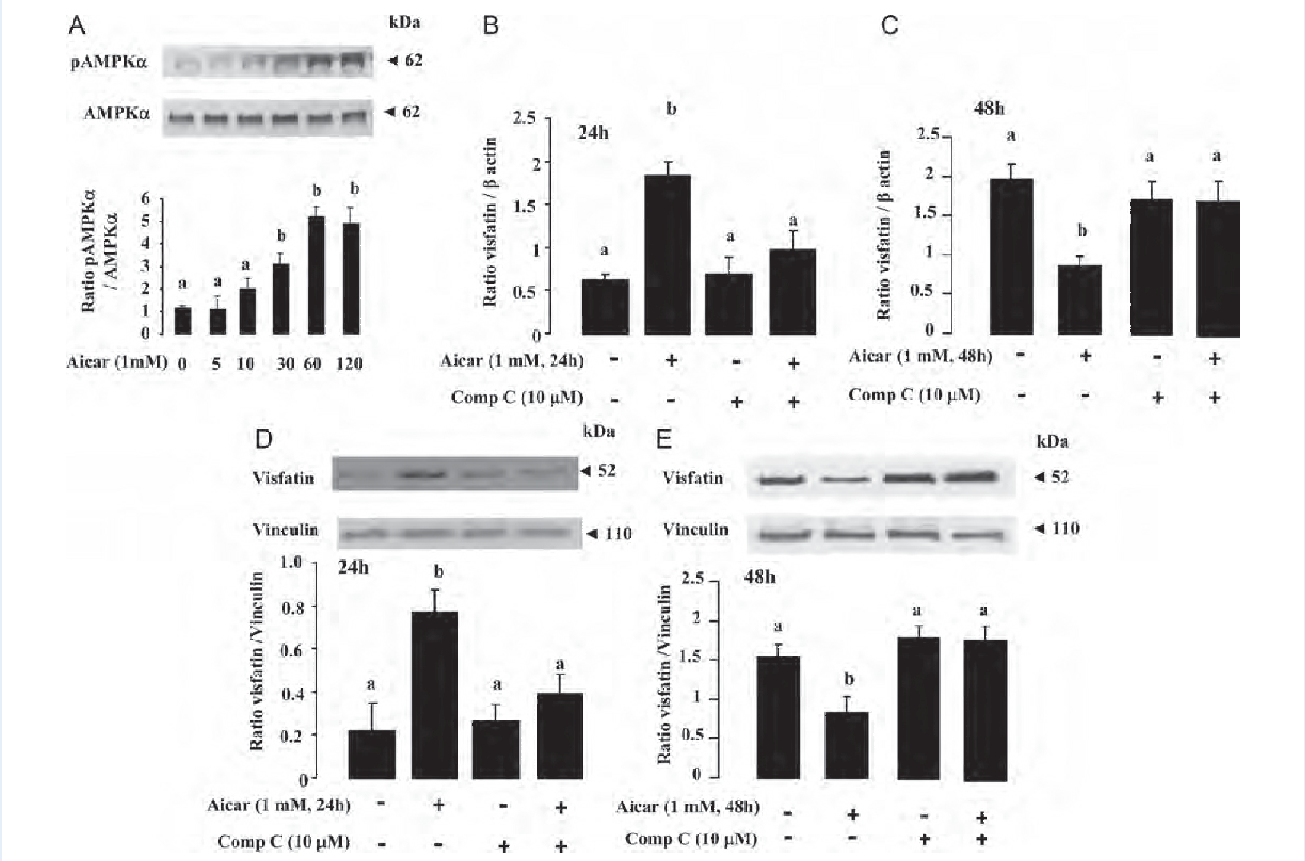

Figure 4 Effect of Aicar on AMPK phosphorylation (**A**) and effect of compound C on visfatin mRNA (**B** and **C**) and protein (**D** and **E**) expression in response to Aicar in KGN cells. (A) Cell lysates were prepared from cells incubated in serum-free medium in the presence of Aicar (1 mM) for various times: 0, 5, 10, 30, 60 or 120 min. Immunoblot was performed as described above. Results are representative of at least four cultures of KGN cells obtained at different passage. Data are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$. (B and C) KGN cells were pre-incubated in the serum-free medium in the presence or absence of Comp C (10 μM) for 60 min and then incubated or not with 1 mM Aicar for 24 h or 48 h in serum-free medium. Visfatin gene expression was measured by quantitative real-time-PCR as indicated in Fig. 2. Results are representative of at least eight cultures of KGN cells obtained at different passage. In each culture, each treatment (in the presence or in the absence of Comp C and/or Aicar for 24 or 48 h) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$. (D and E) Protein levels of visfatin were analysed in the same conditions as described above (B and C part). VLC was used as a loading control. Results are representative of at least eight cultures of KGN cells obtained at different passage. In each culture, each treatment (in the presence or in the absence of Comp C and/or Aicar for 24 or 48 h) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

numerous studies have shown that AMPK activation can also lead to increased SIRT1 activity (Fulco *et al.*, 2008; Canto *et al.*, 2009; Caton *et al.*, 2010), with two of these studies demonstrating that MetF increases the NAD^+/NADH ratio and SIRT1 abundance and activity (Canto *et al.*, 2009; Caton *et al.*, 2010). As shown in Fig. 5A, we observed that MetF treatment for 12 and 24 h increased SIRT1 activity in KGN cells. Similar results were obtained in hGCs (data not shown). To determine whether SIRT1 mediates the positive effects of MetF on visfatin expression, we treated cells with Sirtinol (a SIRT1 inhibitor). As expected, Sirtinol inhibited SIRT1 activity as compared with basal state (no stimulation) or in response to MetF (Fig. 5A). Furthermore, as shown in Fig. 5B, treatment of KGN cells with Sirtinol significantly reduced visfatin expression in response to 24 h MetF treatment. Similar results were observed on the visfatin protein levels (Fig. 5C). The viability of KGN cells as determined by trypan blue staining was not affected by sirtinol pretreatment (data not shown). Thus, MetF-induced visfatin expression is not only AMPK- but also SIRT1-dependent.

Effect of human recombinant visfatin on basal and IGF-1 or FSH-induced progesterone and estradiol production by KGN and hCGs cells

We next determined the effects of visfatin treatment on steroidogenesis in KGN and primary hGCs (Fig. 6). Cells were incubated in serum-free medium with human recombinant visfatin (Rh visfatin) (10 ng/ml) for 48 h in the presence or absence of IGF-1 (10 nM) or FSH (10 nM). As expected, IGF-1 or FSH treatment alone increased progesterone and estradiol secretion in KGN cells (Fig. 6A and B, Pierre *et al.*, 2009) and primary hGCs cells (Fig. 6C and D, Chabrolle *et al.*, 2009). In KGN cells, in basal state and in the presence of IGF-1, visfatin treatment (10 ng/ml) increased by at least 2-fold secretion of progesterone (Fig. 6A, $P < 0.001$) and estradiol (Fig. 6B, $P < 0.001$), whereas no effect of visfatin was observed in the presence of FSH. In primary hGCs, similar effects were observed except that no effect of visfatin was observed in the basal state (Fig. 6C and D).

We next confirmed the effect of visfatin on IGF-1-induced steroid production using FK866, a specific inhibitor of enzymatic activity of vis-

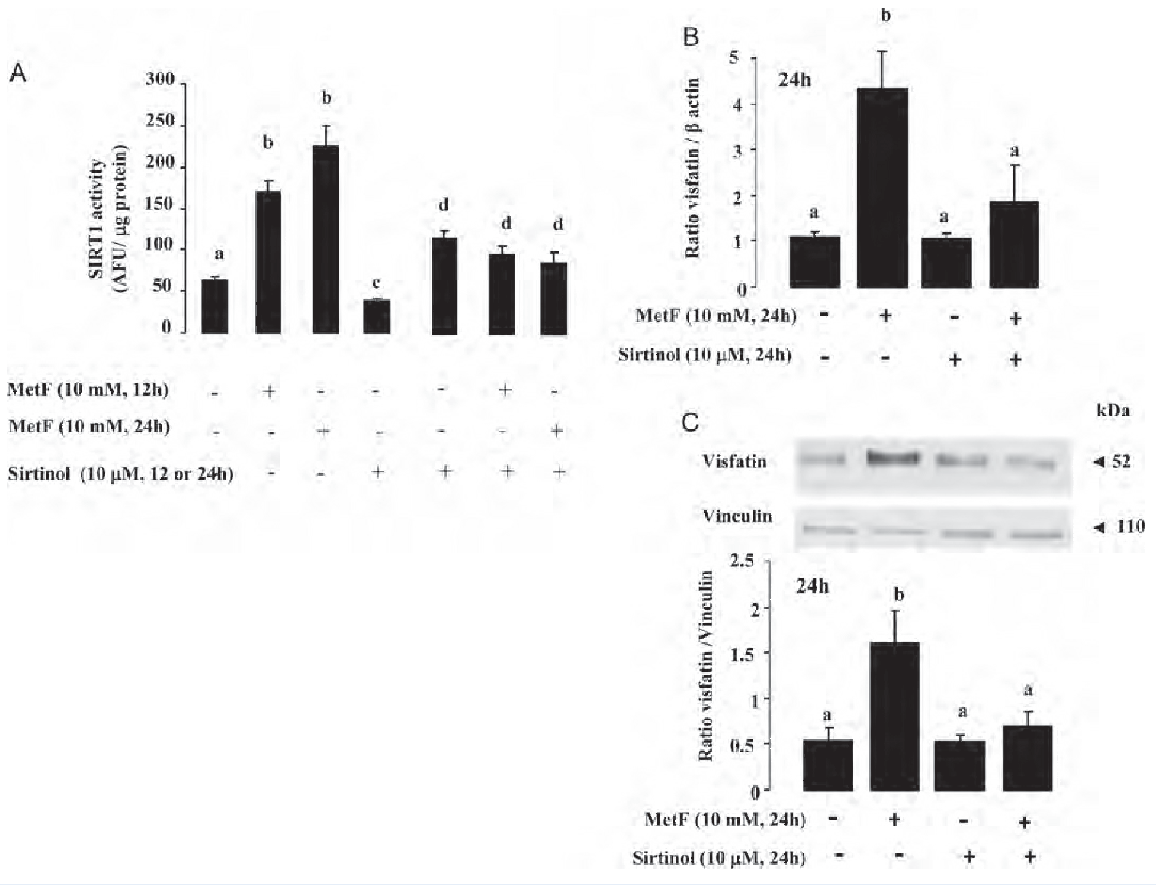

Figure 5 Effect of MetF and Sirtinol on SIRT1 activity (**A**) and visfatin gene expression in KGN cells. KGN cells were incubated in the serum-free medium in the presence or absence of Sirtinol (10 µM) and MetF (10 mM) for 12 and 24 h. SIRT1 activity was then examined as described in Materials and Methods (A) or visfatin gene expression was measured by quantitative real-time-PCR as indicated in Fig. 2 (**B**). (**C**) Protein levels of visfatin were analysed in the same conditions as described above in B. VLC was used as a loading control. Results are representative of at least eight cultures of KGN cells obtained at different passages. In each culture, each treatment (in the presence or in the absence of Sirtinol and/or MetF for 24) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

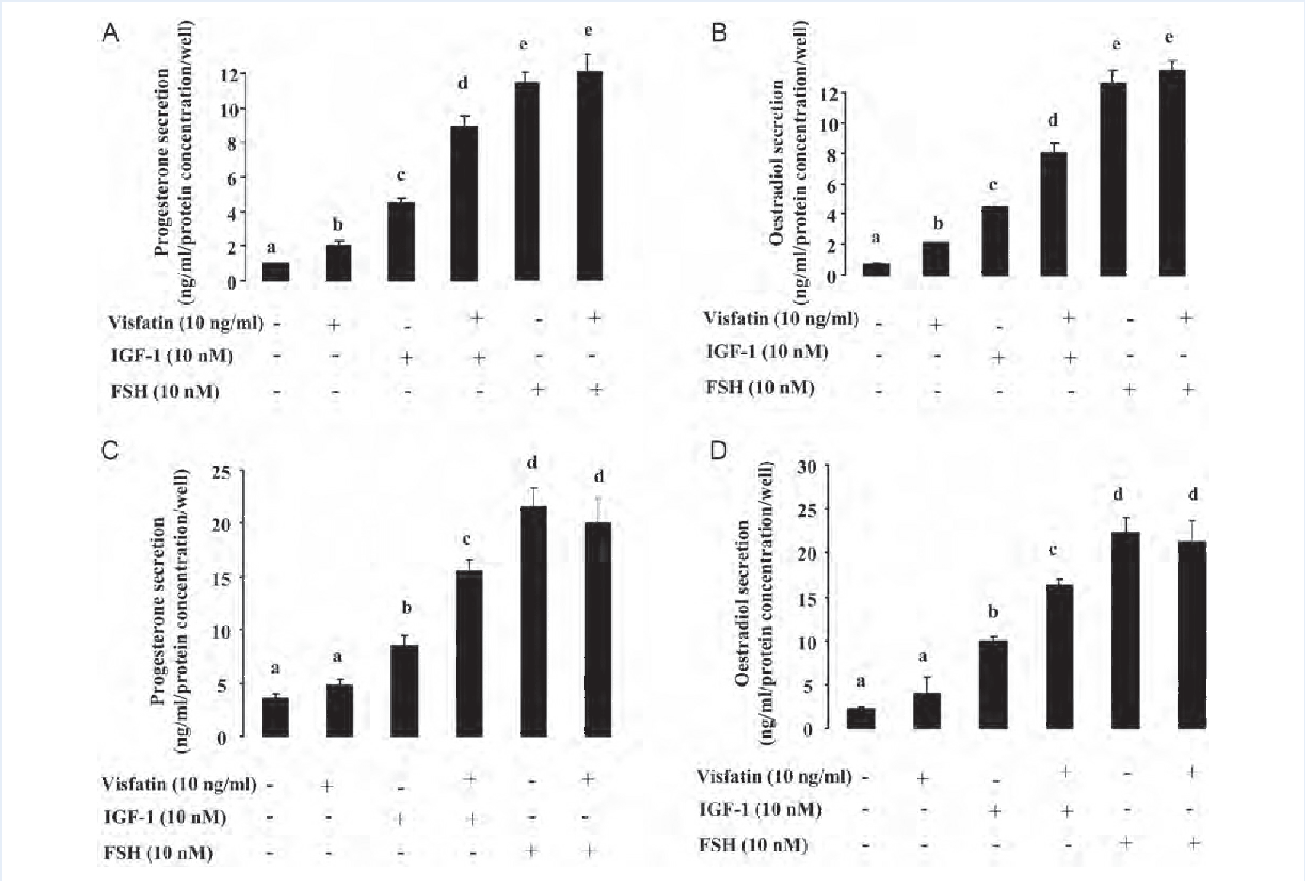

Figure 6 Effect of recombinant human visfatin on basal and IGF-1 or FSH-induced progesterone and estradiol secretion by KGN (**A**, **B**) and primary hGCs (**C**, **D**). (A and C) hGCs (KGN and primary hGCs) were cultured in medium with serum and then in serum-free medium in the absence or in the presence of visfatin (10 ng/ml) ± IGF-1 (10 nM) or FSH (10 nM) for 48 h as described in Materials and Methods. The culture medium was collected, and progesterone (A, C) and estradiol (B, D) production were measured by RIA, and the data are represented as the progesterone or estradiol concentration (ng/ml)/cellular protein concentration/well. Results are mean ± SEM of the six cultures of primary hGCs and four independent experiments (at four different passages) for KGN cells. For primary hGCs, each culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment was applied in quadruplicate. Bars with different letter indicate significant differences ($P < 0.05$).

fatin/NAMPT (Fig. 7). Cells were incubated in serum-free medium with human recombinant visfatin (Rh visfatin) (10 ng/ml) for 48 h in the presence or absence of IGF-1 (10 nM) and/or FK866 (10 nM). As a preliminary experiment, we showed that the dose of FK866 (10 nM) inhibited NAMPT activity causing a significant decrease in the NAD^+ levels (data not shown). As shown in Fig. 7A and B, FK866 totally eliminated the effect of visfatin on progesterone and estradiol production in basal state (no stimulation) and in response to IGF-1. In primary hGCs, FK866 also abolished IGF-1-induced progesterone and estradiol secretion (Fig. 7C and D). Thus, visfatin treatment increased IGF-1- but not FSH-stimulated progesterone and estradiol production in hGCs (KGN and hGC).

Effect of human recombinant visfatin on human granulosa cell proliferation

We next examined the effect of rh visfatin on the DNA synthesis of primary hCGs (Fig. 8A) and KGN cells (data not shown). [^{3}H]-thymidine incorporation by hCGs was determined after 24 h of culture in serum-free medium in the presence or absence of rh visfatin (10 ng/ml) and FK866 (10 nM) ± IGF-1 (10 nM). Visfatin did not affect basal proliferation, whereas IGF-1 treatment alone increased DNA synthesis significantly by about 5-fold in hCGs ($P < 0.05$, Fig. 8A). In these latter cells, visfatin treatment significantly increased [^{3}H]-thymidine incorporation induced by IGF-1 by about 1.3-fold ($P < 0.05$). As shown in Fig. 8A, FK866 eliminated the positive effect of visfatin on the IGF-1-induced cell proliferation (Fig. 8A). Similar results were observed in KGN cells (data not shown). Thus, rh visfatin increased IGF-1-induced DNA synthesis of primary hGCs.

Effect of human recombinant visfatin on signalling pathways in primary hGCs

Because adipokines like adiponectin and resistin can modulate phosphorylation of MAPK ERK1/2 and p38, AKT and AMPK, we studied the pattern of these signalling pathways in response to visfatin in fresh primary hCGs cells. Cells were serum starved overnight and incubated with human recombinant visfatin (10 ng/ml) for different

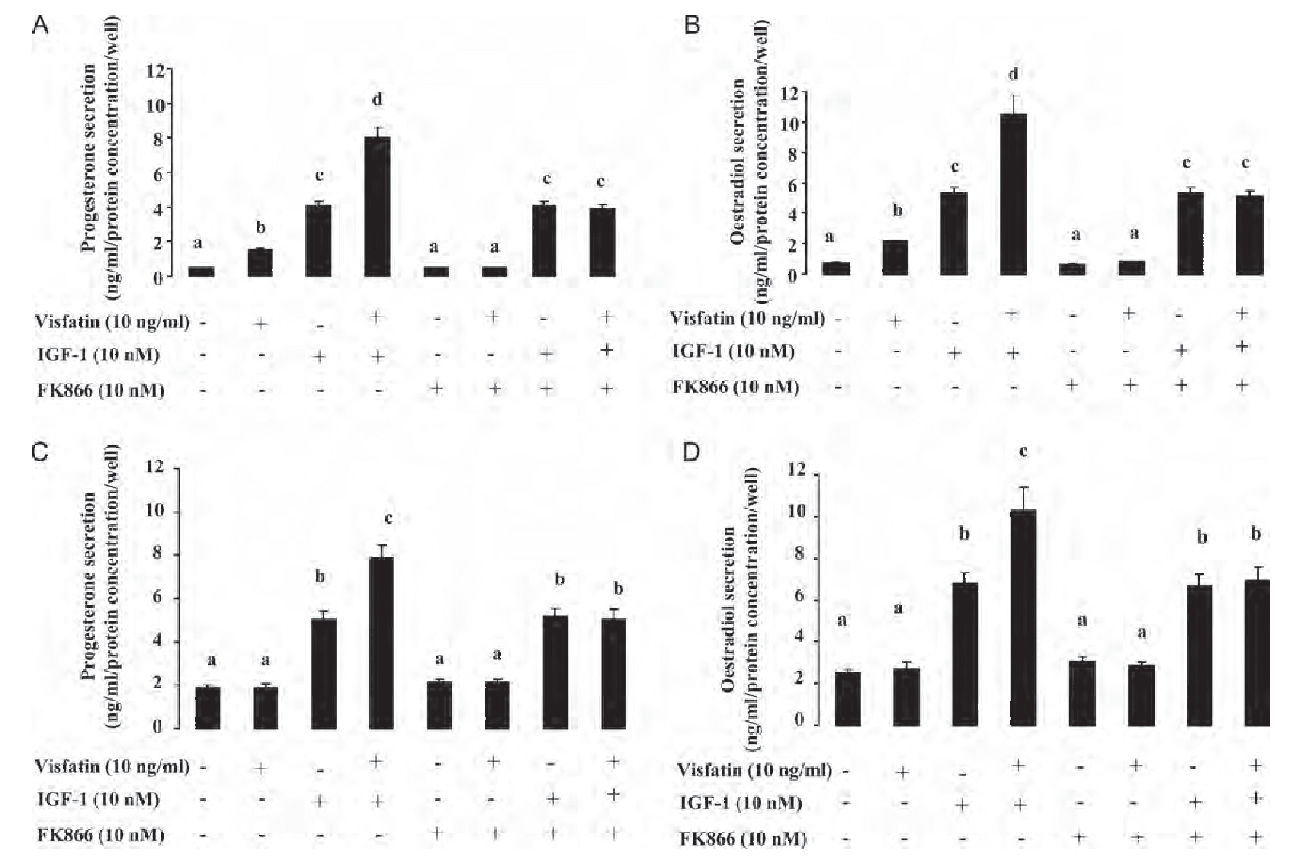

Figure 7 Effect of recombinant human visfatin in the presence or absence of the FK866 visfatin inhibitor, on basal and IGF-1-induced progesterone and estradiol secretion by KGN (**A**, **B**) and primary hGCs (**C**, **D**). (A and C) hGCs (KGN and primary hGCs) were cultured in a medium with serum and then in serum-free medium in the absence or presence of FK866 (10 nM) and visfatin (10 ng/ml) ± IGF-1 (10 nM) for 48 h as described in Materials and Methods. The culture medium was collected, and progesterone (A, C) and estradiol (B, D) production were measured by RIA, and the data are represented as the progesterone or estradiol concentration (ng/ml)/cellular protein concentration/well. Results are mean ± SEM of the six cultures of primary hGCs and four independent experiments (at four different passages) for KGN cells. For primary hGCs, each culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or in the absence of IGF-1 ± FK866) was applied in quadruplicate. Bars with different letter indicate significant differences ($P < 0.05$).

times (1, 5, 10, 30 and 60 min). As shown in Fig. 8B–D, rh visfatin activated quickly (at 1 min of stimulation, $P < 0.05$) the MAPK ERK1/2, p38 and AKT signalling pathways. However, it did not affect AMPK phosphorylation (data not shown). Thus, rh visfatin activates Akt and MAPK-ERK1/2 and -p38 signalling pathway in primary hGCs.

Discussion

In this study, we report for the first time that visfatin is present in human ovarian follicles and in the human ovarian granulosa-like tumour cell line, KGN. In primary hGCs and KGN cells, visfatin expression is regulated by MetF through the AMPK and SIRT1 signalling pathways. Furthermore, recombinant human visfatin increased IGF-1-induced thymidine incorporation and steroid production, suggesting that visfatin could affect ovarian folliculogenesis.

We have also shown the presence of visfatin in human oocytes. Other adipokines, including leptin and adiponectin, have already been found in oocytes of various species, including man (Chabrolle *et al.*, 2007; Madeja *et al.*, 2009). Their role in oocytes is still unclear. Energy homeostasis of the oocyte is a crucial determinant of fertility. It depends on numerous enzymes that are dependent on NAD biosynthesis. Because visfatin is the rate-limiting enzyme in the NAD biosynthesis pathway from nicotinamide, it could influence oocyte quality. Visfatin is a secreted cytokine-like protein (Ognjanovic *et al.*, 2005). Thus, it will be interesting to know if the ovarian cells are able not only to produce but also secrete visfatin and if this local production has a real role in the ovarian functions. Several studies found a similar concentration of visfatin in follicular fluid and plasma, suggesting that ovarian cells do not secrete much visfatin (Shen *et al.*, 2010).

In our study, we have shown that two insulin sensitizers, rosiglitazone and MetF, known to regulate human granulosa cell steroidogenesis modulated visfatin expression in primary hGCs and KGN cells. They increased it after 24 h of stimulation and inhibited it after 48 h of stimulation. These effects were confirmed at the protein level only for MetF. Rosiglitazone affects visfatin mRNA expression only at the higher concentration used (10 μM), whereas a dose–response effect was

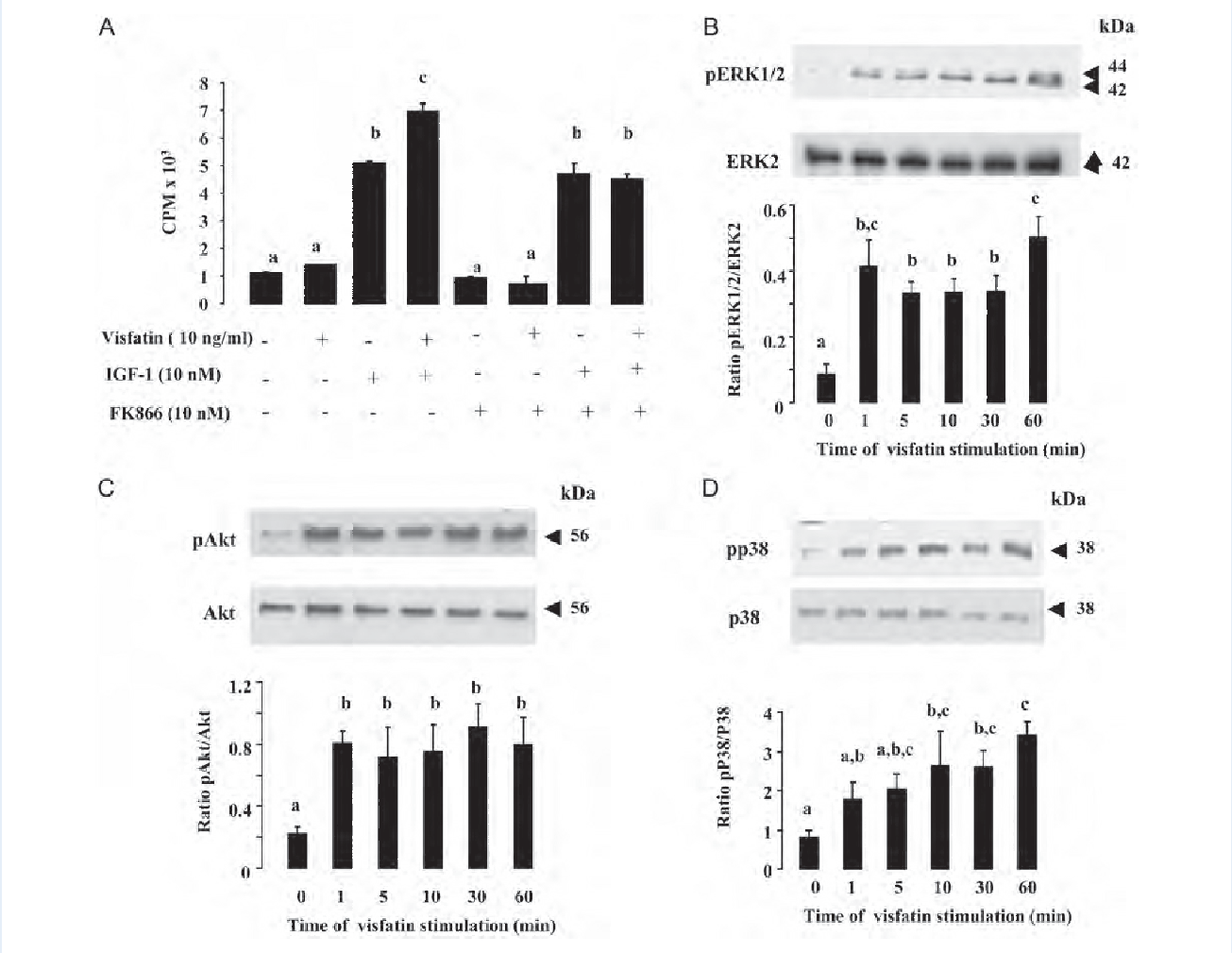

Figure 8 Effect of recombinant human visfatin on thymidine incorporation (**A**) and signalling pathway activation (**B**, **C**, **D**) in primary hCGs. (A) Thymidine incorporation was determined in hGCs cultured for 24 h in the presence or absence of human recombinant visfatin (10 ng/ml) ± IGF-1 (10 nM) and FK866 (10 nM) as described in Materials and Methods. Results are representative of at least four cultures of GCs. Each culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or in the absence of IGF-1 or FK866) was applied in triplicate. The results are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$). (B, C, D) Cell lysates were prepared from primary hGCs incubated with visfatin 10ng/ml for various times: 0, 1, 5, 10, 30 or 60 min. Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-phospho-MAPK ERK1/2 (A), anti-phospho-Akt (B), anti-phospho-p38 (C) and then with anti-ERK2, Akt or P38 protein antibodies. Representative blots from four different cultures are shown. Each culture was made using cells obtained from different follicles from one patient. In each culture, each treatment (different time of visfatin stimulation) was applied in duplicate. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

observed for MetF. Thus, we cannot exclude that the rosiglitazone effect is non-specific. However, rosiglitazone is known to increase visfatin expression in visceral fat of Otsuka Long Evans Tokushima Fatty rats (Choi *et al.*, 2005) and in human macrophages (Mayi *et al.*, 2010). The antidiabetic thiazolidinedione rosiglitazone activates the peroxisome proliferator-activated receptor gamma (PPARγ) that, once activated, forms a heterodimer with the retinoic X receptor (Semple *et al.*, 2006). The binding of this heterodimer to specific DNA sequences, called PPAR response elements (PPRE), results in the regulation of its target genes (Semple *et al.*, 2006). In primary human macrophages, a functional PPRE was identified within the visfatin promoter. In hGCs, PPAR gamma is strongly expressed. Thus, rosiglitazone could increase visfatin expression through the PPRE. We observed no effect of rosiglitazone on visfatin protein levels, suggesting that these effects are only transcriptional. Conversely, MetF treatment regulated mRNA and protein visfatin levels in hGCs. Indeed, it increased visfatin expression after 12 (data not shown) or 24 h of stimulation and decreased it after 48 h of stimulation. A dose–response of MetF was observed for these effects. MetF treatment has already been shown to increase visfatin expression in other cell types than ovarian cells. For example, in mice, administration of MetF increased visfatin expression in white adipose tissue (Caton *et al.*, 2010).

We investigated the molecular mechanisms involved in the regulation of visfatin expression. The search for the specific intracellular targets of MetF is still ongoing. However, AMPK is one of potential targets of MetF's action on glucose and steroid metabolism (Tosca *et al.*, 2006, 2007). It is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells. In GCs, AMPK is expressed and its activation modulates steroid production in different species (Tosca *et al.*, 2005, 2006, 2007). As previously shown (Richardson *et al.*, 2009), we have observed that MetF activates AMPK in hGCs. Furthermore, in our study, we showed that an inhibitor of AMPK, Compound C, abolished the MetF effects on visfatin mRNA and protein expression. In contrast, an activator of AMPK, Aicar, increased and decreased visfatin expression after 24 and 48 h of stimulation, respectively, suggesting that MetF regulates visfatin expression through AMPK in hGCs. We also studied the involvement of the histone deacetylase, SIRT1, in the MetF effects on visfatin expression. Like AMPK, SIRT1 regulates energy homeostasis and is expressed in the ovary (Morita *et al.*, 2012). Furthermore, MetF has already been shown to activate SIRT1 in skin cells (Lee *et al.*, 2010). To our knowledge, our study is the first to show that MetF activates SIRT1 activity in hGCs. Furthermore, we have observed that a specific inhibitor of SIRT1, sirtinol, abolished MetF-induced visfatin mRNA and protein expression, suggesting that not only AMPK but also SIRT1 is involved in this process. Previous studies have reported that AMPK can activate SIRT1 in skeletal muscle and cultured myocytes (Fulco *et al.*, 2008; Canto *et al.*, 2009). Thus, it remains to be determined if MetF-induced visfatin expression through AMPK is SIRT1 dependent and what are the effects on the granulosa cell functions.

Our study is the first to demonstrate that recombinant human visfatin (10 ng/ml) significantly increased IGF-1-induced progesterone and estradiol secretion in both primary hCGs and KGN cells. Furthermore, this effect disappeared when cells were incubated with FK866, a specific inhibitor of visfatin activity. In contrast, we observed no effect of visfatin on the steroid production induced by FSH in hGCs. Plasma visfatin concentration is about 3–30 ng/ml (Shen *et al.*, 2010; Olszanecka-Glinianowicz *et al.*, 2012), suggesting that the dose that we used was not pharmacological. However, we obtained similar results with visfatin 100 ng/ml (data not shown). This effect of visfatin on IGF-1-induced steroid production is in good agreement with the literature. Indeed, studies on human osteoblasts showed that visfatin exerted insulin-mimetic effects through stimulated phosphorylation of insulin receptor, insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) (Xie *et al.*, 2007). It remains to be seen whether visfatin can phosphorylate insulin receptor or IGF-1R in hGCs. In the basal state (no stimulation with IGF-1), we have shown that visfatin activates MAPK ERK1/2 and Akt. However, it is not known whether visfatin increases IGF-1-induced steroid secretion through IGF-1R phosphorylation and/or MAPK ERK1/2 or Akt. We also investigated the effect of rh visfatin on steroid production in response to FSH in primary hGCs, and we observed no effect of visfatin (data shown). This result suggests a specific effect of visfatin on the IGF-1-induced steroidogenesis. In basal state (no IGF-1 stimulation), we detected no effect of visfatin on steroid production in primary hCGs, whereas visfatin treatment increased progesterone and estradiol secretion in KGN cells. In contrast, in both primary hGCs and KGN cells, visfatin increased IGF-1-induced cell proliferation, and this effect was totally eliminated when cells were incubated with the potent NAMPT catalytic inhibitor, FK866. Previous reports have shown that visfatin is a survival factor for various cell types. Indeed, several studies have revealed that visfatin inhibits apoptosis in different cell types (Lim *et al.*, 2008; Rongvaux *et al.*, 2008; Cheng *et al.*, 2011). Furthermore, NAMPT activity of visfatin seems to be required for cell protection because FK866 prevents visfatin-mediated cell protection (Yang *et al.*, 2007). Further studies are needed to elucidate the molecular mechanism involved in the visfatin effect on the IGF-1-induced cell proliferation in hGCs.

In conclusion, we have shown the presence of visfatin in human ovarian follicles, including oocyte and CC. In primary hGCs and KGN cells, visfatin expression (mRNA and protein) is regulated by MetF through AMPK activation and SIRT1 activity. Furthermore, we observed that rh visfatin increased IGF-1-induced steroid secretion and cell proliferation through NAMPT activity in hGC and KGN cells. These findings significantly increase our understanding of the role of visfatin in hGCs. However, further investigations are needed to understand the role of visfatin on other human ovarian cells, including theca cells and oocytes and also its potential involvement in PCOS.

Acknowledgements

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Authors' roles

M.R. participated together with J.D. in the design of the study. The experiments were carried out by M.R., M.C., L.C., C.R., F.G., D.R. and J.D. Data analysis was performed by M.R., M.C., L.C. and J.D. The manuscript was written by M.R. and J.D. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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Article 3

Resistin decreases insulin-like growth factor I–induced steroid production and insulin-like growth factor I receptor signaling in human granulosa cells

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Objective: To identify resistin in human ovarian follicles and investigate the effect and the molecular mechanisms associated with resistin on steroidogenesis in human granulosa cells (GCs).
Design: The effects of recombinant human resistin on the secretion of progesterone (P) and estradiol (E_2) by cultured human GCs were investigated.
Setting: Academic institutions.
Patient(s): Twenty infertile and healthy women undergoing IVF.
Intervention(s): Primary human GC cultures stimulated with recombinant human resistin (10 ng/mL).
Main Outcome Measure(s): Determination of messenger RNA (mRNA) and protein expression of resistin in fresh human GCs by reverse transcriptase-polymerase chain reaction (RT-PCR), immunoblot and immunohistochemistry, respectively; measurement of P and E_2 levels in the conditioned media by radioimmunoassay; determination of cell proliferation by tritiated thymidine incorporation; and analysis of signaling pathways activation by immunoblot analysis.
Result(s): Human GCs and theca cells express resistin. In primary human GCs, resistin decreases P and E_2 secretion in response to insulin-like growth factor I (IGF-I). This was associated with a reduction in the P450 aromatase and P450scc (cholesterol side-chain cleavage cytochromes P450) (P450scc) protein levels but not those of 3β-hydroxysteroid dehydrogenase (3β-HSD) or steroidogenic acute regulatory protein (StAR) and with a decrease in IGF-I-induced IGF-I receptor and mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Resistin treatment does not affect IGF-I-induced cell proliferation and basal steroidogenesis (there is no IGF-I or follicle-stimulating hormone stimulation). In the basal state, resistin rapidly stimulates Akt and MAPK ERK1/2 and p38 phosphorylation in primary human GCs.
Conclusion(s): Resistin is present in human GCs and theca cells. It decreases P and E_2 secretion, P450scc and P450 aromatase protein levels, and IGF-IR signaling in response to IGF-I in primary human GCs. (Fertil Steril® 2013;100:247–55. ©2013 by American Society for Reproductive Medicine.)
Key Words: Adipokines, growth factors, human ovary, signaling, steroidogenesis

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Resistin is an adipokine also known as inflammatory zone 3 (FIZZ3). It is a cysteine-rich, 12.5 kd polypeptide secreted by adipose tissue in rodents (1) and macrophages in humans (2). Resistin has been described as a potential factor in obesity-mediated insulin resistance and type 2 diabetes. In human and rodents, serum resistin levels are higher

in obese compared with lean individuals (3, 4). There is evidence that resistin impairs glucose intake by adipocytes and promotes insulin resistance (1), proinflammatory changes in vascular endothelium (5), and angiogenesis (6). The molecular mechanism of resistin is unclear and seems to depend on the cell type. Indeed, recent studies show that resistin can signal either through the Toll-like receptor-4 (TLR4) in rat hypothalamus (7) or through the tyrosine kinase-like orphan receptor-1 in 3T3-L1 cells (8). An isoform of decorin has also been identified as a resistin receptor involved in white adipose tissue expansion (9).

It is well known that metabolic disorders, including obesity, can affect reproductive functions. However, the factors and molecular mechanisms involved are still obscure. Some data suggest that resistin could regulate male and female fertility. Indeed, resistin is expressed in various reproductive tissues such as the hypothalamus (10), pituitary (11, 12), and gonads (13–15). In the hypothalamus, resistin mRNA is detectable in the arcuate nucleus where gonadotropin-releasing hormone (GnRH) neurons are present. Pituitary resistin is developmentally regulated, reduced in the ob/ob mouse, severely down-regulated by food deprivation (24 hours), and up-regulated by dexamethasone (12, 16). In the rat testis, resistin is expressed in interstitial Leydig cells and, to a lesser extent, in Sertoli cells within seminiferous tubules (13). Testicular expression of resistin is modulated by gonadotropins and fasting in vivo, and it significantly enhances basal and stimulated testosterone secretion in vitro (13). In the bovine ovary, resistin is expressed in the follicle and more precisely in the granulosa, cumulus, theca, and oocyte. In contrast, in mice resistin (mRNA and protein) is undetectable in granulosa cells (GCs).

Recombinant human resistin modulates steroidogenesis and proliferation induced by insulin-like growth factor I (IGF-I) in a species-dependent manner (14). In cultured human theca cells, 17α-hydroxylase activity is increased by resistin in the presence of forskolin or forskolin $\pm$ insulin, suggesting a role of resistin in stimulation of androgen production by theca cells (17). Moreover, some studies have reported a higher serum concentration of resistin in women with polycystic ovary syndrome (PCOS) (18–20), which is known to be associated with hyperinsulinemia, hyperandrogenism, and insulin resistance (21). However, others have reported no variation in serum resistin levels between PCOS and control patients, and have suggested a role of adipokines at the tissue or cellular level (22, 23). Thus, the role of resistin in the pathogenesis of PCOS is still unclear. A recent study showed the presence of resistin in human GCs (24), but the role and the molecular mechanism of resistin in these cells are not yet known. Thus, we identified resistin more precisely in human follicles and investigated the effects and the molecular mechanisms of human recombinant resistin on human GC steroidogenesis in vitro in response to IGF-I and follicle-stimulating hormone (FSH).

MATERIALS AND METHODS

Patients

This collaborative study was performed at the Institut National de la Recherche Agronomique and the Department of the Biology of Reproduction, CHRU Bretonneau (Tours, France). It was approved by the hospital's ethics committee (CHRU Bretonneau, France), and no patients received any monetary compensation for participating in the study; all participants gave written informed consent before the use of cells. Twenty infertile women (36 $\pm$ 4 years old) undergoing IVF with intracytoplasmic sperm injection (ICSI) were recruited in 2011. The causes of infertility were mechanical or male factor infertility without any known endocrinopathy like PCOS, hypotrophism and hypertrophism, or hyperprolactinemia. From each patient, one cycle was included in the study.

Hormones and Reagents

Recombinant human resistin was purchased from Biovendor Research and Diagnostic Products. Purified ovine FSH-20 (oFSH; lot no. AFP-7028D, 4,453 IU/mg, FSH activity = 175 times the activity of oFSH-S1) was a gift from the U.S. National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone Pituitary Program, Bethesda, Maryland. The recombinant human IGF- used for culture treatment was from Sigma-Aldrich.

Antibodies

Rabbit polyclonal antibodies to human resistin were obtained from Biovendor Research and Diagnostic Products. Rabbit polyclonal antibodies to IGF-I receptor β (IGF-IR β; C20), extracellular signal-regulated kinase 2 (ERK2; C14) and p38 (C20) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser 473), and phospho-AMP-activated protein kinase (AMPKα; Thr172) were purchased from New England Biolabs. Rabbit polyclonal antibodies against AMPKα were obtained from Upstate Biotechnology. The cholesterol side-chain cleavage cytochromes P450 (P450scc), steroidogenic acute regulatory protein (StAR), and 3β-hydroxysteroid dehydrogenase (3β-HSD) antibodies were a gift from Dr. Dale Buchanan Hales (University of Illinois, Chicago, IL) and Dr. Van Luu-The (CHUL Research Center and Laval University, Canada), respectively. Mouse monoclonal antibodies to vinculin and P450 aromatase were purchased from Sigma-Aldrich and Serotec, respectively. The phosphotyrosine (PY20) antibodies were obtained from BD Biosciences. All antibodies were diluted at 1/1,000 in Western blotting. To confirm that the observed effects are dependent on IGF-I, we used the 1H7 antibody, which is a monoclonal antibody directed against the α-subunit of the IGF-IR purchased from Santa Cruz Biotechnology.

Isolation and Culture of Human Granulosa Cells

Human GCs were obtained from preovulatory follicles of women undergoing IVF after oocyte retrieval. The ovarian stimulation protocol, IVF, and ICSI procedures used have already been reported (25, 26). After isolation of cumulus-oocyte complexes (COCs), the follicular fluids were centrifuged for 10 minutes at 400 $\times$ g to recover the cell pellet. The cumulus cells were collected after the COCs' dissociation

for ICSI and then were frozen at −80°C. Human GCs were isolated from the red blood cells with 20 minutes of centrifugation at 400 × *g* on two layers of discontinuous Percoll gradient (40%, 60% in Ham's F-12 medium; GIBCO-BRL/Life Technologies). To obtain better purification of hCGs, the 40% fraction was treated with hemolytic medium (NH_4Cl 10 mM in Tris HCL, pH 7.5; Sigma). The cells were then washed with fresh medium (Ham's F-12) and counted in a hemacytometer; cell viability was determined using trypan blue dye exclusion. Finally, the human CGs were cultured in McCoy's 5A medium supplemented with 20 mM HEPES, penicillin (100 IU/mL), streptomycin (100 mg/mL), L-glutamine (3 mM, 0.01% bovine serum albumin, 0.1 μM androstenedione, 5 mg/L transferrin, 20 μg/L selenium) and 5% fetal bovine serum (FBS). After 48 hours, the cells were starved for 24 hours and incubated in fresh medium cultured with or without treatment for an appropriate time. All cultures were performed in a water-saturated atmosphere containing 95% air/5% CO_2 at 37°C. In each experiment, all treatments were applied on three or four independent culture cells.

Resistin Plasma and Follicular Fluid Levels

Resistin was measured in fasting blood and in follicular fluid samples collected the day of the transvaginal oocyte retrieval using a commercially available competitive enzyme-linked immunoassay (ELISA) kit (Phoenix Pharmaceuticals) with a low sensitivity limit of 1.21 ng/mL (range: 0–500 ng/mL) and intra-assay and inter-assay coefficient of variation of <5% and <14%, respectively. The plasma sample was diluted twofold with ELISA buffer before the assay.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA from human GCs and visceral or subcutaneous adipose tissue (Vis AT, Sc AT) were extracted with TRIzol reagent according to the manufacturer's procedure (Invitrogen). Total RNA (1 μg) was denatured and reverse transcripted in a 20 μL reaction mixture containing 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Amersham), 50 pmol of oligo(dT), 15.5 IU of ribonuclease inhibitor, and 15 IU of Moloney murine leukemia virus (M-MLV) reverse transcriptase. The mixture was incubated for 1 hour at 37°C, then polymerase chain reaction (PCR) was performed in 50 μL reaction mixture containing 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 IU of Taq polymerase and 2 μL of the RT mix. We used specific primer pairs for resistin (forward 5′-TGGTGTCTAGCAAGACCCTG-3′ and reverse 5′-GCAGTGA CATGTGGTCTCGG-3′) and β actin (forward 5′-ACGGAAC CACAGTTTATCATC-3′ and reverse 5′-GTCCCAGTCTTCAAC TATACC-3′). The samples were denatured and processed (95°C, 1 minute; 58°C, 1 minute; 72°C, 1 minute), with a final extension step at 72°C for 10 minutes. The PCR products were analyzed on an agarose gel (1.5%) stained with ethidium bromide, and the DNA fragment of interest was sequenced by Genome Express. The RT-PCR consumables were purchased from Sigma (France), and the Moloney murine leukemia virus reverse transcriptase and RNase inhibitor (RNasin) were obtained from Promega.

Protein Extraction and Western Blot

Proteins were extracted from primary human GCs and adipose tissues (Sc and Vis AT) as previously described elsewhere (27, 28). The lysates were centrifuged at 12,000 × *g* for 30 minutes at 4°C, and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay. The protein extracts were denatured, and samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The blots were blocked with Tris-buffered saline buffer supplemented with 0.1% Tween 20 and 5% milk for 30 minutes at room temperature, then the membranes were then incubated for one night at 4°C with specific antibodies at 1/1,000 final dilution. The blots were washed several times and incubated at room temperature for 1 hour, 30 minutes with a horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse IgG (dilution 1/5,000). The signal was detected by a electrochemiluminescence kit using a G:Box SynGene apparatus (Ozyme) with the GeneSnap software (release 4.01.02; Syngene). The results are expressed as the intensity signal in arbitrary units after normalization allowed by the presence of ERK2, p38, Akt, AMPK total (for ERK1/2, p38, Akt, and AMPK phosphorylation, respectively), and vinculin was used as an internal standard for resistin. The effect of recombinant human resistin was observed in the culture of four independent human GCs. Each primary culture was derived from a different patient.

Immunohistochemistry

Human ovary sections were deparaffinized, hydrated, and microwaved for 5 minutes in antigen unmasking solution (Vector Laboratories), and then were allowed to reach room temperature. The sections were washed for 5 minutes in a phosphate-buffered saline (PBS) bath and then were incubated in peroxidase-blocking reagent for 10 minutes at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako). After two washes in a PBS bath for 5 minutes, the nonspecific background was eliminated by blocking with 5% lamb serum in PBS for 20 minutes, followed by incubation overnight at 4°C with PBS containing rabbit primary antibody raised against either resistin (1:100). The sections were washed twice for 5 minutes each time in a PBS bath and were incubated for 30 minutes at room temperature with "ready to use" labeled polymer-HRP anti-rabbit (DakoCytomation Envision Plus HRP system; Dako). The sections were then washed twice in PBS, and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System, DakoCytomation). The negative controls involved replacing primary antibodies with rabbit IgG.

Progesterone and Estradiol Radioimmunoassay

The steroid concentration in the culture medium of human GCs cells was measured by a radioimmunoassay protocol

after an overnight serum starvation and 48 hours of treatment as previously described elsewhere (27, 28). The intra-assay and interassay coefficients of variation were less than 10% and 11%, respectively. The limit of detection of progesterone and estradiol were 12 pg/tube and 1.5 pg/tube, respectively. The intra-assay and inter-assay coefficients of variation were less than 7% and 9%, respectively. The results are expressed as the concentration of steroids (ng/mL)/mg protein/well. The mean ± standard error of the mean (SEM) of the data obtained from three to four independent cultures and in each condition (resistin, IGF-I, 1H7, FSH) were applied in quadruplicate.

Statistical Analysis

All experimental data are expressed as the mean ± SEM. The statistical analysis comprised a *t* test or one-way analysis of variance (ANOVA) to compare the different conditions. If ANOVA revealed statistically significant effects, the means were compared by a Fisher's test. $P<.05$ was considered statistically significant.

RESULTS

Resistin Concentration in the Plasma and Follicular Fluid of Women

Resistin levels were measured in the follicular fluid and plasma of nine infertile women the day of the oocyte retrieval. As shown in Figure 1, the resistin levels were statistically significantly higher (about twofold) in plasma than in follicular fluid, despite the small number of patients ($P<.05$, n = 9).

Resistin Expression in Human Follicles

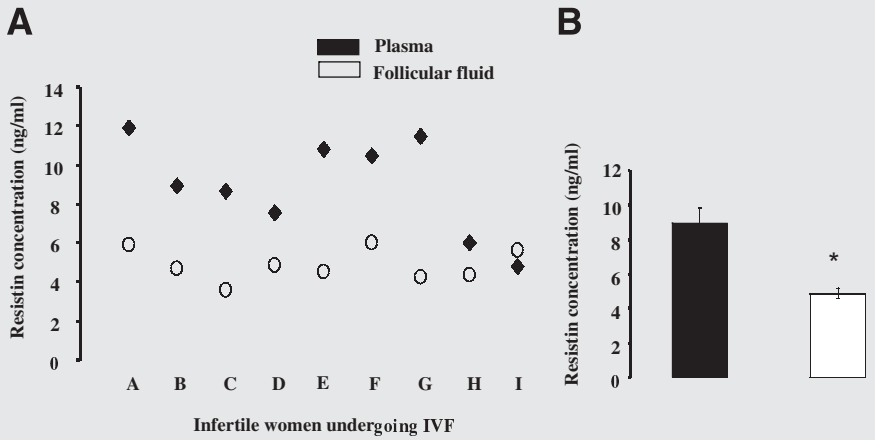
We analyzed the expression (mRNA and protein) of resistin in fresh human GC, human cumulus cells, and in the human GC line KGN. As shown in Figure 2A, the RT-PCR analysis revealed the amplification of one complementary DNA (cDNA) of 300 pb corresponding to resistin (see Fig. 2A). We used as a positive control the visceral and subcutaneous adipose tissues (ATv, ATsc). The transcript of resistin is present in human GCs, cumulus cells, and KGN cells. Similar results were observed at the protein level, as determined by an immunoblotting (see Fig. 1B). We also observed by immunohistochemical analysis that resistin is present in theca cells in the large follicle and the oocytes in the primary follicle (see Fig. 1). Thus, resistin is expressed in oocytes and GCs at all stages of development as well as in theca cells in large follicles.

Effect of Human Recombinant Resistin on Basal and IGF-I or FSH-Induced Steroid Secretion by Primary Human GCs

We had previously shown that adipokines can modulate steroid production in primary human GCs (26, 27), so we investigated whether human recombinant resistin at a physiologic concentration (10 ng/mL) could affect steroidogenesis in primary human GCs. Human GCs were incubated for 48 hours in serum-free medium with human recombinant resistin in the absence or presence of FSH (10^{-8} M) and/or IGF-I (10^{-8} M). As expected, IGF-I and FSH treatment alone significantly increased progesterone (P4) and estradiol (E_2) production by primary human GCs (Fig. 3A and B) (26). We found that IGF-I increased P4 secretion by about fourfold and E_2 production by human GCs by about 4.3-fold. The IGF-I-induced production of P4 and E_2 was statistically significantly eliminated by recombinant human resistin treatment (Fig. 3A and B).

To confirm that the observed effects depend on IGF-I, we used the 1H7 antibody that is a monoclonal antibody directed against the α-subunit of IGF-IR. This antibody has been described as an inhibitor of the biologic activity of IGF-I (29, 30). We incubated cells with the 1H7 antibody (10 μg/mL) and evaluated the effect of resistin in IGF-I-induced steroid

FIGURE 1



Measurement of the concentration of resistin in plasma and follicular fluid of women treated with IVF. Plasma (◆) and FF (○) resistin levels after collecting blood and follicular fluid on the day of the oocyte retrieval from nine infertile women undergoing IVF. Each concentration was determined by ELISA as described in the *Materials and Methods* section. The individual data (A) or the mean of these data (B) are represented. *$P<.05$.

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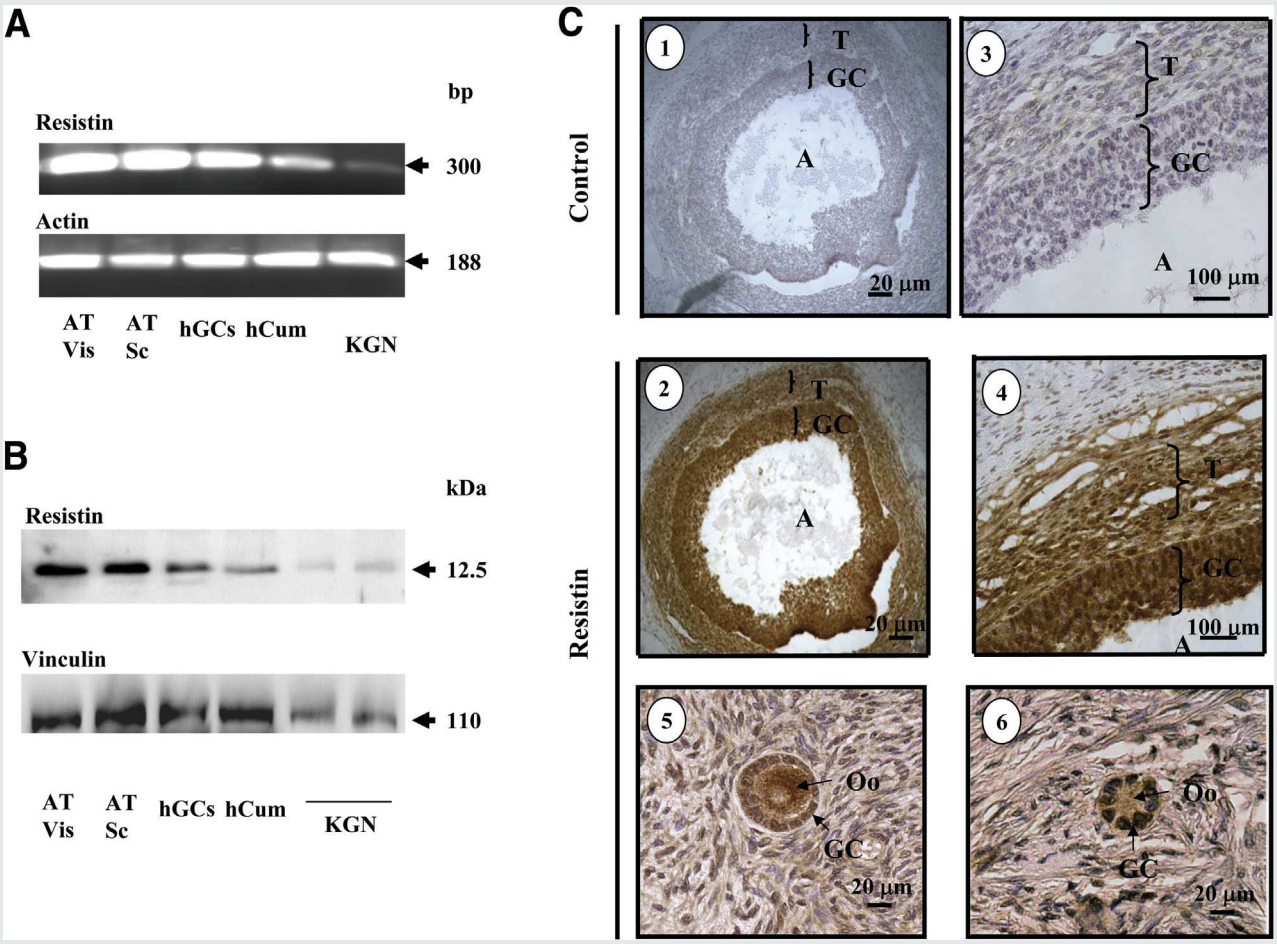
FIGURE 2



Expression of resistin in human ovarian follicle. (A) Total RNA from fresh human GCs, cumulus cells obtained from women undergoing IVF, and a human ovarian granulosa tumor-derived cell line (KGN) was extracted, as described in *Materials and Methods*. We performed RT-PCR with primers designed to amplify fragments of resistin (300 pb). Human visceral (AT Vis) and subcutaneous (AT Sc) adipose tissues were used as a positive control for resistin expression (mRNA [A] and protein [B]). Tissues or cells from four different patients were used and two replications per patient were performed. (B) Protein extracts (50 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with specific antibodies against resistin. Equal protein loading was verified by reprobing membranes with an antivinculin antibody. Tissues or cells from four different patients were used, and two replications per patient were performed. (C) Localization of resistin in human ovarian follicles by immunohistochemistry. The two panels at the bottom are higher magnifications of part of the panels on the top. We performed DAB-immunoperoxidase staining on paraffin-embedded human ovary using antibodies against resistin (2, 4, 5, and 6), or no primary antibodies but rabbit IgG (1 and 3). The immunospecific staining is *brown*. The sections were counterstained with hematoxylin. Resistin was detected in the granulosa cells (GCs) and theca cells (T) of large follicles and in GCs and oocyte (Oo) in primary follicle. A: antrum. Bars = 100 μm or 20 μm. Immunohistochemical analysis was performed on two different human ovary slides from each of four patients.

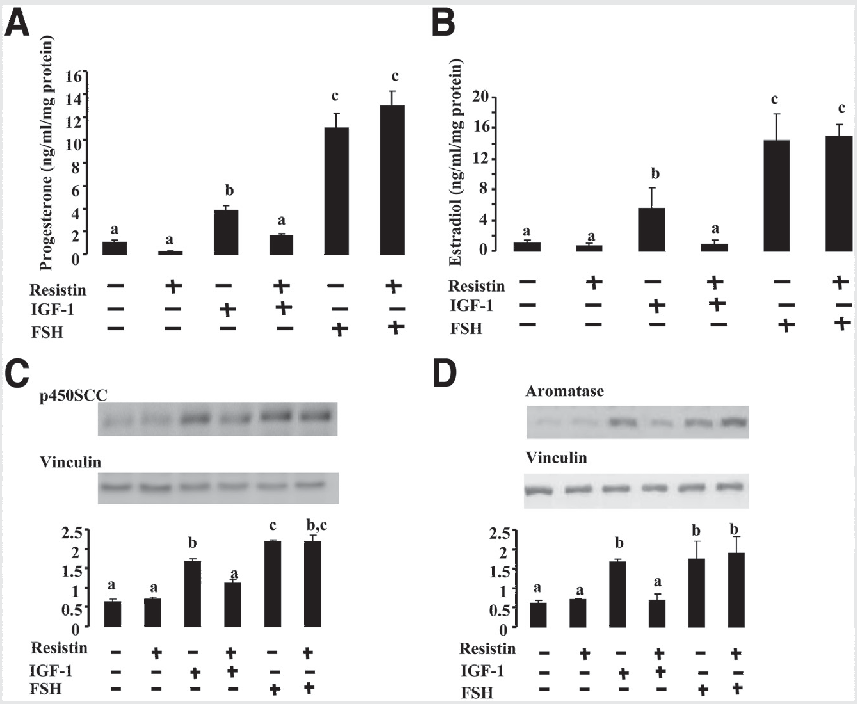
Reverchon. Resistin in human granulosa cells. Fertil Steril 2013.

secretion. We observed that this antibody inhibits the effect of IGF-I on steroid secretion by primary human GCs in the absence or presence of resistin (Supplemental Fig. 1, available online). We observed that recombinant human resistin did not affect steroid secretion by human GCs in the absence or presence of FSH ($P<.05$) (see Fig. 3A and B). Similar results were observed with recombinant human resistin at 100 ng/mL (data not shown).

We also determined whether recombinant human resistin can alter human GC proliferation by [^{3}H]-thymidine incorporation and cell viability by staining with trypan blue. Recombinant human resistin did not affect cell proliferation or cell viability in the basal state or in response to IGF-I or FSH (data not shown). Thus, recombinant human resistin abolished IGF-I-induced P_4 and E_2 production without any effect on the proliferation and viability of human GCs.

To better understand how recombinant human resistin affects human GC steroidogenesis, we investigated the effect of recombinant human resistin on the protein levels of three important enzymes of steroidogenesis, P450scc, 3β-HSD, and P450 aromatase, and the amount of StAR, which is an important cholesterol transporter. As shown in Figure 3C and D, resistin treatment (10 ng/mL) strongly decreased the protein levels of P450scc and P450 aromatase in the presence of IGF-I, whereas no statistically significant effect was

FIGURE 3



Effect of recombinant human resistin on basal and FSH or insulin-like growth factor I (IGF-I)-stimulated P (A) and E_2 (B) secretions by primary human GCs and on the amount of the P450scc (C) and P450 aromatase (D) protein in primary human GCs. Human GCs were cultured for 48 hours in a medium with serum and then in serum-free medium in the absence or presence of resistin (10 g/mL) ± FSH (10^{-8} M) or IGF-I (10^{-8} M) as described in the *Materials and Methods* section. The culture medium was collected and P (A) and E_2 (B) production was measured by radioimmunoassay. Results are mean ± standard error of the mean (SEM) of the three independent groups of GCs from five or six patients. Different letters indicate statistically significant differences ($P<.05$). (C, D) Protein extracts from human GCs, cultured for 48 hours in the absence or presence of resistin (10 ng/mL) ± FSH (10^{-8} M) or insulin-like growth factor I (IGF-I) (10^{-8} M), were submitted to SDS-PAGE, as described in the *Materials and Methods* section. The membranes were probed with antibodies against P450scc (C) and P450 aromatase (D). Equal protein loading was verified by reprobing membranes with an antivinculin antibody. Results are representative of at least three independent experiments. The blots were quantified, and the P450scc or P450 aromatase-to-vinculin ratio is shown. The results are expressed as mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences ($P<.05$).

Reverchon. Resistin in human granulosa cells. Fertil Steril 2013.

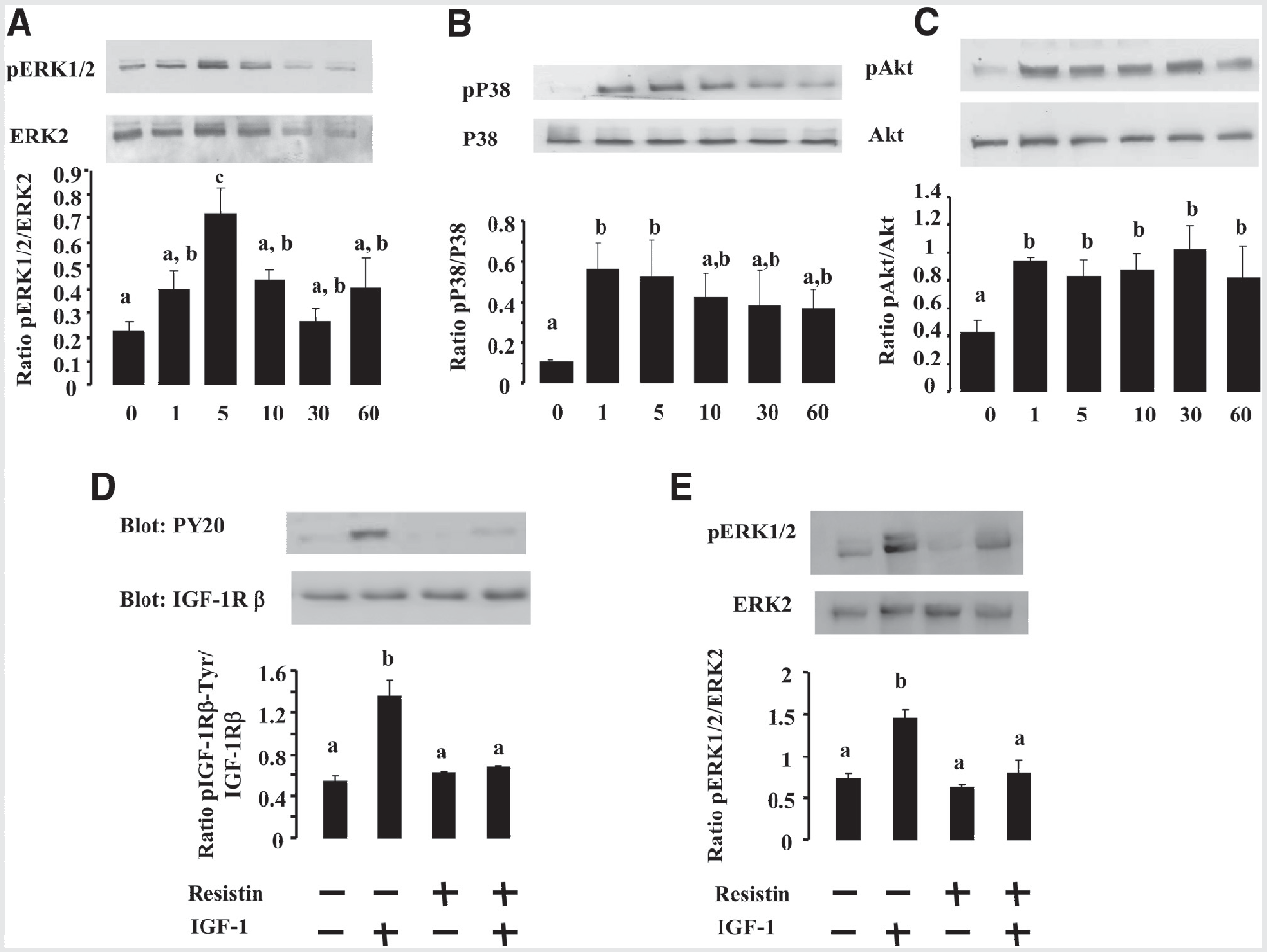
observed in the presence of FSH or in the basal state (no stimulation). Conversely, resistin treatment (10 ng/mL) did not affect the amount of StAR or 3β-HSD in the absence or presence of IGF-I or FSH (data not shown).

Effect of Human Recombinant Resistin in Human GCs on Signaling Pathways

It is well known that adipokines such as resistin can activate various signaling pathways in different cell types, including GCs (14). Here, we measured the effect of resistin (10 ng/mL) on the phosphorylation levels of Akt, AMPK, and mitogen-activated protein kinase (MAPK) ERK1/2 and p38 in the absence (Fig. 4A–4C, and data not shown) or presence of IGF-I (Fig. 4E, and data not shown). As shown in Figure 4A–4C, recombinant human resistin (10 ng/mL) rapidly activated (at 1 or 5 minutes, $P<.05$) the Akt and MAPK ERK1/2 and p38 signaling pathways. This activation was transient for MAPK p38 and ERK1/2 and was maintained for 60 minutes for Akt. No statistically significant variation in phosphorylation was observed for the AMPK signaling pathway in response to recombinant human resistin. Thus, recombinant human resistin activates MAPK (ERK1/2 and p38) and Akt signaling pathway in human GCs.

We next investigated the molecular mechanisms involved in the effect of resistin on steroid production in response to IGF-I in human GCs. More precisely, we determined whether resistin treatment can alter IGF-IR signaling. Human GCs were incubated in serum-free medium supplemented with recombinant human resistin (10 ng/mL) for 48 hours (conditions used to measure P_4 and E_2 production) in the absence or presence of IGF-I (10^{-8} M) treatment for 10 minutes. As expected, IGF-I treatment alone increased by about 2.5-fold and by about 2.1-fold ($P<.05$) tyrosine phosphorylation of IGF-IR receptor β subunit (Fig. 4D) and phosphorylation levels of MAPK ERK1/2 (Fig. 4E), respectively. Treatment with only recombinant human resistin did not affect basal phosphorylation of IGF-IRβ and MAPK ERK1/2 whereas it totally eliminated IGF-I-induced tyrosine phosphorylation of IGF-IR β subunit and phosphorylation of MAPK ERK1/2 (Fig. 4D and E). To confirm that the observed effects are dependent on IGF-I, we incubated cells with the 1H7 antibody (10 μg/mL) and evaluated the effect of resistin in IGF-I-induced tyrosine phosphorylation of IGF-IR β subunit

FIGURE 4



Effect of human recombinant resistin on the MAPK ERK1/2 (A), p38 (B), and Akt (C) phosphorylation levels and on phosphorylation of IGF-IR β subunit (D) and MAPK ERK1/2 (E) in response to IGF-I in primary human GCs. Human GC lysates were prepared from cells incubated with 10 ng/mL resistin for various times: 0, 5, 10, 30, 60, or 120 minutes. Lysates (50 mg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-MAPK ERK1/2 (A), p38 (B), or Akt (C) and then with anti-ERK2, p38 or Akt total protein antibodies. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated MAPK ERK1/2/ERK2, phosphorylated MAPK p38/p38 or phosphorylated Akt protein ratios are shown. The results are represented as mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences ($P<.05$). (D, E) Primary human GCs were cultured in a medium with serum and then in serum-free medium in the absence or in the presence of resistin (10 ng/mL) ± IGF-I (10^{-8} M) for 48 hours (conditions used to measure P and E_2 production). Cells were lysed, and lysates were directly subjected to immunoblotting with antibodies recognizing phosphotyrosine (PY20) (panel D) or with anti-phospho-MAPK ERK1/2 (panel E) antibodies. IGF-IRβ and MAPK ERK2 levels were evaluated by reprobing the membranes with IGF-IRβ and ERK2 total antibodies, respectively. Representative blots from four different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (resistin in the presence or absence of IGF-I) was applied in duplicate. The blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as mean ± SEM.

Reverchon. Resistin in human granulosa cells. Fertil Steril 2013.

and phosphorylation of MAPK ERK1/2. We observed that this antibody inhibits the effect of IGF-I on IGF-IR β subunit and MAPK ERK1/2 phosphorylation by primary human GCs in the absence or presence of resistin (Supplemental Fig. 2, available online).

DISCUSSION

In the present study, we report the localization of resistin in the human ovarian follicle and we investigate for the first time its effects and its molecular mechanisms in vitro in GCs. By RT-PCR, immunoblot and immunohistochemistry, we show that the mRNA and the protein for resistin are found in human GCs. Moreover, by ELISA assay we detected lower levels of resistin in plasma than in follicular fluid. In primary human GCs, we observed that human recombinant resistin decreased IGF-I-induced progesterone and estradiol production without any variation of StAR and 3betaHSD protein levels. However, this was associated with a reduction in the protein amount of P450scc and P450 aromatase and an inhibition of the IGF-I-induced tyrosine phosphorylation of IGF-IR β subunit and phosphorylation of MAPK ERK1/2.

We also showed that resistin treatment alone activated various signaling pathways including MAPK (ERK1/2 and p38) and Akt in primary human GCs.

We found resistin in human ovarian follicles, especially in granulosa, cumulus, and theca cells and oocytes. Similar results were observed in bovine species that also express resistin in oocytes (14, 15). In contrast, resistin is not present in rat GCs, suggesting that it is expressed in a cell-specific manner according to the species. In the cells of the white adipose tissue, resistin is also located differently in man and rodents (1, 2). In our present study, we observed in nine patients a lower concentration of resistin in human follicular fluid than in plasma on the day of the oocyte retrieval. Our results are in good agreement with a previous study that reported lower resistin levels in follicular fluid than in plasma in both control and PCOS groups (31). In a previous study, we showed opposite results for other adipokines including chemerin and adiponectin. Using the same samples as those analyzed in the present study, we have observed that chemerin levels are higher in follicular fluid than in plasma (26). Similar results were found for adiponectin measured on other samples (27).

The limitation of all these studies is that the follicular fluid from the different follicles for each individual patient was pooled, and thus the adipokine levels were not measured in each individual follicle. However, the overall follicular resistin levels from our study and those observed by Seow et al. (31) are very low compared with the serum levels, despite the samples being pooled. Thus, these results suggest that resistin does not have a paracrine effect in the process of oocyte development in women undergoing IVF.

We showed that resistin is not only expressed in human GCs but also active in these cells because resistin treatment (10 ng/mL) increased MAPK ERK1/2, p38, and Akt phosphorylation in primary human GCs. These signaling pathways were also activated in response to resistin in rat and bovine cultured GCs (14). They are all involved in the functions of GCs, including steroidogenesis and proliferation (32–34). We did not observe an effect of resistin on AMPK phosphorylation, although recombinant human resistin is able to activate AMPK in bovine GCs (14). It is possible that the stimulation time used in our experiment was not enough long to activate AMPK in human GCs. The concentration of resistin tested in vitro was physiologic, as in humans the average plasma resistin levels range from 4 to 8 ng/mL (23, 35).

In this study, we demonstrated that recombinant human resistin significantly reduced P and E_2 secretions in response to IGF-I but not to FSH in primary human GCs. It has already been shown that recombinant resistin can alter in vitro steroid production by rat and bovine GCs (14). Other adipokines have been also reported to affect GC steroidogenesis. For example, leptin and chemerin treatment decrease IGF-I-induced steroid production by human GCs (26, 36), whereas opposite effects are observed with adiponectin treatment (27). Chemerin is known to decrease insulin sensitivity in different tissues; they could inhibit IGF-I action in the human GCs. We also have shown that resistin strongly reduces tyrosine phosphorylation of the IGF-IR β subunit and phosphorylation of MAPK ERK1/2 in primary human GCs. We reported elsewhere that we had observed similar results for chemerin (26).

The molecular mechanism of resistin action is still obscure. However, in rheumatoid arthritis it has been shown that resistin can use the IGF-IR signaling pathway (37). In 3T3-L1 adipocytes, resistin inhibits several components of insulin signaling, including insulin receptor (IR) phosphorylation, IR substrate 1 (IRS-1) phosphorylation, phosphatidylinositol-3-kinase (PI3K) activation, phosphatidylinositol triphosphate production, and activation of protein kinase B/Akt (38). The reduction in IGF-I-induced MAPK ERK1/2 phosphorylation by resistin treatment could help to explain the inhibition of steroidogenesis in primary human GCs. Indeed, several studies have demonstrated that the MAPK ERK1/2 signaling pathway positively regulates in vitro steroid production by human and rat GCs (39, 40).

Our study has shown that the decrease in IGF-I-induced steroid production in response to resistin is associated with a reduction in the amount of P450scc and P450 aromatase, which are limiting steroidogenesis enzymes. Indeed, P450scc converts cholesterol into pregnenolone, a progesterone precursor in ovarian cells, and P450 aromatase converts androgen into estrogen. We have also shown that chemerin treatment inhibits P450 aromatase protein levels in response to IGF-I in primary human GCs (26). Wang et al. (41) also have demonstrated that chemerin inhibits FSH-induced P450 aromatase expression and E_2 secretion in rat-cultured follicles and GCs in vitro (41). All these results suggest that resistin, like other adipokines, can regulate GC steroidogenesis in vitro. Through immunohistochemistry analysis, we have shown that resistin is also expressed in theca cells. In cultured human theca cells, 17α-hydroxylase activity is increased by resistin in the presence of forskolin or forskolin $\pm$ insulin, suggesting a role of resistin in stimulation of androgen production by theca cells (17).

We have demonstrated that resistin is not only expressed in granulosa but also in cumulus and theca cells and in oocytes from human ovarian follicles. Furthermore, in human GCs in vitro it can activate the MAPK (ERK1/2 and p38) and Akt signaling pathways. We have shown for the first time that human recombinant resistin decreased IGF-I-induced P and E_2 production by human GCs, and that this was associated with a reduction in the P450scc and P450 aromatase levels and an inhibition of tyrosine phosphorylation of IGF-IR and MAPK phosphorylation. However, further investigations are required to determine whether the resistin expression in ovarian cells is altered in some forms of infertility, as in PCOS.

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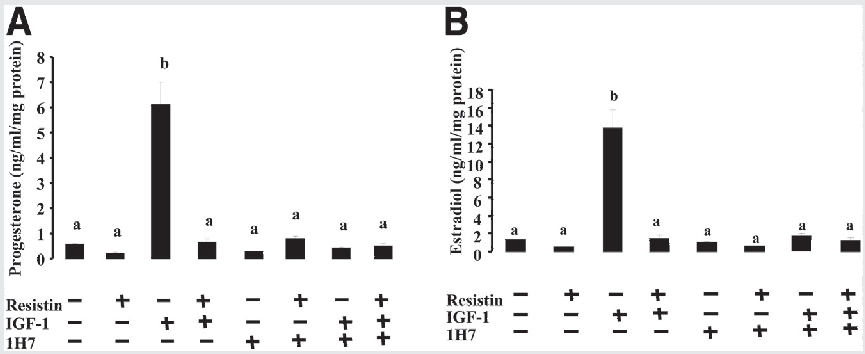
SUPPLEMENTAL MATERIALS AND METHODS

Thymidine Incorporation into Granulosa Cells

Primary human granulosa cells (GCs) (2×10^5 viable cells/500 μL) were cultured in 24-well dishes in McCoy's 5A medium and 10% fetal bovine serum (FBS) for 48 hours. After serum starvation overnight, the human GCs were incubated for 24 hours with 1 μCi/μL of [3H] thymidine (Amersham Life Science) in the presence or absence of resistin and/or IGF-I (10^{-8} M). Excess thymidine was then removed by washing twice with phosphate-buffered saline (PBS), fixed with cold 50% trichloroacetic acid for 15 minutes, and lysed by NaOH 0.5 N. The radioactivity, expressed as counts per minute (cpm), was determined by scintillation fluid (Packard Bioscience) and counting in a β-photomultiplier. The values are representative of three independent cultures, with each condition in triplicate.

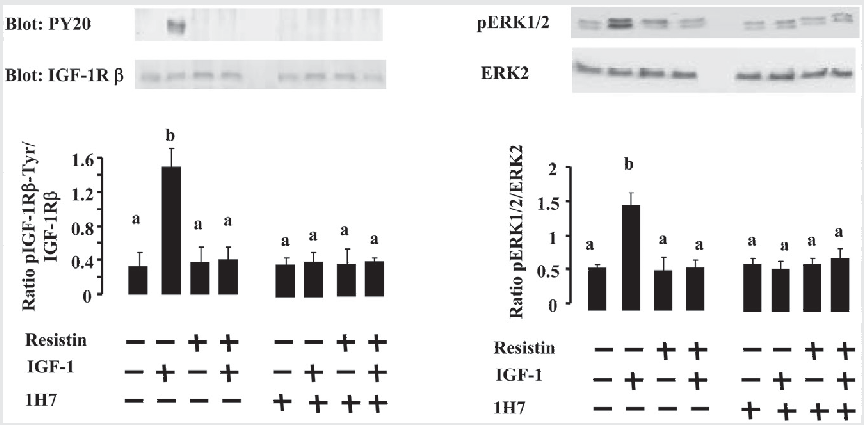
SUPPLEMENTAL FIGURE 1



Effect of recombinant human resistin in the presence or absence of IGF-IRβ 1H7 antibody on basal and FSH or insulin-like growth factor I (IGF-I)-stimulated P (A) and E_2 (B) secretions by primary human GCs. Human GCs were cultured for 48 hours in a medium with serum and then in serum-free medium in the absence or presence of resistin (10 g/mL) ± IGF-I (10^{-8} M) ± IGF-IRβ IH7 antibody (10 μg/mL), as described in the *Materials and Methods* section. The culture medium was collected, and P (A) and E_2 (B) production was measured by radioimmunoassay. Results are mean ± standard error of the mean (SEM) of the three independent groups of GCs from five or six patients. Different letters indicate statistically significant differences ($P<.05$).

Reverchon. Resistin in human granulosa cells. Fertil Steril 2013.

SUPPLEMENTAL FIGURE 2



Effect of human recombinant resistin in the presence or in the absence of IGF-IRβ IH7 antibody on phosphorylation of IGF-IR β subunit (A) and MAPK ERK1/2 (B) in response to IGF-I in primary human GCs. Primary human GCs were cultured in a medium with serum and then in serum-free medium in the absence or in the presence of resistin (10 ng/mL) $\pm$ IGF-I (10^{-8} M) $\pm$ IGF-IR 1H7 antibody (10 μg/mL) for 48 hours (conditions used to measure P and E_2 production). Cells were lysed, and the lysates were directly subjected to immunoblotting with antibodies recognizing phosphotyrosine (PY20) (panel A) or with anti-phospho-MAPK ERK1/2 (panel B) antibodies. The IGF-IRβ and MAPK ERK2 levels were evaluated by reprobing the membranes with IGF-IRβ and ERK2 total antibodies, respectively. Representative blots from four different cultures are shown. Each culture was performed by using cells obtained from different follicles from one patient. In each culture, each treatment (resistin in the presence or absence of IGF-I $\pm$ IGF-IR 1H7 antibody) was applied in duplicate. The blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as mean $\pm$ SEM.

Reverchon. Resistin in human granulosa cells. Fertil Steril 2013.

Etudes des adipocytokines, résistine et chemerine chez la vache au niveau du tissu adipeux et de l'ovaire (Articles 4 et 5)

Dans ce travail, nous avons étudié les adipocytokines résistine et chemerine chez la vache. Pour la résistine, une précédente étude réalisée au laboratoire a montré sa présence dans l'ovaire bovin et son influence sur les fonctions ovariennes (Maillard et al., 2011). Ici, nous avons déterminé la concentration plasmatique de la résistine et son rôle dans le tissu adipeux durant la lactation chez la vache laitière. Nous avons plus précisément comparé la concentration plasmatique en résistine avec celle de l'insuline, du glucose, et des acides gras non estérifiés (AGNE) avant vêlage et au début de la lactation où la mobilisation des réserves adipeuses est élevée. Lors de la seconde lactation nous avons en plus étudié les niveaux des transcrits et protéiques de la résistine dans le tissu adipeux après vêlage et à 5 mois de gestation où une reconstitution des réserves adipeuses est observée. Enfin nous avons analysé l'effet de la résistine sur la lipolyse *in vitro* dans des explants de tissu adipeux prélevés un ou deux mois après vêlage.

Concernant la chemerine nous avons étudié dans l'ovaire bovin les différents paramètres cités précédemment lors des études dans l'ovaire humain. De plus, ici nous avons recherché la présence et l'expression des récepteurs de la chemerine et étudié l'effet *in vitro* de la rh chemerine sur la maturation ovocytaire.

Les différents points présentés sont les suivants :

Etude de la concentration plasmatique de résistine et de son expression dans le tissu adipeux sous-cutané de vache laitière à différentes périodes de la lactation (Article 4)

A) Concentration plasmatique de résistine durant la première et la deuxième lactation

B) Analyse des niveaux d'ARNm et protéiques de la résistine et niveau protéique de l'adiponectine dans le tissu adipeux de vache à une semaine avant vêlage (SAV) et 5 mois de gestation (5 MG).

C) Etude des niveaux de phosphorylation de IRβ, IRS-1, IRS-2 et IGF-1Rβ dans le tissu adipeux de vache à une SAV et 5 MG

D) Expression du gène de la résistine dans des adipocytes mature bovin

E) Effet de la résistine recombinante bovine sur la libération de glycérol à partir d'explants du tissu adipeux sous cutané et sur le niveau d'ARNm d'ATGL (adipose triglycéride lipase) et HSL (Hormone-sensitive lipase) dans des explants de tissu adipeux

Etude de l'impact de la rh chemerine sur les fonctions ovariennes bovines (Article 5)

A) Caractérisation de la chemerine et de ces récepteurs dans l'ovaire bovin

B) Régulation de l'expression de la chemerine et de ses récepteurs dans des cultures *in vitro* de cellules de la granulosa bovine

C) Effet de la rh chemerine sur la sécrétion de stéroïdes basal ou induite par IGF-1 ou FSH dans les cellules primaires de la granulosa bovine

D) Effet de la rh chemerine sur la teneur en cholestérol et le niveau protéique de HMG co-réductase dans les cellules primaires de la granulosa

E) Effet de la chemerine sur la prolifération et la viabilité des cellules primaires de la granulosa bovine

F) Voies de signalisation activées en réponse à la chemerine dans les cellules primaires de la granulosa bovine

G) Effet de la rh chemerine sur la maturation ovocytaire bovine de COCs et la sécrétion de P4 lors d'une maturation *in vitro*

H) Effet de la rh chemerine sur le niveau de phosphorylation de la voie MAPK3/1 dans l'ovocyte et les cellules du cumulus des COCs chez le bovin lors d'une maturation *in vitro*

A) Concentration plasmatique de résistine durant la première et la deuxième lactation (Article 4)

Lors de la première lactation nous avons déterminé la concentration plasmatique de la résistine, des acides gras non estérifiés (AGNE), glucose et insuline chez 8 vaches laitières Prim Holstein à partir de 4 semaines avant vêlage jusqu'à 22 semaines après parturition (**Fig**

1). Ces concentrations varient grandement avant et après lactation (**Table 2**). Comme attendu les concentrations plasmatiques de glucose et d'insuline sont plus faibles alors que la concentration plasmatique d'AGNE est plus élevée une semaine après parturition qu'avant vêlage (P<0,001, **Fig 1 B-D**). La concentration plasmatique de résistine apparait plus faible avant vêlage (environ 40 ng/ml) avant d'augmenter et d'atteindre un seuil une semaine après vêlage (environ 90 ng/ml). Sa concentration va par la suite diminuer progressivement jusqu'à un nouveau seuil pré-vêlage 6 semaines avant parturition (**Fig 1 A, Table 2**). La concentration de résistine ne varie pas entre les semaines 6 et 22 après parturition (**Table 2**). Nous avons observé une corrélation significative entre les concentrations plasmatiques de résistine et celles d'AGNE (r=0,43, P<0,001) entre 4 semaines avant vêlage et 22 semaines post-partum (**Table 3**).

Nous avons ensuite analysé des paramètres zootechniques concernant la production laitière, la prise alimentaire, le poids et la balance énergétique à partir du vêlage jusqu'à 22 semaines après parturition durant la première lactation. Nous avons constaté une corrélation significative entre la résistine plasmatique et le rendement laitier (r=-0,52, P<0,0001) la résistine plasmatique et l'ingestion de matière sèche (r=-0,65, P<0,001) et la résistine plasmatique et la balance énergétique (r=-0,62, P<0,0001) (**Table 5**).

Durant la seconde lactation, nous avons mesuré les mêmes paramètres que précédemment et plus précisément le jour où a été effectuée la biopsie de tissu adipeux, c'est à dire une semaine après parturition et à 5 mois de gestation. Les concentrations plasmatiques de résistine et d'AGNE sont supérieures (Fig 2 A et B) alors que les concentrations plasmatiques de glucose et d'insuline sont plus faibles (Fig 2 C et D) une semaine après vêlage qu'à 5 mois de gestation. Ceci peut s'expliquer par le fait que les vaches laitières Prim Holstein mobilisent leurs réserves pour la production laitière une semaine après vêlage alors qu'à 5 mois de gestation leurs réserves corporelles sont en reconstitution.

B) Analyse des niveaux d'ARNm et protéiques de la résistine et niveau protéique de l'adiponectine dans le tissu adipeux de vache à une semaine avant vêlage et 5 mois de gestation (Article 4)

Du tissu adipeux a été prélevé lors de la deuxième lactation à une semaine avant vêlage (SAV) et à 5 mois de gestation (MG). Nous observons que les niveaux de messager et protéique de la résistine sont plus élevés une SAV qu'à 5 MG (**Fig 3A et B**). A l'opposé les niveaux protéiques d'adiponectine sont plus faibles à SAV qu'à 5 MG (**Fig 3C**).

C) *Etude des niveaux de phosphorylation de IRβ, IRS-1, IRS-2 et IGF-1Rβ dans le tissu adipeux de vache à une SAV et 5 MG (Article 4)*

Nous avons analysé l'activation des récepteurs IRβ, IRS-1, IRS-2 et IGF-1Rβ dans le tissu adipeux une SAV et à cinq MG. Nous observons que les niveaux de phosphorylation de tyrosine d'IRβ, IRS-1 et IRS-2 sont plus faibles ($P<0,05$) à une SAV qu'à cinq MG (**Fig 4 A C et D**). Le niveau de phosphorylation d'IGF-1Rβ n'est pas modifié (**Fig 4 B**). De plus, le niveau protéique des récepteurs étudiés ne varie entre une SAV et 5MG.

D) *Expression du gène de la résistine dans des adipocytes mature bovin (Article 4)*

Nous avons étudié le niveau d'ARNm de résistine dans le tissu adipeux sous-cutané chez des vaches laitières adultes. Par PCR en temps réel nous avons observé que l'expression du messager de la résistine est plus élevée dans les adipocytes matures que dans les cellules stromales vasculaires (**Fig 6A**). Les cellules stromales vasculaires regroupent plusieurs types cellulaires comme des pré-adipocytes, des cellules immunitaires, des fibroblastes et des cellules endothéliales. Etant donné que chez l'humain la résistine est majoritairement produite par les macrophages (Kaser et al., 2003 ; Patel et al., 2003), nous avons vérifié que la présence de la résistine dans les adipocytes matures n'était pas due à une contamination par des cellules immunitaires. Pour cela nous avons quantifié le niveau d'ARNm de CD68 (le principal marqueur de surface des macrophages), il apparaît être élevé dans les cellules stromales vasculaires et faible dans les adipocytes matures (**Fig 6A**). Ce résultat suggère donc que la résistine est bien produite par les adipocytes bovins matures. Nous avons confirmé ces observations par immunohistochimie (**Fig 6 B**). En effet, nous avons réalisé des immunohistochimies sur du tissu adipeux sous-cutané de vaches âgées de 32 mois avec un anticorps dirigé contre la résistine, le même anticorps utilisé précédemment. Nous observons une forte présence de la résistine dans ce tissu (**Fig 6 C**).

E) *Effet de la résistine recombinante bovine sur la libération de glycérol à partir d'explants du tissu adipeux sous-cutané et sur le niveau d'ARNm d'ATGL (adipose triglycéride lipase) et HSL (Hormone-sensitive lipase) dans des explants de tissu adipeux (Article 4)*

Nous avons déterminé l'effet de plusieurs concentrations de résistine recombinante bovine en présence ou non d'insuline (10^{-8}M), sur la lipolyse dans des explants de tissu adipeux sous-cutané bovin en mesurant la teneur en glycérol et le niveau de messager de ATGL (adipose triglycéride lipase) et de HSL (hormone-sensitive lipase) après 4h d'incubation. La résistine bovine recombinante augmente la teneur en glycérol de manière dose-dépendante (**Fig 7 A**).

Une concentration de 100 ng/ml de résistine recombinante bovine annule l'effet inhibiteur de l'insuline sur la teneur en glycérol (**Fig 7 A**). De plus, la résistine recombinante bovine (100 ng/ml) augmente le niveau d'ARNm de ATGL et HSL à l'état basal et en réponse à IGF-1 dans des explants de tissu sous-cutané bovin (**Fig 7 B**).

Etude de l'impact de la rh chemerine sur les fonctions ovariennes bovines

A) Caractérisation de la chemerine et de ses récepteurs dans l'ovaire bovin (Article 5)

D'autres adipocytokines comme la leptine, l'adiponectine et la résistine ont déjà été identifiées dans l'ovaire bovin. Par RT-PCR, nous avons amplifié des fragments d'ADNc correspondant à la chemerine, CMKLR1, GPR1 et CCRL2 dans le corps jaune, le cortex, les petits et gros follicules et dans les cellules de la granulosa provenant de petits ou de gros follicules (Fig 1 A). Par PCR quantitative nous avons constaté que la chemerine est plus exprimée dans les cellules de la granulosa provenant des petits follicules par rapport à celle des gros follicules (Fig 1B). L'immunohistochimie confirme les résultats précédents et révèle la présence de la chemerine et de ces trois récepteurs dans les cellules de la thèque (Fig 1 D).

B) Régulation de l'expression de la chemerine et de ces récepteurs dans les cellules de la granulosa bovine (Article 5)

Nous avons testé l'effet de l'insuline (10^{-8}M), IGF-1 (10^{-8}M), de la metformine (10^{-6}M) et la rosiglitazone (10^{-8}M) sur l'expression de la chemerine, CMKLR1, GPR1 et CCRL2 dans des cultures primaires de cellules de la granulosa bovine. La PCR en temps réel révèle qu'après 24h de stimulation ces 4 acteurs (insuline, IGF-1, metformine et rosiglitazone) augmentent l'expression de la chemerine alors qu'ils diminuent l'expression de ses récepteurs (Fig 2). Nous avons obtenu des résultats similaires avec 6 ou 12 h de stimulation (données non montrées). Nous avons également étudié l'effet d'autres adipocytokines sur l'expression de la chemerine et de ces trois récepteurs *in vitro* dans des cellules de la granulosa bovine. Les cellules ont été incubées pendant 24h avec soit la résistine (100 ng/ml), l'adiponectine (10 µg/ml), la leptine (10 ng/ml) ou le TNF-α (10 ng/ml). Après une étude par PCR en temps réel nous avons observé que seul le TNF-α augmente l'expression du messager de la chemerine (Fig 3 A) et seule l'adiponectine l'expression du messager du récepteur CMKLR1 (Fig 3 B). Nous obtenons des effets similaires après des stimulations de 6 et 12h. Aucune autre adipocytokine n'affecte l'expression de la chemerine ou de ces récepteurs (données non montrées).

C) Effet de la rh chemerine sur la sécrétion de stéroïdes basale ou induite par IGF-1 ou FSH dans les cellules de la granulosa bovine (Article 5)

Les cellules ont été incubées pendant 48h soit avec différentes concentrations de rh chemerine (0, 12, 25, 50, 100 et 200 ng/ml) ou avec de la rh chemerine (200 ng/ml)

supplémentée ou non avec de la FSH (10^{-8}M) ou IGF-1 (10^{-8}M). Nous avons observé que la chemerine diminue la sécrétion de P4 et d'E2 de manière dose-dépendante (Fig 4 P<0,001). Elle réduit d'environ 2 fois également la sécrétion de stéroïdes en présence de FSH ou IGF-1 (Fig 4 P<0,001). Afin de tester si l'effet de la chemerine sur la sécrétion d'E2 et de P4 passe par le récepteur CMKLR1, nous avons utilisé un anticorps spécifique dirigé contre CMKLR1 (1 µg/ml) que nous avons incubé une heure avant d'appliquer les différents traitements (chemerine +/- IGF-1 ou FSH). En présence de l'anticorps anti-CMKLR1 nous abolissons totalement l'effet inhibiteur de la chemerine sur la sécrétion de stéroïdes basale ou induite par l'IGF-1 ou FSH (Fig 5). En utilisant des IgG de lapin dans les mêmes conditions nous ne retrouvons pas ces effets inhibiteurs de la chemerine recombinante humaine (Fig 5). Pour mieux comprendre le mode d'action de la chemerine sur la sécrétion de stéroïdes nous avons étudié son effet sur les principaux acteurs de la stéroïdogenèse. La rh chemerine (200 ng/ml) diminue de deux fois le niveau protéique de la StAR et P450 aromatase en présence de FSH et d'IGF-1(Fig 6A). La rh chemerine n'a pas d'effet au niveau basal, ni sur les autres enzymes de la stéroïdogenèse comme la 3βHSD, et la P450 SCC (données non montrées). Une fois de plus les effets de la rh chemerine ont été totalement abolis en présence de l'anticorps Anti-CMKLR1 (1 µg/ml).

Ainsi, l'effet de la chemerine sur la sécrétion de stéroïdes implique la voie de signalisation de CMKLR1 et s'explique par son effet inhibiteur sur la production des protéines StAR et P450 aromatase.

D) Effet de la rh chemerine sur la teneur en cholestérol et le niveau protéique de HMG co-réductase dans les cellules de la granulosa bovine (Article 5)

Les cellules de la granulosa bovine ont été cultivées et stimulées de la même manière que précédemment (stéroïdogenèse). Nous observons que la rh chemerine (200 ng/ml) diminue de moitié la teneur en cholestérol et le niveau protéique de l'HMG co-réductase en présence ou en absence de FSH (10^{-8}M) ou IGF-1 (10^{-8}M). Egalement, lorsque que ces cellules sont incubées pendant 48h en présence de l'anticorps dirigé contre le récepteur CMKLR1 les effets de la rh chemerine sont abolis.

Ces résultats pourraient expliquer l'effet de la chemerine sur la stéroïdogenèse par son inhibition sur la synthèse de cholestérol *via* le récepteur CMKLR1 dans les cellules de la granulosa bovine.

E) Effet de la chemerine sur la prolifération et la viabilité des cellules de la granulosa bovine (Article 5)

Les cellules de la granulosa bovine ont été incubées pendant 24h en présence de [^{3}H]-thymidine avec de la rh chemerine (200 ng/ml) supplémentée ou non avec FSH (10^{-8}M) ou IGF-1 (10^{-8}M). La chemerine ne présente aucun effet significatif sur la prolifération cellulaire basale ou induite par l'IGF-1 ou la FSH (données non montrées). Avec un test au bleu trypan nous avons constaté qu'elle n'affecte pas non plus la viabilité des cellules.

Ainsi, la chemerine diminue la production de stéroïdes par les cellules primaires de la granulosa bovine sans affecter ni leur prolifération ni leur viabilité.

F) Voies de signalisation activées par la chemerine dans les cellules de la granulosa bovine (Article 5)

Nous avons observé que la rh chemerine diminue la phosphorylation de la voie de signalisation MAPK ERK1/2 jusqu'à 30 minutes (**Fig 8 A**) et après 48h de stimulation en absence ou en présence d'IGF-1 (10^{-8}M) ou de FSH (10^{-8}M) (**Fig 8 B**).

G) Effet in vitro de la rh chemerine sur la maturation ovocytaire bovine et la sécrétion de **P4** *par les complexes-cumulo-ovocytaires (COCs) (Article 5)*

Une fois collectés, les ovocytes de vaches sont théoriquement matures après 22h de culture, ici nous avons étudié leur état de maturation après 10 et 22h de culture en présence ou en absence de chemerine. Sans traitement après 10h de stimulation 40% des ovocytes ont atteint le stade GVBD (Germinal Vesicle Break Down) (**Fig 9 B**) et après 22h de stimulation la plupart ont dépassé le stade GVBD (environ 85% des ovocytes ont atteint le stade de métaphase II (Fig 9 B). Cependant en présence de rh chemerine (100, 200 ou 400 ng/ml) après 22h de stimulation 30 à 50 % des ovocytes restent au stade GV (**Fig 9 B et C**). La rh chemerine bloque la division méiotique des COCs bovine *in vitro* de manière dose-dépendante. Egalement, la rh chemerine diminue de manière significative la sécrétion de P4 par les COCs après 22h de stimulation (**Fig 10 A**). Ce résultat pourrait expliquer l'effet bloquant de la rh chemerine sur le développement ovocytaire puisque la sécrétion de P4 par les cellules du cumulus est connue pour favoriser la maturation ovocytaire bovine.

H) Effet de la rh chemerine sur le niveau de phosphorylation de la voie MAPK ERK1/2 dans l'ovocyte et les cellules du cumulus des COCs chez le bovin lors d'une maturation in vitro (Article 5)

Dans les conditions témoins après 22h d'incubation le niveau de phosphorylation de la voie de signalisation MAPK ERK1/2 augmente comme attendu dans l'ovocyte et les cellules du cumulus (**Fig 10 B et C**). L'ajout de rh chemerine (200 ng/ml) dans le milieu de maturation diminue de moitié le niveau de phosphorylation de MAPK ERK1/2 dans l'ovocyte et de quatre fois dans les cellules du cumulus provenant des COCs (**Fig 10 B et C**).

Conclusions et discussion

Dans ces articles nous avons montré que la résistine, exprimée par les adipocytes matures bovin est impliquée dans la mobilisation des réserves adipeuses et que la chemerine inhibent *in vitro* la stéroidogenèse des cellules de la granulosa et la maturation ovocytaire chez la vache.

Chez les vaches laitières Prim Holstein, le début de la lactation se caractérise par une augmentation des besoins énergétiques due aux besoins de la glande mammaire en nutriments pour la production de lait (Bauman & Griinari, 2000; Bell et al, 1995). Ces adaptations métaboliques sont coordonnées avec des modifications de la concentration plasmatique de métabolites et d'hormones clés (Bauman & Griinari, 2000; Bell et al., 1995). Par exemple récemment il a été montré que les AGNE activent la voie de signalisation AMPK afin d'augmenter l'oxydation des lipides et de diminuer la synthèse de lipides dans les hépatocytes bovins. Ceci pourrait générer plus d'ATP pour compenser la balance énergétique négative chez les vaches laitières (Gu et al., 2006). L'activation de la voie AMPK est régulée par plusieurs adipocytokines comme l'adiponectine dans les hépatocytes bovins (Gu et al., 2006), la chemerine dans les CGH (Reverchon et al., 2012) et la résistine dans les CGB (Maillard et al., 2011). Par conséquent, ces acteurs jouent un rôle clé dans le contrôle de la masse grasse. Dans cette étude nous avons observé pour la première fois que le profil de la concentration plasmatique de résistine est parallèle à celui des AGNE, il est plus élevé une semaine après parturition qu'avant le vêlage ou six semaines après la parturition. En accord avec ces résultats nous avons trouvé que l'expression de la résistine au niveau messager et protéique dans le tissu adipeux sous-cutané est plus élevé une semaine avant vêlage qu'à 5 mois de gestation. Ainsi, nous pouvons supposer que la plus forte concentration de résistine une semaine après vêlage est due à une production du tissu adipeux de résistine. La lactation chez

la vache laitière est connue pour être associée avec une forte perte de la masse graisseuse. Dans notre étude les animaux perdent plus de 1 kg par jour de leur masse corporelle en début de la lactation. Les profils de concentrations plasmatiques d'AGNE et de résistine sont similaires durant la période peri-partum. Cependant le seuil plasmatique de résistine le plus bas a été atteint à 4 semaines post-partum alors que celui d'AGNE à 6 semaines post-partum. Lors de l'étude *in vitro* sur des explants de tissu adipeux prélevés 1 à 2 mois après vêlage nous avons montré que la résistine recombinante bovine (rb) (100 ng/ml) augmente la teneur en glycérol et l'expression des gènes ATGL et HSL. La lipase hormono-sensible (HSL) est responsable de l'hydrolyse des triglycérides dans le tissu adipeux de vache lors d'une stimulation avec des catécholamines (Sumner et al., 2007). Peu de temps après la parturition l'expression de HSL est diminuée et le niveau plasmatique des AGNE est élevé (Sumner et al., 2007). En début de lactation la sécrétion de GH est également élevée, elle stimule la mobilisation des AGNE en inhibant la lipogenèse induite par l'insuline dans le tissu adipeux et en augmentant la lipolyse en réponse aux signaux beta adrénergiques (Etherton & Bauman, 1998). La régulation du récepteur de l'hormone de croissance dans le tissu adipeux en début de lactation est peu connue (Butler et al., 2003; Rhoads et al., 2004). Chez le rat et l'humain, la GH augmente l'expression du gène de la résistine dans le tissu adipeux ou le niveau sérique de résistine (Delhanty et al, 2002; Nozue et al., 2007). Ainsi, nous pouvons émettre l'hypothèse que la résistine pourrait participer aux effets *in vivo* de la GH dans le tissu adipeux de vaches laitières. De plus, nous avons constaté que la résistine augmente l'expression de messager de ATGL et de HSL dans des explants de tissu adipeux suggérant que la résistine pourrait agir indépendamment de la GH. Dans cette étude, nous avons obtenu une corrélation négative entre le niveau plasmatique de résistine et la balance énergétique et entre le niveau plasmatique de résistine et la matière sèche ingérée entre 1 et 2 semaines post-partum. En parallèle nous avons observé que les niveaux de protéines du tissu adipeux de l'adiponectine sont inférieurs 1 semaine post-partum comparé à 5 mois de gestation. Une étude a également montré que la concentration plasmatique de leptine et le niveau des deux récepteurs de l'adiponectine (AdipoR1 et AdipoR2) sont plus faibles dans le tissu adipeux sous cutané une semaine avant vêlage que 3 semaines post-partum (Lemor et al., 2009). L'adiponectine tout comme la leptine a un rôle insulino-sensibilisateur chez plusieurs espèces. De plus, il est connu qu'au début de la lactation les niveaux plasmatiques d'insuline sont diminués comparé au niveau avant la parturition (Blum et al., 1973). Avec toutes ces données nous pouvons émettre l'hypothèse que l'augmentation du niveau plasmatique de résistine et la diminution de l'adiponectine et de la leptine durant la lactation pourraient contribuer à réduire

la sensibilité à l'insuline. Plusieurs expérimentations seront nécessaires pour démontrer cette hypothèse. Nos résultats montrent que les niveaux protéiques d'IR, IRS-1, IRS-2, Akt, MAPK ERK1/2 et P38, AMPK, P70S6K, et IGF-1R ne changent pas à 1 semaine après vêlage et 5 mois de gestation. Cependant nous avons observé une diminution significative de la phosphorylation des tyrosines d'IR, IRS-1, IRS-2, Akt, P70S6K, S6, et MAPK ERK1/2 ce qui pourrait expliquer la diminution significative du niveau plasmatique d'insuline une semaine post-partum.

Dans l'étude sur la chemerine nous avons montré sa présence ainsi que celle de ces récepteurs CMKLR1, GPR1, et CCRL2 au niveau messager et protéique dans les différents compartiments ovariens bovins. La chemerine a déjà été identifiée dans l'ovaire de rate et d'humain (Reverchon et al., 2012; Wang et al., 2013; Zabel et al., 2005). Dans le follicule bovin la chemerine et ses récepteurs sont présents dans les cellules de la thèque et de la granulosa, les cellules du cumulus et l'ovocyte. La concentration plasmatique de chemerine chez l'humain varie entre 100 et 200 ng/ml (Bozaoglu et al., 2010; Hu & Feng, 2011). Chez le bétail, la concentration plasmatique n'est pas encore connue. Dans notre étude, nous avons stimulé les cellules avec une concentration de chemerine proche de celle observée dans le plasma humain (200 ng/ml). Les séquences d'ADNc des gènes de la chemerine et de son récepteur CMKLR1 sont très proches chez l'humain, la souris et le porc (Song et al., 2010). Ainsi nous avons utilisé de la chemerine recombinante humaine (rh chem) pour les expérimentations. Dans notre étude nous avons constaté que l'insuline, l'IGF-1 et les deux insulino-sensibilisateurs (metformine et rosiglitazone) augmentent l'expression de RARRES2 et diminuent celle de ses 3 récepteurs (CMKLR1, GPR1 et CCRL2) dans des cultures primaires de CGB. En accord avec nos résultats, dans la littérature il a déjà été montré qu'une hyperinsulinémie à court terme augmente la production de chemerine *in vitro* par les adipocytes et que l'insuline induit la libération de chemerine par les adipocytes chez la souris (Bauer et al., 2012). Par contre, l'effet stimulateur de la metformine n'est pas en accord avec les résultats de deux études ; un traitement de six mois par la metformine chez des patientes atteintes du SOPK diminue la concentration sérique de chemerine (Tan et al., 2009). Chez des rats soumis à un régime induisant l'insulino-résistance, la metformine diminue l'expression de la chemerine dans le tissu adipeux (Pei et al., 2012). Ces données suggèrent un effet spécifique de la metformine sur l'expression de la chemerine selon l'espèce et le tissu. De plus, le TNF-α et l'adiponectine augmentent de manière significative l'expression de RARRES2 et CMKLR1, respectivement. D'autres études ont déjà montré que le TNF-α

augmente le niveau de chemerine bioactive dans les adipocytes (Parlee et al., 2010; Song et al., 2010). Le TNF-α est connu pour son effet inhibiteur sur la stéroïdogenèse dans les cellules de la granulosa bovine (Spicer, 1998), or nos résultats montrent que la chemerine inhibe fortement la production de P4 et E2. Ceci suggère que la chemerine pourrait être un médiateur de l'effet inhibiteur du TNF-α dans les cellules de la granulosa bovine. En effet, la chemerine inhibe la production de stéroïdes à l'état basal et en réponse à la FSH ou IGF-1 *via* son récepteur CMKLR1. Nous avions déjà observé un effet inhibiteur de la chemerine dans une précédente étude au laboratoire sur des cultures humaines de cellules de la granulosa (Reverchon et al., 2012). Egalement nous avons observé que la rh chemerine diminue le niveau protéique de StAR et P450 aromatase induit par la FSH ou IGF-1. La StAR est responsable du transport du cholestérol et la P450 aromatase participe à la synthèse de l'E2, ce qui représente un élément de réponse à l'effet inhibiteur de la chemerine sur la sécrétion de stéroïdes. La chemerine active plusieurs voies de signalisation, ici nous avons montré que dans les cellules de la granulosa bovines à court terme ou à long terme la chemerine diminue la phosphorylation de la voie MAPK ERK1/2. Or, cette voie de signalisation a été montrée être un régulateur positif dans la sécrétion de stéroïdes induite par l'IGF-1 ou la FSH dans des cultures de cellules de la granulosa de rate, d'humain et de bovin (Seto-Young et al., 2003; Tosca et al., 2005; Tosca et al., 2007). L'effet de la chemerine sur la voie MAPK ERK1/2 contribue probablement à l'effet inhibiteur de la chemerine sur la stéroïdogenèse des cellules de la granulosa bovine. Nous savons que la stéroïdogenèse est dépendante de la disponibilité de son précurseur, le cholestérol. Chez le porc, le cholestérol joue un rôle primordial dans l'action de la FSH sur les cellules de la granulosa (Barañao & Hammond, 1986). Dans des cellules de la granulosa bovine, HMGCR, une enzyme clé dans le contrôle de la synthèse de cholestérol *de novo* est importante pour la production de P4 en réponse à l'IGF-1 (Spicer et al, 1996). Notre étude montre que la rh chemerine diminue la teneur en cholestérol et le niveau protéique de HMGCR dans les cellules de la granulosa bovine. De plus, nous avons montré que l'expression du facteur de transcription SREBP-2 qui est connu réguler plusieurs gènes impliqués dans la synthèse du cholestérol dont HMGCR est réduite après un traitement avec la rh chemerine de 24h. Ces résultats sont une piste de plus, pour comprendre l'impact de la chemerine sur la stéroïdogenèse puisqu'elle pourrait agir sur la biodisponibilité du cholestérol et sur sa synthèse *de novo*. Tous les résultats précédemment obtenus sont abolis en présence d'un anticorps dirigé contre CMKLR1. Ceci nous permet de penser que le récepteur CMKLR1 est au centre d'un dialogue entre la chemerine et IGF-1R et FSH-R dans la régulation de la stéroïdogenèse des cellules de la granulosa bovine. Cependant, il faudra

s'intéresser aux autres récepteurs de la chemerine GPR1 et CCRL2 afin de déterminer leur rôle dans les fonctions des cellules de la granulosa. Enfin dans cet article, nous avons étudié l'impact de la rh chemerine sur la maturation ovocytaire bovine *in vitro*. Il apparaît que la rh chemerine induit un blocage de la maturation ovocytaire au stade GV après 22h de stimulation *in vitro*. De plus, la chemerine réduit la sécrétion de P4 et la phosphorylation de la voie de signalisation MAPK ERK1/2 dans l'ovocyte et les cellules du cumulus. Ainsi, la chemerine pourrait agir négativement sur la maturation ovocytaire *via* son effet inhibiteur sur la sécrétion de P4, en effet plusieurs études ont montré l'effet stimulateur de la P4 sur la maturation des ovocytes (Borman et al, 2004; Zhang & Armstrong, 1989). De plus il est connu que l'activation de la voie MAPK ERK1/2 intervient durant les premières heures de la maturation chez plusieurs espèces dont le bovin. Chez les bovins cette activation se produit au moment du stade GVBD (Fissore et al., 1996). Ces résultats laissent à penser que la chemerine pourrait agir sur la maturation ovocytaire en diminuant la phosphorylation de la voie MAPK ERK1/2 dans l'ovocyte et les cellules du cumulus. Maintenant, il serait intéressant d'étudier les effets de la chemerine sur des ovocytes dénudés (sans les cellules du cumulus) afin de déterminer l'implication des cellules du cumulus durant la maturation *in vitro* des ovocytes bovins.

Article 4

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Resistin in Dairy Cows: Plasma Concentrations during Early Lactation, Expression and Potential Role in Adipose Tissue

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Abstract

Resistin is an adipokine that has been implicated in energy metabolism regulation in rodents but has been little studied in dairy cows. We determined plasma resistin concentrations in early lactation in dairy cows and investigated the levels of resistin mRNA and protein in adipose tissue and the phosphorylation of several components of insulin signaling pathways one week post partum (1 WPP) and at five months of gestation (5 MG). We detected resistin in mature bovine adipocytes and investigated the effect of recombinant bovine resistin on lipolysis in bovine adipose tissue explants. ELISA showed that plasma resistin concentration was low before calving, subsequently increasing and reaching a peak at 1 WPP, decreasing steadily thereafter to reach pre-calving levels at 6 WPP. Plasma resistin concentration was significantly positively correlated with plasma non esterified fatty acid (NEFA) levels and negatively with milk yield, dry matter intake and energy balance between WPP1 to WPP22. We showed, by quantitative RT-PCR and western blotting, that resistin mRNA and protein levels in adipose tissue were higher at WPP1 than at 5 MG. The level of phosphorylation of several early and downstream insulin signaling components (IRβ, IRS-1, IRS-2, Akt, MAPK ERK1/2, P70S6K and S6) in adipose tissue was also lower at 1 WPP than at 5 MG. Finally, we showed that recombinant bovine resistin increased the release of glycerol and mRNA levels for ATGL (adipose triglyceride lipase) and HSL (hormone-sensitive lipase) in adipose tissue explants. Overall, resistin levels were high in the plasma and adipose tissue and were positively correlated with NEFA levels after calving. Resistin is expressed in bovine mature adipocytes and promotes lipid mobilization in adipose explants *in vitro*.

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Introduction

In the dairy cow, late gestation and early lactation are periods marked by major changes in the sensitivity and responses of tissues to hormones involved in homeostasis, such as insulin [1]. Indeed, during these periods, there is a moderate decrease in peripheral tissue insulin sensitivity, promoting the mobilization of non esterified fatty acids (NEFAs) and amino acids and facilitating the preferential use of nutrients by the fetus or mammary gland [2]. The decrease in insulin sensitivity occurring in adipocytes during late gestation and early lactation in dairy cows remains poorly understood. Insulin acts by binding to the insulin receptor (IR), a tyrosine kinase receptor, on cells. Following insulin binding, the IR phosphorylates various substrates, including IRS-1 and IRS-2, which interact with several intracellular proteins to activate different signaling pathways, including the PI3K/Akt and MAPK ERK1/2 pathways [3]. Sadri *et al.* studied the expression of genes encoding components of the insulin receptor signaling pathway in adipose tissue during the dry period and in early lactation, in dairy cows [4]. They observed a significant decrease in insulin-responsive glucose transporter (GLUT4) gene expression in subcutaneous adipose tissue around the time of parturition. However, the levels of phosphorylation of IR signaling components have never been investigated. Adipokines — factors secreted by the adipose tissue — may be involved. In particular, resistin is known to decrease insulin sensitivity in rodents, whereas its effect in humans is unclear [5]. Resistin is a protein consisting of 108 amino acids in humans, 114 amino acids in mice, and 109 amino acids in cattle; it belongs to the "resistin-like molecules" or "FIZZ" (found in inflammatory zone) family [6]. It consists of homodimers linked by disulfide bridges. Resistin is produced directly by the adipocytes in mice, whereas it is produced by macrophages and transported to adipocytes in humans [7,8]. Plasma resistin levels are correlated with the degree of insulin resistance in mice, whereas conflicting results have been reported concerning this aspect in humans [6]. In bovine species, the localization in adipose tissue and the role of resistin in lipolysis are still unknown.

In mice, plasma and adipose tissue levels of resistin decrease in response to thiazolidinediones (insulin sensitizers) and increase during obesity [9]. Very little is currently known about the mode of action of resistin. No receptor has yet been clearly identified and the signaling pathways used remain unclear. Recent studies have

Table 1. Oligonucleotide primers sequences.

Abbrev.	name	gene ID	forward 5′-3′	reverse 5′-3′	Size bp	efficiency
PPIA	Cyclophilin A	NM_178320	GCATACAGGTCCTGGCATCT	TGTCCACAGTCAGCAATGGT	217	2.01
RETN	Resistin	NM_183362	AGTCCACAGAGAGGCACCTG	TGGTGACCTCCTGGATCTTC	133	2.04
RPL19	Ribosomal protein L19	BC102223	AATCGCCAATGCCAACTC	CCCTTTCGCTTACCTATACC	156	2.20
ACT	Beta Actin	D12816	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATACC	180	2.05
ATGL	Adipose Triglyceride Lipase	FJ798978	AAGCTGGTGCCAACATCATC	TAGCAATCAGCAGGCAGAAT	130	2.01
HSL	Hormone-Sensitive Lipase	NM_00108	GAGACTGGCATCAGTGTGAC	TTGCTAGAGACGATAGCACCT	199	1.98
CD68	CD68	NM_001045902	GAGGCAATAGGAGACTACAC	TGAATCCGAAGCTGAGCTGT	220	2.01
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	NM_001034034	TTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCAC	119	2.18

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suggested that resistin may bind a receptor tyrosine kinase called ROR1 (receptor tyrosine kinase-like orphan receptor) in murine pre-3T3-L1 adipocytes [10], or to TLR4 (Toll-like receptor 4) in the hypothalamus of mice [11]. The adipose tissue of dairy cows also produces several adipokines [4,12,13,14], including resistin [15]. Komatsu *et al*. showed that levels of resistin gene expression in the adipose tissue were significantly higher in lactating than in non lactating cows, whereas the opposite pattern was observed in the mammary gland [15]. However, plasma resistin concentration has never been determined during lactation in the dairy cow and the role of resistin in bovine adipose tissue has never been studied.

We investigated the profile of plasma resistin, insulin, glucose and non esterified fatty acid (NEFA) concentrations around the time of parturition and at the start of the first lactation in dairy cows. For the second lactation in the same animals, we then investigated mRNA and protein levels for resistin and the phosphorylation rates of several insulin receptor signaling components *in vivo* in subcutaneous adipose tissue in early lactation and mid-gestation. Finally, for the fifth lactation in the same animals, we analyzed the effects of bovine recombinant resistin on lipolysis *in vitro* in adipose tissue explants performed between one and two months after calving.

Materials and Methods

Animals

All experimental protocols were approved by an ethics committee (“Comité d’Ethique en Expérimentation Animale Val de Loire” CEEA VdL, protocol registered as n°2012-09-6), and were carried out in accordance with the guidelines of the French Council for Animal Care.

First experiment. Eight Holstein dairy cows were studied during the first months of their first and second lactations. Dairy cows were managed in loose housing conditions throughout the study. Cows were fed *ad libitum*, throughout the first four months of lactation after calving, with a complete mixed diet composed of 64.5% maize silage, 10% soybean, 15% concentrate, 10% dehydrated alfalfa and 0.5% calcium oxide (CaO). From the fifth month of lactation, the diet consisted of 74.64% maize silage, 10% soybean, 8% concentrate, 7% dehydrated alfalfa, 0.16% CaO and 0.2% mineral and vitamin mixture. Cows were artificially inseminated from seven weeks postpartum (7 WPP), half a day after the detection of estrus.

Second experiment. The same dairy cows as for the first experiment were studied during their second lactation. Biopsies of adipose tissue were performed at 1 week postpartum (WPP1) and 5 months of gestation (5 MG), as described below in the section dealing with sample collection.

Third experiment. Subcutaneous adipose tissue for adipose tissue explants was collected from the same dairy cows as for the first and second experiment (n = 8) between one and two months after calving during their fifth lactation. Animals were slaughtered at a local abattoir (INRA, PRC Unit, Nouzilly).

Body weight, milk yield, feeding and Energy Balance

After each milking, cows were automatically weighted (software RIC version RW1.7). Only the morning live body weight was used for weight analyses because the afternoon body weight was more variable. All cows were milked twice daily. At the entrance of the milking parlour, the cows were identified by an electronic collar and milk yield of each cow was automatically recorded (software Manufeed 500 pro, vc5 version 2.011.14).

Primiparous cows were fed *ad libitum* with two total mixed rations according to their stage of lactation using the INRA French feeding system [16] as described in [16]. Dry matter intake was determined from the intake of fresh matter and the dry matter content of each feed of the ration. The chemical composition of each feed is the same as described in [16]. The feeding values of the different feeds were calculated using chemical composition according the methods defined in INRA feeding systems [16]. Energy balance corresponds to the difference between energy needs for body maintenance, pregnancy and lactation, and energy intake.

Sample collection

Blood samples were collected from the tail vein immediately before food distribution, once per week (from 4 weeks before calving until 22 weeks after calving). They were centrifuged at 3000×g for 10 min at 4°C and plasma was stored at −20°C until its use for assays.

During the second lactation, adipose tissue biopsies were carried out on the same animals at 1 week post partum (WPP 1) and 5 months of gestation (5 MG; at this stage of gestation, the animals were still lactating). Cows were fasted for 12 hours before surgery and anesthesia was induced by intravenous (IV) injections of 12 to 14 mg of xylazine (Rompun, Bayer, Leverkusen, Germany) and subcutaneous (SC) injections of 20 mg lidocaine (Lurocaïne, Vetoquinol, Lure, France). Subcutaneous fat was collected from the dewlap under the neck, immediately frozen in liquid nitrogen

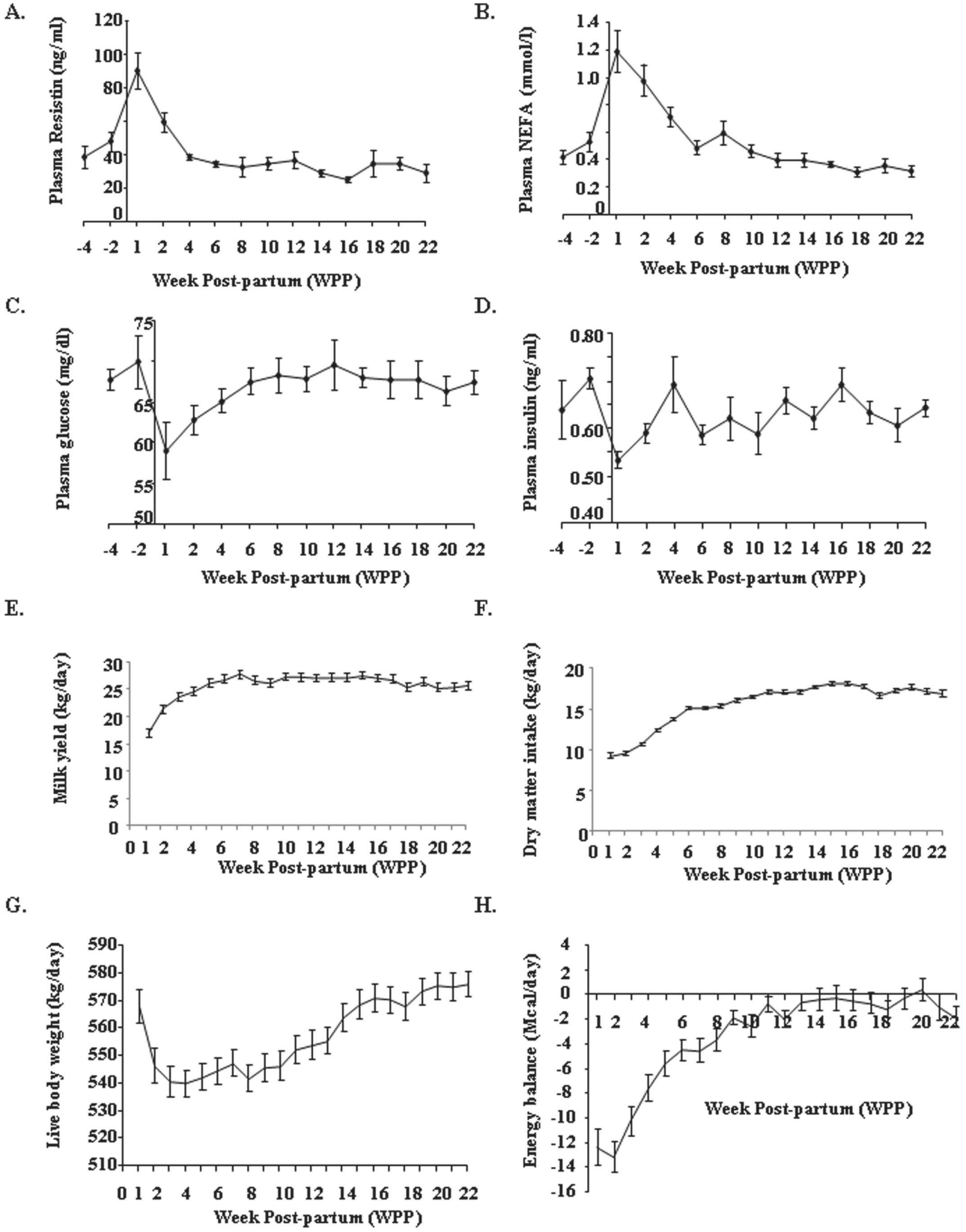
A.
Plasma Resistin (ng/ml)
120
100
80
40
20
0
-4 -2 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
B.
Plasma NEFA (mmol/l)
1.4
1.2
1.0
0.4
0.2
0
-4 -2 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
C.
Plasma glucose (mg/dl)
75
65
60
55
50
-4 -2 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
D.
Plasma insulin (ng/ml)
0.80
0.60
0.50
0.40
-4 -2 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
E.
Milk yield (kg/day)
30
25
20
15
10
5
0
0 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
F.
Dry matter intake (kg/day)
20
15
10
5
0
0 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
G.
Live body weight (kg/day)
590
580
570
560
550
540
530
520
510
0 1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)
H.
Energy balance (Mcal/day)
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
1 2 4 6 8 10 12 14 16 18 20 22
Week Post-partum (WPP)

Figure 1. Plasma resistin, NEFA, glucose and insulin levels and zootechnical parameters during the 1st lactation. Changes in plasma (A) resistin, (B) NEFA, (C) glucose, and insulin (D) concentrations in dairy cows, from 1 month before calving until 22 weeks after calving ($n = 8$ animals). Evolution of (E) milk yield, (F) dry matter intake, (G) live body weight and (H) energy balance between week post-partum (WPP) 1 and WPP22. Results are presented as means ± SEM.
doi:10.1371/journal.pone.0093198.g001

and stored at −80°C until use. Blood samples were collected on the day of the biopsy.

Plasma metabolites and insulin assays

Non esterified fatty acids (NEFA) and glucose were determined by enzymatic colorimetry, on a multiparameter analyzer (KONE Instruments Corporation, Espoo, Finland). Plasma insulin was determined by RIA, as previously described [17].

Resistin ELISA assay

Plasma bovine resistin levels were determined with a commercially available bovine resistin enzyme-linked immunosorbent assay (ELISA) (reference: E90847Bo (96 tests), distributed by Euromedex, France; supplier: USCNLife), according to manufacturer's protocol, with an intra-assay coefficient of variation < 6%.

Immunohistochemistry

Adipose tissue samples from the left side of the carcass were fixed by incubation with Bouin's solution for 24 h at room temperature, dehydrated, embedded in paraffin, and cut into 5 µm-thick sections. The paraffin was then removed from the sections, which were hydrated and microwaved in antigen-unmasking solution for 5 minutes (Eurobio, Les Ulis, France) and then allowed to cool to room temperature. Sections were then washed in PBS for 5 minutes and immersed in peroxidase-blocking reagent for 10 minutes at room temperature, to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). Adipose tissue sections were incubated for 20 min in PBS supplemented with 5% lamb serum, to eliminate nonspecific background. They were then washed in a PBS bath for five minutes and incubated overnight at 4°C with PBS supplemented with a rabbit primary antibody raised against human resistin (ab14323) from Abcam. According to the manufacturer, this antibody should recognize bovine resistin. Sections were washed twice, for 5 minutes each, in a PBS bath, and were then incubated for 30 minutes at room temperature with a "ready-to-use" polymer-HRP-conjugated anti-rabbit antibody (DakoCytomation Envision Plus HRP System, Dako, Ely, UK). Finally, sections were washed twice in PBS and staining was detected by incubation at room temperature with 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB+Substrate Chromogen System, DakoCytomation). We used primary antibodies against rabbit IgG as negative controls.

Total RNA extraction

Total RNA was extracted from 250 mg of dewlap subcutaneous adipose tissue on ice, with an Ultraturax homogenizer and 8 ml of QIAzol lysis reagent (Qiagen, Courtaboeuf, France). Chloroform (1.6 ml) was added to each sample. Tubes were shaken for 15 seconds and left at room temperature for 5 minutes before centrifugation (5000×*g*, 15 minutes, 4°C). The aqueous phase was mixed with an equal volume of ethanol 70% (v:v) and total RNA was purified with an RNeasy Midi Kit (Qiagen, France), according to the manufacturer's protocol. An RNase-free DNaseI (Qiagen) treatment was performed during the purification process. The RNA was eluted in RNase-free water, and the solvent was allowed to evaporate off, without heating, for 1.5 hours in a Thermo Savant SPD1010 SpeedVac System. The RNA was then stored at −80°C until use. The amount of RNA was determined with a NanoDrop Spectrophotometer (Nyxor Biotech, Paris, France) and RNA quality was assessed with an Agilent 2100 Bioanalyzer, using an RNA 6000 Nano assay protocol (Agilent Technologies, Massy, France). The RNA integrity number (RIN) for each RNA sample is shown in the Table S1.

Real-time quantitative RT-PCR (RT-qPCR)

Reverse transcription was performed as previously described [18]. Resistin cDNAs were quantified by real-time PCR with SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) and 250 nM specific primers (Invitrogen™ by Life Technologies™, Table 1), in a total volume of 20 µl, in a MyiQ Cycle device (Bio-Rad). Samples were tested in duplicate on the same plate, and PCR amplification with water, instead of cDNA, was performed systematically as a negative control. After incubation for 2 minutes at 50°C and denaturation for 10 minutes at 95°C, samples were subjected to 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C, and the melting curve was determined. Primer efficiency (E), determined for the bovine resistin, ATGL and HSL primers on serial dilutions of a pool of the cDNAs obtained, was 1.90, 1.85 and 2.0, respectively. The geometric mean of four reference genes (PPIA, RPL19, ACTB and GAPDH) was used to normalize gene expression. The relative amounts of gene transcripts (R) were calculated according to the equation R = (Egene−Ct gene)/[geometric mean (EPPIA−CtPPIA; ERPL19−CtRPL19; EACTB−CtACTB; EGAPDH−CtGAPDH)], where Ct is the cycle threshold and E the PCR efficiency for each primer pair

Recombinant proteins and antibodies

Recombinant bovine resistin was produced by Cliniscience (Nanterre, France); the reference number of the product used was: Recombinant Bovine Resistin-E Coli-CSB-EP019573BO. Recombinant human insulin used for culture treatment was obtained from Sigma (St Louis, MO, USA). Rabbit polyclonal antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser 473), Akt, phospho-P70S6 kinase (Ser424/Thr421), phospho-S6 (Ser235/236), P70S6, S6 and phospho-AMPK alpha Thr172 were obtained from New England Biolabs Inc. (Beverly, MA). Rabbit polyclonal antibodies against AMPKalpha, IRS-1 and IRS-2 were purchased from Upstate Biotechnology Inc. (Lake, Placid, NY, USA). Rabbit polyclonal antibodies against IGF-1R beta subunit (C20), IR beta subunit (C19), ERK2 (C14) and p38 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against vinculin were purchased from Sigma (St. Louis, MO, USA). PY20 antibodies were obtained from BD Biosciences (Le Pont de Claix, France). Rabbit monoclonal antibodies to human Adiponectin (C45B10) were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). Rabbit polyclonal antibodies against human resistin were obtained from Abcam (reference: ab14323). On immunoblots, this antibody recognized a band at about 12.5 kDa in bovine adipose tissue (Figure S1). It also recognized the recombinant bovine resistin, which migrated at the

Table 2. Statistical analysis of resistin, NEFA, glucose and insulin plasma levels.

Item	Animal	Period	Animal*Period	Period Post Partum					
	4 wk before calving to WPP22			4 to 2 wk before calving	WPP1 to WPP2	WPP4 to WPP6	WPP8 to WPP10	WPP12 to WPP14	WPP16 to WPP22
Resistin (ng/ml)									
mean	40.15			43.25	75.10	36.42	33.03	32.76	30.26
P	0.0001	0.0001	0.52	B	A	C	C	C	C
NEFA (mmol/l)									
mean	0.54			0.47	1.08	0.60	0.52	0.40	0.33
P	0.0004	0.0001	0.0011	B,C	A	B	B	C,D	D
Glucose (mg/dl)									
mean	67.12			68.89	61.12	66.38	68.17	70.15	67.40
P	0.0001	0.0001	0.03	A	B	A	A	A	A
Insulin (ng/ml)									
mean	0.63			0.68	0.57	0.65	0.60	0.63	0.65
P	0.0025	0.0010	0.27	A	B	C	C	C	C

Arithmetic mean values for each studied period are presented. P values of the effects of animal, period, interaction between animal and period for each studied parameter, and P values of the comparison between lsmeans of each parameter and for each of the six studied periods are described. Different letters indicate significant differences at $p<0.05$.

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Table 3. Statistical analysis of the relationships between quantitative parameters (NEFA, glucose, insulin and resistin plasma levels during the period 4 weeks before calving to 22 weeks post partum) using Pearson correlations with the CORR procedure of the SAS software.

	NEFA	Glucose	Resistin	Insulin
NEFA	1.00			
Glucose	−0.36	1.00		
P	<0.0001			
Resistin	0.43	−0.13	1.00	
P	<0.0001	0.21		
Insulin	−0.30	0.30	0.05	1.00
P	0.004	0.003	0.650	

doi:10.1371/journal.pone.0093198.t003

same molecular weight. We therefore conclude that the band detected in the bovine adipose tissue is probably bovine resistin. All antibodies were diluted 1/1000 for western blotting.

Isolation of bovine stromal vascular cells and mature adipocytes

Adipose tissue samples were collected from the left side of the carcass immediately after exsanguination. Incisions were made dorsal to the 12th and 13th rib, and a sample approximately 10 cm^3 in volume and containing a portion of subcutaneous adipose tissue was obtained. Immediately after collection, the samples were placed in sterile ice-cold PBS and transported to the laboratory. Briefly, subcutaneous adipose tissue was separated from the visible collagenous connective tissue. All excised adipose samples were then cut into approximately 2-mm^3 cubes. The samples were digested in Dulbecco's modified Eagle's medium (DMEM; 5.5 mM glucose; PAA, France) supplemented with 2 mg/ml collagenase (Sigma, France) and 2% BSA. They were incubated, with shaking at 230 rpm, for 45 minutes in a 37°C water bath. The digested cell suspension was then filtered through a sterile nylon mesh with 1000-µm pores into a clean 50 ml centrifuge tube. The unwanted connective tissue was retained on the mesh. The cells passing through the filter were centrifuged at 200×*g* for 10 minutes and the floating mature adipocytes in the uppermost layer were collected and washed twice by centrifugation (200×*g*, 10 min). The final pellet (the stromal vascular cell fraction) and the isolated mature adipocytes were then frozen at −80°C until use.

Adipose tissue explant culture

Subcutaneous adipose tissue was obtained from the same location as for stromal vascular cell and mature adipocyte isolation from dairy cows ($n = 8$ animals during their fifth lactation (same animals as those used for experiment 1 and 2), n = 2 animals per experiment), from a local abattoir. We placed about 3 g of tissue in sterile sodium chloride solution (0.9%) to remove the excess blood, and connective tissue was removed at the initial sampling stage. The remaining tissue was immediately transferred to a 50 ml tube containing DMEM supplemented with l-glutamine (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 100 µg/ml streptomycin and 50 µg/ml gentamicin, used as the basal medium. The tube was immersed in water at 37°C water in a thermo-flask dewar and transported to the laboratory. We dissected 200 mg of the subcutaneous adipose tissue, cutting it into 10 small pieces in sterile conditions. The tissue samples were incubated in triplicate with 3 ml of basal medium, basal medium with various concentrations of recombinant bovine resistin (1, 10 and 100 ng/ml), in the presence or absence of insulin (10^{-8} M), for 4 h, at 37°C, under an atmosphere containing 5% CO_2. The tissue explants (200 mg) were then collected, immediately frozen in liquid nitrogen and stored at −80°C.

In vitro lipolysis assay

The rate of lipolysis was determined by monitoring glycerol release from the adipose tissue explants into the incubation medium with a determination kit for free glycerol (Sigma, St. Louis, MO, USA).

Protein extraction and western blotting

Tissue lysates (adipose tissue) were prepared on ice with an Ultraturax homogenizer in lysis buffer A, consisting of 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0.5% Nonidet P-40 supplemented with various protease inhibitors (2 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate), as previously described [19]. The proteins extracted (80 µg) were denatured, subjected to SDS-PAGE in a 12% polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with specific antibodies, as previously described [20,21]. Proteins were detected by enhanced chemiluminescence (Western Lightning *Plus*-ECL, Perkin Elmer), with a G:Box SynGene (Ozyme) and GeneSnap software (release 7.09.17). The signals detected were quantified with GeneTools software (release 4.01.02). The results are expressed as the intensity signal after normalization, in arbitrary units, as indicated in the figure legends.

Immunoprecipitation

After normalization for the protein concentration (500 µg) of cell lysates, IR, IRS-1, IRS-2 and IGF-1R were immunoprecipitated from the supernatants with 5 µg of the appropriate antibodies, by incubation overnight at 4°C. The immunocomplexes were precipitated by incubation with 40 µl of protein A-agarose for 1 h at 4°C. The pellets were washed twice with a 1 in 2 dilution of buffer A and then boiled for 4 min in reducing Laemmli buffer containing 80 mm dithiothreitol. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose mem-

Table 4. Statistical analysis of live body weight, milk yield, dry matter intake and energy balance.

Item	Animal	Period	Animal*Period	Period Post Partum				
	WPP1 to WPP20			WPP1 to WPP2	WPP4 to WPP6	WPP8 to WPP10	WPP12 to WPP14	WPP16 to WPP22
Live body weight (kg/day)								
mean	557.30			548.07	543.75	549.20	564.53	572.80
P	0.0001	0.0001	0.0001	A	A	A	B	C
Milk yield (kg/day)								
mean	25.96			21.97	27.04	27.23	27.46	26.06
P	0.0004	0.0001	0.10	A	B,C	B,C	B	C
Dry matter intake (kg/day)								
mean	15.50			10.36	14.78	16.61	17.76	17.18
P	0.0001	0.0001	0.004	A	B	C	D	C,D
Energy balance (Mcal/day)								
mean	−3.43			−10.86	−4.59	−1.08	−0.79	−0.44
P	0.0001	0.0001	0.06	A	B	C	C	C

Arithmetic mean values for each studied period are presented. P values of the effects of animal, period, interaction between animal and period for each studied parameter, and P values of the comparison between lsmeans of each parameter and for each of the five studied periods are described. Different letters indicate significant differences at $p<0.05$.

doi:10.1371/journal.pone.0093198.t004

Table 5. Statistical analysis of the relationships between quantitative parameters (resistin plasma levels, milk yield, dry matter intake and energy balance during the period 1 to 20 WPP) using Pearson correlations with the CORR procedure of the SAS software.

	Resistin	Milk Yield	Dry matter intake	Energy Balance	Live Body Weight
Resistin	1.00				
Milk Yield	−0.52	1.00			
P	<0.0001				
Dry matter intake	−0.65	0.75	1.00		
P	<0.0001	<0.0001			
Energy Balance	−0.62	0.27	0.77	1.00	
P	<0.0001	0.002	<0.0001		
Live Body Weight	−0.10	0.46	0.49	0.08	1.00
P	0.34	<0.0001	<0.0001	0.35	

doi:10.1371/journal.pone.0093198.t005

branes. Blots were blocked in 2% BSA and probed with the various antibodies, as indicated in the figure legends.

Statistical analysis

In experiment 1, data for the plasma concentrations of NEFA, glucose, insulin and resistin were analyzed from 4 weeks before calving until 22 weeks post partum (wpp), in a linear mixed model, with the MIXED procedure of SAS software [22] for repeated measurements. For the analysis of these metabolic data, the period studied was divided into six subperiods: period 1: 4 and 2 weeks before calving; period 2: 1 and 2 WPP; period 3: 4 and 6 WPP; period 4: 8 and 10 WPP; period 5: 12 and 14 WPP; period 6: between 16 and 22 WPP. The model included period and animal as fixed effects and the interactions between period and animal. If significant effects were detected, the lsmeans per subperiod were compared, considering $p<0.05$ to be significant. The relationships between quantitative parameters (NEFA, glucose, insulin and resistin concentrations) were investigated by Pearson's correlation analyses, with the CORR procedure of SAS software [22].

For experiments 2 and 3, all the other experimental data (mRNA and protein levels, and phosphorylation levels) are presented as means ± SEM. One-way analysis of variance (ANOVA) was used to assess the significance of differences (Statview version 5.0, SAS Institute, Inc.). If ANOVA revealed

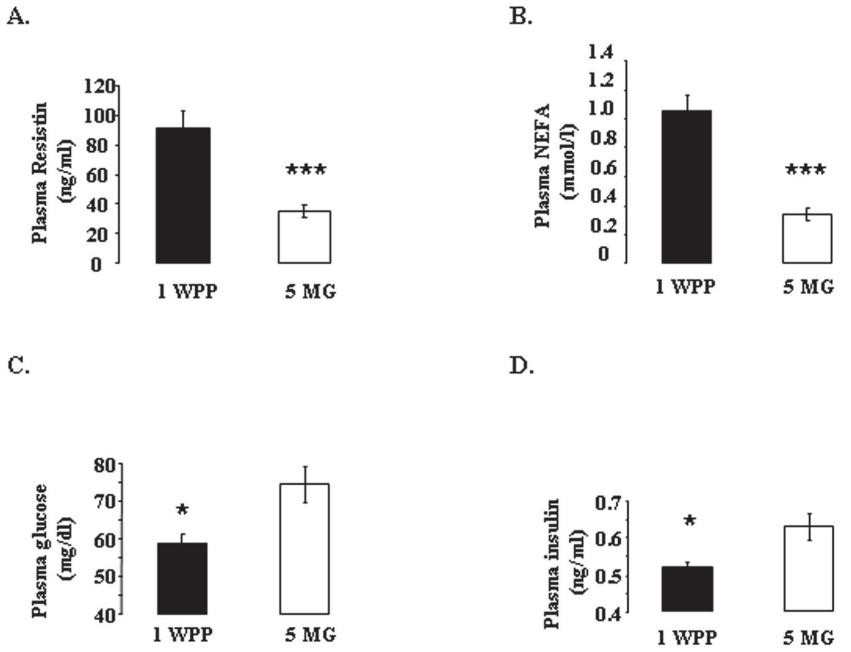

Figure 2. Plasma resistin, NEFA, glucose and insulin levels at 1 week post partum (WPP 1) and 5 months of gestation (5 MG) during the second lactation. Plasma resistin, NEFA, glucose and insulin concentrations measured on the day of adipose tissue biopsy (1 WPP and 5 MG) in Holstein dairy cows. Animals ($n=8$ in each group) were fasted for 12 hours before surgery Results are presented as means ± SEM. Results are considered significantly different if $p<0.05$. * and *** indicate significant differences at $p<0.05$ and $p<0.0001$, respectively.
doi:10.1371/journal.pone.0093198.g002

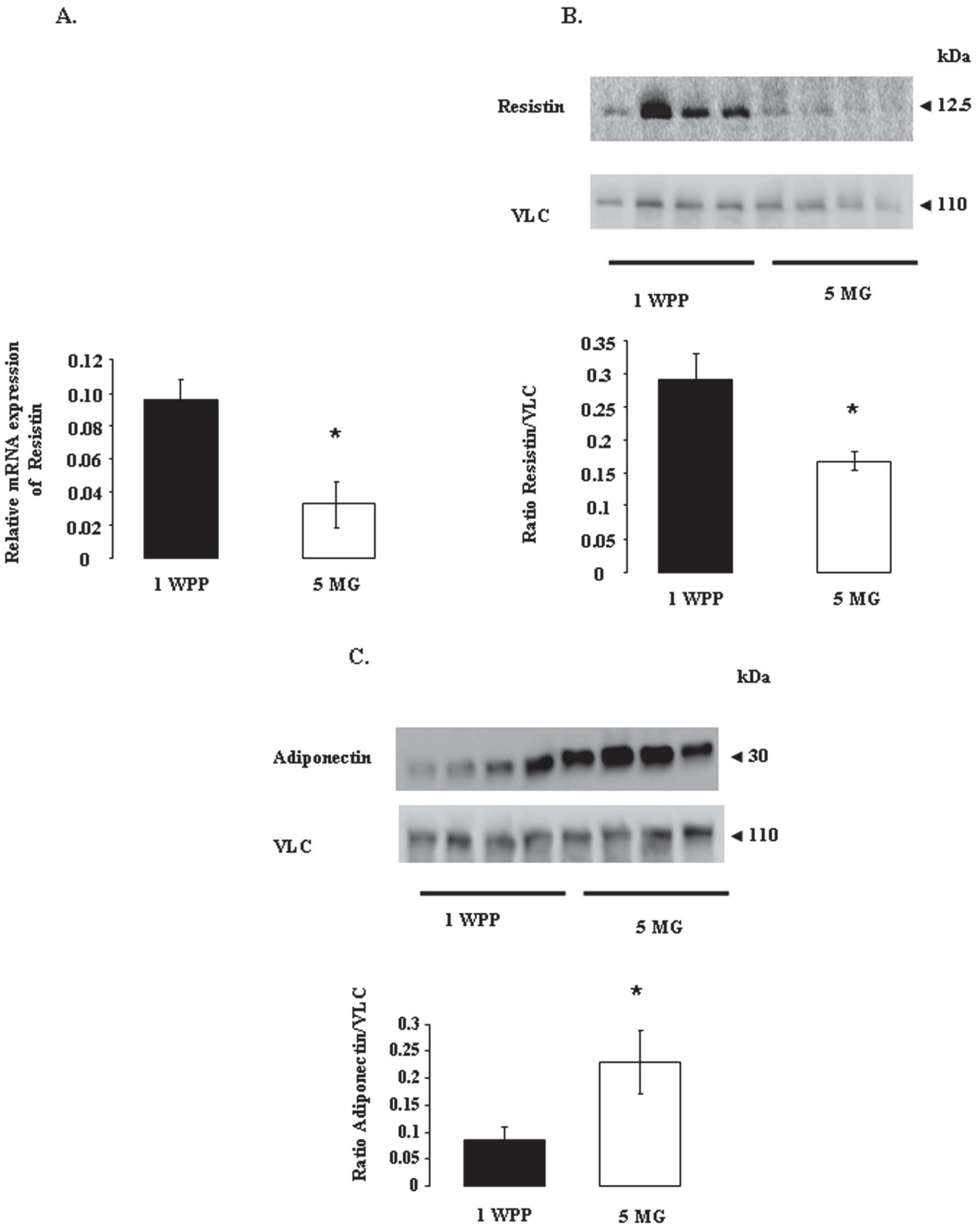

Figure 3. Resistin mRNA and protein levels and adiponectin protein levels in bovine adipose tissue at one week post partum (WPP 1) and 5 months of gestation (5 MG). A. Resistin gene expression was assessed by quantitative reverse transcription-polymerase chain reaction on bovine adipose tissue at one week post partum (WPP 1, $n=8$ animals) and 5 months of gestation (5 MG, $n=8$ animals), as described in the materials and methods. Relative expression was measured relatively to the geometric mean of 4 reference gene expression [cyclophilin A (PPIA),

GAPDH, Actin B (ACTB), and ribosomal protein L19 (RPL19)] by real-time reverse-transcription PCR. Results are represented as means ± SEM. * indicates a significant difference ($p<0.05$). **B and C**. Resistin (B) and adiponectin (C) protein levels were analyzed by western blotting on bovine adipose tissue at one week post partum (1 WPP, $n=8$ animals) and 5 months of gestation (5 MG, $n=8$ animals), as described in the materials and methods. Vinculin (VLC) was used as a loading control. Results are represented as means ± SEM. * indicates a significant difference ($p<0.05$).
doi:10.1371/journal.pone.0093198.g003

significant effects, the means were compared in Fisher's test, considering $p<0.05$ to be significant. Different letters indicate significant differences.

Results

Plasma resistin levels during the first and second lactation

In the first experiment, we determined the plasma concentrations of resistin, NEFAs, glucose and insulin in eight Holstein dairy cows, from four weeks before calving to 22 weeks post partum, during their first lactation (figure 1). These concentrations differed significantly before and after lactation ($p<0.001$, Table 2). As expected, plasma glucose and insulin concentrations were lower, whereas plasma NEFA concentration was higher one week after calving than before calving ($p<0.05$, figure 1B to D). Plasma resistin concentrations were low before calving (about 40 ng/ml), subsequently increasing and reaching a peak one week after calving (about 90 ng/ml), decreasing steadily thereafter to reach pre-calving levels at 6 weeks post-partum (Figure 1A, Table 2). Plasma resistin concentration did not change between 6 and 22 weeks post partum (Table 2). A significant correlation between plasma resistin and NEFA concentrations ($r=0.43$, $p<0.0001$) was observed between four weeks before calving and 22 weeks post partum (Table 3). By contrast, there was no significant correlation between plasma resistin and glucose or insulin concentrations during this period (Table 3).

As shown in figure 1E to H, zootechnical parameters concerning milk production, food intake, body weight and energy balance were analyzed from calving to 22 weeks post partum (wpp) during first lactation. As expected, all those parameters significantly varied during the lactation (wpp effect: $p<0.0001$, Table 4). A significant correlation between plasma resistin and milk yield ($r=-0.52$, $p<0.0001$), plasma resistin and dry matter intake ($r=-0.65$, $p<0.0001$) and plasma resistin and energy balance ($r=-0.62$, $p<0.0001$) was observed between 1 and 22 weeks post partum (Table 5). By contrast, there was no significant correlation between plasma resistin and live body weight (Table 5).

In the second experiment, we measured the same plasma variables during the second lactation and, more precisely, on the days on which adipose tissue biopsies were carried out, one week postpartum (1 WPP) and at five months of gestation (5 MG). Plasma resistin and NEFA concentrations were higher (figure 2A and B), whereas plasma glucose and insulin concentrations were lower (Figure 2C and D) one week post partum than at 5 MG. At 1 WPP, fat is being mobilized, whereas at 5 MG, body reserves are being reconstituted.

Resistin mRNA and protein levels and adiponectin protein levels in the adipose tissue of dairy cows at one week post partum and 5 months of gestation

We determined resistin mRNA levels in adipose tissue at 1 WPP and 5 MG during the second lactation. Levels of resistin mRNA were higher at 1 WPP than at 5 MG (Figure 3A). We confirmed this result at the protein level by western blotting (figure 3B). At the opposite, we showed that adiponectin protein levels were lower at 1 WPP than at 5 MG (Figure 3C).

Phosphorylation of IRβ, IRS-1, IRS-2 and IGF-1Rβ in the adipose tissue of dairy cows at one week post partum and 5 months of gestation

Early steps in insulin and IGF-1 receptor signaling (IRβ, IGF-1Rβ, IRS-1 and IRS-2) were first compared in adipose tissue at 1 WPP and 5 MG (Figure 4A to D). Our results suggest that the tyrosine phosphorylation levels of IRβ, IRS-1 and IRS-2 were lower ($P<0.05$) at 1 WPP than at 5 MG (Figure 4A, C and D). By contrast, the IGF-1R beta subunit displayed similar levels of tyrosine phosphorylation at these two stages (Figure 4B). Furthermore, we found that the levels of the IRβ, IGF-1Rβ, IRS-1 and IRS-2 proteins did not differ between 1 WPP and 5 MG (normalized with respect to VLC, data not shown).

Phosphorylation of MAPK ERK1/2 and p38, Akt, AMPK, P70S6K and S6 in the adipose tissues of dairy cows at one week post partum and 5 months of gestation

We then studied various signaling pathways: the MAPK ERK1/2 and P38, Akt, AMPK, P70S6K and S6 pathways (Figure 5). Our results suggest that the level of phosphorylation of MAPK ERK1/2 (Figure 5A), Akt (Figure 5B), P70S6K and S6 (Figure 5E and F) was lower at 1 WPP than at 5 MG, whereas the phosphorylation of MAPK P38 and AMPK (figure 5C and D) did not differ between the two stages. We also found that the levels of Akt, AMPK, MAPKs ERK2 and P38, P70S6K and S6 (normalized with respect to VLC) proteins did not differ between 1 WPP and 5 MG (data not shown).

Expression of the resistin gene in mature bovine adipocytes

We analyzed the levels of resistin mRNA in the subcutaneous adipose tissues of adult dairy cows. As described in the materials and methods, we isolated stromal vascular cells and mature adipocytes and investigated resistin gene expression (by assessing mRNA and protein levels) in both these cell fractions. The quantification of resistin mRNA levels by real-time RT-PCR, indicated that resistin mRNA levels were higher in mature adipocytes than in stromal vascular cells (Figure 6A). Stromal vascular cells include many different types of cell, such as pre-adipocytes, immune cells, fibroblasts, and endothelial cells. In humans, resistin is produced by macrophages rather than adipocytes [7,8]. We checked that the presence of resistin in the mature bovine adipocytes was not due to immune cell contamination, by also assessing mRNA levels for CD68 (the main cell surface marker of macrophages). CD68 mRNA levels were high in the stromal vascular cells and very low in mature adipocytes (Figure 6A), suggesting that in the resistin was indeed produced by the bovine mature adipocytes. Immunoblotting confirmed that resistin was produced in larger amounts in mature bovine adipocytes than in stromal vascular cells (Figure 6B). We further explored resistin production in bovine adipose tissue, by

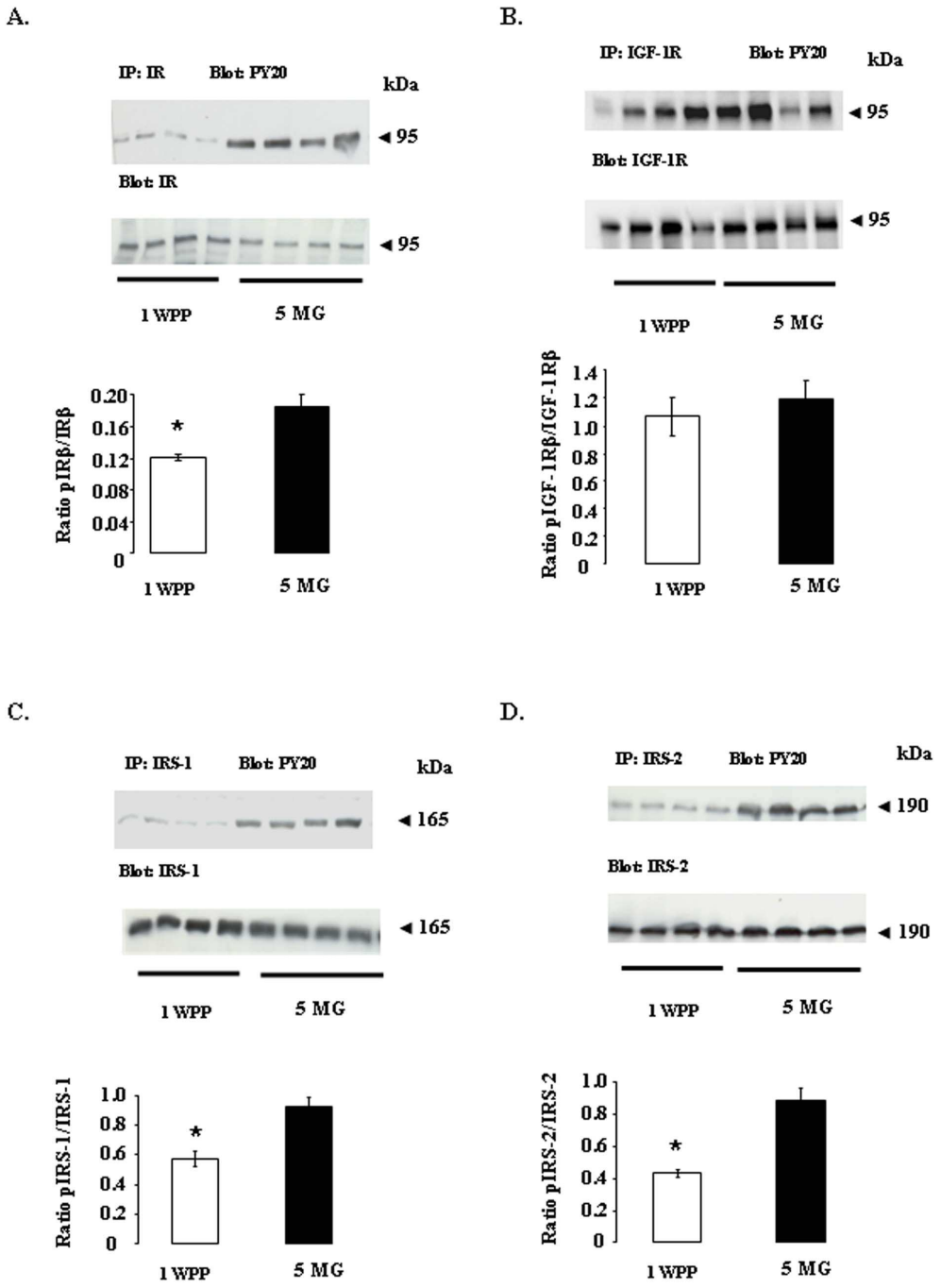

Figure 4. Levels of tyrosine phosphorylation for IR, IGF-1R, IRS-1 and IRS-2 in bovine adipose tissue at one week post partum (1 WPP) and 5 months of gestation (5 MG). Western blots showing the tyrosine phosphorylation levels of IRβ (A), IGF-1Rβ (B), IRS-1 (C) and IRS-2 (D) in adipose tissue lysates from dairy cows at one week post partum (1 WPP, $n = 8$ animals) and 5 of months gestation (5 MG, $n = 8$ animals). Immunoprecipitation (IP) was performed before gel electrophoresis. The antibody used is indicated as follows: IP: molecule X; the immune sera used to determine protein phosphorylation levels are indicated to the left of the gels (e.g., PY20 is directed against anti-phosphotyrosine residues). The levels of phosphorylation of IR, IGF-1R, IRS1 and IRS-2 were normalized with respect to the corresponding total protein, with specific antibodies, as indicated to the left of the gels. The gels show protein bands, which are underlined for each group, with four dairy cows per group. From left to the right, the groups are 1 WPP and 5 MG. Below each gel, the histograms show the mean ± SEM for a total of $n = 8$/group. *indicates a significant difference ($p < 0.05$).
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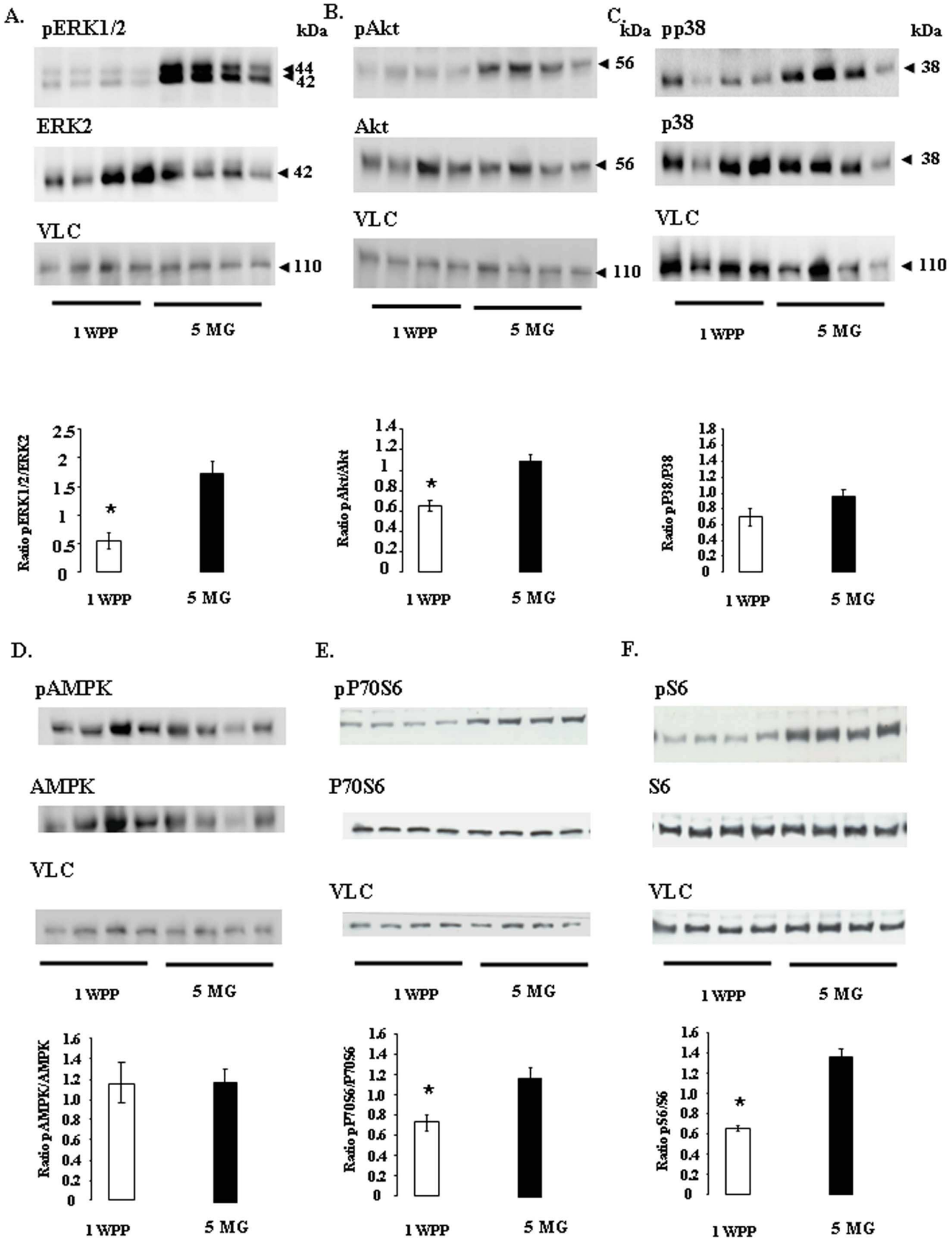
carrying out an immunohistochemical study with a specific antibody directed against human resistin (the same antibody used for immunoblotting). We observed intense immunostaining for resistin in white adipose tissue (Figure 6C). As a negative control, we replaced the primary antibody with PBS or normal serum. No immunoreaction was observed for the negative controls.


A.
pERK1/2
kDa
44
42
ERK2
42
VLC
110
1 WPP
5 MG
Ratio pERK1/2/ERK2
2.5
2
1.5
1
0.5
0
*
1 WPP
5 MG
B.
pAkt
kDa
56
Akt
56
VLC
110
1 WPP
5 MG
Ratio pAkt/Akt
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
1 WPP
5 MG
C.
pp38
kDa
38
p38
38
VLC
110
1 WPP
5 MG
Ratio P38/P38
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1 WPP
5 MG
D.
pAMPK
AMPK
VLC
1 WPP
5 MG
Ratio pAMPK/AMPK
1.6
1.4
1.2
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0.8
0.6
0.4
0.2
0
1 WPP
5 MG
E.
pP70S6
P70S6
VLC
1 WPP
5 MG
Ratio pP70S6/P70S6
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
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*
1 WPP
5 MG
F.
pS6
S6
VLC
1 WPP
5 MG
Ratio pS6/S6
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
1 WPP
5 MG

Figure 5. Levels of phosphorylation of MAPK ERK1/2, Akt, MAPK P38, AMPK, p70S6K and S6 kinase in the adipose tissue of dairy cows at one week post partum (1 WPP) and 5 months of gestation (5 MG). Western blots showing the levels of phosphorylation of MAPK ERK1/2 (A), Akt (B), MAPK P38 (C), AMPK (D), P70S6K (E) and S6 (F) in adipose tissue lysates from dairy cows at one week post partum (1 WPP) and 5 months of gestation (5 MG). The levels of phosphorylation of these kinases were normalized by dividing the values obtained for immunoblots of phosphorylated protein by those for immunoblots of the corresponding total protein. Protein levels for MAPK ERK1/2, Akt, MAPK P38, AMPK, P70S6K and S6 were normalized with respect to vinculin levels. The histograms below each gel show the mean ± SEM for two gels, with $n = 4$/group; the groups are 1 WPP and 5 MG. *indicates a significant difference ($p<0.05$).
doi:10.1371/journal.pone.0093198.g005

Effect of recombinant bovine resistin on the release of glycerol from subcutaneous adipose tissue explants and on ATGL (Adipose Triglyceride Lipase) and HSL (Hormone-Sensitive Lipase) mRNA levels in adipose tissue explants

In the last experiment, we determined the effect of different concentrations of recombinant bovine resistin, in the presence or absence of insulin (10^{-8}M), on lipolysis in bovine subcutaneous adipose tissue explants, by measuring glycerol release and levels of mRNA for ATGL (adipose triglyceride lipase) and HSL (hormone-sensitive lipase) after four hours of incubation. Recombinant bovine resistin treatment increased glycerol release in a dose-dependent manner (Figure 7A). Treatment with 100 ng/ml recombinant bovine resistin also abolished the inhibitory effect of insulin on the glycerol release (Figure 7A). As shown in Figure 7B and C, treatment with 100 ng/ml recombinant bovine resistin increased levels of mRNA for ATGL and HSL both in the basal state and in response to insulin (10^{-8} M).

Discussion

In dairy cows, the onset of lactation increases the total energy requirements due mainly to the nutrient needs of the mammary gland for milk synthesis (Bauman, 2000, Bell, 1995). The hyperphagia required to meet those demands develops slowly, consequently mobilization of endogenous reserves is observed [22,23]. These metabolic adaptations are coordinated by changes in the plasma concentration of key hormones [22,23]. For example, the secretion of growth hormone (GH) is elevated in early lactation [22,24] and promotes the mobilization of nonesterified fatty acids from adipose tissue and their oxidative use by the rest of the body [23]. Cathecolamine-induced lipolysis in adipose tissue (AT) depots is also considered to be the key metabolic pathway for providing endogenous energy in times of high energy demand in the peripartal dairy cow [25,26]. Recently it has been shown that NEFAs activate the AMPKα signaling pathway to increase lipid oxidation and decrease lipid synthesis in bovine hepatocytes, which in turn, could generates more ATP to relieve the negative energy balance in transition dairy cows [27]. AMPK activation is regulated by various adipokines including adiponectin in bovine hepatocytes [28] and resistin in bovine granulosa cells [29]. Consequently it plays a key role in the control of body fat mass. Here, we show for the first time that plasma resistin concentrations increase one week after calving in a similar manner to NEFA levels in dairy cows. We also found that resistin mRNA and protein levels in adipose tissue were higher one week post partum than at five months of gestation. Conversely, the level of phosphorylation of several components of the insulin receptor signaling pathway in adipose tissue was significantly lower one week after calving than at 5 MG. We also showed that resistin was produced in bovine mature adipocytes and that recombinant bovine resistin increased the release of glycerol and levels of mRNA for ATGL and HSL in adipose tissue explants. Our data suggest that the high levels of resistin in the plasma and adipose tissue observed immediately after calving may contribute to lipid mobilization during early lactation in dairy cows.

Resistin is considered to be a potential factor underlying obesity-mediated insulin resistance and type 2 diabetes. In humans and rodents, serum resistin levels are about 2 to 15 ng/ml [30–32], but considerable variability has been noted between species and types of assay. In this study, we obtained values for plasma resistin concentration of 30 to 90 ng/ml in dairy cows. Resistin is produced principally by adipocytes in mice, whereas it is produced predominantly by peripheral blood mononuclear cells, macrophages and bone marrow cells in humans [7,8]. The production of resistin in bovine adipose tissue has already been reported, but the cell type responsible for this production was not identified [15]. We detected resistin in mature bovine adipocytes.

We also demonstrated that plasma resistin concentration was significantly higher one week after calving than before calving or six weeks postpartum. Consistent with these results, we found that resistin mRNA and protein levels in subcutaneous adipose tissue were higher at 1 WPP than at 5 MG, suggesting that the high plasma concentrations of resistin at 1 WPP are generated by the adipose tissue. Lactation in dairy cows is known to be associated with many metabolic changes, including the loss of a large amount of adipose tissue. In our study, the animals lost more than 1 kg of body weight/day during early lactation. These results are consistent with those of Jarrige (1989) that indicates a mobilization of body fat from 15 to 60 kg after parturition [33]. As expected, plasma NEFA concentration was also found to have increased considerably at 1 WPP, reflecting a high level of lipid mobilization [34]. The plasma concentration profiles of NEFAs and resistin were similar during the peri-partum period. However, the nadir for plasma resistin was reached at 4 WPP while those for plasma NEFAs at 6 WPP.

In vitro, in bovine adipose tissue explants from animals at about the same physiological status (between one and two months after calving), we showed that recombinant bovine resistin at a concentration of 100 ng/ml increased the release of glycerol and the expression of the ATGL and HSL genes. This concentration is physiologically relevant because we measured a plasma resistin concentration of about 90 ng/ml at one week post-partum when plasma NEFAs were high. In adipose tissue of cows, hydrolysis of triacylglycerols is mediated by hormone-sensitive lipase (HSL) under stimulation of catecholamines [35]. Concomitantly with the decrease in HSL expression, plasma NEFA levels are high during the early postpartum period [35]. Resistin induces lipolysis in human adipocytes [36]. The secretion of GH is also high in early lactation. Growth hormone stimulates the mobilization of NEFAs from adipose tissue by inhibiting insulin-mediated lipogenesis and increasing the lipolytic response to beta adrenergic signals [37]. However, the regulation of GH receptor expression in the adipose tissue of early lactation dairy cows is unclear [38,39]. In rodents or human, GH increases resistin gene expression in white adipose

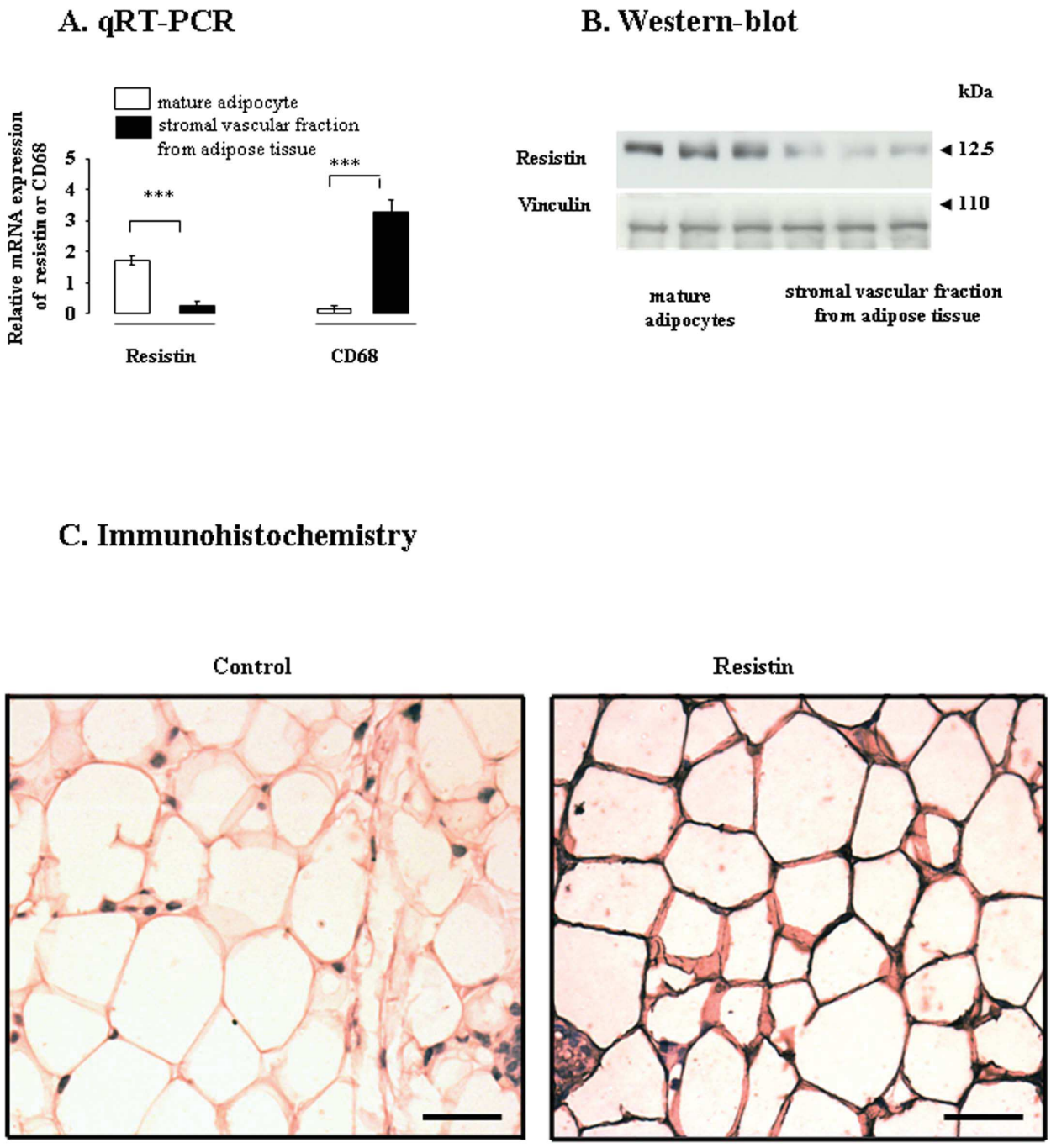

Figure 6. Immunolocalization and levels of resistin protein in bovine adipocytes. Levels of mRNA (A) and protein (B) for resistin in isolated mature bovine adipocytes and in isolated bovine stromal vascular cells, as determined by qRT-PCR and western blotting, respectively. mRNA levels for CD68 (the main cell surface marker of macrophages) was also measured in in isolated mature bovine adipocytes and in isolated bovine stromal vascular cells (A). Relative expression was measured relatively to the geometric mean of 4 reference gene expression [cyclophilin A (PPIA), GAPDH, Actin B (ACTB), and ribosomal protein L19 (RPL19)] by real-time reverse-transcription PCR. Vinculin (VLC) was used as the loading control for immunoblotting. The results are representative of five cell preparations (adipose tissue from one cow was used for one cell preparation). Results are represented as means ± SEM. * indicates a significant difference ($p<0.05$). C. Subcutaneous white adipose tissue from 32-month-old cows was fixed and sectioned, and then subjected to immunochemical analysis to determine of the distribution of resistin. Bar: 100 μm. The image shown is representative of three experiments on three different animals.
doi:10.1371/journal.pone.0093198.g006

tissue or serum resistin levels [40,41]. Thus, we can hypothesis that resistin could participate to the in vivo GH effects on the adipose tissue of dairy cows. However, we observed that resistin induces in vitro mRNA expression of ATGL and HSL mRN on adipose tissue explants suggesting that resistin could also act independently of GH.

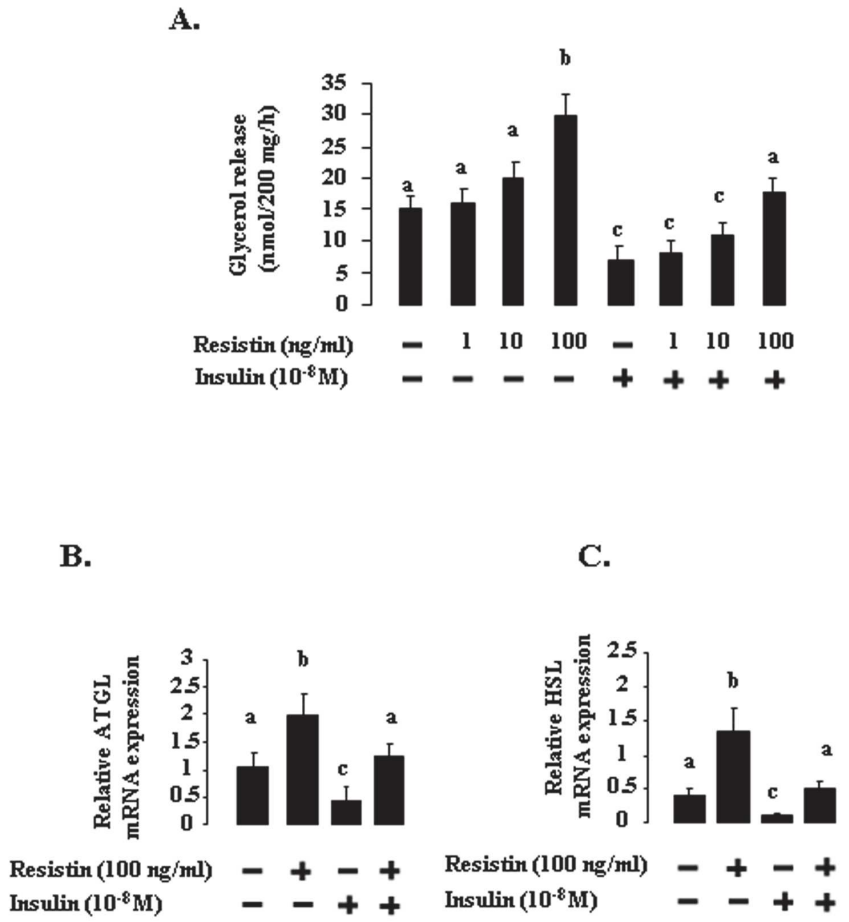

Figure 7. A. Effect of recombinant bovine resistin, in the presence or absence of insulin, on the release of glycerol from subcutaneous adipose tissue explants ($n = 4$ experiments). Data are expressed as nanomoles of glycerol per 200 milligrams of tissue per 1 h. Each value is the mean ± SEM, and different letters indicate significant differences at the $P<0.05$ level. B. ATGL mRNA and C. HSL mRNA in subcutaneous adipose tissue explants incubated in the presence or absence of recombinant bovine resistin (100 ng/ml) ± insulin (10-8M) ($n = 4$ experiments). Relative expression was measured relatively to the geometric mean of 4 reference gene expression [cyclophilin A (PPIA), GAPDH, Actin B (ACTB), and ribosomal protein L19 (RPL19)] by real-time reverse-transcription PCR. Data are presented as means ± SEM. Different letters indicate significant differences at the $P<0.05$ level.
doi:10.1371/journal.pone.0093198.g007

We found that plasma insulin and glucose concentrations followed patterns typical of the peri-partum period, declining sharply at 1 WPP [2]. By contrast, plasma resistin levels and the levels of resistin mRNA and protein in adipose tissue increased during this period. Komatsu *et al.* also reported higher levels of resistin production in adipose tissue and lower plasma insulin concentrations in dairy cows at peak lactation (around 8 WPP – 10 WPP) than in dry animals [15]. Plasma concentrations of two other adipokines, leptin (the most studied) and adiponectin, have been analyzed in dairy cows. Leptin regulates food intake, energy partitioning and adipose tissue deposition during both short- and long-term changes in nutritional state [12]. In dairy cows, plasma leptin concentrations are high before calving, proportionally to body condition score (BCS); they then decrease at calving and then remain low even when energy status improves [12,42]. In our study we observed a significant negative correlation between plasma resistin levels and energy balance and plasma resistin levels and dry matter between WPP1 and WPP2.After calving, hypoleptinemia may contribute to peripheral insulin resistance [12]. Indeed, unlike resistin, leptin is known to increase insulin sensitivity [43]. Plasma adiponectin concentrations have recently been investigated in dairy cows. Adiponectin, like leptin, increases insulin sensitivity in various species. Mielenz *et al.* (2013) showed, by western blotting and ELISA, that, in multiparous Holstein-Frisian dairy cows, plasma adiponectin concentration decreased from day −21 antepartum, reaching a trough at day 1, and increasing thereafter, with the highest values attained on day 14 postpartum [44]. Giesy *et al.* (2012) also obtained similar results [45]. The profile of adiponectin protein levels in adipose tissue reported here are at odds with the variations of plasma adiponectin concentration previously described by [44–47]. Indeed, our results show that adipose levels of adiponectin protein are lower at 1 WPP than at 5 MG. Koltes and Spurlock (2012) and very recently Saremi *et al.* (2014) observed a decrease of the adiponectin mRNA in subcutaneaous adipose tissue throughout the transition period [48,49]. Moreover, Lemor *et al.*, 2009 showed that plasma leptin concentrations and the levels of two adiponectin receptors (AdipoR1 and AdipoR2) in subcutaneous adipose tissue were lower one week before calving than three weeks post partum [13]. It is well known that at the beginning of lactation plasma insulin levels are decreased compared to the pre partum level [50] because of reduction of pancreatic function [51], and insulin

response to glucose infusion is reduced [52]. However, we can also hypothesize that an increase in plasma resistin levels and a decrease in plasma leptin and adiponectin levels towards lactation may contribute to the decrease in insulin sensitivity. However, further experiments are necessary to demonstrate this hypothesis.

As pointed out above, the molecular mechanism underlying the decrease in insulin sensitivity in peripheral tissues (adipocytes and muscles) during early lactation in dairy cows is not yet well understood. However, it is well established that bovine adipose tissue adapts pre-partum with a shift towards NEFA mobilization rather triglyceride accumulation [53]. Using tail-head subcutaneous fat, Sadri *et al.* (2010) showed a decrease in the abundance of mRNA for GLUT4 and GLUT1 on day 1 post partum, potentially reflecting a physiological adaptation of the adipose tissue [4]. However, they observed no change in gene expression for IRS-1, IR and P85 or P110 (regulatory and catalytic subunits of PI3K). Our findings confirm these results at the protein level for IR, IRS-1, IRS-2, Akt, MAPK ERK1/2 and P38, AMPK, P70SK, S6K and IGF-1R, at 1 WPP and 5 MG. We also observed a significant decrease in the tyrosine phosphorylation of IR, IRS-1, IRS-2, P70S6K, S6, Akt and MAPK ERK1/2 that can be explained by the strong decrease in the plasma insulin levels one week after calving. However, IGF-1Rbeta, P38 MAPK and AMPK displayed similar levels of phosphorylation at both these stages. The insulin receptor (IR) and the insulin-like growth factor-1 receptor (IGF-1R) belong to the same subfamily of receptor tyrosine kinases [3,54]. In our study we showed that plasma insulin concentrations are low one week postpartum and increase after. We did not measure IGF-1 plasma concentrations but it is well known that plasma IGF-I concentrations are also low during the week following parturition [55]. Here, we observed that IR beta subunit tyrosine phosphorylation was decreased WPP1 as compared to 5MG whereas tyrosine phosphorylation of IGF-1R was similar in both physiological states. These data suggest that even if IR and IGF-1R are two tyrosine kinases receptors very closed they are differently regulated in bovine adipose tissue. However, the physiological meaning of this different activation between IR and IGF-1R remains to be determined. The p38 MAPK signalling pathway is not specific to insulin or IGF-1. It allows cells to interpret various external signals and respond by generating a plethora of different biological effects. This can explain why P38 MAPK phosphorylation was unchanged between WPP1 and 5MG. AMPK plays an important role in the regulation of energy metabolism. Previous studies have reported higher levels of AMPK phosphorylation in bovine adipose tissue on day 1 postpartum than on day 21 prepartum [56,57]. Based on these results, antilipolytic effects of AMPK for dairy cows have been addressed [56]. However, consistent with our results, Locher et al (2012) reported no variation of AMPK phosphorylation between days 1 and 21 postpartum and according to the degree of NEB. They suggested that AMPK activation is dependent on the lipolysis. In our study even if plasma NEFAs are significantly different between WPP1 and 5MG we observed no change in the AMPKα phosphorylation. However, in our study we determined phosphorylation of both AMPKα isoforms (α1 and α2) (not only AMPKα1 as in [56]) and the location of subcutaneaous adipose tissue was also different (region of the tailhead for [56] vs neck for our study).

Conclusion

In our study we have showed that resistin is present *in vivo* in mature bovine adipocytes. We also observed that plasma resistin levels were high one week after calving and positively correlated with plasma NEFA levels and negatively with milk yield, dry matter intake and energy balance between WPP1 to WPP22. Finally, we have shown that recombinant bovine resistin increased the mobilization of lipids *in vitro* in adipose explants. Further studies are necessary to determine if resistin is *in vivo* involved in the adipose tissue mobilization during early lactation.

Supporting Information

Figure S1 Resistin protein production in mature bovine adipocytes, with recombinant bovine resistin used as the control.
(TIF)

Table S1 Value of the RNA integrity number (RIN) for each RNA sample used in the study.
(DOC)

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Author Contributions

Conceived and designed the experiments: MR JD. Performed the experiments: MR JD CR DG. Analyzed the data: MR JD DG. Contributed reagents/materials/analysis tools: JD DG. Wrote the paper: MR JD. Perfomed western-blot and qRT-PCRs: MR JD CR. Performed adipose tissue biopsies: JC. Collected adipose tissue of dairy cows: SE CR. Took care of animals: EB.

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Article 5

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CHEMERIN (RARRES2) Decreases In Vitro Granulosa Cell Steroidogenesis and Blocks Oocyte Meiotic Progression in Bovine Species[1]

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ABSTRACT

?2 **CHEMERIN, or RARRES2, is a new adipokine that is involved in the regulation of adipogenesis, energy metabolism, and** ?3 **inflammation. Recent data suggest that it also plays a role in reproductive function in rats and humans. Here we studied the expression of CHEMERIN and its three receptors (CMKLR1, GPR1, and CCRL2) in the bovine ovary and investigated the in vitro effects of this hormone on granulosa cell steroidogenesis** ?4 **and oocyte maturation. By RT-PCR, immunoblotting, and immunohistochemistry, we found CHEMERIN, CMKLR1, GPR1, and CCRL2 in various ovarian cells, including granulosa and theca cells, corpus luteum, and oocytes. In cultured bovine granulosa cells, INSULIN, IGF1, and two insulin sensitizers—metformin and rosiglitazone—increased *rarres2* mRNA expression whereas they decreased *cmklr1, gpr1,* and *cclr2* mRNA expression. Furthermore, TNF alpha and ADIPONECTIN significantly increased *rarres2* and *cmklr1* expression, respectively. In cultured bovine granulosa cells, human recombinant CHEMERIN (hRec, 200 ng/ml) reduced production of both progesterone and estradiol, cholesterol content, STAR abundance, CYP19A1 and HMGCR proteins, and the phosphorylation levels of MAPK3/MAPK1 in the presence or absence of FSH (10^{-8} M) and IGF1 (10^{-8} M). All of these effects were abolished by using an anti-CMKLR1 antibody. In bovine cumulus-oocyte complexes, the addition of hRec (200 ng/ml) in the maturation medium arrested most oocytes at the germinal vesicle stage, and this was associated with a decrease in MAPK3/1 phosphorylation in both** ?5 **oocytes and cumulus cells. Thus, in cultured bovine granulosa cells, hRec decreases steroidogenesis, cholesterol synthesis, and MAPK3/1 phosphorylation, probably through CMKLR1. Moreover, in cumulus-oocyte complexes, it blocked meiotic progression at the germinal vesicle stage and inhibited MAPK3/1 phosphorylation in both the oocytes and cumulus cells during in vitro maturation.**

adipokine, cholesterol, follicle, granulosa, hormone action, oocyte maturation, ovary, reproduction, signaling pathways

INTRODUCTION

CHEMERIN (16 kDa), also called retinoic acid receptor responder protein 2 (RARRES2) or tazarotene-induced gene 2 (TIG2), is an adipokine mainly expressed by white adipocytes but also by macrophage, plasmacytoid dendritic cells, and ?6 natural killer cells in rodents and humans [1, 2]. It is produced and released as an inactive precursor called prochemerin that is quickly converted to its active form by proteolytic cleavage of its C-terminus [3]. Three G protein-coupled receptors are able to bind chemerin with high affinity, namely CMKLR1 (chemokine receptor-like 1 or ChemR23, [4]), GPR1 (C protein-coupled receptor1, [5]), and CCRL2 (C-C chemokine receptor-like 2, [6]). CHEMERIN binding to CMKLR1 triggers calcium mobilization, receptor and ligand internalization, and cell migration [4]. In contrast to CMKLR1, very few studies have investigated signal transduction pathways coupled to GPR1 in response to CHEMERIN. CHEMERIN binding to CCRL2 does not induce signaling pathways or ligand internalization but could control CHEMERIN bioavailability and improve the interaction between CHEMERIN and CMKLR1 on adjacent cells [3, 7]. CHEMERIN is involved in the regulation of immunity, adipogenesis, lipolysis, and glucose metabolism [8–10]. Plasma CHEMERIN levels are associated with body mass index and plasma triglycerides [10], and are high in obese patients and in those with diabetes [8, 10]. Plasma CHEMERIN levels are also increased in patients with polycystic ovary syndrome (PCOS) [11]. CHEMERIN regulates INSULIN sensitivity in rodents and humans. Furthermore, in sheep, a recent study shows that a CHEMERIN analog in vivo regulates INSULIN secretion related to glucose metabolism and the release of triglycerides [12].

Recently, some data have suggested that CHEMERIN could affect female and male reproductive functions. Indeed, CHEMERIN is present in the ovary and testis in rodents and humans [13–15] but also in the rat placenta and in human umbilical cord blood [16]. In rats, elevated CHEMERIN levels suppress follicle development [17]. Furthermore, serum CHEMERIN levels fall as pregnancy progresses, suggesting a regulatory role of CHEMERIN in maternal-fetal metabolism [18]. In rat testes, CHEMERIN and its three receptors are developmentally regulated and highly expressed in Leydig cells [15]. Moreover, in vitro treatment with CHEMERIN suppressed the human chorionic gonadotropin (hCG)-induced testosterone production from primary Leydig cells [15]. Our laboratory has recently shown that CHEMERIN and CMKLR1 are present in the human ovary, especially in granulosa cells (GCs) and in the human GC line KGN [14]. In primary human GCs, we observed that CHEMERIN decreases IGF1-induced steroidogenesis and cell proliferation through a decrease in the activation of IGF1R-signaling pathways [14]. In a 5a-dihydrotestosterone-induced rat model, mRNA and protein expression levels of CHEMERIN and CMKLR1 were significantly higher in the ovaries; this could help to explain the induction of antral follicle growth arrest [13, 17]. The same authors have also shown that CHEMERIN decreases FSH-induced steroidogenesis in rat follicle and GC culture [13, 19]. In patients with PCOS, serum CHEMERIN levels and subcutaneous or omental adipose tissue were significantly increased [11]. However, treatment with metformin, an insulin sensitizer, for 6 mo strongly reduced serum CHEMERIN levels

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in PCOS patients. PCOS is the most common cause of anovulation and infertility, affecting 5% to 10% of women of reproductive age [20]. It is characterized by hyperandrogenism, chronic anovulation, and, occasionally, obesity [20]. In addition to its reproductive consequences, PCOS is a metabolic disorder associated with insulin resistance and hyperinsulinemia [21].

In dairy cows, the decrease in fertility associated with increases in milk yield is related, in part, to the intensity of selection on milk production [22]. Dairy cows have been selected to produce more milk, mostly through their ability to mobilize fat to support milk production. This results in a strong loss of adipose tissue after calving that is associated with alterations in blood metabolite and hormone profile, including adipokines such as LEPTIN and ADIPONECTIN [23–25], which in turn could influence fertility. Plasma CHEMERIN during lactation has not yet been determined in dairy cow. However, adipose CHEMERIN mRNA abundance is raised during adipose tissue mobilization in Holstein cows [26], but whether this elevated CHEMERIN affects ovarian follicular function in cattle is unknown. In the present study, we identified CHEMERIN and its receptors in the bovine ovary and investigated the effects of human recombinant CHEMERIN (hRec) in cultured GC steroidogenesis and during in vitro oocyte maturation.

MATERIALS AND METHODS

Ethics

All the procedures were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

Hormones and Reagents

Recombinant human IGF1, INSULIN, and metformin were obtained from Sigma. Purified ovine FSH (lot no. AFP-7028D, 4453 international units/mg, FSH activity = 175 × activity of oFSH-S1 used for culture treatment was a gift from National Institute of Diabetes and Digestive and Kidney, National Hormone Pituitary Program, Bethesda, MD). Recombinant human CHEMERIN and TNFα were purchased from R&D. Recombinant human LEPTIN was obtained from Biovendor (Euromedex). Recombinant bovine ADIPONECTIN and RE-SISTIN were obtained from Clinisciences. Rosiglitazone was a gift from B. Staels (Lille, France). Thymidine methyl-[^{3}H] was obtained from Amersham Life Science.

Antibodies

Rabbit polyclonal antibodies to PRKAA1 and HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) were purchased from Upstate Biotechnology Inc. Rabbit polyclonal antibodies to phospho-MAPK3/1 (Thr202/Tyr204), phospho-MAPK14 (Thr180/Tyr182), phospho-AKT1 (Ser 473), AKT1, and phospho-PRKAA1 (Thr172) were obtained from New England Biolabs Inc. Mouse monoclonal antibodies to VCL and CYP19A1 were obtained from Sigma and Serotec, respectively. Rabbit polyclonal antibodies against CYP11A1, STAR, and HSD3B were generously provided by Dr. Dale Buchanan Hales (University of Illinois, Chicago, IL) and Dr. Van Luu-The (CHUL Research Center and Laval University, Quebec, Canada), respectively. Rabbit polyclonal antibodies to MAPK3 (C14), MAPK14 (C20), CHEMERIN, GPR1, CCRL2, and CMKLR1 were purchased from Santa Cruz Biotechnology. All the antibodies were used at 1:1000 dilution in the Western blot analyses.

Collection of Bovine Tissue and Primary Culture of Bovine GCs

Bovine tissues—corpus luteum (CL), ovarian cortex(Cx), small (SF)and large follicles (LF), and GCs—were recovered from a local slaughterhouse. Tissue and cell samples for mRNA and protein extraction were frozen in liquid nitrogen and stored at −80°C. Bovine ovaries were obtained from adult cows collected in a slaughterhouse and transported aseptically before dissection. Then, GCs were recovered from the small antral follicles (3 to 5 mm) in modified McCoy 5A medium followed by 5 min centrifugation. Cells were washed, counted in a hemocytometer, and cultured in McCoy 5A supplemented with 20 mM Hepes, penicillin (100 units [U]/ml), streptomycin (100 mg/L), L-glutamine (3 mM), 0.1% bovine serum albumin (BSA), 5 mg/L transferrin, 20 mg/L selenium, and 10% fetal bovine serum (FBS) (PAA Laboratories). Approximately 4×10^5 viable cells were seeded in each plastic multiwell containing 1 ml medium. After 24 h of culture, cells were starved with McCoy 5A medium containing 1% of FBS for one night and then incubated in fresh culture medium with or without test reagent for the appropriate time. All the cultures were performed in a water-saturated atmosphere of 95% air/5% CO_2 at 37°C.

RNA Isolation and RT-PCR

As described previously [14], total RNA was extracted by using Trizol reagent according to the manufacturer's procedure (Invitrogen). Reverse transcription (RT) and polymerase chain reaction (PCR) were then carried out. Briefly, 1 μg of total RNA was reverse transcribed for 1 h at 37°C in 20 μl final volume of reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Amersham), 50 pmol of oligo (dT) 15, 5 U of ribonuclease inhibitor, and 15 U of Moloney murine leukemia virus reverse transcriptase. As described in Table 1, 2 μM of each set of specific primers were used. PCR conditions were DNA denaturation at 95°C for 5 min, 95°C for 1 min, 58°C for 1 min, and 72°C min for 35 cycles before a final extension at 72°C for 10 min. For this, 2 μl of RT reaction mixture were added in 25 μl final volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 U of Taq polymerase. The results were viewed on 1.5% agarose gel stained with ethidium bromide, and the amplified DNA was extracted and sequenced by the Genome Express company. Consumables for RT-PCR were obtained from Sigma, and Moloney murine leukemia virus reverse transcriptase and RNase inhibitor were from Promega.

Real-Time Polymerase Chain Reaction (PCR)

After the RT, the bovine cDNAs of GCs were diluted 1:5. Real-time PCR was made in 20 μl final volume containing 10 μl iQ SYBR Green supermix (Bio-Rad), 0.25 μl of each primer (10 μM), 4.5 μl of water, and 5 μl of template. The cDNA templates were amplified and detected with the MYIQ Cycler real-time PCR system (Bio-Rad) with the following protocol: 1 cycle for 5 min at 95°C to denature the sample and then 40 cycles 1 min at 95°C for denaturation, 1 min at 60°C for hybridization, 1 min at 72°C for stretching, and finally 1 cycle for 5 min at 72°C for final elongation. We used the following control genes for normalization: *actb* (F 5′-ACGGAACCACAGTTTAT CATC-3′ and R 5′-GTCCCAGTCTTCAACTATACC-3′), *rpl19* (F 5′-AATCGCCAATGCCAACTC-3′ and R 5′-CCCTTTCGCTTACCTATACC -3′), and *ppia* (F 5′-GCATACAGGTCCTGGCATCT-3′ and R 5′-TGTCCA CAGTCAGCAATGGT-3′). We determined the primer efficiency by diluting the pool of cDNA of different samples. The efficiency was between 1.7 and 2. We detected the amplification signal with a fluorophore reporter, SYBR Green. Then we determined the cycle threshold (Ct) to quantify the sample, with all the samples being used in duplicate. The cycle threshold represents the minimal number of cycles necessary to ensure that the fluorescence emitted by SYBR green exceeds the detection threshold. The logarithms of Ct obtained were calculated, and the linear regression equation of these points allowed for the efficiency of each primer pair to be calculated (E = 10-1/k-1, where K = slope). This Ct value is compared with that of a housekeeping gene, and the ratio indicates the adipokine expression, the values being obtained by subtracting the Ct value of chemerin from that of the reference gene.

Protein Extraction and Western Blot Analysis

Primary bovine GCs and all the other tissues or cells were homogenized as previously described [27]. Lysates were incubated on ice for 30 min before centrifugation at 12 000 × *g* for 20 min at 4°C. The pellet was eliminated, and the samples were stored at −80°C. The protein concentration for each condition was measured using a bicinchoninic acid protein assay. Samples were denatured and submitted to electrophoresis on 12% SDS-polyacrylamide gel at 90 V before being transferred onto nitrocellulose membranes (Schleicher and Schuell). Then the membranes were blocked for 30 min with Tris-buffered saline-Tween-5% milk and incubated with specific primary antibodies (dilution 1:1000) for 16 h at 4°C. After several washes, membranes were incubated for 90 min with the secondary antibodies horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) at 1:5000 final dilution. Proteins

Table 1. Oligonucleotide primers sequences.

Abbreviation	Name	Forward 5′–3′	Reverse 5′–3′	Size (bp)
ppia	Cyclophilin A	GCATACAGGTCCTGGCATCT	TGTCCACAGTCAGCAATGGT	217
Gpr1	GPR1	CTGTCATTTGGTTCACAGGA	AACAACCTGAGGTCCACATC	571
Rpl19	Ribosomal protein L19	AATCGCCAATGCCAACTC	CCCTTTCGCTTACCTATACC	156
actb	Beta actin	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATACC	180
Cmklr1	CMKLR1	CGGCCATGTGCAAGATCAGC	CAGGCTGAAGTTGTTAAAGC	400
Ccrl2	CCRL2	CGTCATGATCACGTGCAAGA	GCAGGAAGTTGCTGATCTTG	131
Rarres2	RARRES2 or Chemerin	GAGGAGTTCCACAAGCATC	ACCTGAGTCTGTATGGGACA	266

were revealed by enhanced chemiluminescence (Western Lightning Plus-ECL; Perkin Elmer) using a G:Box SynGene (Ozyme) with the GenSnap software (release 7.09.17). Quantification was performed with the GeneTools software (release 4.01.02).

Cholesterol Content

The cholesterol content was quantified with the cholesterol E-test assay kit (Wako). Standard solutions were prepared from 0 to 50 mg/ml. Four microliters of each sample and of the standard solution were loaded onto a 96-well plate, followed by the addition of 300 µl of reaction mixture in each well. The absorbance was recorded using a spectrophotometer (wavelength, 600 nm). The cholesterol concentrations were corrected according to the protein contents of the samples measured by a bicinchoninic acid protein assay.

Immunohistochemistry

Bovine ovary sections were deparaffinized, hydrated, and microwaved in antigen-unmasking solution for 5 min before being allowed to cool to room temperature. Sections were then washed in PBS for 5 min and immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DakoCytomation; Dako). Ovary sections were washed for 5 min in PBS followed by incubation for 20 min in PBS with 5% lamb serum in order to eliminate nonspecific background labeling. Sections were incubated overnight at 4°C with PBS containing rabbit primary antibody raised against either CHEMERIN (RARRES2), CMKLR1, GPR1, or CCRL2 at a dilution of 1:100. Sections were washed twice for 5 min each time in a PBS bath, followed by 30 min incubation at room temperature with a ready-to-use labeled polymer-horseradish peroxidase anti-rabbit (DakoCytomation Envision Plus HRP System; Dako). Finally sections were washed twice in PBS, and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB+Substrate Chromogen System; Dako). We used primary antibodies with rabbit IgG as negative controls.

Thymidine Incorporation into GCs

Primary bovine GCs were cultured for 24 h in McCoy 5A medium and 10% FBS. Cells were plated in a 24-well plate (2×10^5 viable cells/well), and four replicates were tested for each experimental condition (CHEMERIN, IGF1, etc.) for each culture. After several washes and one night serum starvation, cells were cultured for 24 h with 1 µCi/µl of [^{3}H]-thymidine (Amersham Life Science) in the presence or absence of chemerin and/or IGF1 (10^{-8} M) and or
?11 FSH (10^{-8} M). After that, thymidine was removed with PBS and fixed with cold 50% trichloroacetic acid for 15 min on ice. Finally, the cells were lysed by 0.5 N NaOH, and the radioactivity was counted in a β-photomultiplier by adding scintillation fluid (Packard Bioscience).

Progesterone and Estradiol Radioimmunoassay

Progesterone and estradiol concentrations were measured in serum-free medium from primary bovine GCs after 48 h of culture by a radioimmunoassay (RIA) protocol as previously described [27]. Cells were plated in a 48-well plate (10^5 viable cells/well), and eight replicates for each experimental condition (CHEMERIN, IGF1, etc.) for each culture were tested. The results
?12 were expressed as: (ng steroid/ml)/protein concentration/well. The intra- and interassay coefficients of variation for progesterone were less than 10% and 11%, respectively. The intra- and interassay coefficients of variation for estradiol were less than 7% and 9%, respectively. The results are the means ± SEM and are representative of six to eight independent cultures with each condition in quadruplicate.

Bovine Oocyte Collection and In Vitro Maturation

Bovine ovaries were collected from a slaughterhouse in sterile NaCl solution and maintained at 37°C until aspiration. The cumulus-oocyte complexes (COCs) were aspirated from follicles 3–8 mm in diameter using an 18-gauge needle connected to a sterile test tube and to a vacuum line (100 mmHg) as previously described [28]. COCs were then selected under a dissecting microscope. Expanded or nonintact COCs were eliminated: only intact COCs were washed in TCM Hepes 199 (Sigma) supplemented with 0.4% BSA and gentamycine (2.5ml/L) under mineral oil (Sigma). The COCs were cultured in TCM 199 (Sigma) with 4 mg/ml BSA supplemented or not with IGF1 (10^{-8} M) and/or chemerin (200 ng/ml) for 10 or 22 h at 39°C in 5% CO_2 in air with saturated humidity. Each oocyte group contained at least 25 oocytes. After maturation, COCs were denuded by pipetting with 0.5% hyaluronidase (Sigma), and the DNA was colored with Hoechst before mounting.

Statistical Analysis

All the experimental results are presented as the mean ± SEM. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) or a *t*-test to test differences. If ANOVA revealed significant effects, it was followed by the Student-Newman-Keuls test.

RESULTS

CHEMERIN and Its Receptors (CMKLR1, GPR1, and CCRL2) mRNA and Protein Expression in the Bovine Ovary

RT-PCR analysis with RNA from dissected CL, Cx, SF and LF, and fresh GCs from SF (GC SF) and LF (GC LF) resulted in the amplification of four cDNAs corresponding to fragments of *chemerin* (*rarres2*) (266 bp), *cmklr1* (400 bp), *gpr1* (571 bp), and *ccrl2* (131 bp) (Fig. 1A). By quantitative RT-PCR, we showed that *rarres2* mRNA was more highly expressed in GC SF than in GC LF (Fig. 1B). Similar results were obtained by using two other reference genes, *rpl19* and *ppia* (Supplemental Fig. S1; all the supplemental data is available online at www.biolreprod.org). Only the ratios with *actb* are shown in Figure 1B. In contrast, chemerin receptors (*cmklr1*, *gpr1*, and *ccrl2*) mRNA expression were similarly expressed in all the ovarian compartments and cells studied (data not shown). Immunoblotting of protein extracts revealed one band corresponding to CHEMERIN (16 kDa), CMKLR1 (43 kDa), GPR1 (41 kDa), and CCRL2 (40 kDa), showing that CHEMERIN and its receptors are produced in the bovine ovary (Fig. 1C). Immunohistochemistry with ovarian sections from bovine follicles confirmed the immunoblot findings and revealed CHEMERIN and its three receptors in oocytes and theca cells (Fig. 1D and Supplemental Fig. S2). Thus, CHEMERIN (RARRES2) and CHEMERIN receptors are present in the different bovine ovarian follicular cells.

Effect of INSULIN, IGF1, Metformin, and Rosiglitazone on rarres2 and Its Three Receptors mRNA Expression in Bovine GCs

We next investigated the effect of INSULIN (10^{-8} M), IGF1 (10^{-8} M), and two insulin sensitizers—metformin (10^{-6}M) and

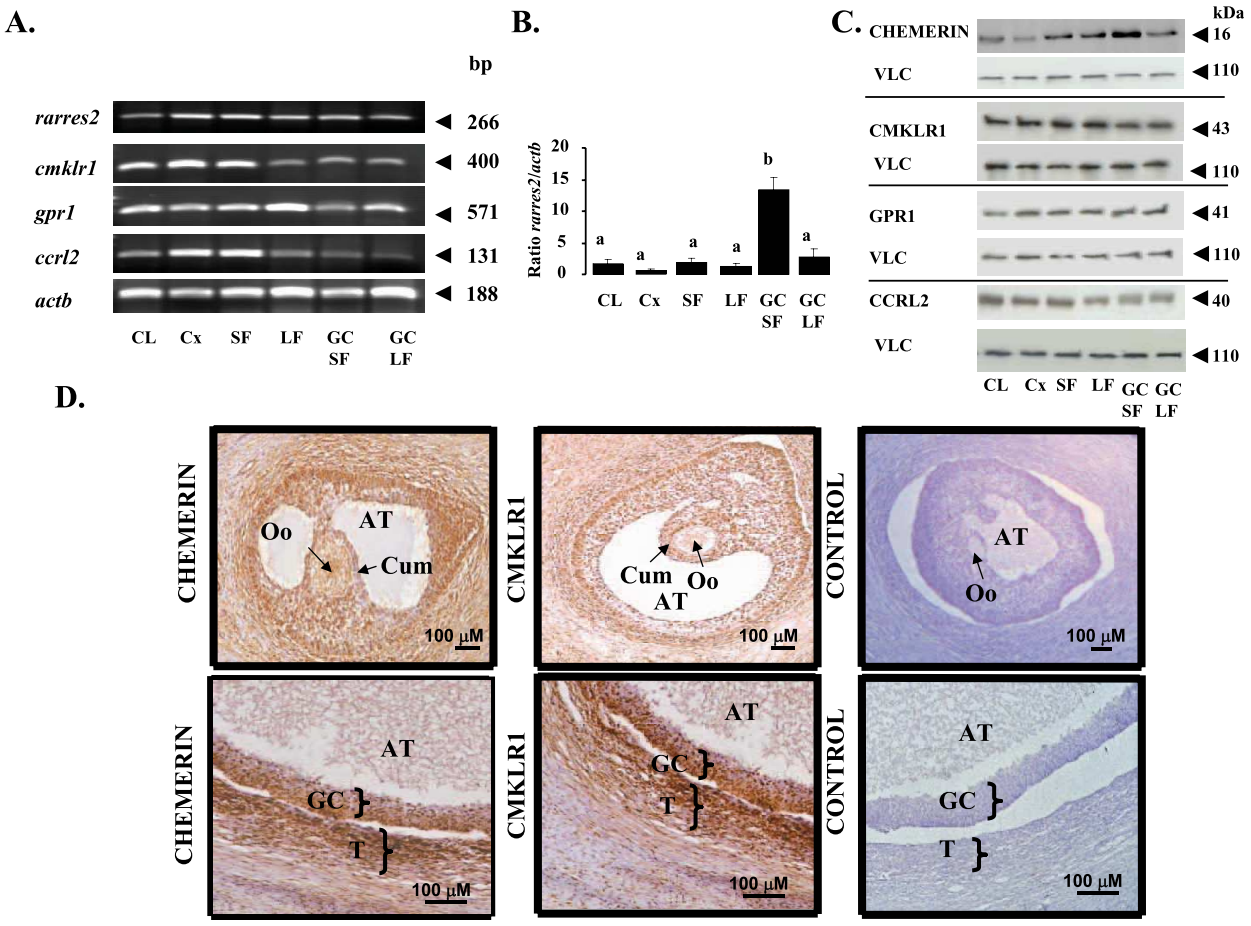

FIG. 1. CHEMERIN (RARRES2) and its receptor CMKLR1, GPR1, and CCRL2 expression in the bovine ovary. **A**) RT-PCR analysis of *rarres2, cmklr1, gpr1,* and *ccrl2* mRNAs in the corpus luteum (CL), ovarian cortex (Cx), small (SF) and large (LF) follicles, and GCs from SF (GC SF) and LF (GC LF). Tissues or cells from seven different Prim Holstein cows were used. **B**) Expression of *rarres2* mRNA in the bovine ovarian follicle. Messenger RNA expression of *rarres2* in CL, Cx, SF LF, and in GC SF and GC LF was measured by quantitative real time-PCR. *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. Tissues or cells from seven different Prim Holstein cows were used. The results are represented as mean ± S.E.M. Different letters indicate significant differences at $P < 0.05$. **C**) Detection of CHEMERIN (RARRES2), CMKLR1, GPR1, and CCRL2 proteins by immunoblotting in CL, Cx, SF, and in GC SF and GC LF. VLC is used as a loading control (n = 7). **D**) Localization of CHEMERIN and CMKLR1 in the bovine ovary by immunohistochemistry. 3,3′-Diaminobenzidine tetrahydrochloride-immunoperoxidase staining was performed on paraffin-embedded bovine ovary using antibodies against CHEMERIN (1:100), CMKLR1 (1:100) or no primary antibodies but rabbit IgG (1:100). Immunospecific staining is brown. The sections were counterstained with hematoxylin. CHEMERIN and CMKLR1 are present in all the bovine ovarian cells. AT, antrum; Oo, oocyte; T, Theca cells; GC, granulosa cells. Bars = 100 µm. Immunohistochemistry was performed on six different cows.

rosiglitazone (10^{-8} M)—on *rarres2*, *cmklr1*, *gpr1*, and *ccrl2* mRNA expression in primary bovine GCs. Overnight-starved cells (with 1% FBS) were incubated for different times (12 and 24 h) with INSULIN (10^{-8} M), IGF1 (10^{-8} M), metformin (10^{-6} M), or rosiglitazone (10^{-8} M). We have previously shown in human GCs that insulin sensitizers, including metformin and rosiglitazone, regulate expression of visfatin, another adipokines, after 12 and 24 h of stimulation [29]. Using real-time quantitative PCR, we showed that after 12 h of stimulation, INSULIN, IGF1, metformin, and rosiglitazone increased *rarres2* mRNA expression (Fig. 2A) whereas they decreased mRNA expression of *cmklr1* (except for rosiglitazone, Fig. 2B), *ccrl2* (Fig. 2C), and *gpr1* (Fig. 2D). Similar results were obtained by using two other reference genes (*rpl19* and *ppia*). Only the ratios with *actb* are shown in Figure 2. The
?13 results were consistent at 24 h of stimulation (data not shown).

Effect of Adipokines RE-SISTIN, ADIPONECTIN, LEPTIN, and TNFα on rarres2, cmklr1, gpr1, and cclr2 mRNA Expression in Bovine GCs

It is well known that bovine or ovine GCs express several adipokines and adipokines receptors including resistin [30], adiponectin and its receptors (ADIPOR1 and ADIPOR2) [31, 32], leptin and its receptor [33], and TNFalpha and its receptors [34]. Furthermore these adipokines have already been shown to modulate in vitro GC steroidogenesis. Consequently, we studied the effect of these adipokines on *rarres2*, *cmklr1*, *gpr1*, and *cclr2* mRNA expression in primary bovine GCs. Overnight-starved cells (with 1% FBS) were incubated for 12 h with RE-SISTIN (100 ng/ml), ADIPONECTIN (10 µg/ml), LEPTIN (10 ng/ml), or TNFα (10 ng/ml). Using real-time quantitative PCR, we observed that after 12 h of stimulation, only TNFα increased *rarres2* mRNA expression (Fig. 3A)

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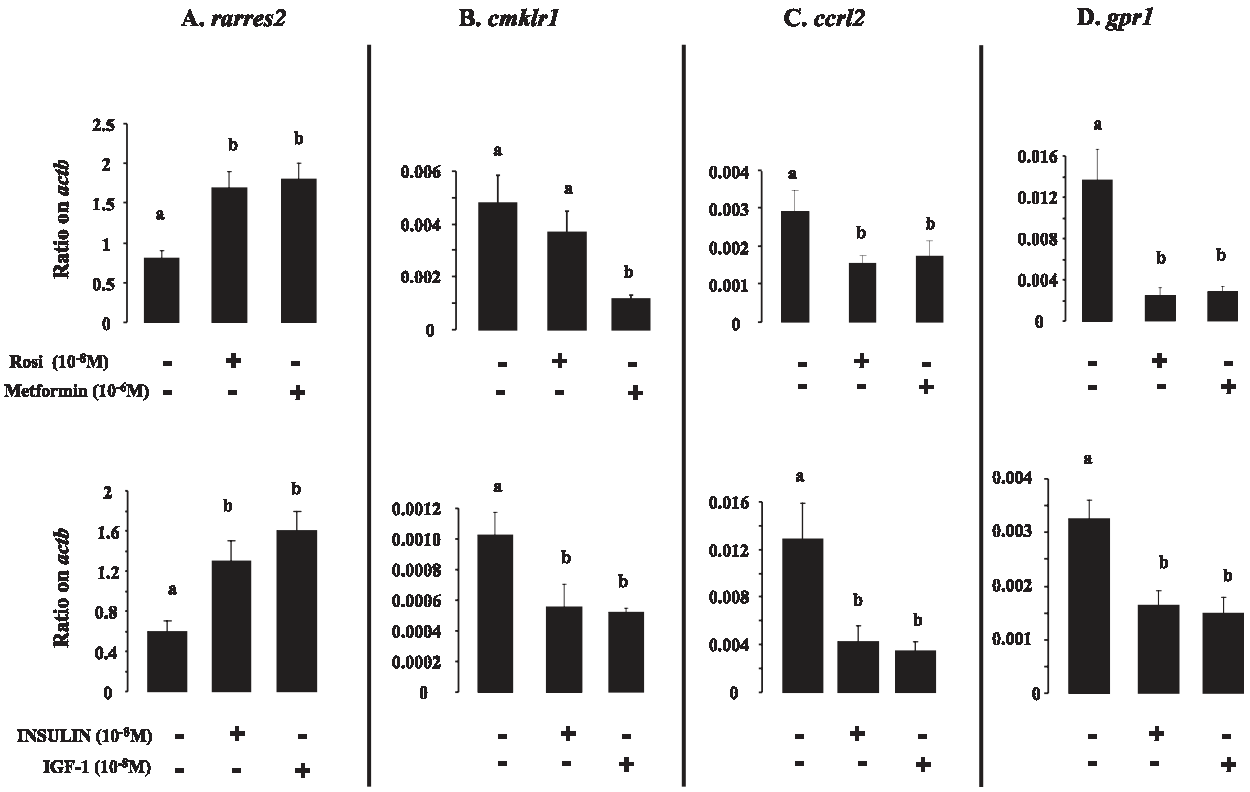

FIG. 2. Effect of INSULIN, IGF1, metformin, and rosiglitazone on *rarres2* (**A**), *cmklr1* (**B**), *ccrl2* (**C**), and *gpr1* (**D**) mRNA expression in primary bovine GCs. The mRNA expression of *rarres2, cmklr1, ccrl2,* and *gpr1* was measured by quantitative real-time PCR in primary bovine GCs after 12 h of stimulation with or without rosiglitazone (Rosi, 10^{-8} M), metformin (10^{-6} M), INSULIN (10^{-8} M), or IGF1 (10^{-8} M). *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. The results represent six to eight cultures of primary bovine GCs. The results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

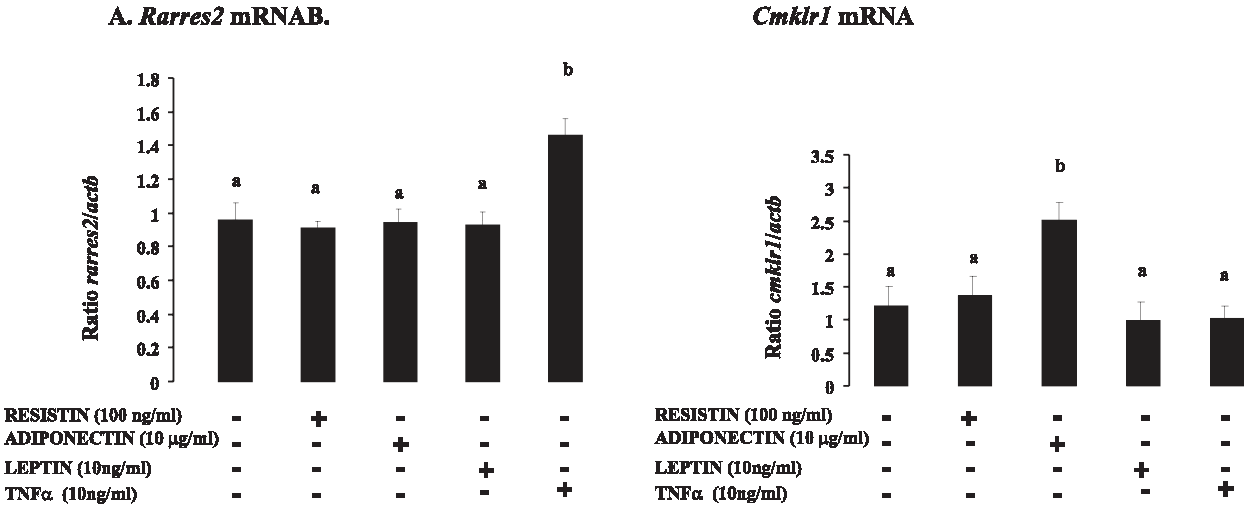

FIG. 3. Effect of adipokines (RE-SISTIN, ADIPONECTIN, LEPTIN, and TNFα) on *rarres2* and *cmklr1* mRNA expression in primary bovine GCs. The mRNA expression of *rarres2* and *cmklr1* was measured by quantitative real-time PCR in primary bovine GCs after 12 h of stimulation with or without RE-SISTIN (100 ng/ml), ADIPONECTIN (10 µg/ml), LEPTIN (10 ng/ml), or TNFα (10 ng/ml). *Actb* was used as a reference gene. Similar results were obtained using two other reference genes, *rpl19* and *ppia*. The results represent six to eight cultures of primary bovine GCs. The results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

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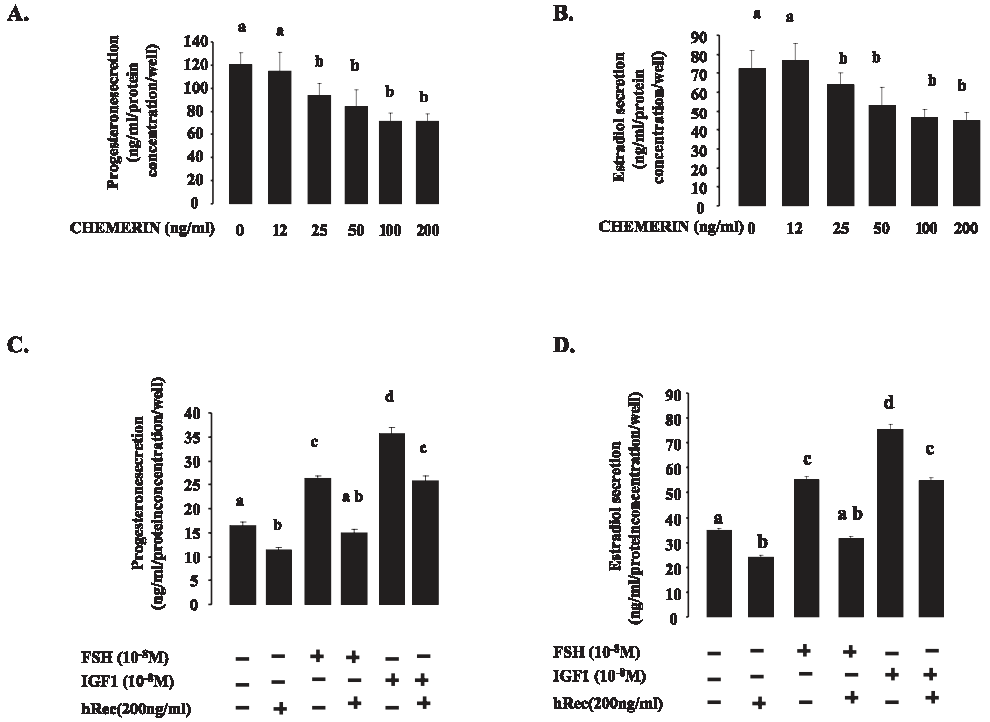

FIG. 4. Effect of CHEMERIN treatment on basal and FSH- or IGF1-stimulated secretion of progesterone and estradiol by bovine GCs. Granulosa cells from small bovine follicles were cultured for 48 h in a medium with serum and then in serum-free medium in the presence or in the absence of various doses of hRec (human recombinant CHEMERIN, **A** and **B**) for 48 h, or in presence or absence of 200 ng/ml hRec, ± 10^{-8} M FSH, or ± 10^{-8} M IGF1 (**C** and **D**) as described in *Materials and Methods*. The culture medium was then collected and analyzed for progesterone (**A** and **C**) and estradiol (**B** and **D**) content by RIA. The results are expressed as (ng/ml)/protein concentration/well. The results are means ± SEM of six independent experiments. Bars with different letters are significantly different ($P < 0.05$).

whereas only ADIPONECTIN increased mRNA expression of *cmklr1* (Fig. 3B). We observed similar results when cells were stimulated for 24 h. We detected no effect of the adipokines on the mRNA expression of *gpr1* and *ccrl2* whatever the time of stimulation (data not shown).

Effects of hRec on Basal and FSH- or IGF1-Stimulated Progesterone and Estradiol Production in Bovine GCs

To investigate the effect of hRec on the production of progesterone and estradiol, bovine GCs were incubated with various concentrations of hRec (0, 12, 25, 50, 100, or 200 ng/ml) for 48 h or with hRec for 48 h in the presence or absence of FSH (10^{-8} M) or IGF1 (10^{-8} M). Secretion of both progesterone (Fig. 4A) and estradiol (Fig. 4B) were inhibited by hRec treatment at a concentration of 25 ng/ml or greater ($P < 0.001$). In the presence of FSH (10^{-8} M) or IGF1 (10^{-8} M), hRec (200 ng/ml, 48 h) decreased progesterone and estradiol secretion by almost twofold ($P < 0.001$) (Fig. 4, C and D).

Effects of a Neutralizing CMKLR1 Antibody on hRec-Inhibited Production of Progesterone and Estradiol in Bovine GCs

We next examined whether the hRec-induced decrease in the production of progesterone and estradiol was mediated by CMKLR1 receptor. We blocked CHEMERIN/CMKLR1 signaling with an anti-CMKLR1 antibody (CMKLR1 Ab, 1μg/ml) beginning 1 h prior to application of hRec in the presence or absence of IGF1 or FSH and persisting throughout 48 h of stimulation. Rabbit IgG was used as an isotype control for the rabbit anti-human CMKLR1 neutralizing antibody. As shown in Figure 5, A and B, CMKLR1 Ab totally abolished the CHEMERIN-induced decrease in the production of progesterone and estradiol in the presence or absence of FSH or IGF1. Incubation with rabbit IgG under the same conditions did not restore these inhibitory effects of hRec (Fig. 5, A and B). Thus, hRec reduces progesterone and estradiol secretion at least through CMKLR1 in bovine GCs. ?14

We next determined whether the inhibitory effects of hRec on steroid production were due to effects on the protein levels of three crucial enzymes of steroidogenesis (HSD3B, CYP11A1, and CYP19A1) or that of STAR, a cholesterol carrier. As shown in Figure 6, A and B, hRec treatment (200 ng/ml, 48 h) reduced by at least twofold the production of steroidogenic acute regulatory protein (STAR) and CYP19A1 proteins not in the absence (basal state) but in the presence of FSH and IGF1 whereas no effect was observed on the protein levels of HSD3B and CYP11A1 (data not shown). These effects were totally abolished in the presence of 1 μg/ml CMKLR1 Ab. Thus, the decrease in steroid secretion in response to hRec may be due to a reduction in the amounts of the CYP19A1 and STAR proteins through activation of CMKLR1.

Effects of hRec on Cholesterol Content and HMGCR Protein Level in Bovine GCs

We next investigated the effect of hRec on the IGF1-or FSH-induced cholesterol content in GCs in the absence or in the presence of the neutralizing CMKLR1 Ab for 48 h (same

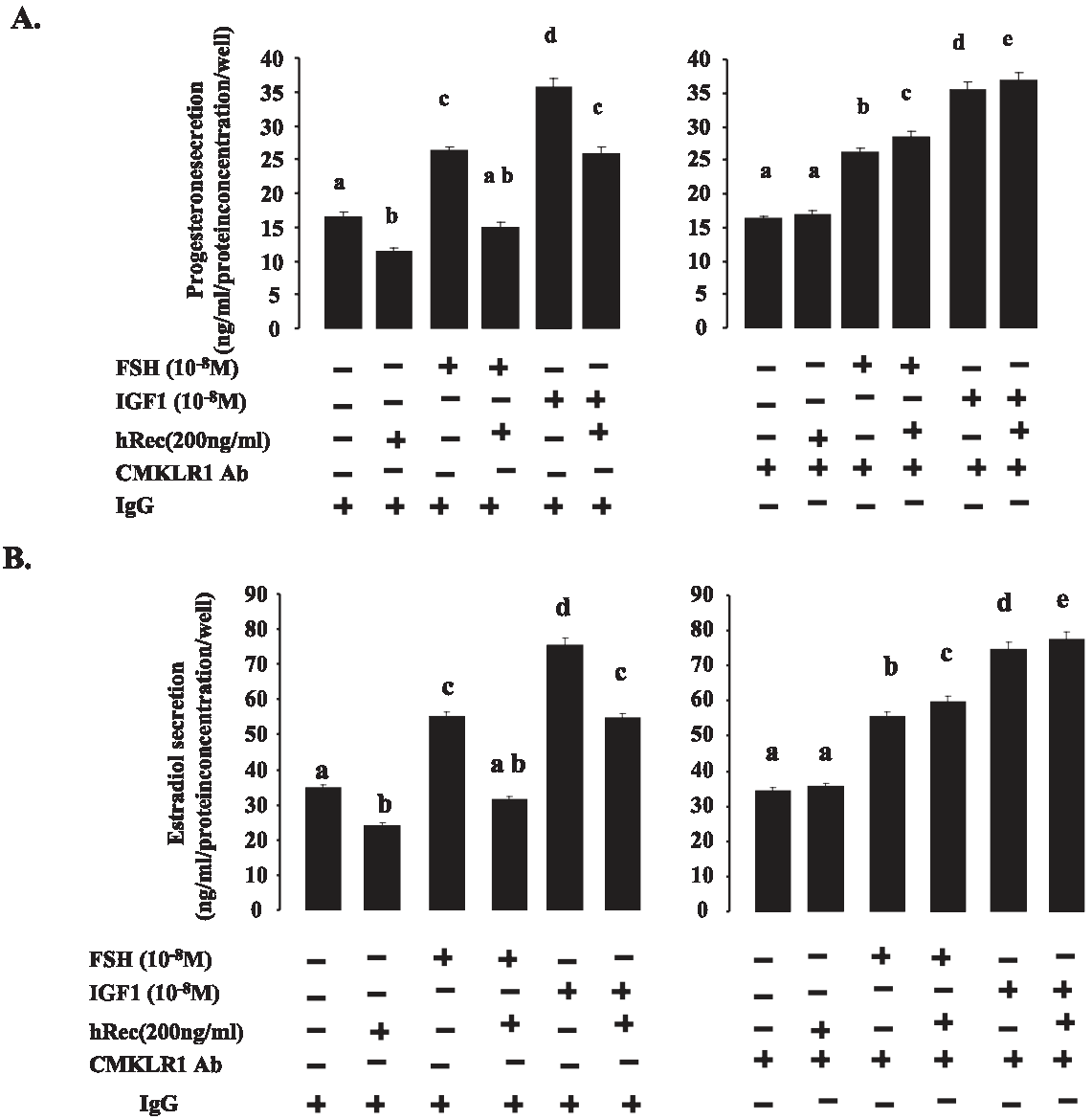

FIG. 5. Effect of CMKLR1 antibody (CMKLR1 Ab) treatment on basal and FSH- or IGF1-stimulated steroid secretion in response to chemerin in bovine GCs. Overnight-starved GCs from small bovine follicles were preincubated with and without 1 μg/ml CMKLR1 Ab for 1 h and then treated for 48 h with or without 200 ng/ml hRec, ± 10^{-8} M FSH, or ± 10^{-8} M IGF1 as described in Figure 4. The culture medium was then collected and analyzed for progesterone (**A**) and estradiol (**B**) content by RIA. The results are expressed as (ng/ml)/protein concentration/well. The results are means ± SEM of six independent experiments. Bars with different letters are significantly different ($P < 0.05$).

conditions used as those for steroidogenesis). As shown in Figure 7A, hRec (200 ng/ml) halved the cholesterol content and the protein level of HMGCR in GCs in the absence or the presence of FSH (10^{-8} M) or IGF1 (10^{-8} M). Incubation of cells with the neutralizing CMKLR1 Ab for 48 h abolished these inhibitory effects. Thus, hRec could decrease steroidogenesis through an inhibition of cholesterol synthesis through CMKLR1 in bovine GCs.

Effects of hRec on Bovine GC Proliferation and Viability

We also examined the effect of hRec on the number of bovine GCs in culture, either by induction of mitosis or by altering cell viability. [^{3}H]-Thymidine incorporation by primary bovine GCs treated with hRec (200 ng/ml) was determined after 24 h of culture in the presence or in the absence of FSH (10^{-8} M) or IGF1 (10^{-8} M). As expected, FSH and IGF1 treatment significantly increased [^{3}H]-thymidine incorporation (data not shown). However, CHEMERIN treatment did not affect cell proliferation in the basal state or in response to IGF1 or FSH (data not shown). As revealed by staining with trypan blue, chemerin treatment (200 ng/ml) had no effect on cell viability in the absence or presence of FSH and IGF1 (data not shown). Thus, hRec decreased steroid

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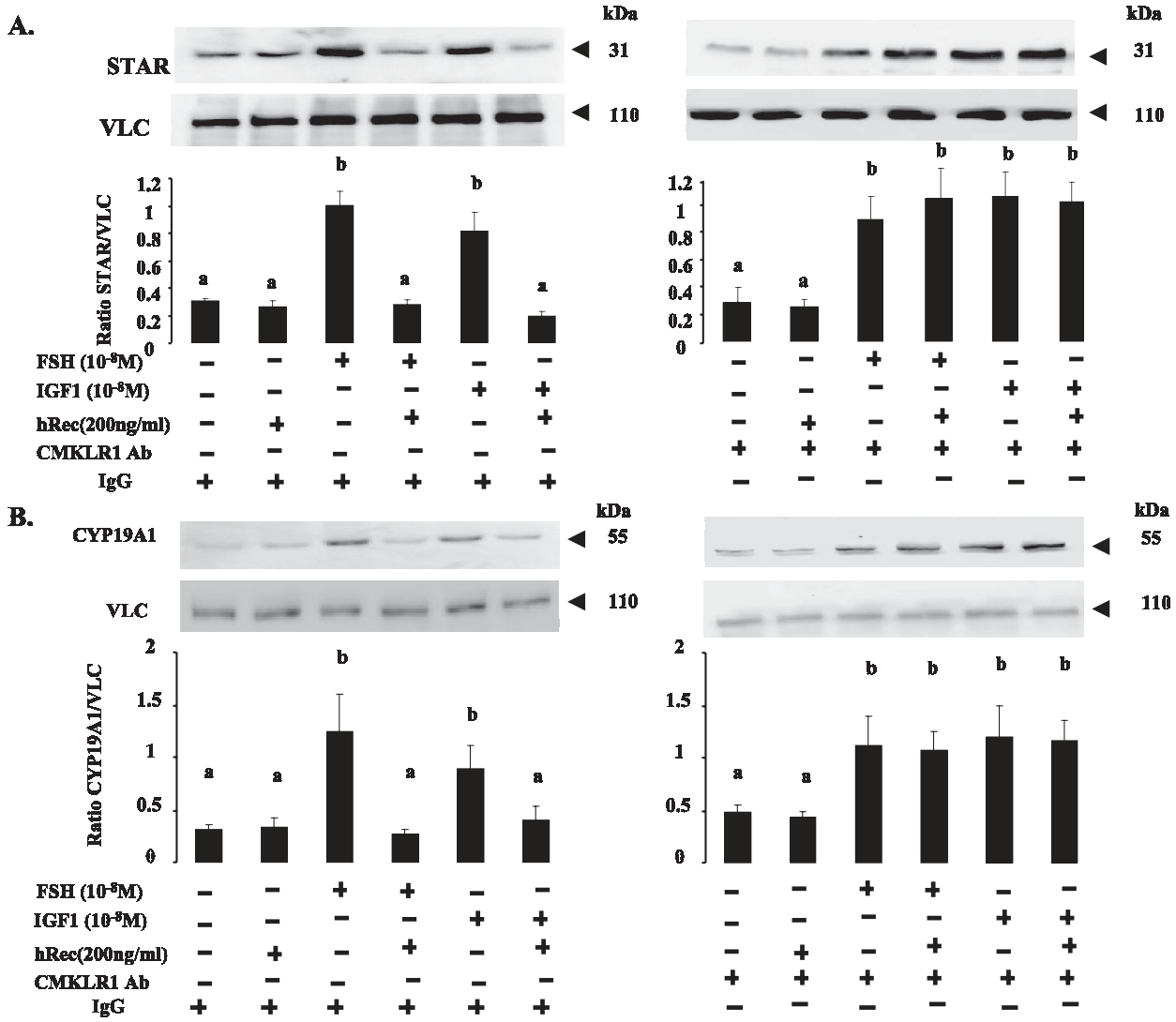

FIG. 6. Effect of CHEMERIN treatment on the amounts of STAR and CYP19A1 proteins and the potential involvement of CMKLR1 in bovine GCs. Protein extracts from bovine GCs were preincubated for 1 h with or without 1 μg/ml CMKLR1 Ab, then cultured for 48 h with or without 200 ng/ml hRec, $\pm 10^{-8}$ M FSH, or $\pm 10^{-8}$ M IGF1, and finally subjected to SDS-PAGE as described in *Materials and Methods*. The membranes were incubated with antibodies raised against the STAR (**A**) and CYP19A1 (**B**) proteins. Equal protein loading was verified by reprobing membrane with an anti-VCL antibody. Blots were quantified, and the STAR and CYP19A1 to αVCL ratios are shown. The results are expressed as means ± SEM of four independent experiments. Bars with different letters are significantly different ($P < 0.05$).

production in response to IGF1 or FSH without affecting the proliferation or viability of bovine GCs.

Signaling Pathways Involved in the hRec Effects in Bovine GCs

It is well known that G-protein coupled receptors (GPCR) activate the MAPK3/MAPK1 signaling pathway that is involved in the regulation of steroidogenesis [35]. As shown in Figure 8A, hRec treatment significantly inhibited phosphorylation of MAPK3/MAPK1 after 10 and 30 min of stimulation in bovine GCs. Under the same conditions, phosphorylation of AKT1, MAPK14, and PRKA was unchanged (data not shown). Incubation of cells with CMKLR1 Ab (1 μg/ml) totally abolished the hRec-induced decrease of MAPK3/MAPK1 phosphorylation in the absence or in the presence of FSH (10^{-8} M, 48 h) or IGF1 (10^{-8} M, 48 h) (Fig. 8B). Incubation with rabbit IgG under the same conditions did not restore the inhibitory effects of hRec (Fig. 8B). Thus, hRec inhibits MAPK3/MAPK1 phosphorylation through CMKLR1 in bovine GCs. ?15

Effects of hRec Treatment on the Nuclear Maturation of Bovine Oocytes in COCs and Progesterone Secretion of Bovine COCs During In Vitro Maturation

We also studied the effects of hRec (200 ng/ml) on the meiotic progression of bovine oocytes in COCs during in vitro maturation (IVM). For the control group, after 10 h of IVM, ?16 about 40% of oocytes underwent germinal vesicle breakdown

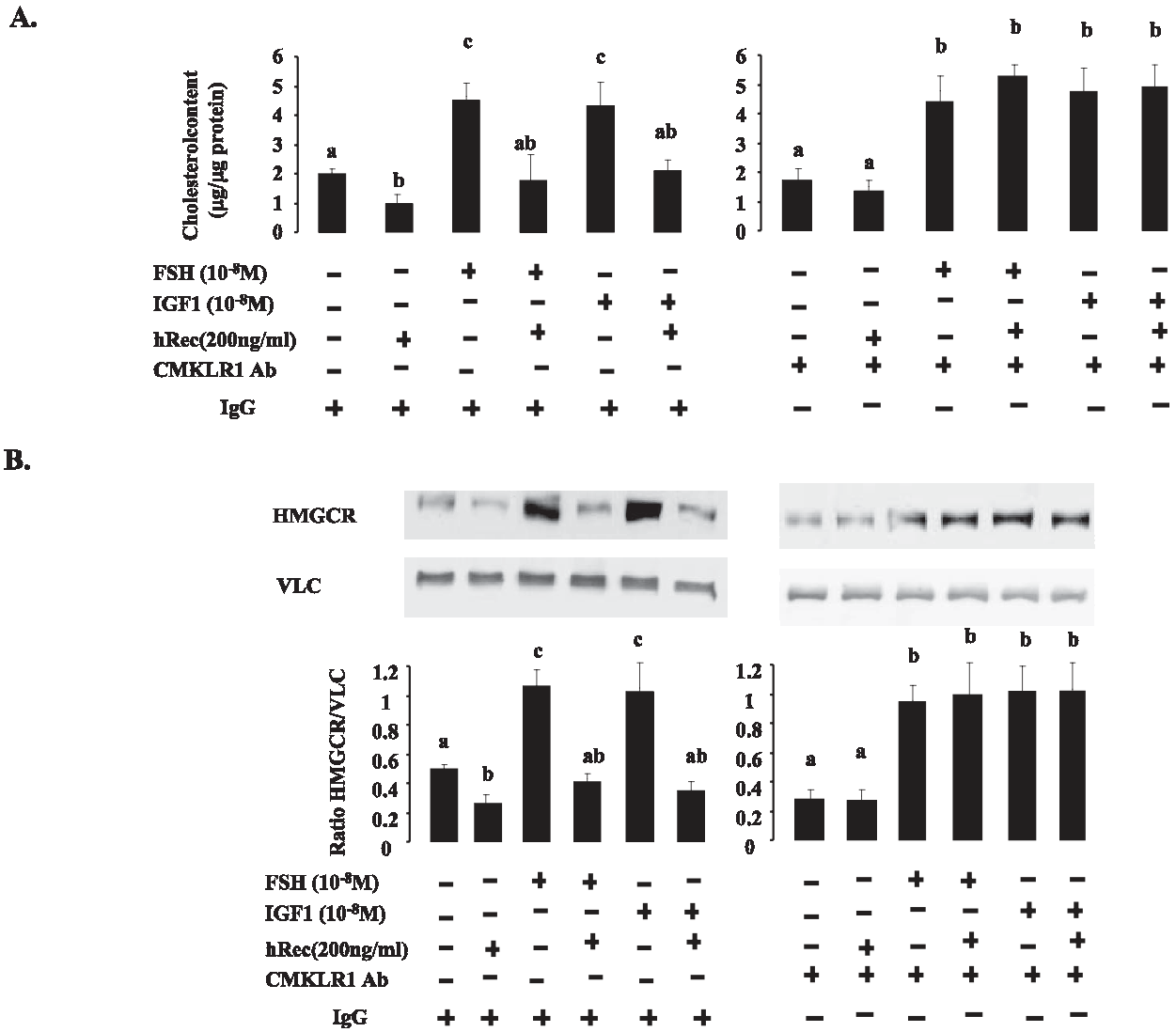

FIG. 7. Effect of CHEMERIN treatment on the cholesterol content and HMGCR protein levels and the potential involvement of CMKLR1 in bovine GCs. Overnight-starved GCs from small bovine follicles were preincubated with/without 1 µg/ml CMKLR1 Ab or IgG for 1 h and then treated for 48 h with or without 200 ng/ml hRec, $\pm 10^{-8}$ M FSH, or $\pm 10^{-8}$ M IGF1 as described in Figure 4. Granulosa cells were lysed, and their cholesterol content was determined as indicated in *Materials and Methods* and expressed as µg/µg total protein. Granulosa cell lysates were also subjected to SDS-PAGE as described in *Materials and Methods*. The membranes were incubated with antibodies raised against the HMGCR proteins. Equal protein loading was verified by reprobing membranes with an anti-VCL antibody. Blots were quantified and the HMGCR to αVCL ratios are shown. The results are expressed as means ± SEM of four independent experiments. Bars with different letters are significantly different ($P < 0.05$).

(GVBD) (Fig. 9B). After 22 h of culture in IVM medium, most oocytes underwent GVBD (Fig. 9B), and about 85% of oocytes had progressed to the metaphase II stage, with less than 10% remaining at the GV stage (Fig. 9B). Conversely, if COCs matured in the presence of 200 ng/ml of CHEMERIN, meiotic progression was inhibited (Fig. 9B). Furthermore, if COCs matured for 22 h in IVM medium supplemented with hRec (100, 200, or 400 ng/ml), 30% to 60% of oocytes remained at the GV stage (Fig. 9, B and C). Thus, hRec treatment of COCs during IVM resulted in meiotic arrest in a dose-dependent manner. Progesterone secretion by cumulus cells is known to play a key role in bovine oocyte maturation [36, 37]. We therefore investigated the effects of CHEMERIN treatment on progesterone secretion by COCs. The addition of hRec to the maturation medium for 22 h significantly decreased progesterone secretion in COCs (Fig. 10A).

Effects of hRec Treatment on MAPK3/1 Phosphorylation Levels in Bovine Oocytes and Cumulus Cells in COCs after IVM

We investigated the molecular mechanisms involved in the effects of hRec on the nuclear maturation of bovine oocytes in COCs by determining the levels of MAPK3/1 phosphorylation in the presence or absence of hRec (200 ng/ml) in COCs allowed to mature in vitro for 22 h. As shown in Figure 10, B and C, the level of MAPK3/1 phosphorylation increased in the

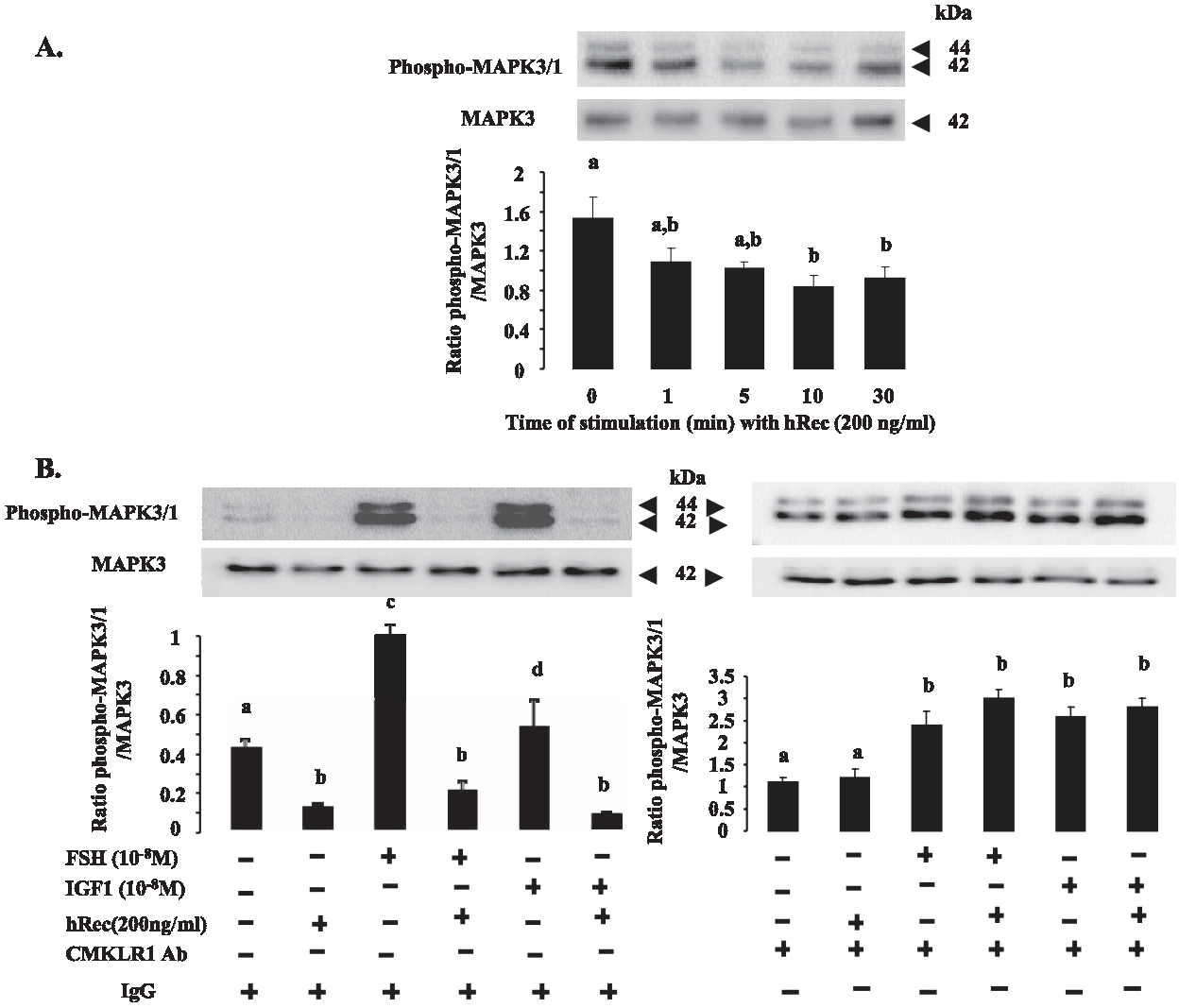

FIG. 8. Effect of CHEMERIN treatment on basal and FSH- or IGF1-stimulated phosphorylation of MAPK3/MAPK1 and the potential involvement of CMKLR1 in bovine GCs. Granulosa cells from small bovine follicles were cultured for 48 h in medium with serum and then in serum-free medium in the presence or absence of hRec (200 ng/ml) for various times (1, 5, 10, and 30 min) (**A**) or for 48 h ± 10^{-8} M FSH or ±10^{-8} M IGF1 with or without 1 h of CMKLR1 Ab or IgG preincubation (the same conditions as described in Fig. 5) (**B**). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-MAPK3/MAPK1 and anti-MAPK3 antibodies. Representative blots from three independent experiments are shown. Bands on the blots were quantified, and the phosphorylated protein to total protein ratio is shown. The results are reported as means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

oocyte and in cumulus cells from COCs during IVM. The addition of hRec (200 ng/ml) to the maturation medium for 22 h decreased MAPK3/1 phosphorylation by a factor of two in oocytes and by a factor of four in cumulus cells from COCs. Thus, hRec treatment during IVM decreased MAPK3/1 phosphorylation in COCs.

DISCUSSION

In this study, we demonstrated for the first time that CHEMERIN (RARRES2) and its three receptors—CMKLR1, GPR1, and CCRL2—are present in the bovine ovarian follicle. In bovine cultured GCs, INSULIN, IGF1, and two insulin sensitizers (metformin and rosiglitazone) increased *rarres2* expression whereas they decreased mRNA expression of *cmklr1*, *gpr1*, and *ccrl2*. In addition, we showed that hRec decreased basal and IGF1 or FSH-induced steroidogenesis, probably through CMKLR1, in cultured bovine GCs. This was associated with a reduction in the cholesterol content and in the levels of STAR, CYP19A1, HMGCR, and MAPK3/1 phosphorylation. Furthermore, we have observed for the first time that the addition of hRec (200 ng/ml) to the maturation medium arrested most of the bovine oocytes at the GV stage in COCs, suggesting that CHEMERIN could regulate not only GC steroidogenesis but also nuclear maturation in bovine oocytes during IVM.

We found CHEMERIN and its receptors CMKLR1, GPR1, and CCRL2 at the mRNA and protein level in all the different bovine ovarian compartments, including CL, Cx, and SF and LF. In bovine follicles, CHEMERIN and CMKLR1 are present

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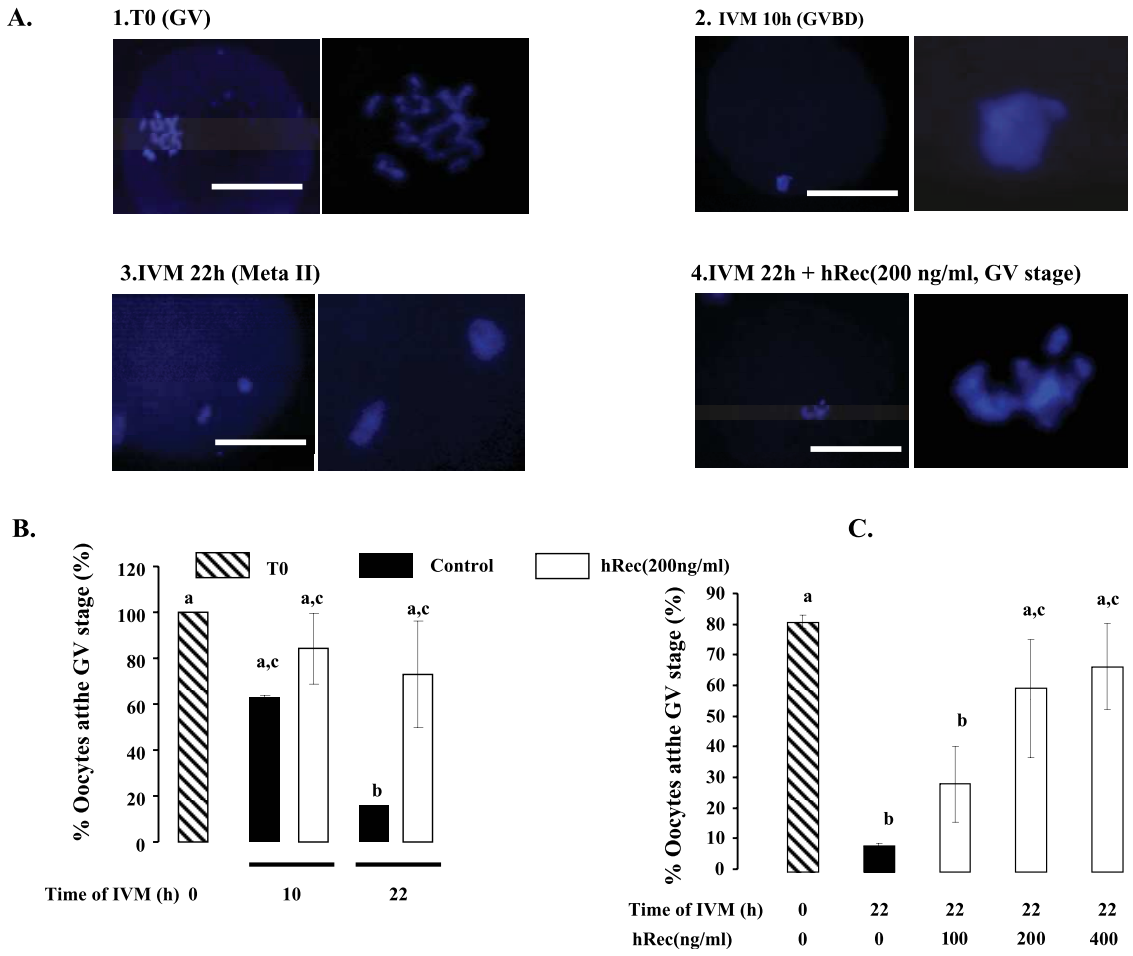

FIG. 9. Effects of CHEMERIN treatment on bovine oocyte nuclear maturation. **A**) Hoechst (blue) staining 1) before (T0, GV stage just after oocyte collection; 2) during (IVM 10 h, GVBD stage; and 3) and after maturation in the absence (IVM 22 h, MII stage) or 4) presence of CHEMERIN at a concentration of 200 ng/ml (IVM 22 h, GV stage). Bars = 50 μm (1–4). At each stage (panel 1 to 4), the right picture is a magnification (×5) of the left picture. **B**) Bovine oocytes were allowed to mature for 10 or 22 h in the presence or absence of hRec (200 ng/ml). The percentage of oocytes at the GV stage in the various conditions is shown. Different letters indicate significant differences with $P < 0.05$. The results are presented as mean ± SEM of three independent experiments. At least 50 bovine oocytes for each set of conditions in each experiment were used. **C**) Bovine oocytes were allowed to mature for 22 h in the presence or absence of various concentrations of hRec (100, 200, or 400 ng/ml). The percentage of oocytes at the GV stage in the various conditions is shown. Different letters indicate significant differences with $P < 0.05$. The results are presented as mean ± SEM of three independent experiments. Fifty bovine oocytes for each set of conditions in each experiment were used.

in theca and GCs, cumulus cells, and in oocytes. CHEMERIN and CMKLR1 have already been localized in the human and rat ovary [2, 14, 19]. Other adipokines such as LEPTIN [38], ADIPONECTIN [31], and RE-SISTIN [30] have been previously identified in bovine follicular cells. Using real-time PCR, we found that *rarres2* was significantly more expressed in GCs from SF than those observed in other follicular cells. The mRNA expression of the three receptors was similar for all follicular cells. In human, CHEMERIN circulates in plasma as different isoforms [39]. Its plasma concentration is about 100–200 ng/ml [40, 41]. In cattle, the plasma CHEMERIN concentrations are not yet known. In our experiments, we stimulated bovine GCs with 200 ng/ml, a concentration close to those observed in human plasma. The DNA sequences of bovine *rarres2* and its receptor, *cmklr1,* are highly homologous to those of humans, mice, and pigs [42], suggesting also a high identity for the amino acid sequences. Thus, we used hRec to determine the effects of chemerin on the bovine follicular cells.

In our study, we examined the effect of INSULIN, IGF1, and two insulin sensitizers (metformin and rosiglitazone) on the mRNA expression of *rarres2* and its three receptors (*cmklr1, gpr1,* and *ccrl2*) in primary bovine GCs. We showed that INSULIN, IGF1, and both insulin sensitizers increased *rarres2* expression, whereas they decreased mRNA expression of the three CHEMERIN receptors. The stimulatory effect of INSULIN on *rarres2* expression is in good agreement with the literature. Indeed, short-term hyperinsulinaemia upregulates adipocyte CHEMERIN production in vitro and INSULIN induces CHEMERIN release from adipocytes within 24 h in mice [43]. Conversely, the stimulatory effect of metformin on CHEMERIN expression observed in our study is not in agreement with two different studies. Indeed, it has been shown that metformin treatment for 6 mo decreased serum CHEMERIN concentration in women with PCOS [11]. In addition, in the white adipose tissue and serum in high-fat-diet-induced insulin-resistant rats, metformin decreased the expression of

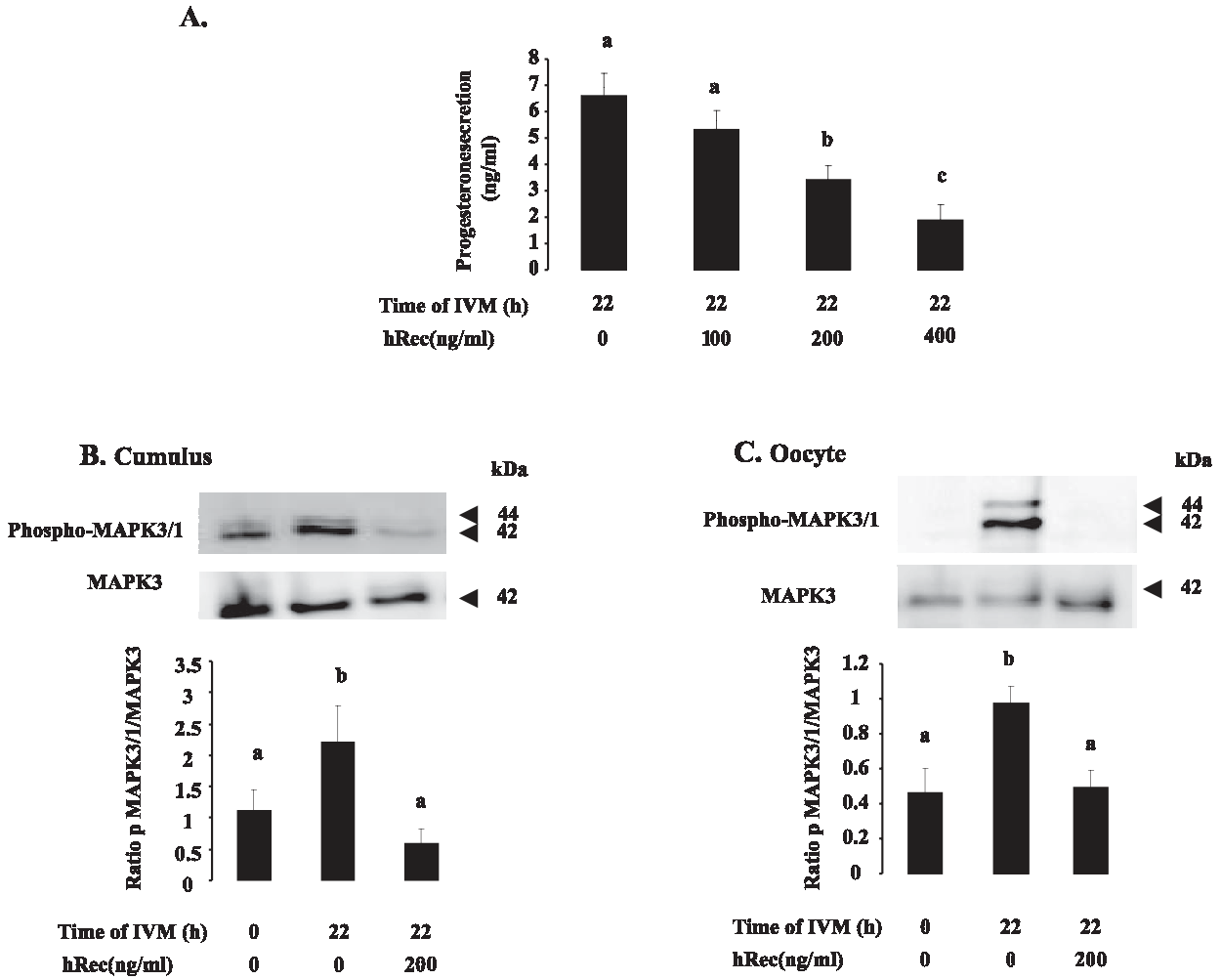

FIG. 10. Effects of CHEMERIN treatment on the level of phosphorylation of MAPK3/1 in bovine cumulus cells and oocyte from COCs and on the progesterone levels in culture medium after IVM. **A**) Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of various doses of CHEMERIN (100, 200, and 400 ng/ml). The culture medium was then collected, and its progesterone content was analyzed by RIA as described in *Materials and Methods*. The results are expressed as ng/ml of 50 COC-equivalent cumulus cells. The results are mean ± SEM for three independent experiments. Different letters indicate significant differences with $P < 0.05$. **B** and **C**) Bovine COCs were cultured for 22 h in maturation medium in the presence or absence of CHEMERIN (200 ng/ml). COCs were then mechanically separated into oocyte and cumulus cells. Denuded oocytes (50 oocytes per lane, **B**) and cumulus cells (**C**) were lysed and subjected to Western blot analysis with antibodies against phospho-MAPK3/1 and MAPK3. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein to total protein ratio is shown. Different letters indicate significant differences with $P < 0.05$. The results are presented as mean ± SEM.

CHEMERIN [44]. Thus, these data suggest a tissue and species dependence of the metformin effect on CHEMERIN expression. We also studied the effect of various adipokines (RESISTIN, ADIPONECTIN, LEPTIN, and TNFα) on *rarres2* and *cmklr1* mRNA expression in bovine GCs. Interestingly, we observed that TNFα and ADIPONECTIN significantly increased *rarres2* and *cmklr1* expression, respectively. Such stimulatory effects on *rarres2* and *cmklr1* expression or CHEMERIN plasma levels have been already described in other cell types. For example, at least two studies showed that TNF-α treatment increased bioactive CHEMERIN levels in adipocytes [42, 45], and TNFα is a proinflammatory cytokine associated with insulin resistance and inflammation that is known to inhibit steroidogenesis in bovine GCs [46]. In our study, we showed that CHEMERIN strongly inhibited steroid production. Thus, CHEMERIN could be involved in the TNFalpha effect in bovine GCs. In good agreement with our results, Wanninger et al. [47] showed that ADIPONECTIN upregulates *cmklr1* mRNA and protein level in primary human hepatocytes. Thus, our results show that *rarres2* and *cmklr1* expression can be regulated by other adipokines in bovine GCs.

In the present study, we investigated the effect of hRec on bovine GC steroidogenesis. As expected, we observed that IGF1 and FSH alone increase progesterone and estradiol secretion by bovine GCs [27]. We showed that hRec significantly inhibited steroid production in the basal state and in response to IGF1 or FSH and that this effect was abolished by using a blocking CMKLR1 Ab. Our results are in good agreement with those described by Wang et al. [19] that reported that mouse recombinant CHEMERIN decreases FSH-induced steroidogenesis in rat GCs [19]. Furthermore, in

previous work in primary human GCs and in the KGN GC line, we showed that hRec impairs steroid production in response to IGF1 but not FSH, suggesting a species effect of CHEMERIN [14]. During ovarian steroid hormone synthesis, cholesterol is first transported into the mitochondrial inner membrane, facilitated by STAR, and then converted to the important sex steroid progesterone under sequential actions of the mitochondrial enzyme CYP11A1 and endoplasmic reticulum enzyme HSD3B. Progesterone is then further enzymatically processed into estrogens through the action of various enzymes, including CYP19A1. In the present study, hRec decreased the protein levels of STAR and CYP19A1 induced by IGF1 or FSH. These latter results could explain the decrease in progesterone and estradiol secretion. CHEMERIN is known to modulate various signaling pathways, including MAPK3/1 and MAPK14 in myoblast and gastric cells [48, 49] and AKT1 and PRKA (AMPK) in muscle [50]. In bovine GCs, we showed that CHEMERIN stimulation in the short-term (1–30 min) or long-term (48 h) decreased MAPK3/1 phosphorylation, whereas no significant effect was observed for MAPK14, AKT1, and PRKA phosphorylation. Some studies reported that the MAPK3/1 signaling is a positive regulator in IGF1- or FSH-induced steroid production in cultured rat, human, and bovine GCs [27, 35, 51]. In primary human GCs and in rat Leydig cells, CHEMERIN treatment inhibited phosphorylation of MAPK3/1 pathways in response to IGF1 or hCG, respectively [14, 15]. Thus, MAPK3/1 is probably a molecular event involved in the inhibitory effect of CHEMERIN on bovine GC steroidogenesis.

Steroidogenesis depends on the supply of its precursor, cholesterol, derived from intracellular and extracellular sources. Even if lipoproteins are the major source of cholesterol for steroidogenic cells [52], cellular cholesterol can be derived from the de novo synthesis pathway in GCs. Indeed, stimulation of cholesterol biosynthesis plays a fundamental role in FSH action in pig GCs [53]. In bovine GCs, stimulation of HMGCR, a key enzyme controlling de novo cholesterol synthesis, is important in progesterone production and cell proliferation in response to IGF1 [54]. In our study, hRec decreased the cholesterol content and the HMGCR protein levels. Taken together, our results suggest that CHEMERIN inhibits steroidogenesis not only through a decrease in the amount of the cholesterol carrier STAR and CYP19A1 but also through a reduction in cholesterol de novo synthesis in bovine GCs. The inhibitory effects of CHEMERIN on bovine GC steroid production, cholesterol content, and MAPK3/1 phosphorylation in response to IGF1 and FSH were abolished by using a CMKLR1 Ab, which suggests that there is a cross-talk between CHEMERIN/CMKLR1-signaling axis and IGF1R and FSH-R in modulating GC function. Further investigations are needed to better understand this potential cross-talk. Moreover, it also appears important to inhibit the two other CHEMERIN receptors, GPR1 and CCRL2, in order to determine their role in the GC functions. In our study, we did not observe any effect of CHEMERIN on cell proliferation and viability in primary bovine GCs, whereas Kim et al. [17] observed an apoptotic effect of chemerin on GC in follicle culture of dihydrotestosterone-treated rats and showed that CHEMERIN suppressed FSH and GDF9 stimulated follicular growth [17]. The dose of CHEMERIN used in both studies was quite similar, so we can suggest that the effect of CHEMERIN on cell proliferation and viability depend on the species and also the culture conditions.

In the present study, we showed that hRec induced an arrest at the GV of the bovine oocyte after 22 h of IVM. We also observed that the addition of hRec to the bovine COCs' maturation medium strongly reduced progesterone secretion and MAPK3/1 phosphorylation in both oocyte and cumulus cells. Several studies have shown that progesterone stimulates oocyte maturation [36, 37]. Thus, CHEMERIN could block bovine nuclear oocyte maturation through an inhibition of progesterone production by COCs. Furthermore, it is well known that MAPK3/1 activation in oocyte occurs during the first hours of maturation in many species, including cattle. In bovine oocytes, it is associated with the GVBD stage [55]. Thus, the decrease in CHEMERIN-induced MAPK3/1 phosphorylation observed in bovine oocyte and cumulus cells could help to explain the inhibitory effect of CHEMERIN on oocyte nuclear maturation. It will be interesting to determine the involvement of cumulus cells in the CHEMERIN effects during IVM. For this, we are investigating the effects of CHEMERIN on oocyte nuclear maturation when the oocytes are separated from their cumulus cells.

In conclusion, we have demonstrated for the first time the presence of CHEMERIN and its three receptors CMKLR1, GRP1, and CCRL2 in the bovine ovary. In primary bovine GCs, CHEMERIN decreased steroidogenesis and cholesterol synthesis in the basal state and in response to IGF1 or FSH through at least CMKLR1. This was associated with a reduction in protein level of STAR, CYP19A1, and HMGCR and phosphorylation of MAPK3/1. Furthermore, we showed that the addition of hRec to bovine COCs' maturation medium arrested most of the oocytes at the GV stage and decreased MAPK3/1 phosphorylation in oocytes and cumulus cells. Further investigations are required to determine the in vivo effects of CHEMERIN in bovine folliculogenesis.

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La visfatine est exprimée dans l'ovaire bovin : est-elle impliquée dans la stéroïdogenèse des cellules de la granulosa ? (article en préparation)

Nous avons également commencé à étudier l'effet de la visfatine au niveau des fonctions ovariennes bovines de la même manière que pour la chemerine (**Article 5**). Nous avons tout d'abord identifié la visfatine dans les différents tissus bovins puis plus particulièrement au niveau des cellules ovariennes. Nous avons aussi déterminé la concentration de visfatine dans le plasma et le liquide folliculaire encore inconnue chez le bovin. Puis comme précédemment nous avons étudié la régulation de l'expression de la visfatine et observé les effets de la rh visfatine sur la sécrétion de stéroïdes et l'activation des voies de signalisation au niveau des cellules primaires de la granulosa bovine.

Les différents points qui seront présentés sont :

A) Dosage de la concentration plasmatique et dans le liquide folliculaire de la visfatine chez la vache

B) Expression de la visfatine dans les tissus chez la vache

C) Régulation de l'expression de la visfatine dans les cellules de la granulosa bovine

D) Effet de la rh visfatine sur la sécrétion de P4 et E2 par les cellules primaires de la granulosa bovine

E) Implication de la voie de signalisation SIRT1 dans l'effet de la visfatine sur les cellules primaires de la granulosa bovine

Caractérisation des SIRT dans l'ovaire bovin

Implication de SIRT1 dans la stéroïdogenèse induite par la visfatine

F) Effet de la rh visfatine sur la phosphorylation de la voie MAPK-ERK1/2

A) Détermination de la concentration dans le plasma et le liquide folliculaire de la visfatine chez la vache

A l'aide d'un ELISA commercial spécifique bovine nous avons mesuré la concentration plasmatique de visfatine à différents stades de la lactation. La concentration plasmatique de visfatine d'environ 150 ng/ml est stable avant et après gestation, seulement à 6 mois de gestation nous constatons une nette augmentation de la concentration en visfatine (**Fig. 31A**). Egalement, nous avons prélevé du liquide folliculaire provenant de petits, moyens et gros follicules issus d'ovaires d'abattoir, nous n'observons aucune différence de concentration en visfatine selon la taille des follicules (**Fig. 31B**).

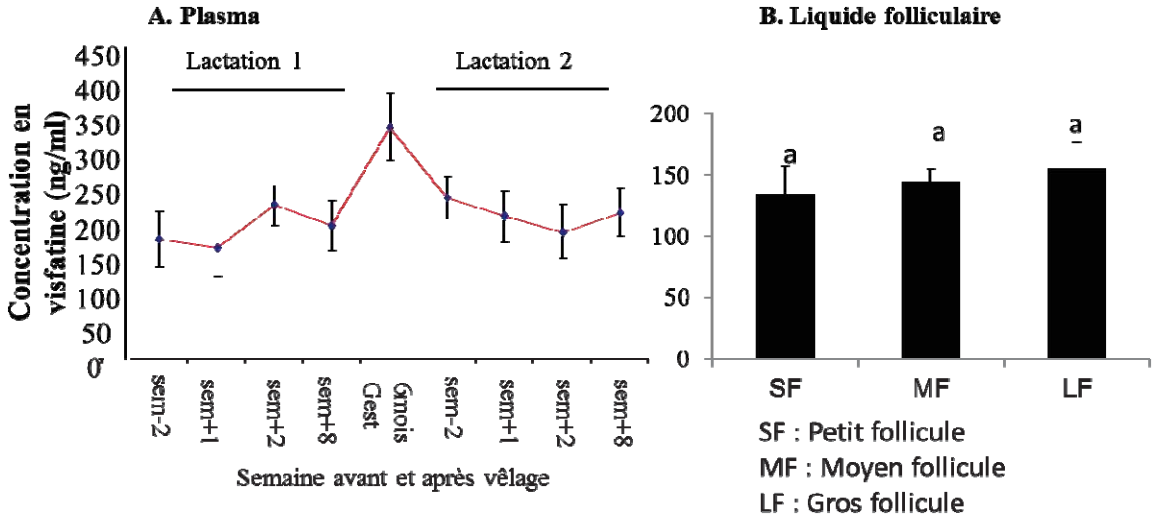

Figure 31: Concentration de la visfatine dans le plasma et le liquide folliculaire chez la vache.

B) Expression de la visfatine dans les tissus chez la vache

Après extraction des ARN totaux provenant de différents tissus bovins, nous avons observé par RT-PCR (**Fig 32A**) et western blot (données non présentées) la présence de la visfatine dans l'hypothalamus, l'hypophyse, et dans les tissus ovariens (le corps jaune, les petits et les gros follicules), la glande mammaire et le tissu adipeux, ce dernier tissu étant utilisé comme témoin positif. Plus particulièrement, au sein de l'ovaire nous avons retrouvé la visfatine dans les cellules de la granulosa provenant à la fois de petits (diamètre < 5mm) mais aussi de gros follicules (diamètre > 7mm) (**Fig 32A**) et également dans l'ovocyte et les cellules de cumulus provenant de complexe cumulo-ovocytaires (COCs) matures et

immatures (**Fig. 32A**). Par immunohistochimie nous avons confirmé les résultats précédents. Nous observons un fort marquage de la visfatine dans le follicule et plus précisément au niveau des cellules de la granulosa, du cumulus et de l'ovocyte et plus faiblement au niveau des cellules de la thèque (**Fig. 32B**). Il reste à déterminer l'activité de la visfatine dans les différentes cellules ovariennes bovines.

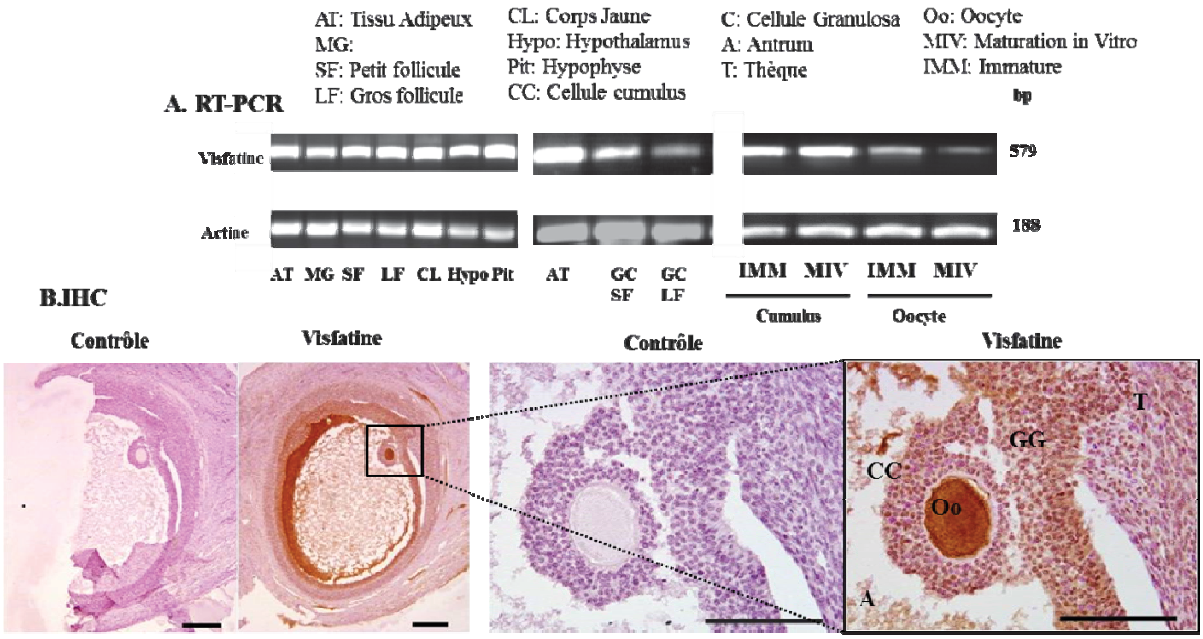

Figure 32: Expression de la visfatine dans les tissus de vache.

(A) Expression de la visfatine dans les tissus de vache par RT-PCR et (B) localisation de la visfatine par immunohistochimie (IHC) dans le follicule ovarien de vache.

C) Régulation de l'expression de la visfatine dans les cellules de la granulosa bovine

Après une nuit de sevrage, des cellules primaires de la granulosa bovine issues de petits follicules ont été stimulées pendant 24 ou 48 h avec de l'insuline (10^{-8}M) ou de l'IGF-1 (10^{-8}M). Nous observons un effet opposé suivant le temps de stimulation. Après 24h de stimulation avec l'insuline ou l'IGF-1 l'expression de la visfatine est diminuée alors qu'après 48h de stimulation son expression est significativement augmentée par l'IGF-1 (**Fig.33**).

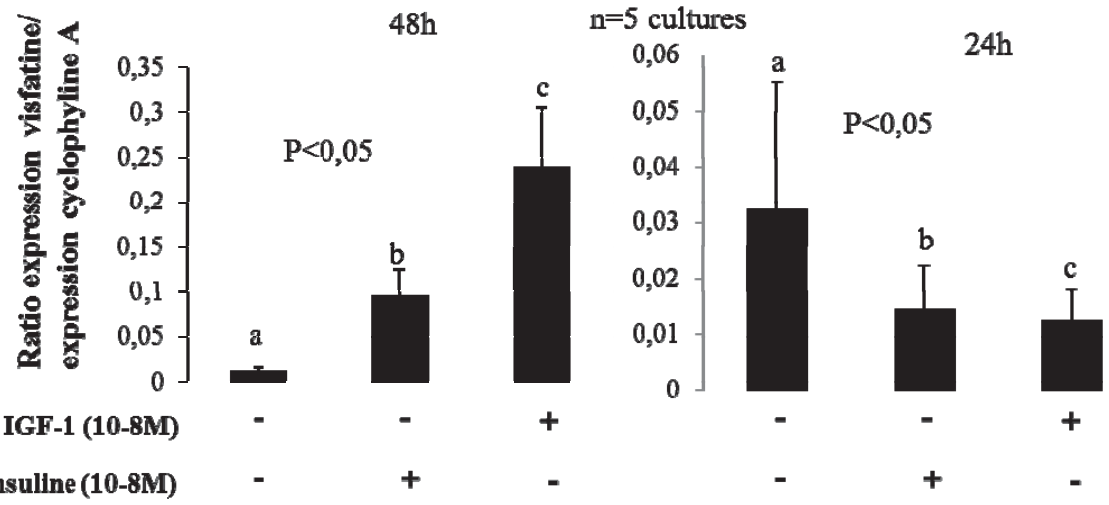

Figure 33: Régulation de l'expression de la visfatine en réponse à l'IGF-1 et l'insuline dans les cellules primaires de la granulosa de vache.

D) Effet de la rh visfatine sur la sécrétion de P4 et E2 dans des cultures primaires de cellules de la granulosa bovine

Après une nuit de sevrage, des cellules primaires de la granulosa bovine ont été stimulées pendant 48 h soit avec de la rh visfatine à différentes doses (0 ; 0,1 ; 1 ; 5 ; 10 ; 100 ng/ml) soit avec de la rh visfatine (10 ng/ml) supplémentée ou non avec de l'IGF-1 (10^{-8}M) ou de la FSH (10^{-8}M). Nous observons que la rh visfatine augmente la sécrétion de P4 et d'E2 à la dose de 10 et 100 ng/ml (**Fig 34A et 35A**).

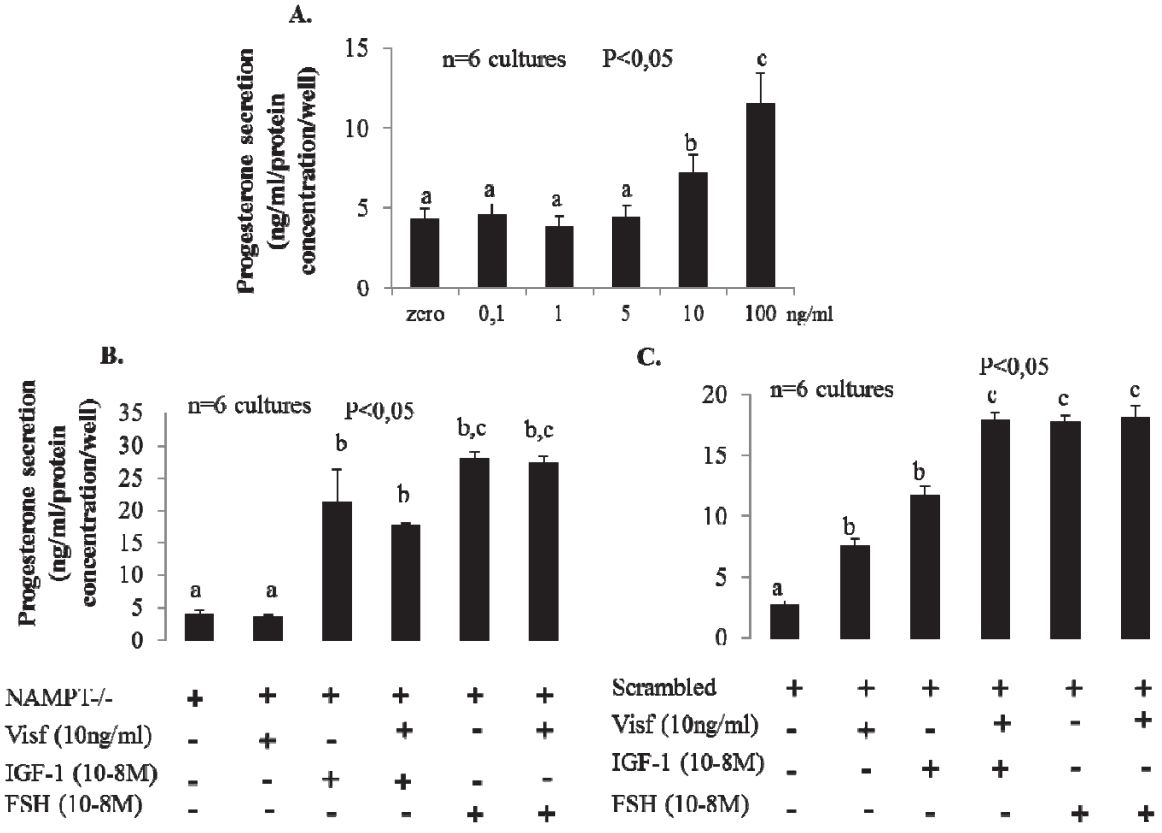

Figure 34: Effet de la rh visfatine sur la sécrétion de P4 par les cellules primaires de la granulosa bovine.

A. Effet dose de la visfatine. B. Effet de la rh visfatine en présence ou en absence d'IGF-1 ou de FSH dans des cellules infectées avec un lentivirus exprimant un shRNA dirigé contre la visfatine (NAMPT-/-) ou contre aucune protéine (Scrambled).

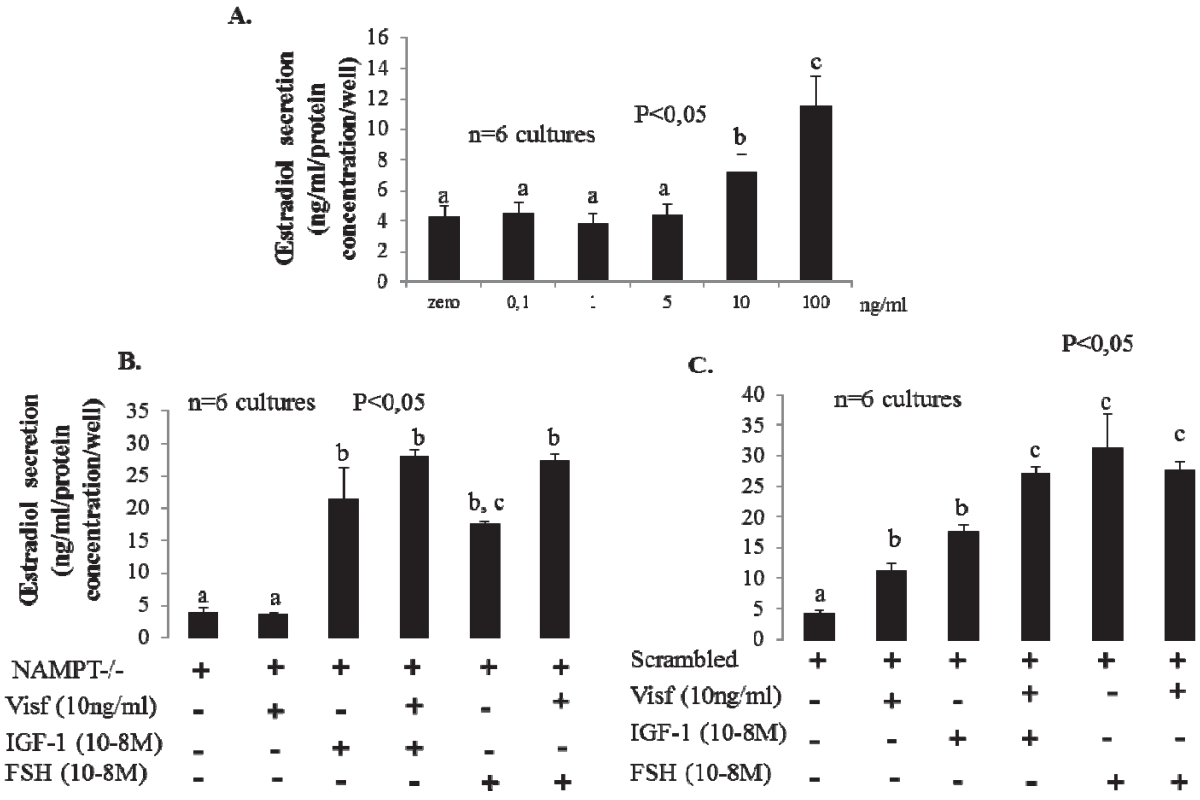

Figure 35: Effet de la rh visfatine sur la sécrétion d'E2 par les cellules primaires de la granulosa bovine.

A. Effet dose de la visfatine. B. Effet de la rh visfatine en présence ou en absence d'IGF-1 ou de FSH dans des cellules infectées avec un lentivirus exprimant un shRNA dirigé contre la visfatine (NAMPT-/-) ou contre aucune protéine (Scrambled).

A la vue de ces résultats nous avons choisi d'utiliser la dose de 10 ng/ml pour la suite des expérimentations. Nous avons confirmé l'action de la rh visfatine sur la sécrétion basale de P4 et E2, de plus, nous notons que la rh visfatine augmente significativement la sécrétion induite par l'IGF-1. Nous avons constaté que la rh visfatine n'a pas d'effet significatif sur la sécrétion de stéroïdes induite par la FSH. Par la suite nous avons confirmé ces résultats en utilisant deux lentivirus un témoin (Scrambled) et un autre inhibant spécifiquement la visfatine (NAMPT-/-) dans les cellules de la granulosa bovine. Nous avons tout d'abord testé si le lentivirus infectait bien l'ensemble des cellules en culture, comme nous pouvons le voir (**Fig 36),** le lentivirus infecte 90% des cellules.

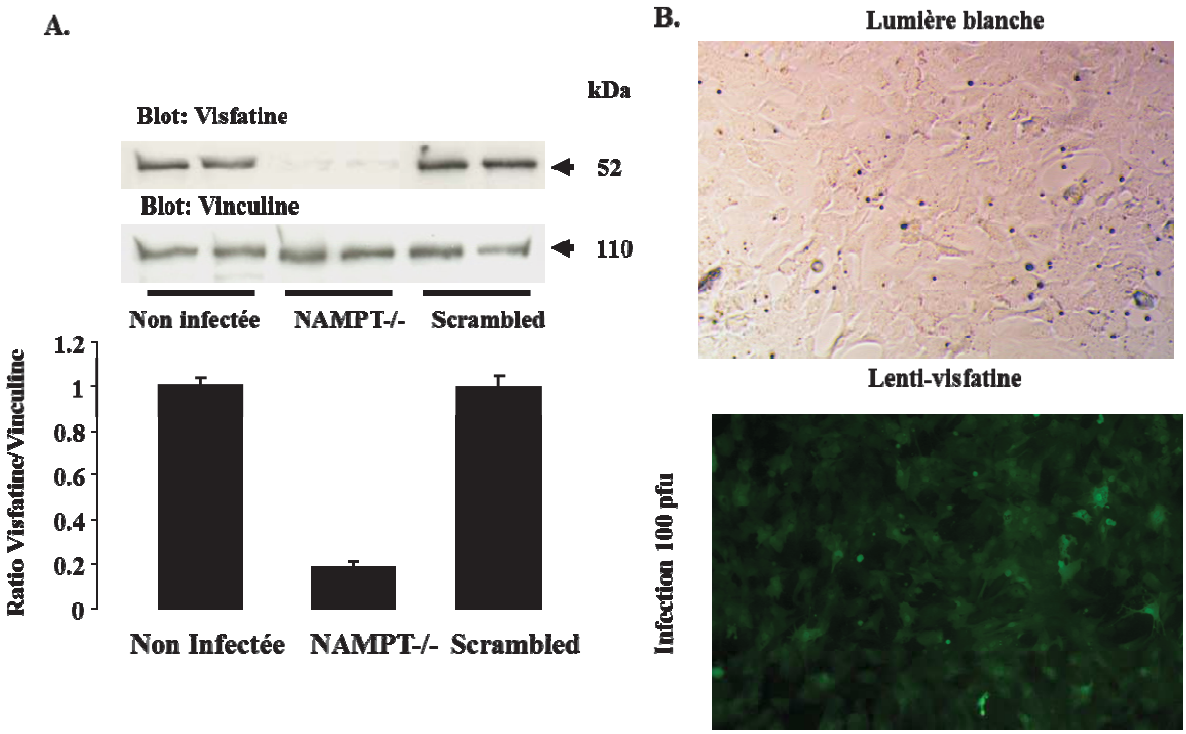

Figure 36: Infection des cellules primaires de la granulosa bovine par les lentivirus NAMPT-/- et scrambled.

A. Expression de la visfatine déterminée par western-blot dans les cellules primaires de la granulosa bovine infectée ou non par le lentivirus NAMPT-/- ou Scrambled.

De plus, nous avons constaté que le lentivirus témoin n'affecte pas l'expression de la visfatine (**Fig 36),** ni la sécrétion de stéroïdes (**Fig 34C et 35C**). Nous avons vérifié par western blot l'effet des lentivirus utilisés ; nous observons que le scrambled (témoin) n'altère pas le niveau protéique de visfatine alors que le NAMPT-/- inhibe très fortement son niveau d'expression (**Fig 36A**). Nous observons que le lentivirus NAMPT-/- inhibe l'effet potentialisant de la visfatine sur la sécrétion de P4 et E2 basale et induite par IGF-1(**Fig 34B et 35B**). Le lentivirus NAMPT-/- n'affecte pas la sécrétion de stéroïdes induite par la FSH. De plus, après un test au bleu trypan nous avons observé que la visfatine n'a pas d'effet sur la viabilité cellulaire. Egalement, l'effet de la rh visfatine sur la prolifération cellulaire basale et induite par IGF-1 ou FSH a été étudié ; aucun effet significatif de la visfatine n'a été observé. Ainsi, l'effet de la visfatine sur la sécrétion de stéroïdes n'est pas dû à un effet sur le nombre de cellules ou sur leur viabilité. La visfatine augmente la sécrétion de stéroïdes dans les cellules de la granulosa bovine.

E) Implication de la voie de signalisation SIRT dans l'effet de la visfatine sur les cellules primaires de la granulosa bovine

Caractérisation de SIRT1 dans l'ovaire bovin

Nous avons tout d'abord caractérisé les sirtuines (SIRTs) dans les différents tissus ovariens bovins. Par PCR en temps réel, nous observons que la SIRT1 est plus fortement exprimée dans les cellules de la granulosa provenant de petits follicules que dans les autres types cellulaires ovariens (corps jaune, gros et petits follicules et cortex) (**Fig 37A**). Nous avons confirmé ce résultat par western blot, en effet le niveau de protéine de SIRT1 est plus élevé dans les cellules de la granulosa provenant de petits follicules que de grands follicules ou du cortex (**Fig 37B, données quantifiées non représentées**). Egalement, nous avons localisé la présence de la SIRT1 par immunohistochimie, nous constatons que la SIRT1 est présente dans le liquide folliculaire, l'ovocyte, les cellules du cumulus, les cellules de la granulosa et aussi dans les cellules de la thèque bovine (**Fig 37C**). Ayant observé la présence de la SIRT1 dans l'ovocyte nous avons étudié son expression en fonction du stade de maturation. Par PCR en temps réel, nous avons remarqué que l'expression de la SIRT1 par rapport à un ovocyte immature est significativement diminuée dans les ovocytes matures *in vitro* alors qu'elle est significativement plus élevée dans les blastocystes (**Fig 37D**). Il reste maintenant à déterminer l'activité des sirtuines dans les différentes cellules ovariennes.

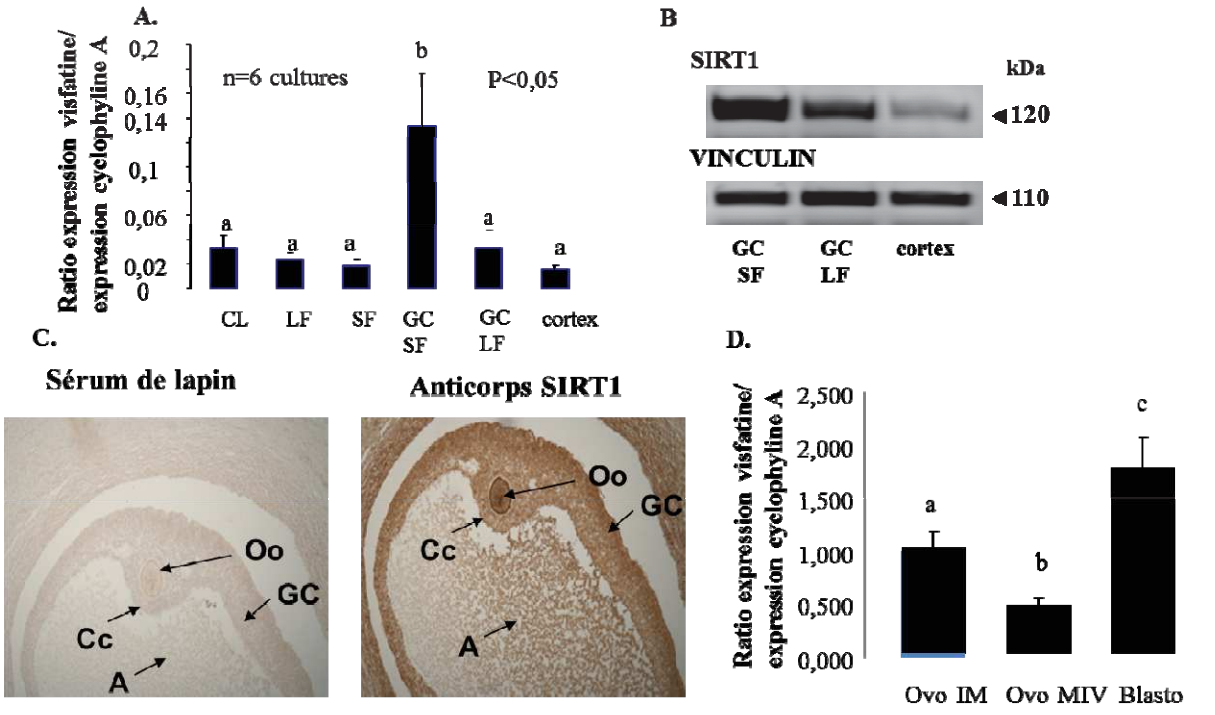

Figure 37: Caractérisation de SIRT1 dans l'ovaire bovin.

A. Expression par RT-PCR quantitative de la visfatine dans les compartiments et cellules ovariennes (CL : Corps jaune, LF : gros follicules, SF : petits follicules, GC SF : cellules de la granulosa de petits follicules, GC LF : cellules de la granulosa de gros follicules). B. Expression de la protéine SIRT1 par western-blot. C. Expression par RT-PCR quantitative du messager de la visfatine dans les ovocytes immatures (Ovo IM) ou maturés *in vitro* (Ovo MIV) et les blastocystes.

Implication de SIRT1 dans la stéroïdogenèse induite par la visfatine

Comme nous avons vu précédemment, la visfatine augmente la sécrétion de stéroïdes. Il a été raporté dans la littérature que la visfatine pouvait activer SIRT1, ainsi nous avons étudié si l'effet bénéfique de la visfatine sur la sécrétion de P4 et E2 implique la voie de signalisation des SIRT1. Pour cela, nous avons reproduit les mêmes conditions d'expérimentation que pour l'analyse sur la sécrétion de stéroïdes (D) en utilisant un inhibiteur spécifique de SIRT1, le CHIC 35 (1 μM). Lorsque nous ajoutons du CHIC 35 dans le milieu de culture, nous abolissons totalement l'effet de la rh visfatine sur la sécrétion de P4 et E2 basale et induite par IGF-1 (10^{-8}M) (**Fig 38B**). L'inhibiteur de SIRT1 (CHIC 35) ne présente pas d'effet significatif sur la sécrétion de stéroïdes induite par FSH (10-8 M) (**Fig 38B**). Nous avons également testé l'effet d'un activateur de la voie de signalisation de SIRT1 (le resvératrol) sur la sécrétion de P4. Comme décrit ci-dessous, les cellules primaires de la

granulosa bovine ont été stimulées pendant 48 h avec le resvératrol (10 µM) (activateur des SIRT1) supplémenté ou non avec IGF-1 (10^{-8}M). Nous retrouvons l'effet stimulateur de l'IGF-1 sur la sécrétion de P4 mais de plus nous observons que le resvératrol augmente la sécrétion de P4 seule et en présence d'IGF-1 (**Fig 38C**). Ainsi, la visfatine augmente la sécrétion de stéroïdes des cellules de la granulosa bovine et cet effet semble impliquer la voie de signalisation SIRT1.

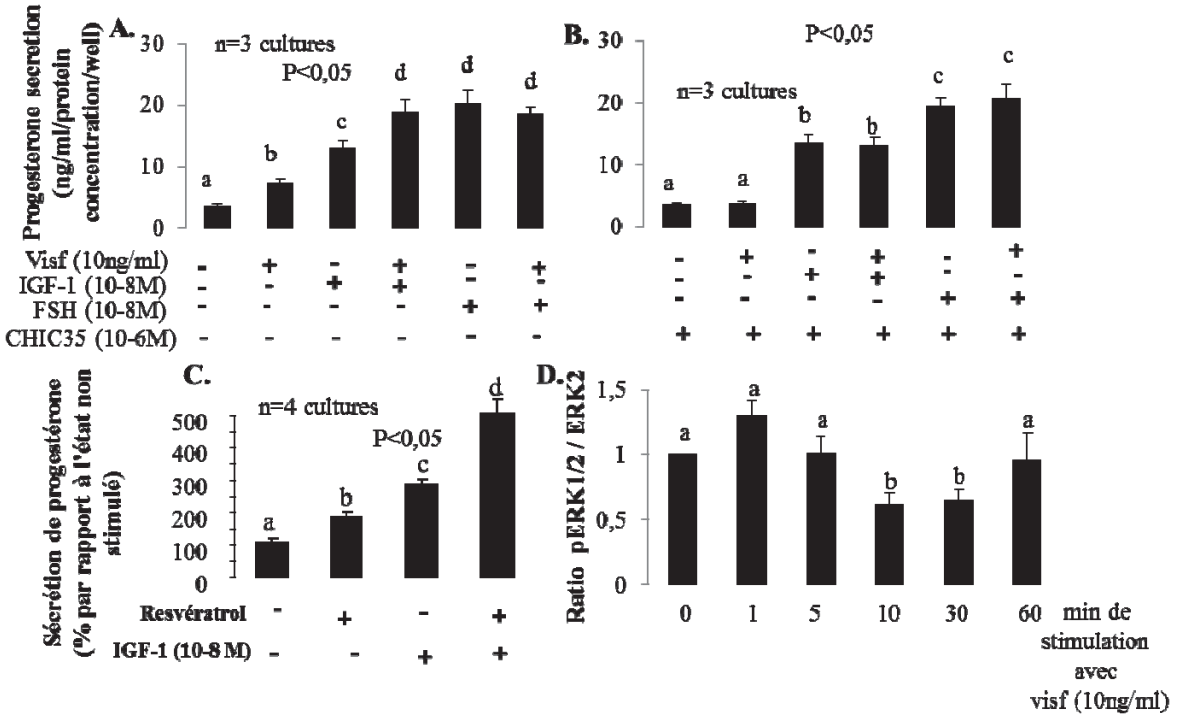

Figure 38: Implication de SIRT1 dans la stéroïdogenèse induite par la visfatine dans les cellules primaires de la granulosa bovine.

A et B. Effet de l'inhibiteur de SIRT1, CHIC 35 (1 µM) sur la production de P4 en absence ou en présence de visfatine ± IGF-1 ou FSH dans les cellules primaires de la granulosa bovine. C. Effet d'un activateur de SIRT1, le resvératrol sur la sécrétion de progésterone en absence ou en présence d'IGF-1. D. Effet d'une stimulation courte (1 à 60 min) de rh visfatine sur la phosphorylation des MAPK ERK1/2 dans les cellules primaires de la granulosa bovine.

F) Effet de la rh visfatine sur la phosphorylation de la voie MAPK-ERK1/2

Nous avons aussi étudié les voies de signalisation activées par la rh visfatine dans les cellules primaires de la granulosa bovine. Nous avons étudié si la rh visfatine active les voies de signalisation MAPK-ERK1/2 et P38 ; Akt et AMPK. Les cellules ont été stimulées avec la rh visfatine à 10 ng/ml durant des cinétiques de 60 minutes. Seul un effet de la rh visfatine a été relevé sur la voie de signalisation MAPK-ERK1/2. La visfatine inhibe la phosphorylation de la voie MAPK-ERK ½ à partir de 10 minutes jusqu' à 30 minutes avant un retour à l'état basal (**Fig 38D**).

Discussion-Perspectives

Discussion-Perspectives

Résumé des résultats

Au cours de cette thèse nous avons recherché la présence et étudié l'effet de trois adipocytokines, chemerine, visfatine et résistine sur la fonction de reproduction femelle, et plus précisément sur les fonctions ovariennes chez la femme et la vache.

Ces trois adipocytokines, chemerine, et son récepteur CMKLR1, la visfatine et la résistine sont présentes *in vivo* au niveau de leur messager et protéine dans l'ovaire humain et bovin, plus particulièrement dans les follicules (cellules de la granulosa, cellules de la thèque et complexe cumulo-ovocytaire) et le corps jaune chez la vache. De plus, la visfatine et la résistine ont été localisées dans l'ovocyte humain et la visfatine et la chemerine ainsi que ces trois récepteurs CMKLR1, CCRL2 et GPR1 dans l'ovocyte bovin. **Chez la femme**, les insulino-sensibilisateurs (metformine et rosiglitazone) régulent l'expression de la visfatine dans les cellules de la granulosa au niveau messager et seulement pour la metformine au niveau protéique. Cette régulation par la metformine s'exerce *via* la voie de signalisation AMPK/SIRT1. Chez la vache, l'expression du gène de la chemerine est régulée positivement par l'insuline, l'IGF-1, la metformine et la rosiglitazone alors que l'expression de ces trois récepteurs CMKLR1, GPR1 et CCRL2 est diminuée en réponse à ces différents agents. De plus, le TNF-α augmente l'expression de la chemerine et l'adiponectine celle de CMKLR1. L'expression du gène de la visfatine (PBEF1) n'est pas significativement régulée par les précédents acteurs.

Chez la femme, ces trois adipocytokines modulent *in vitro* la sécrétion de stéroïdes par les cellules de la granulosa. La chemerine et la résistine diminuent la production de stéroïdes en réponse à l'IGF-1 alors que la visfatine l'augmente. Pour induire de tels effets, la chemerine et la résistine affectent des enzymes clés de la stéroïdogenèse comme la P450 aromatase et la P450scc. De plus, la chemerine, la visfatine et la résistine diminuent l'activation de la voie de signalisation MAPK-ERK1/2 connue pour être impliquée dans la sécrétion de stéroïdes. D'autres voies de signalisation sont aussi activées telles que les voies Akt et MAPK-P38 et la voie AMPK seulement par la chemerine. Egalement, la chemerine et la résistine diminuent la phosphorylation de la sous unité beta du récepteur IGF-1 induite par

IGF-1. L'effet des adipocytokines sur la prolifération des cellules de la granulosa est variable, en effet la chemerine diminue la prolifération cellulaire induite par IGF-1 alors que la visfatine l'augmente et la résistine n'a aucun effet. **Chez la vache**, la chemerine et la visfatine modulent aussi *in vitro* la stéroïdogenèse des cellules de la granulosa. La chemerine diminue la sécrétion de P4 et E2 à l'état basal et en réponse à l'IGF-1 ou la FSH et la visfatine l'augmente en absence ou en présence de l'IGF-1. Dans ce modèle, la chemerine affecte la sécrétion de stéroïdes en diminuant la teneur en cholestérol, l'abondance de la protéine de transport du cholestérol StAR, les protéines P450 aromatase et HMGCR et le niveau de phosphorylation de la voie de signalisation MAPK-ERK1/2 en présence ou en absence d'IGF-1 et FSH. Tous les effets observés de la chemerine impliquent le récepteur CMKLR1. La visfatine quant à elle induit ces effets probablement *via* la voie de signalisation SIRT1. Ces deux adipocytokines n'affectent dans nos conditions ni la viabilité ni la prolifération des cellules de la granulosa bovine. Dans les complexes cumulo-ovocytaires bovin la chemerine bloque la majorité des ovocytes au stade GV *in vitro* et ce résultat est associé avec une diminution de la sécrétion de P4 par les cellules du cumulus et de la phosphorylation de la voie de signalisation MAPK-ERK1/2 dans l'ovocyte et les cellules du cumulus.

Chez la vache laitière un dosage des trois adipocytokines a été réalisé par un ELISA commercial spécifique bovin dans le plasma et le liquide folliculaire. Le test n'a pas été concluant pour la chemerine mais pour la visfatine sa concentration autour de 150 ng/ml semble similaire dans le plasma et le liquide folliculaire. Pour la résistine sa concentration plasmatique augmente autour de la parturition entre 40 ng/ ml et 90 ng/ ml. Sa concentration plasmatique est faible avant vêlage puis augmente jusqu'à atteindre un pic une semaine après vêlage avant de diminuer progressivement pour atteindre la concentration d'avant vêlage six semaines après la parturition. La concentration plasmatique de résistine est corrélée positivement avec le niveau plasmatique d'AGNE et négativement avec la production laitière, la matière sèche ingérée et la balance énergétique entre les semaines 1 et 22 après parturition. De plus, dans le tissu adipeux les niveaux messager et protéique de résistine sont plus élevés une semaine après parturition qu'à 5 mois de gestation. Enfin, la résistine augmente la libération de glycérol et le niveau d'ARNm de ATGL et HSL dans des explants de tissu adipeux suggérant un rôle de la résistine dans la lipolyse du tissu adipeux chez le bovin.

Ces résultats nous amènent à poser plusieurs questions :

Les actions des adipocytokines sur les fonctions ovariennes sont-elles autocrines, paracrines ou endocrines?

Comme décrit précédemment, nous avons montré la présence au niveau messager et protéique (en utilisant différentes techniques, RT-PCR, Western Blot, Immunohistochimie) des adipocytokines, chemerine, visfatine et résistine dans l'ovaire humain ainsi que la présence de la chemerine et de la visfatine dans l'ovaire bovin. Plusieurs travaux sont en accord avec nos résultats des trois premiers articles, la présence de la chemerine a déjà été montré dans l'ovaire humain par Northern Blot (Nagpal et al., 1997) ainsi que l'expression de la résistine dans les cellules ovariennes bovines et même dans l'ovocyte (Maillard et al., 2011; Spicer et al., 2011). Chez la rate, la résistine n'est pas retrouvée dans les cellules de la granulosa. Egalement, l'expression de l'adiponectine varie suivant les différents types cellulaires ovariennes et en fonction de l'espèce. En effet, l'adiponectine est quasi-indétectable dans les cellules de la granulosa sauf chez la vache. **Ces données suggèrent que les adipocytokines sont exprimées de manière spécifique suivant le type cellulaire étudié et l'espèce.** De plus, nous avons observé que les adipocytokines influencent certaines fonctions ovariennes (Articles 1, 2 et 3). La question qui se pose maintenant est : **l'action des adipocytokines au niveau ovarien est-elle due à une production locale et par conséquent à un effet autocrine/paracrine ou à un effet endocrine (production du tissu adipeux ou autres tissus périphériques).**

Une action locale autocrine/paracrine ?

Nous avons réalisé des dosages à la fois dans le plasma et le liquide folliculaire de la chemerine et de la résistine chez la femme et de la visfatine chez la vache laitière. Chez la femme nous avons observé que la concentration de chemerine est plus forte dans le liquide folliculaire que dans le plasma un jour avant la ponction ovocytaire. Nous obtenons des résultats opposés pour la résistine. La chemerine a déjà été dosée dans le fluide d'ascites d'ovaires de patientes atteintes de cancer ovariens où sa concentration est élevée (1,8-7 nM)

(Wittamer et al., 2003). Dans notre équipe, Chabrolle et collaborateurs ont montré que l'adiponectine présente également une plus forte concentration dans le liquide folliculaire que dans le plasma (Chabrolle et al., 2009). Etant donné que la concentration de ces deux adipocytokines (chemerine, adiponectine) apparaît plus importante dans le liquide folliculaire que dans le plasma, cela suggère que les cellules ovariennes pourraient sécréter ces adipocytokines. Cependant, il faut rester prudent avec ces résultats car le kit ELISA utilisé lors de notre expérimentation dose à la fois la chemerine active et inactive ainsi, la concentration de chemerine mesurée pourrait ne pas correspondre à la quantité réelle de chemerine active. **Nous pouvons toutefois émettre l'hypothèse que les adipocytokines exprimées par les cellules ovariennes pourraient être secrétées et avoir une action locale.** Les résultats obtenus chez la poule vont dans ce sens. En effet, l'adiponectine est fortement produite par les cellules de la thèque et très peu par les cellules de la granulosa. En revanche, les cellules de la granulosa expriment deux à trois fois plus les récepteurs AdipoR1 et AdipoR2 que les cellules de la thèque (Chabrolle et al., 2007). De plus, la production d'ARNm d'adiponectine par les cellules de la thèque augmente au cours de la maturation folliculaire. Ainsi, l'adiponectine produite par la thèque pourrait exercer un effet paracrine sur les cellules de la granulosa de poule.

Pour la résistine, nos données chez la femme sont en accord avec une précédente étude où la concentration de résistine apparait plus faible dans le liquide folliculaire que dans la circulation. Nous avons également réalisé le dosage de la visfatine dans le plasma et le liquide folliculaire de vache avec un kit ELISA commercial spécifique bovin. Même si l'analyse du plasma et du liquide folliculaire n'a pas été réalisée sur la même vache (le plasma est issu des animaux de notre troupeau alors le liquide folliculaire analysé provient d'ovaires prélevés sur des vaches d'abattoir), la concentration de visfatine apparaît similaire dans les deux compartiments analysés (environ 150 ng/ml). Des résultats semblables ont été observés chez l'humain. En effet, Shen et al ne trouvent pas de différence de concentration de visfatine entre le plasma et le liquide folliculaire (Shen et al., 2010). Ces données nous laissent penser que la résistine et la visfatine ne sont peut-être pas ou peu sécrétées par les cellules ovariennes**.**

Afin de vérifier si les différents types cellulaires ovariens sécrètent ou non les adipocytokines, il est envisagé au laboratoire de réaliser des cultures primaires de cellules de la granulosa ou de la thèque ou de complexe cumulo-ovocytaires et de doser dans le milieu de culture la concentration des adipocytokines (adiponectine, chemerine, visfatine résistine…). D'autre part, l'invalidation spécifique dans les cellules ovariennes

de ces adipocytokines ou de leurs récepteurs, si connu, apparaît indispensable pour déterminer si ces molécules produites au niveau ovarien ont un rôle important dans la reproduction chez la femelle. A notre connaissance aucune souris qui n'exprime plus la chemerine, la visfatine ou la résistine dans l'ovocyte ou les cellules de la granulosa n'a été développée.

Une action endocrine ?

L'irrigation sanguine de chaque ovaire s'effectue par une artère ovarienne qui naît de l'aorte. La adipocytokines sont produites par le tissu adipeux mais peuvent l'être aussi par d'autres tissus périphériques. Ainsi, il est tout à fait possible que les adipocytokines agissent sur les cellules ovariennes par voie endocrine. Dans ce cas là, elles pourraient contribuer à expliquer certaines infertilités liées à un dysfontionnement métabolique comme par exemple dans le cas de l'obésité ou du syndrome des ovaires polykystiques (SOPK). En effet, plusieurs travaux montrent une variation de concentration plasmatique de la chemerine, de la visfatine et de la résistine dans le cas de ces pathologies.

Quels pourraient être les effets des adipocytokines in vivo au niveau de l'ovaire et du tractus génital

Au cours de cette thèse, nous avons observé que les adipocytokines chemerine, visfatine et résistine sont capables d'affecter *in vitro* les sécrétions de stéroïdes par les cellules de la granulosa ainsi que leur prolifération. Cependant, ces adipocytokines pourraient agir *in vivo* sur d'autres fonctions au niveau de l'ovaire et du tractus génital.

L'apoptose des cellules ovariennes

Dans les conditions de nos études nous n'avons pas observé d'effet *in vitro* de nos trois adipocytokines (chemerine, visfatine et résistine) au niveau de l'apoptose des cellules de la granulosa humaine et bovine. Pour la chemerine, nos résultats sont opposés à ceux observés dans les cellules de la granulosa de rate par Kim et al.,. En effet ces derniers auteurs ont montré que la chemerine induit *in vivo* l'apoptose des cellules de la granulosa et l'arrêt de la croissance folliculaire chez la rate en inhibant la protéine inhibitrice de l'apoptose liée au chromosome X appelée XIAP (Kim et al., 2013). Ainsi, il est possible que la chemerine ait

des fonctions différentes à la fois *in vitro* et *in vivo*. Cependant, plusieurs travaux ont relaté un effet des adipocytokines sur l'apoptose des cellules ovariennes. Par exemple, des souris déficientes en leptine (souris ob/ob) présentent une augmentation du nombre de follicules atrétiques et de l'activité apoptotique des cellules de la granulosa qui se traduit par une hausse de l'expression de marqueurs apoptotiques comme FAS et FasL dans ces cellules (Hamm et al., 2004). De plus, dans les ovaires de poule, la leptine inhibe l'expression de plusieurs agents pro-apoptotiques (Bax, ASK-1 et p53) et augmente l'expression de Bcl-2 un agent anti-apoptotique. De plus, la leptine inhibe l'expression de PCNA (un marqueur de la phase S de la mitose) (Sirotkin & Grossmann, 2007). Chez la rate, la leptine inhibe *in vivo* l'apoptose des cellules ovariennes (Almog et al., 2001). Ainsi, la leptine pourrait contrôler la folliculogenèse en régulant l'équilibre prolifération/apoptose des cellules ovariennes (Sirotkin et al., 2008). L'adiponectine *via* son récepteur AdipoR1 présente aussi des effets anti-apoptotiques dans les cellules de la granulosa humaine (Pierre et al., 2009). En revanche, dans certaines conditions pathologiques comme la pré-éclampsie, il a été montré que l'adiponectine peut induire l'apoptose *via* AdipoR1 (Jarvenpaa et al., 2009). Toutes ces données indiquent que les adipocytokines peuvent agir sur la mort cellulaire.

La maturation ovocytaire et développement embryonnaire

Nous avons observé dans nos études que l'addition de rh chemerine dans le milieu de culture des complexes cumulo-ovocytaires bovine bloque la plupart des ovocytes au stade de vésicule germinale. De plus ceci est associé à une diminution de la phosphorylation de la voie de signalisation MAPK ERK1/2 dans l'ovocyte et les cellules du cumulus (Article 5). Ainsi, la chemerine pourrait être un inhibiteur de la maturation méiotique de l'ovocyte. **Il reste à déterminer si les effets de la chemerine observés nécessitent les cellules du cumulus et si cet effet est spécifique à l'espèce bovine ou si ils sont également retrouvés dans d'autres espèces**. D'autre part, il serait intéressant d'étudier plus en détails les mécanismes moléculaires de la chemerine impliqués dans cette inhibition de la maturation méiotique. En particulier nous avons analysé seulement la voie MAPK ERK1/2 mais il serait bon aussi de regarder la voie AMPK. En effet il a été montré dans le laboratoire que cette kinase serait un marqueur de la maturation ovocytaire.

Plusieurs études ont montré que d'autres adipocytokines sont impliquées dans la maturation ovocytaire. Chez la souris et l'humain lors de procédure de fécondation *in vitro*

l'adiponectine améliore la maturation ovocytaire et le développement embryonnaire précoce (Richards et al., 2012). Chez la truie, l'adiponectine améliore la maturation ovocytaire *via* la voie de signalisation MAPK-P38 ainsi que le développement embryonnaire précoce *in vitro* (Chappaz et al., 2008). De plus, l'ajout de leptine (1 ou 10 ng/ml) dans des complexes cumulo-ovocytaires bovins augmente la proportion d'ovocytes atteignant le stade de blastocyste. La leptine augmente le niveau d'ARNm de STAT3 (transducteur de signal et activateur de la transcription), de XIAP (baculoviral inhibitor of apoptosis protein repeat-containing 4 ou BIRC4) et du récepteur de la leptine dans les ovocytes bovins et réduit le niveau d'ARNm de Bax dans les blastocystes bovins. Ainsi, la leptine augmente les chances de l'ovocyte à parvenir au stade de blastocyste en réduisant la mort cellulaire (Boelhauve et al., 2005). Chez les souris âgées, une injection de visfatine au moment de l'ovulation améliore la qualité ovocytaire et le développement embryonnaire (Choi et al., 2012)**. Ainsi, les adipocytokines sont non seulement capables d'affecter la stéroïdogenèse et la prolifération/apoptose des cellules de la granulosa mais aussi la maturation ovocytaire et le développement embryonnaire. Les mécanismes moléculaires impliqués restent cependant à déterminer.**

La lutéogenèse et la lutéinisation

Nous avons mis en évidence la présence des adipocytokines (chemerine et visfatine) dans le corps jaune chez la vache. Le corps jaune joue un rôle central dans la régulation du cycle oestrien et le maintien de la gestation. Les cellules lutéales ont pour fonction principale de produire de la P4 afin d'assurer une éventuelle nidation. Nous avons observé au cours de ma thèse que les adipocytokines (chemerine, visfatine et résistine) régulent la stéroïdogenèse. Il serait donc utile de suivre l'évolution de l'expression des adipocytokines et de leurs récepteurs au cours de la lutéogenèse et en particulier au cours de la lutéinisation des cellules de la granulosa. Pour fonctionner le corps jaune a besoin d'un apport constant de cholestérol. Ce cholestérol provient d'une synthèse *de novo*, par l'hydrolyse des stocks d'ester de cholestérol ou par les lipoprotéines exogènes, LDL et HDL (lipoprotéines de haute densité, source majeure). Au cours de notre travail nous avons montré que la chemerine est impliquée dans la régulation de la synthèse de cholestérol. Il serait donc très intéressant d'étudier le rôle de la chemerine dans la lutéogenèse et la lutéinisation.

Un rôle au niveau de l'utérus et de l'endomètre

Afin de comprendre certaines infertilités, il serait intéressant de rechercher la présence et d'étudier le rôle de la chemerine, visfatine et résistine au niveau du tractus génital femelle comme par exemple au niveau de l'utérus ou de l'endomètre. L'adiponectine et la leptine ainsi que leurs récepteurs respectifs ont été détectés dans l'utérus et plus précisément au niveau de l'endomètre (Dos Santos et al., 2012). Une forte expression des gènes codant pour AdipoR1 et AdipoR2 a été observée chez la femme durant le milieu de la phase sécrétoire du cycle menstruel. (Čikoš et al., 2010). De plus, récemment une étude indique que dans l'endomètre de femme suivie dans le cadre d'une FIV, l'expression d'AdipoR1 et AdipoR2 est diminuée de 60% en cas d'échec d'implantation comparé à l'expression dans l'endomètre de femme fertile (Dos Santos et al., 2012). Ainsi, les trois adipocytokines étudiées au cours de cette thèse pourraient aussi affecter la réceptivité utérine et par conséquent affecter le développement embryonnaire lors de la période pré-implantatoire.

Quelles sont les interactions entre les adipocytokines dans l'ovaire ?

La plupart des études sur les adipocytokines ont été réalisées *in vitro* en testant l'effet d'une adipocytokine à la fois dans des conditions bien définies. Seulement à l'échelle de l'organisme les adipocytokines interagissent toutes entre elles et avec un grand nombre d'autres acteurs. Il est nécessaire de prendre en considération ces potentielles interactions afin de mieux comprendre les mécanismes d'action des adipocytokines sur les gonades. De précédentes études ont déjà montré des interactions entre les adipocytokines. Dans la lignée cellulaire BeWo (modèle de cellule du cytotrophoblaste humain) la sécrétion de visfatine est augmentée après 48 h d'incubation avec le TNF-α de manière dose-dépendante (Ma et al., 2010). Chez des rats soumis à une restriction alimentaire suivie d'une réalimentation normale, l'expression de la chemerine dans le tissu adipeux est significativement augmentée probablement due à l'insuline (Stelmanska et al., 2013). Récemment dans le laboratoire, nous avons montré *in vitro* que l'intelectine1 (ITLN1) (une adipocytokine intervenant dans l'insulino-résistance, l'inflammation et la réponse immunitaire) augmente de manière dose-dépendante l'expression de la visfatine dans les cellules primaires de la granulosa humaine

(Cloix et al., 2014). Or nous avons montré que la visfatine est nécessaire pour transmettre l'effet bénéfique de l'ITLN1 sur la sécrétion de stéroïdes induites par IGF-1. De plus, ITLN1 augmente le niveau protéique de StAR et CYP19A1 induit par IGF-1 *via* l'expression de la visfatine, ce qui pourrait expliquer l'augmentation de la sécrétion de P4 et d'E2 en réponse à l'IGF-1 dans les cellules de la granulosa humaine. Cette étude montre également que l'ITLN1 *via* l'induction de l'expression de la visfatine augmente la phosphorylation de la voie de signalisation MAPK ERK1/2 induite par l'IGF-1. Ainsi, nous montrons que l'effet de l'ITLN1 sur la signalisation d'IGF-1R est dépendant de l'expression de la visfatine dans les cellules de la granulosa humaine. Ici au cours de cette thèse nous avons constaté que les différentes adipocytokines peuvent avoir des effets opposés sur une même fonction au sein du même type cellulaire. Par exemple, chez la femme, dans les cellules de la granulosa, la résistine et la chemerine diminuent la sécrétion de stéroïdes induite par l'IGF-1 (articles 1 et 3) alors que l'adiponectine et la visfatine l'augmentent (article 2 et Chabrolle et al., 2009). Qu'en est-il lorsque différentes adipocytokines sont combinées ?

Les adipocytokines sont-elles des senseurs énergétiques pour l'ovaire?

Il a déjà été montré dans de précédentes études et dans cette discussion que la concentration sérique et le niveau d'expression des adipocytokines dans le tissu adipeux varient fréquemment en fonction de la balance énergétique de l'individu et donc de l'état des réserves (= quantité de tissu adipeux). Les liens entre la nutrition, l'obésité, l'insulino-résistance et le diabète de type 2 avec les adipocytokines sont étroits. Les adipocytokines en fonction de leur expression ovarienne et/ou de leur concentration plasmatique pourraient transmettre des informations avertissant les cellules cibles reproductrices de la possibilité ou non de mener à terme une gestation. Il serait intéressant de déterminer l'expression des adipocytokines et de leurs récepteurs au niveau de l'ovaire dans différentes situations nutritionnelles (à jeûn, régimes enrichis en lipides…) et de la comparer par rapport à celles des tissus périphériques. D'autre part, il a été montré que les adipocytokines (chemerine, visfatine, résistine…) peuvent exercer leur action *via* la voie de signalisation AMPK qui est reconnue comme un senseur énergétique. L'AMPK est exprimée dans les cellules ovariennes et des variations de son activation sont associées à des modifications de la stéroïdogenèse et de la maturation ovocytaire. Ainsi, les adipocytokines *via* l'AMPK ou d'autres voies de signalisation pourraient jouer un rôle majeur dans l'interface entre la reproduction et le métabolisme énergétique.

Quelle est l'implication des adipocytokines dans certaines pathologies ovariennes ou infertilités? Rôle des insulino-sensibilisateurs au niveau ovarien ?

Le SOPK

Les adipocytokines sécrétées par le tissu adipeux voient leur expression et leur concentration plasmatique modifiées en fonction de l'état nutritionnel. Elles sont impliquées dans les phénomènes d'obésités, d'insulino-résistance et de diabète de type 2. Comme décrit dans l'introduction, le syndrome des ovaires polykystiques (SOPK) est l'une des causes les plus fréquentes d'infertilité dans les pays développés. Chez ces patientes plusieurs dysfonctionnements du tissu adipeux ont été observés comme une surproduction d'adipocytokines pro-inflammatoires tel que le TNF-α. Ainsi, les adipocytokines pourraient être un lien entre l'obésité et la pathologie du SOPK. En effet, plusieurs études ont constaté que les niveaux sériques d'adipocytokines sont modifiés chez les patientes atteintes du SOPK en comparaison à des sujets témoins. Cependant plusieurs de ces études n'arrivent pas à la même conclusion et un lien direct entre les adipocytokines et la pathologie du SOPK n'a pas encore été pleinement établi (cf Tableau 7).

Adipocytokines	Concentration sérique chez les SOPK comparées aux contrôles	Références bibliographiques
Leptine	augmente	Brzechffa et al., 1996, Vicennati et al., 1998 ; El Orabi et al., 1999 ; Brannian et Hansen ; 2002 ; Pehlivanov et Mitkov ;2009 ; Yildizhan et al., 2011
	similaire	Chapman et al., 1997 ; Lauglhin et al., 1997 ; Mantzoros et al., 1997 ; Micic et al., 1997 ; Rouru et al., 1997 ; Gennarelli et al., 1998 ; Carmina et al., 2009 ; Svendsen et al., 2012
Adiponectine	diminue	Ardawi et Rouzi ., 2005 ; Escobar-Morreale et al., 2006 ; Pinhas-Hamiel et al., 2009 ; Manneras-Holm et al., 2011 ; Shin et al., 2011.
	similaire	Orio et al., 2003 ; Spranger et al., 2004 ; Lecke et al., 2011
Résistine	similaire	Panidis et al., 2004 ; Escobar-Morreale et al., 2006 ; Seow et al., 2007 ; Olszanecka-Glinianowicz et al., 2001 ; Zhang et al., 2011
	diminue	Munir et al., 2005
Visfatine	augmente	Tan et al., 2006 ; Chan et al., 2006 ; Kowalska et al., 2007 ; Panidis et al., 2008 ; Carmina et al., 2009 ; Ozkaya et al., 2010 ; Plati E et al., 2010 ; Seow et al., 2011 ; Dikmen E et al., 2011
	similaire	Guducu et al., 2012 ; Lajunen et al., 2012 ; Olszanecka-Glinianowicz et al., 2012
Chemerine	augmente	Tan et al., 2009 ; Guzel EC et al., 2014
Omentine	diminue	Tan et al., 2008 ; Choi et al., 2011
	similaire	Guzel EC et al., 2014

Tableau 7: Concentration sérique des adipocytokines chez des femmes avec le SOPK comparées à des patientes témoins

La chemerine est la seule adipocytokine pour laquelle les résultats ne sont pas contradictoires dans la littérature, sa concentration plasmatique est augmentée chez les femmes SOPK comparé aux témoins. De plus, son expression au niveau messager et protéique est augmentée dans le tissu adipeux. Chez des rats traités, par la 5 α-dihydrotestosterone (pour induire le SOPK), le niveau d'expression de la chemerine et de son récepteur CMKLR1 est significativement plus élevé dans l'ovaire au niveau messager et protéique (Wang et al., 2013).

Il s'avère que des molécules insulino-sensibilisatrices, utilisées dans le traitement du SOPK modifient l'expression d'adipocytokines dans le tissu adipeux mais également dans l'ovaire. Par exemple un traitement avec la metformine une molécule insulino-sensibilisatrice utilisée pour le traitement du diabète de type 2 diminue fortement la concentration sérique de leptine chez les patientes obèses ou non atteintes du SOPK (Marciniak et al., 2009; Morin-Papunen et al., 1998). D'autre part, une étude a montré que les niveaux de visfatine sont significativement réduits chez les patientes SOPK après 3 mois de traitement avec la metformine (Ozkaya et al., 2010). Plusieurs études montrent que la metformine pourrait agir directement au niveau des cellules ovariennes. Au cours de ma thèse, nous avons montré que la metformine régule l'expression des adipocytokines dans les cellules de la granulosa suggérant un effet direct de la metformine au niveau de l'ovaire.

Le diabète gestationel

Les adipocytokines sont aussi impliquées dans d'autres pathologies affectant la fertilité femelle. Par exemple, le diabète gestationnel est une complication de la gestation qui est caractérisée par une intolérance au glucose apparue au cours de la gestation chez une femme n'ayant jamais eu de diabète précédemment (Miehle et al, 2012). Il se développe quand la réserve des cellules β du pancréas n'est pas suffisante pour compenser la diminution de la sensibilité à l'insuline durant la gestation. Le rôle des adipocytokines dans cette pathologie est là encore loin d'être élucidé, plusieurs études sont contradictoires comme décrit dans le tableau ci-dessous (Tableau 8).

Adipocytokines	Concentration sérique chez les GDM comparées aux témoins	Références bibliographiques
Leptine	augmente	Ategbo JM et al., 2006 ;Chen D et al., 2010 ; Kauztky willer et al., 2001., Vitoratos N et al., 2001
	similaire	Simmons, D. & Breier, B.H. (2002)
	diminue	Festa A et al., 1999 ; MClachlan KA et al., 2006
Adiponectine	diminue	Altinova AE et al., 2007 ; Cortelazzi D et al., 2007 ; Kinalski M et al., 2005 ; Mazaki Tovi S et al., 2009 ; Ranheim T et al., 2004 ; Retnakaran R et al., 2004., Park S et al., 2013 ; Fereirra AF et al., 2011 ; Doruk M et al., 2014
Resistine	augmente	Kuzmicki M et al., 2009
	similaire	Karatas A et al., 2014 ; Lobo TF et al., 2013
Visfatine	augmente	Lewandowski KC et al., 2007 ; Kaygusuz I et al., 2013 ; Fereirra AF et al., 2011 ; Gok DE et al., 2011 ; Mazaki-Tovi S et al., 2009 ; Krzyzanowska K et al., 2006
	similaire	Karatas A et al., 2014 ; Telejko B et al., 2009
	diminue	Chan TF et al., 2006, Park S et al., 2013 ; Rezvan N et al., 2012 ; Akturk M et al., 2008 ;
Chemerine	diminue	Hare KJ et al., 2014
	similaire	Pfau D et al., 2010

Tableau 8: Concentration sérique des adipocytokines chez des femmes avec un diabète gestationnel (GDM) comparées à des patientes témoins

La pré-eclampsie (PE)

La pré-éclampsie est une complication de la gestation qui est caractérisée par un développement d'une hypertension associé à une protéinurie. Cette pathologie affecte 2-5% des gestations chez la femme et elle est un facteur majeur dans les causes de mortalité fœtale.

Elle se développe dans la deuxième moitié de la grossesse dès 20 semaines de gestation jusqu'à 6 semaines après l'accouchement (Miehle et al., 2012). Comme pour le diabète gestationnel, les concentrations plasmatiques des adipocytokines (leptine, adiponectine,

chemerine, résistine et visfatine) observées lors d'une pré-éclampsie sont très variables selon les études (cf tableau ci-dessous).

Adipocytokines	Concentration sérique chez les PE comparées aux témoins	Références bibliographiques
Leptine	augmente	Adali E et al., 2009 ; Haugen et al., 2006
	similaire	Martinez Abundis E et al., 2000
	diminue	Laml T et al., 2001
Adiponectine	augmente	Hendler I et al., 2005 ; Kajantie E et al., 2005 ;Naruse K et al., 2005 ; Ramsay JE et al., 2003 ; Fasshauer M et al., 2008 ; Takemura Y et al., 2007
	diminue	Cortelazzi D et al., 2007 ; D'anna R et al., 2006
Résistine	augmente	Haugen F et al., 2006
	similaire	Hendler I et al., 2005
	diminue	Chen D et al., 2005 ; Lappas M et al., 2005
Visfatine	augmente	Fasshauer M et al., 2008 ; Zorba E et al., 2012 ; Adali E et al., 2009 :
	similaire	Mazaki-Tovi S et al., 2010
	diminue	Hu W et al., 2008
Chemerine	augmente	Stepan H et al., 2011 ; Duan DM et al., 2011

Tableau 9: Concentration sérique des adipocytokines chez des femmes avec une pré-éclampsie (PE) comparées à des patientes témoins

Les adipocytokines et l'infertilité des vaches laitières hautes productrices

Les vaches en début de lactation comblent difficilement leurs besoins énergétiques de production et d'entretien par la ration. Il se développe alors chez ces animaux un bilan énergétique négatif avec une perte importante des réserves adipeuses. Cet état de déficit énergétique affecterait la reproduction principalement de deux façons: 1. En modifiant le mécanisme de contrôle des hormones liées à la reproduction (Axe hypothalamo-hypophysaire) et 2. En agissant directement sur le métabolisme de l'ovaire.

Lors d'un état de bilan énergétique négatif, la sécrétion pulsatile (par « pics») de l'hormone GnRH est diminuée ou inhibée, entraînant un état d'anoestrus (pas d'expression de chaleurs). En réponse à la GnRH, la sécrétion pulsatile de LH peut elle aussi être diminuée. Un niveau adéquat de sécrétion basale est possiblement présent. Cependant, l'amplitude du pic de sécrétion de LH étant positivement associée avec le bilan énergétique, le pic de sécrétion pré-ovulatoire peut être difficilement obtenu en présence d'un bilan énergétique négatif. Le follicule mature n'ovule alors pas. Cette situation pourrait favoriser la formation de kyste ovarien.

Au niveau ovarien, en réponse à la sécrétion de somatotropine (hormone de croissance, ou GH) et d'insuline, il y a normalement, sécrétion par l'ovaire de facteurs de croissance tel que l'IGF-1. Lors de carence en énergie, il se produit un découplage: la GH augmente mais l'IGF-1 diminue ce qui entraîne une mauvaise croissance des follicules primordiaux et, consécutivement, le développement de follicules matures et de corps jaunes moins fonctionnels. Ceci signifie qu'il y aura une moins grande production d'oestrogènes et donc une expression diminuée des chaleurs. La sécrétion de P4 sera elle aussi moins importante. Par conséquent, le taux de conception risque de diminuer puisque le succès à la première saillie est influencé positivement par le taux de P4 pré-ovulatoire.

Nous avons montré au cours de cette thèse que la résistine plasmatique chez la vache suit la concentration d'AGNE et par conséquent augmente fortement après vêlage. De plus nous avons observé que la résistine diminue la sécrétion des stéroïdes par les cellules humaines de la granulosa. Ainsi une forte augmentation de la résistinémie chez la vache après vêlage (qui pourrait être observé chez des vaches laitières hautes productrices qui mobilisent très fortement leurs réserves adipeuses) pourrait contribuer à expliquer la moins bonne fertilité chez ces animaux. Afin de déterminer si les adipocytokines sont de bons indicateurs de la fertilité, il serait intéressant de déterminer leur niveau plasmatique chez des vaches avec des fertilités différentes. Dans ce sens dans le laboratoire, des études de comparaison de profil plasmatique de résistine, d'adiponectine et de visfatine sont réalisées au cours de la lactation dans deux lots de vaches qui ont été sélectionnées à l'état homozygote pour soit un haplotype « favorable » ou « défavorable » pour un QTL de fertilité femelle situé sur le chromosome 3.

Qu'elle est l'influence des adipocytokines sur les fonctions centrales de la reproduction ?

Nous avons largement évoqué au cours de ce travail de thèse des actions des adipocytokines (chemerine, visfatine et résistine) sur les fonctions de la reproduction à un niveau ovarien. Dans ce tissu, les trois adipocytokines étudiées régulent la stéroïdogenèse des cellules de la granulosa et pour la chemerine chez la vache la maturation ovocytaire. En perspective de ce travail, nous avons testé l'hypothèse selon laquelle la chemerine pourrait également contrôler la fonction de reproduction à un niveau plus central par une action directe sur l'hypophyse responsable de la sécrétion de LH et de FSH, hormones contrôlant la fonction ovarienne.

Par RT-PCR et western blot, nous avons localisé la chemerine mais aussi ses trois récepteurs, CMKLR1, GPR1 et CCRL2 dans l'hypothalamus et l'hypophyse de vache et de souris (données non montrées). Il serait utile maintenant de déterminer par immunohistochimie dans quel type cellulaire sont exprimés les composants du système chemerine. Nous avons étudié l'effet de la rh chemerine (chemerine recombinante humaine) sur la sécrétion de LH par des cellules primaires hypophysaires de vaches. Après une nuit de sevrage les cellules ont été incubées pendant 24 h avec de la rh chemerine à 20 ou 200 ng/ml puis pendant 30 min ou 1 h avec ou sans GnRH (10^{-8}M). Nous observons que la rh chemerine diminue la sécrétion de LH à la fois à l'état basal et en réponse au GnRH à la dose de 20 et de 200 ng/ml (**Fig 39A**). Des résultats similaires ont été observés sur la lignée gonadotrope murine LbetaT2 (données non représentées). Nous avons également testé si la chemerine affecte la prolifération des cellules LbetaT2. Comme montré dans la figure 38B, la rh chemerine diminue de manière significative l'incorporation de thymidine tritiée des cellules LβT2 dès la dose de 12,5 ng/ml de rh chemerine (**Fig 39B**).

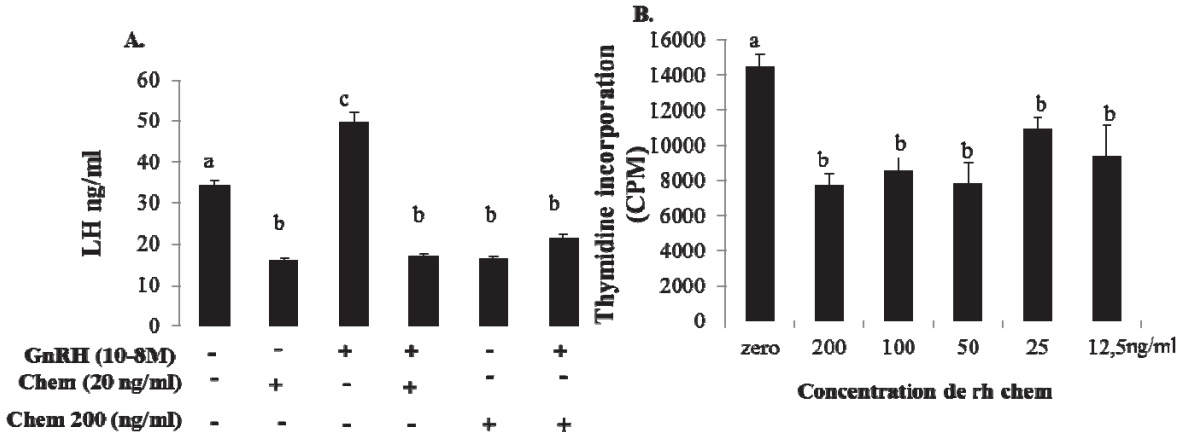

Figure 39: Effet de la chemerine recombinante humaine (rh Chem) sur la sécrétion de LH par les cellules primaires hypophysaires de vache et sur l'incorporation de thymidine tritiée des cellules LBetaT2. Des lettres différentes indiquent des différences significatives à $p<0,05$. Les résultats ont été obtenus à partir de 6 cultures différentes.

Les trois adipocytokines qui ont fait l'objet de cette thèse, chemerine, visfatine et résistine ont été peu étudiées au niveau central. La résistine a été identifiée au niveau de l'axe hypothalamo-hypophysaire (Morash et al., 2002), la visfatine a été retrouvée dans le fluide cérébro-spinal (Hallschmid et al., 2009) alors que la chemerine n'a pas encore été localisée. D'autres adipocytokines comme la leptine et l'adiponectine influencent la sécrétion de GnRH libérée par l'hypothalamus (Wen et al., 2008) et les sécrétions des gonadotrophines LH et FSH par l'hypophyse (Yu et al., 1997, Lu et al., 2008). De plus, il a été montré une libération pulsatile de leptine qui est synchrone à la fréquence des pulses de LH en milieu de phase folliculaire chez les patientes saines et en début de phase folliculaire chez des patientes avec le SOPK. Dans le laboratoire, nous avons effectué des prélèvements sanguins sériés toutes les dix minutes pendant six heures sur plusieurs vaches afin de déterminer l'existence ou non d'une pulsatilité de la résistine et de la visfatine plasmatique (adipocytokines pour lesquelles nous disposons un kit ELISA fonctionnel), et si oui comment elle se situe par rapport à celle de la LH. Nous souhaitons également déterminer les concentrations de ces adipocytokines au moment du pic pré-ovulatoire chez la vache.

Chez le rongeur (rate ou souris), il serait intéressant de réaliser des injections intracérébro-ventriculaires d'adipocytokines afin de connaître leurs effets sur la cyclicité ovarienne et la fertilité. Des travaux similaires ont déjà été réalisés dans le laboratoire avec des inhibiteurs et activateurs de l'AMPK. Au niveau hypothalamique, des études *in vitro* pourraient être menées sur la lignée neuronale hypothalamique murine GT1-7 afin de déterminer les effets des adipocytokines sur la sécrétion de GnRH. Toutes ces informations nous aideront à

comprendre le rôle des adipocytokines dans les fonctions de la reproduction au niveau de l'axe hypothalamo-hypophysaire.

Conclusion Générale

Au cours de cette thèse, nous avons montré la présence de la chemerine, visfatine et résistine au niveau des cellules ovariennes chez la femme et la vache. Chez ces deux espèces, ces trois adipocytokines régulent la sécrétion de P4 et d'E2 dans les cellules primaires de la granulosa. Chez le bovin, la chemerine recombinante humaine arrête le processus méiotique entamé par les complexes cumulo-ovocytaires au cours de la maturation *in vitro* puisque les ovocytes sont bloqués au stade vésicule germinale. Ces résultats montrent que ces trois adipocytokines pourraient avoir *in vivo* un rôle au niveau des fonctions ovariennes et par conséquent intervenir dans certaines infertilités associées à un dysfonctionnement métabolique. Par ailleurs, d'autres études sont à venir afin d'élucider le rôle des adipocytokines au niveau central et leur potentielle implication dans la régulation de la fonction de reproduction.

Autres articles et revues publiés au cours de la thèse

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Review

Nutritional signals and reproduction

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ABSTRACT

There is extensive evidence that nutrition influences reproductive function in various mammalian species (agricultural animals, rodents and human). However, the mechanisms underlying the relationship between nutrition, energy metabolism and reproductive function are poorly understood. This review considers nutrient sensors as a molecular link between food molecules and consequences for female and male fertility. It focuses on the roles and the molecular mechanisms of some of the relevant hormones, such as insulin and adipokines, and of energy substrates (glucose, fatty acids and amino acids), in the gonadotropic axis (central nervous system and gonads). A greater understanding of the interactions between nutrition and fertility is required for both better management of the physiological processes and the development of new molecules to prevent or cure metabolic diseases and their consequences for fertility.

Contents

1. Introduction

In humans and other mammals, nutrition and especially energy metabolism influence reproductive function. Indeed, when the energy needs are not covered, as in the case of under-nutrition, the onset of puberty is delayed, and there may be ovulation disorders,

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Table 1
Description of some nutrient effects (glucose, fatty acids, amino acids) on the hypothalamus–pituitary–gonadal axis.

	Hypothalamus GnRH	Pituitary LH/FSH
Glucose	↘ glucose = ↘ GnRH secretion (for review Wade et al., 1996)	↘ glucose = ↘ LH secretion (Wade et al., 1996) Positive correlation between circulating glucose levels and pulsatile LH release (Sheep, Ohkura et al., 2000) Glucose ↘ GnRH induced LH secretion (Pig, Barb et al., 1991)
Fatty acids	Peripheral injection of methyl palmoxyrate (an inhibitor of fatty acid oxidation): ↘ GnRH secretion and then ↘ LH secretion (rodent, for review Wade et al., 1996)	Central infusion of lipids ↗ LHβ expression (in vivo rat, Garrel et al., 2011) A. Oleic, A. Linoleic: ↗ LH secretion (in vitro, LβT2 mouse gonadotrop lineage, Garrel et al., 2011) Free fatty acids ↗ LHβ mRNA but suppress FSHβ mRNA in LβT2 cells (Sharma et al., 2013) Free fatty acids ↗ LH secretion and pulsatility (pig, Barb et al., 1991)
Amino acids	mTOR inactivation = ↘ Kiss1 expression (in vivo, rat, Roa et al., 2009)	Injection L leucine intracerebroventricular: ↑ LH secretion (in vivo, rat, Roa et al., 2009) mTOR inactivation: ↘ LH (in vivo, rat, Roa et al., 2009)

	Ovary		Testis		
	Granulosa/theca cells	Oocyte	Leydig cells	Sertoli cells	Germ cells
Glucose	High levels of glucose: ↘ steroidogenesis (Chabrolle et al., 2008)	↘ glucose: ↘ oocyte quality meiotic maturation (mice, Downs, 1995; cattle, Sutton-McDowall et al., 2010)	↗ glucose (diabetes in human): ↘ Testosterone production (Ballester et al., 2004)	↘ glucose: ↘ lactate (substrate of germs cells, in vitro rat Riera et al., 2009)	↗ glucose in STZ treated animals (rodents): alteration seminiferous tubules, alteration morphology spermatozoa (Seethalakshmi et al., 1987)
Fatty	acids	PUFA supplementation: ↗ steroid production (cow, Staples et al., 1998)	PUFA supplementation: ↗ oocyte quality (sheep, cattle, Zeron et al., 2002; Marei et al., 2009)	LXR disruption: ↘ testosterone (mice,Steffensen et al., 2006)	-LXRbeta-deficient mice:↗ lipid in Sertoli cells (mice,Robertson et al., 2005)
SHP	disruption: Germs cells enter meiosis earlier (mice, Volle et al., 2007a,b)				
	PPARγ inactivation in granulosa cells: ↘ ovulation (mice, Kim et al., 2008)		SHP disruption: ↗ testosterone (mice, Volle et al., 2007a,b)	Fatty acids ↘ phagocytosis of germ cells (in vitro, mice, Gillot et al., 2005)	PPARγ activator: ↗ mobility, capacitation, acrosome reaction (human, Aquila et al., 2006)
Amino	acids	↘ mTOR activation: ↘ proliferation (in vivo, mouse, Yu et al., 2011)	↘ mTOR activation: ↘ number of follicle ovulated (in vivo, mouse, Yu et al., 2011)	↘ mTOR activation: ↘ testosterone (human, for review Tartarin and Froment, 2013)	↘ mTOR activation: ↘ FSH-induced protein synthesis (rat in vitro for review Tartarin and Froment, 2013)
	↘ mTOR activation: ↘ sperm production (human, for review Tartarin and Froment, 2013)				

LBetaT2: murine gonadotrope cell line, PUFA: polyunsaturated fatty acids, STZ: Streptozocin mTOR: mammalian target of rapamycin, LXR: Liver X receptor, PPARγ: peroxisome proliferator-activated receptor γ SHP: small heterodimer partner, nd: not determined.

and an increased risk of embryonic mortality. Several studies report associations between individual metabolic disturbances and various indicators of male and female fertility (Palmer et al., 2012; Ramlau-Hansen et al., 2007; Wise et al., 2010). However, the mechanisms underlying the relationship between nutrition, energy metabolism and reproductive function are poorly understood.

The reproductive axis (hypothalamus, pituitary and gonad) appears to have a number of "nutrient sensing" mechanisms that may link nutrient status and fertility. This review examines the evidence for the presence in the gonadotropic axis of pathways that sense nutrient flux. In particular, we will consider hormones produced by the pancreas, such as insulin, or produced by adipose tissue, such as adipokines; we will also describe the involvement of glucose sensors (GLUT), amino-acid sensors (factors that activate mammalian target of rapamycin complex I (mTORC1)), fatty acid receptors (peroxisome proliferator activated receptors) and energy sensors (adenosine monophosphate activated kinase (AMPK)).

2. Glucose

Glucose is an important metabolic regulator of reproductive function and it is able to modulate the functioning of cells at different levels of the hypothalamus–pituitary gonadal axis (Table 1). Glucose deprivation decreases LH secretion in several species (Wade et al., 1996). It can be sensed by hypothalamic neurons (Ohkura et al., 2000). In the gonads, glucose is essential for the maintenance of spermatogenesis in vivo and the quality of oocytes (Rato et al., 2012; Sutton-McDowall et al., 2010). There is evidence in mice that glucose within the oocyte regulates meiotic maturation (Downs, 1995). It is also important for the development of the embryo. Hyperglycemia results in profound reproductive and developmental consequences. The isoforms of glucose transporters (GLUT), responsible for mediating passive glucose transport (GLUT1, 3 and 8) are expressed in the testis and in particular in Sertoli cells (Carosa et al., 2005; Galardo et al., 2008). GLUT1, GLUT3 and the insulin-sensitive glucose transporter GLUT4, are also expressed in the ovary in various species and their expression is regulated by intraovarian factors during follicular development, maturation and ovulation (Purcell and Moley, 2009). Zhang et al. showed that gonadotropin can up-regulate the GLUT content in rat ovary (Zhang et al., 2012). GLUTs in cells act as glucose sensors. Glucokinase is a hexokinase that phosphorylates glucose (Matschinsky, 1990), a step necessary for glucose metabolism (Fig. 1). Male and female germ cells do not possess all the enzymatic machinery necessary use glucose. Sertoli cells in males metabolize glucose, mostly to lactate that can be used by germ cells (Alves et al., 2013). Some reports indicate that in females, glucose is,

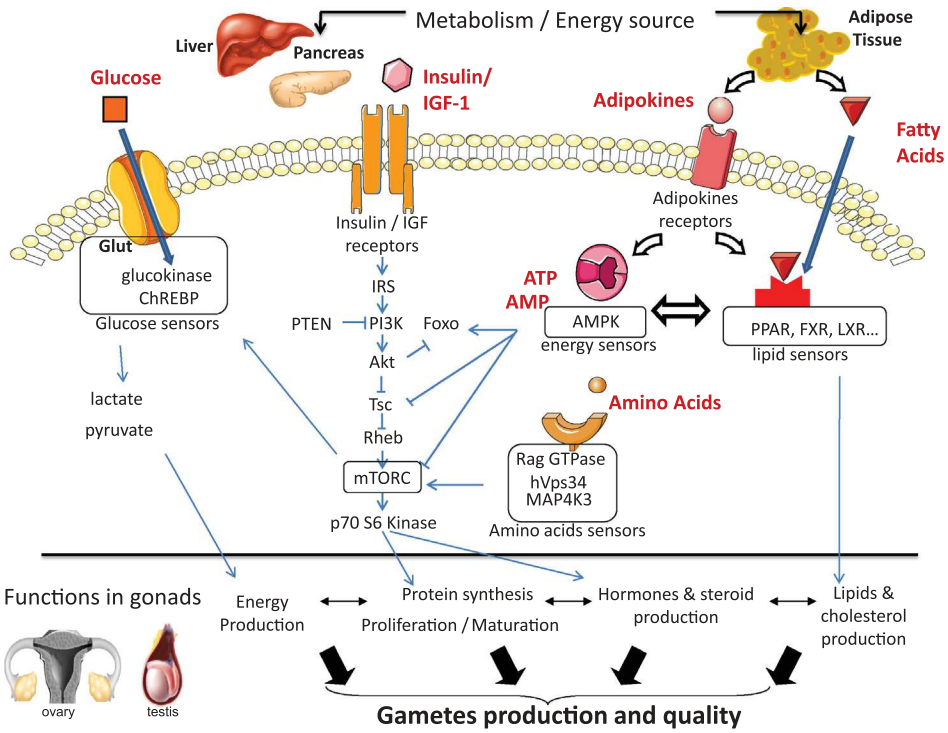

Fig. 1. Molecular interactions between metabolites and sensors controlling gamete production and quality. Insulin produced by beta cells of the pancreas and and IGF-1 produced by liver cells act on gonads through their corresponding tyrosine kinase receptors, called insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R). Upon activation, IR and IGF1R phosphorylate common substrates that activate a network of downstream effectors, including members of the phosphatidylinositol 3-kinase (PI3K)/Akt/P70S6K pathway. This pathway is associated with protein synthesis, proliferation, cell survival and differentiation of both somatic and germ cells, all important processes for gamete production and quality. P70S6K is phosphorylated by mTORC, which itself can be activated in distinct ways: in response to growth factors or to branched chain amino acids through intracellular amino-acid sensing mechanisms including the human vacuolar protein sorting-34 (hVps34), mitogen-activated protein kinase kinase kinase kinase-3 (MAP4K3), or the Rag subfamily of Ras small GTPases (Rag GTPase). Molecules produced by the adipose tissue, called adipokines, affect the function of somatic germ cells: upon binding to their receptors, adipokines activate the adenosine monophosphate-activated kinase, a nutrient and energy sensor that maintains energy homeostasis. AMPK regulates steroidogenesis in somatic cells; it negatively regulates the transcription of various lipid sensors, including peroxisome proliferator-activated receptors (PPARs), that play an important role in lipid and cholesterol production. Glucose is a key nutrient providing energy directly or indirectly through lactate or pyruvate production. Several glucose sensors including glucose transporters, glucokinase (that mediates phosphorylation of glucose to glucose-6-phosphate (G6P), the first step of both glycogen synthesis and glycolysis) and the carbohydrate response element-binding protein (ChREBP, a lipogenic glucose-sensing transcription factor) are present in reproductive cells. In red, metabolite and hormone signals. In boxes: sensors. IRS: insulin receptor substrate; PI3K: phosphatidylinositide 3-kinases; PTEN: phosphatase and tensin homolog; Foxo: forkhead box O; TSC: tuberous sclerosis complex; Rheb: Ras homolog enriched in brain; mTORC: mammalian target of rapamycin complex; P70S6K: ribosomal protein S6 kinase; ChREBP: carbohydrate-responsive-element-binding protein; PPAR: peroxisome proliferator-activated receptor; FXR: Farnesoid X receptor; LXR: Liver X receptor.

Table 2
Description of some metabolic hormone effects (insulin/IGF-1, adipokines (leptin, adiponectin and resistin) on the hypothalamus–pituitary–gonadal axis.

	Hypothalamus		Pituitary		
	GnRH		Kisspeptide(Kiss)		LH/FSH
Insulin/IGF-1	↗ in vitro GnRH secretion (GT1-7, Longo et al., 1998)		↗ Kiss expression, in vivo (rat, Hiney et al., 2010)		↗ in vitro LH and FSH release (primary culture in rodent, Adashi et al., 1981)
Leptin	↗ in vitro GnRH release (explants, Yu et al., 1997)		leptin deficiency: ↘ Kiss expression (in vivo mice, Quennell et al., 2011)		↗ in vitro LH secretion (primary culture in rodent, Ogura et al., 2001, in LβT2 Avelino-Cruz et al., 2009)
Adiponectin	↘ GnRH secretion in vitro (GT1-7 and rat primary cells) Cheng et al., 2011,		↘ Kiss expression in vitro (GT1-7) and in vivo (rat) Wen et al., 2012		↘ in vitro LH secretion (LβT2) and rat primary cells Rodriguez-Pacheco et al., 2007; Lu et al., 2008
Resistin	nd		nd		nd
	Ovary		Testis		
	Granulosa and theca cells (steroidogenesis)	Oocyte (in vitro maturation)	Leydig cells (testosterone Secretion)	Sertoli cells	Germ cells (proliferation)
Insulin/IGF-1	↗ in vitro (rodent, Davoren and Hsueh, 1984)	↗ (Xenopus, El-Etr et al., 1979)	↗ in vitro and in vivo (rodent, Baker et al., 1996; Froment et al., 2004)	↗ in vitroproliferation, glucose transport, lactate secretion (Oonk et al., 1989; Borland et al., 1984)	↗ in vivo (rat, Soder et al., 1992), spermatozoa mobility (rodent, Baker et al., 1996; Froment et al., 2004)
Leptin	↘ in vitro (primary culture, rodent, human, cattle, Ghizzoni et al., 2001; Spicer et al., 2000)	↗ (pig and cattle, Craig et al., 2004; Paula-lopes et al., 2007)	↘ in vitro (rodent, sheep, Herrid et al., 2008)	nd	↗ in vitroln vivo inactivation of leptin = aspermia (mice)
Adiponectin	↗ in vitro in granulosa (rodent, human, chicken) and ↘ in bovine theca cells Chabrolle et al., 2007, 2009; Lagaly et al., 2008	↗ (pig, mouse, human, Chappaz et al., 2008; Richards et al., 2012)	↘ in vitro (rat) Caminos et al., 2008; Pfaehler et al., 2012	nd	Inactivation of AdipoR2: aspermia (in vivo, mice) Bjursell et al., 2007
Resistin	In vitro, ↗ (rat) or ↘ (bovine and human) Maillard et al. (2011), Reverchon et al. (2013)	nd	↗ in vitro Nogueiras et al., 2004	nd	nd

GT1-7: murine neuronal hypothalamic cell line, LβT2: murine gonadotrope cell line, nd: non-determined.

transferred into the oocyte by cumulus cells via the GLUT system and then through gap junctions. It has been suggested that this intercellular pathway may partly mediate the effects of high-glucose availability on oocyte quality (Wang et al., 2012). Glucose is thus essential for reproductive functions as well as being a central nutrient in metabolism generally. Its status is sensed locally in the cells of the hypothalamo–pituitary–gonadal axis by a number of intracellular mechanisms which are probably inter-related. These include insulin signaling pathways.

3. Insulin signaling pathways

Glucose is made available in the body by insulin, which also helps to lower the level of circulating glucose by promoting its storage. Insulin and insulin-like growth factors (IGF-I and IGF-II) are hormones with a similar structure that exert their effects through activation of their own tyrosine kinase receptors, IR and IGF-IR, respectively. Both receptors phosphorylate various common substrates [Insulin Receptor Substrates (IRSs), Src Homology Collagen (Shc and others)] and activate similar signaling pathways including the Ras-Raf-MAPK and PI3K/Akt pathways (Dupont and LeRoith, 2001). IR and IGF-1R are expressed and active in the hypothalamus–pituitary–gonadal axis (Table 2).

Specific genetic invalidation of IR (Bruning et al., 2000) or various components of insulin pathways such as IRSs (Insulin Receptor Susbtrates) (Burks et al., 2000; Fantin et al., 2000; Griffeth et al., 2013; Tamemoto et al., 1994), PTEN (phosphatase and tensin homolog) (Reddy et al., 2008), and the transcription factor FOXO (forkhead box O winged helix) (Castrillon et al., 2003) in mice revealed the important role of this signaling pathway in control of fertility in both the central nervous system and in the gonads (Fig. 1). For example, over 90% of female mice invalidated for IRS-2 are sterile (Burks et al., 2000); their ovaries have a very small number of follicles and show an absence of corpora lutea; and their pituitaries are abnormally small and contain smaller than wild-type numbers of gonadotrope cells. Constitutive activation of the phosphatidylinositol 3-kinase (PI3K) pathway specifically in granulosa cells by conditional inactivation of PTEN led to a precocious menopause, due to the absence of negative feedback, stimulation of ovulation and accumulation of corpora lutea (Fan et al., 2008). Similar observations were recently reported in mice with knockout of Tsc1 (Tuberous sclerosis complex 1) in granulosa cells: they showed increased follicular growth associated with higher than control activity of mTORC1 a factor downstream from PI3K in the pathway and involved in amino-acid sensing (Huang et al., 2013). Tsc1 is a tumor suppressor that negatively regulates mTORC1.

In males, insulin and IGF-I ligands and receptors are expressed in somatic cells (Leydig, Sertoli and peritubular/myoid cells); insulin receptors are also found in germ cells in various species (Vannelli et al., 1988). Several investigations suggest that insulin or IGF-I regulates testosterone production by Leydig cells, which stimulate both the secretion activity of Sertoli cells and germ cell proliferation and survival (Baker et al., 1996; Froment et al., 2004). In vitro and recently in vivo experiments in mice have demonstrated that FSH-driven differentiation of somatic and germ cells in gonads requires insulin/IGF signaling for (Pitetti et al., 2013). In humans, the absence or excess of insulin interferes with ovulation, sperm quality and the functioning of the hypothalamo–pituitary–gonadal axis (Diamanti-Kandarakis et al., 2008; Schoeller et al., 2012). In cases of excess insulin, the female egg or follicle may not completely mature, thereby preventing ovulation and increasing ovarian cyst formation. Thus, insulin signaling pathways play crucial roles in reproductive functions at various points in the gonadotropic axis. These

signaling pathways are regulated by various factors including hormones, called adipokines, produced by adipose tissue. The adipokines are believed to be very important in the interaction between energy balance and reproductive function.

4. Adipokine signaling pathways

Adipose tissue is a specialized endocrine and paracrine organ. It responds to nutrients and energy demands by secreting a variety of specific factors called adipokines. These adipokines are involved in the regulation of insulin sensitivity and adipokine balance is fundamental to preventing obesity, metabolic syndrome, and cardiovascular diseases (Raucci et al., 2013). During the last decade, new roles have been identified in the field of fertility and reproduction for adipokines (Campos et al., 2008; Reverchon et al., 2013a,b). Normal levels of adipokines are essential for maintaining the integrity of hypothalamus–pituitary–gonadal axis, regular ovulatory processes, steroidogenesis, successful embryo implantation, and physiological pregnancy. There is now a large volume of evidence for the role of the adipokine leptin in reproductive function (Hausman et al., 2012). However, new adipokines have been recently discovered (notably, adiponectin, resistin, chemerin, and apelin) and they appear to contribute to mediating reproductive success. Consequently, we will focus on the more recently documented adipokines including adiponectin and resistin.

4.1. Adiponectin

There is evidence that adiponectin expression and plasma levels in rodents and humans are dependent on the amount and form of fatty acids and carbohydrate consumed (de Oliveira et al., 2012). Adiponectin is a collagen-like 30-kDa protein hormone and is the most abundant secretory product of white adipose tissue (Maeda et al., 1996). It circulates as a multimer with three major oligomers (trimer, hexamer and high-molecular weight forms) and its concentration in humans is high, about 5–20 μg/ml (Kadowaki and Yamauchi, 2005). The circulating levels of adiponectin are inversely related to the degree of adiposity and are positively associated with insulin sensitivity in both healthy and diabetic patients. In various species, adiponectin and its receptors are present in various reproductive organs including pituitary, hypothalamus, testis, ovary, oviduct, placenta and uterus, and endometrium (Dos Santos et al., 2012; Palin et al., 2012). Adiponectin exerts its action by binding mainly to two specific receptors, AdipoR1 and AdipoR2. Both AdipoR1 and AdipoR2 are seven-transmembrane receptors that are structurally and functionally distinct from G protein-coupled receptors (Kadowaki and Yamauchi, 2005). They are biologically active and bind to adaptor proteins, APPL1 and 2 containing a pleckstrin-homology domain, a phosphotyrosine-binding domain and a leucine zipper motif. Engagement of AdipoRs and APPL1 induces an intracellular signaling cascade (Deepa and Dong, 2009). Through the stimulation of AdipoR1 or AdipoR2, adiponectin activates adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-α (PPARα) and mitogen-activated protein kinase (MAPK) (Kadowaki and Yamauchi, 2005) (Fig. 1). Adiponectin is involved in the regulation of various processes, including glucose utilization, lipid synthesis, energy homeostasis, vasodilatation and atherogenic activity (Kadowaki and Yamauchi, 2005). Furthermore, there is evidence suggesting that adiponectin may also directly regulate reproductive function.

Clearly, overexpression of circulating adiponectin in female mice impairs fertility (Combs et al., 2004), whereas the absence of adiponectin has no effect (Ma et al., 2002). AdipoR2-deficient male mice have reduced testes weight associated with an atrophy of the seminiferous tubules and aspermia (Bjursell et al., 2007). Results from animal studies indicate that circulating levels of adiponectin are tightly regulated during puberty, sexual differentiation, gestation and lactation.

4.1.1. The central nervous system and the pituitary

It has been suggested that adipose-derived and locally produced adiponectin acts as a neuromodulator of reproductive processes (Table 2). Neumeier et al. reported that adiponectin is able to cross the blood–brain barrier (Neumeier et al., 2007), and subsequent studies showed that both AdipoRs and adiponectin are expressed in the human and rat brain and pituitary (Psilopanagioti et al., 2009). The expression of all adiponectin system components was observed in porcine and rodent hypothalamic structures responsible for GnRH secretion (Kubota et al., 2007). Globular adiponectin significantly decreases GnRH release from mouse hypothalamic GT1-7 cells via AMPK activation. More specifically, low levels of adiponectin may contribute to chronically elevated LH levels (Wen et al., 2008).

The expression of both adiponectin receptors in the pituitary suggests that adiponectin has a local modulatory effect on central endocrine axes and that it participates in central control of metabolic homeostasis. Short-term exposure (4 h) of rat primary pituitary cells to adiponectin reduces the stimulatory effect of GnRH on LH release (Rodriguez-Pacheco et al., 2007). These results have been confirmed by Lu et al., 2008 who demonstrated that adiponectin has an inhibitory effect on basal and GnRH-stimulated LH release, but not on FSH release by the mouse gonadotrope cell line LβT2 (Lu et al., 2008).

4.1.2. The ovary, embryo and uterus

In the ovary, adiponectin has been detected in follicular fluid, the oocyte, the corpus luteum and theca cells, whereas it is only very weakly expressed in granulosa cells (Chabrolle et al., 2007). AdipoR1 and AdipoR2 are found in oocytes, corpus lutea, theca and granulosa cells (Ledoux et al., 2006). In porcine granulosa cells in vitro, recombinant porcine adiponectin induces the expression of genes associated with periovulatory remodeling of the ovarian follicle (Ledoux et al., 2006). We have showed that in rat, chicken and human granulosa cells, adiponectin increases IGF-I-induced progesterone and estradiol production (Chabrolle et al., 2007, 2009). In rat granulosa cells, adiponectin increases IGF-I-induced IGF-IR-beta subunit tyrosine phosphorylation and ERK1/2 phosphorylation suggesting cross-talk between IGF-1R and adiponectin receptors (Chabrolle et al., 2007). In the human granulosa cell line KGN, AdipoR1 is involved in cell survival whereas AdipoR2, through MAPK ERK1/2 activation, has been implicated in the regulation of steroid production (Pierre et al., 2009). In mouse and human in vitro fecondation procedures, adiponectin enhances oocyte maturation and early embryo development (Richards et al., 2012). In pigs, adiponectin enhances oocyte maturation though the p38MAPK pathway and in vitro embryo development (Chappaz et al., 2008). Adiponectin receptors are also detected in the uterus. Higher AdipoR1 and AdipoR2 gene expression has been observed in women during the mid-secretory phase of the menstrual cycle. Adiponectin may also affect uterine receptivity and the development of embryos during the preimplantation period (for review (Cikos, 2012)).

4.1.3. The testis

In the rat testis, adiponectin is mainly expressed by interstitial Leydig cells whereas its receptors, mainly AdipoR1, are present in the seminiferous tubules (Caminos et al., 2008). Recombinant adiponectin significantly inhibits both basal and human choriogonadotropin-stimulated testosterone secretion ex vivo, but it had no effects in the relative levels of several Sertoli cell-expressed mRNAs, such as those for the stem cell factor and anti-Müllerian

hormone (AMH) (Caminos et al., 2008). Furthermore, incubation of rat Leydig cells with recombinant adiponectin decreases testosterone biosynthesis, and this is associated with diminished expression of the cholesterol transporter steroidogenic acute regulatory protein (StAR) (Pfaehler et al., 2012). In chicken testis, sexual maturation is associated with the up-regulation of adiponectin receptor (AdipoR1 and AdipoR2) gene expression (Ocon-Grove et al., 2008). Thus, adiponectin can modulate both female and male reproductive functions.

4.2. Resistin

In contrast to adiponectin, resistin increases insulin resistance in rodents but its role in human metabolism is less well documented. However, some reports indicate that resistin is expressed in the hypothalamus–pituitary–gonadal axis and regulates various reproductive functions. Resistin is a protein, of 108 amino acids in humans and 114 amino acids in mice, and belongs to the family of "Resistin-like molecules" or "FIZZ" (found in inflammatory zone) (Schwartz and Lazar, 2011). It is constituted by homodimers linked by disulfide bridges. In mice, resistin is produced directly by adipocytes, whereas in humans, it is expressed in the bone marrow and in the macrophages that transport it to adipocytes. In mice, the plasma concentration of resistin decreases under the influence of thiazolidinediones (insulin sensitizers), and conversely, is high in obesity. Very little information is currently available about the mode of action of resistin. No receptor has been clearly identified, and the signaling pathway used remains equally obscure. Recent studies suggest that resistin could bind to the receptor tyrosine kinase called ROR1 (receptor tyrosine kinase-like orphan receptor) in murine pre-3T3-L1 adipocytes (Sanchez-Solana et al., 2012) or to the receptor TLR4 (Toll-like receptor 4) in the hypothalamus of mice (Benomar et al., 2012).

The role of resistin in the hypothalamus and pituitary remains to be determined. In the rat pituitary, resistin expression at birth is low and then increases until the age of 28 days. Its expression also increases in the mother at weaning and in males during puberty. Indeed, its concentration in the pituitary is two to three times higher in males than in females. Resistin in vitro downregulates the expression of the receptors AdipoR1 and AdipoR2 in rat pituitary cells in culture (Nogueiras et al., 2004). In ovary, resistin is present in follicular fluid, oocytes, theca cells and the corpus lutea of the rat and cow (Maillard et al., 2011); it is found in the granulosa cells of women and cows, but not rats. In human cultured theca cells, resistin increases the expression of 17 alpha hydroxylase in the presence of forskolin in the presence or absence of insulin, implicating resistin in androgen production (Munir et al., 2005)., Resistin modulates steroidogenesis and proliferation of granulosa cells in response to IGF-1 differently in cows, humans and rodents revealing species-specific effects (Maillard et al., 2011; Reverchon et al., 2013a,b). In the rat testis, resistin is found in Leydig cells and Sertoli cells within the seminiferous tubules. Its expression is regulated by fasting, gonadotropins and leptin. In addition, its abundance varies during postnatal development and is highest in adults (Nogueiras et al., 2004).

5. The fatty acids

There is considerable evidence that dietary fatty-acid supplementation can influence fertility by affecting various elements of the gonadotropic axis (Wathes et al., 2007). Central infusion of polyunsaturated fatty acid (PUFA) to rats in vivo increases the expression of the beta subunit of LH; in vitro, oleic and linoleic acid increase LH secretion by primary pituitary cells and by the murine gonadotrope cell line, LbetaT2 (Garrel et al., 2011). Fatty acids also affect gametogenesis. The PUFA composition of the cell membranes of the sperm and oocyte is important during fertilization (Aitken and Baker, 1995). Leydig and Sertoli cells have important roles in lipid metabolism in the male, including regulating the cholesterol level necessary both for the production of steroids and for ensuring the appropriate composition of spermatozoa membranes. Constant membrane synthesis is required for spermatozoa production, and a large substrate pool of phospholipids is needed to maintain spermatozoa quality. Accumulating evidence indicates that fatty acid β-oxidation in the female is essential for oocyte maturation and during the earliest stages of preimplantation embryo development (Dunning and Robker, 2012). Fatty acids are also precursors for the synthesis of both prostaglandin and steroids, factors that have multiple roles in the regulation of reproductive function. Inactivation of genes involved in lipid metabolism (for example, LXR) or of those involved in the cholesterol efflux lead to subfertility or sterility suggesting that correct lipid metabolism is critical for male and female reproduction (Lobaccaro et al., 2013; Steffensen et al., 2006).

5.1. Fatty acid receptors

Fatty acid receptors have been identified in the gonads (ovaries or testes), but their expression in germ cells has not been clearly described. The invalidation in mice of several of these nuclear receptors (RXR (Retinoid X Receptor), PPAR (Peroxisome Proliferator-Activated Receptor), LXR (Liver X Receptor), small heterodimer partner (SHP), and a target of FXR (Farnesoid X Receptor)) shows that they are involved in reproductive function (male or female), mainly by modulating steroidogenesis and/or the cell cycle (Steffensen et al., 2006; Volle et al., 2007a,b; Yang et al., 2008). Male mice deleted for RXR-beta, LXR and SHP show a decline in fertility associated with lipid accumulation in the testis and lower than control testosterone levels. The SHP receptor is mainly expressed in the Leydig cells and inhibits steroidogenesis gene expression (Volle et al., 2007a,b). LXR receptors naturally bound by oxysterols regulate the metabolism of cholesterol and lipids; they act as sensors of both cholesterol and glucose (Vernet et al., 2008).

PPARγ is activated in the presence of ligands, such as natural long-chain fatty acids (linoleic acid), various derivatives of prostaglandins (prostaglandin J2), and synthetic ligands. A specific invalidation of PPARγ in the female mouse granulosa cells causes a reduction in litter size (Kim et al., 2008). PPARs are also expressed in the testis where lipid metabolism and especially the β-oxidation of fatty acids is important for testicular function. Although the inactivation of PPARγ appears to have no effect on male fertility, incubation of human ejaculated sperm with rosiglitazone (synthetic ligand of PPARγ, used as a drug in the treatment of insulin resistance) improves capacitation, sperm motility, and the acrosome reaction of sperm (Aquila et al., 2006). The absence of both LXRs from mature male mice (5 months old) leads to infertility. LXRβ deficiency decreases expression of AMH in Sertoli cells, and lipid accumulation (Selva et al., 2004). There is further evidence confirming that LXRs are involved in lipid metabolism and the regulation of testosterone production.

6. The amino acid sensors

Amino acids and/or proteins may also have direct or indirect effects on reproductive functions. Intracerebroventricular injection of L-leucine into pubertal rats induces an increase in the secretion of LH. The protein source used in the manufacture of the food provided to chickens can affect reproduction (Menge, 1967): Excess or insufficient protein concentration reduced ovulation rates and egg hatchability. In cows, the concentrations of glycine and alanine in

follicular fluid are good predictors of oocyte quality in terms of capacity of development until the blastocyst stage after in vitro fertilization (Sinclair et al., 2008).

In mammals, the TORC complex is believed to act as an amino acid sensor. The "Tor" protein for "target of rapamycin" (also known as FRAP or RAFT) is a serine threonine kinase found in mammals (referred to as mTOR for mammalian TOR), fish and birds but also in yeast and plants (for review: (Stanfel et al., 2009)). mTOR regulates various cellular processes including cell growth, the cell cycle, cell survival, and autophagy. It is found in two complexes, called mTORC1 and mTORC2, according to the regulatory subunits present (regulatory subunit Raptor or Rictor subunit, respectively). The mTORC1 complex is activated when the energy status is favorable; particular amino acids, including leucine and glutamine, are essential for mTORC1 activity via Rag GTPase, hVps34 and MAP4K3 proteins (Sancak et al., 2008). In conditions of amino acid depletion, mTORC1 activity is quickly abolished (Hara et al., 1998).

6.1. The CNS and pituitary

Central mTOR signaling may contribute to the control of the onset of puberty and gonadotropin secretion, through regulation of the hypothalamic Kiss1 system (Roa et al., 2009). In the arcuate nucleus of the hypothalamus, the activation of mTORC1 induces a decrease in neuropeptide Y (NPY) expression causing, in turn, a decrease in food intake. Thus, mTORC1 appears to integrate the anorexigen signals, such as those involving the metabolic hormones (insulin, leptin, amino acids) present in the cerebrospinal fluid.

6.2. The ovary

The mTOR protein is produced in most cells of the ovary and most abundant in the surface epithelium of the ovary, in the granulosa cells and in immature oocytes. In rat, the activated mTOR pathway stimulates proliferation of granulosa and theca cells (induced by FSH or LH) (Kayampilly and Menon, 2007). Intraperitoneal administration of rapamycin (a pharmacological inhibitor of mTOR) to mice limits proliferation of granulosa cells and leads to a dose-dependent decrease in the number of follicles ovulated (Yu et al., 2011). Work with a human culture model has demonstrated that treatment with rapamycin results in decreased activation of primordial follicles and greater oocyte loss in growing follicles (McLaughlin et al., 2011). The use of sirolimus (rapamycin) in patients increases the duration of amenorrhea (Boobes et al., 2009); and about 50–60% of those treated with sirolimus but only 20–30% of the control group presented ovarian cysts.

6.3. The testis

Administration of sirolimus to men leads to lower plasma testosterone levels and increased secretion of gonadotropin hormones (both luteinizing hormone and follicle-stimulating hormone) (for review: (Huyghe et al., 2007)); this leads to a decline in the amount of semen produced (Bererhi et al., 2003) and even the absence of sperm production in some patients (Skrzypek and Krause, 2007). This reduction in male fertility has been described to be reversible after cessation of the sirolimus treatment (Skrzypek and Krause, 2007). The decrease in testosterone concentrations is however not clearly related to a direct action on Leydig cells which produce androgens. In primary rat Leydig cells, pretreatment with rapamycin increases the expression of the StAR protein, which transports cholesterol into mitochondria. Rapamycin treatment of primary cultures of mouse spermatogonia reduces the activation of the c-kit receptor (Feng et al., 2000); this pathway is activated by the binding of its SCF ligand (stem cell factor) and activates proliferation and maturation of primordial germ cells. Thus, the proliferation of germline stem cells under the control of growth factors, such as SCF, may be deregulated if the mTORC1 complex is either disabled or hyperactivated.

7. The energy sensor: AMPK

Unlike mTORC1, AMPK is inactivated during periods of favorable energy status. AMPK is a heterotrimeric protein consisting of alpha, beta, and gamma subunits (Hardie and Carling, 1997). It can be activated by any cellular stress that increases AMP levels: this is due to the allosteric binding of AMP to sites in the gamma subunit. It is also activated by phosphorylation of Thr172 in the alpha subunit by any of the serine/threonine kinase 11 (STK11/LKB1), calcium/calmodulin-dependent protein kinase kinase (CAMKK), and transforming growth factor-β-activated kinase (TAK1). A number of hormones that regulate energy homeostasis (leptin, ghrelin, adiponectin, and resistin) can tissue-specifically stimulate or inhibit AMPK kinase (Andersson et al., 2004; Kang et al., 2011; Minokoshi et al., 2002; Yamauchi et al., 2002). The effects of AMPK activation are pleiotropic in metabolically relevant tissues, but also in reproductive tissues including the hypothalamus, pituitary, and ovary (Dupont et al., 2012; Tosca et al., 2008).

7.1. The CNS and pituitary

AMPK has been extensively studied in the brain because of its role in the regulation of food intake. In the hypothalamus, the functioning of neurons contributing to kisspeptin and GnRH secretion also appears to be AMPK-dependent. Intracerebroventricular injection of AICAR (an activator of AMPK) reduces the numbers of kisspeptin-positive neurons by 60% in the ARC (arcuate nucleus of the hypothalamus); whereas the injection of compound C doubles the numbers of kisspeptin-positive neurons (Wen et al., 2012). Furthermore, intracerebroventricular injection of AICAR in rats activates hypothalamic AMPK, and increases the duration of estrus and decreases the inter-estrus interval (Coyral-Castel et al., 2008). Thus, AMPK activation may act directly at the hypothalamus to affect fertility by modulating GnRH release and the estrous cycle. AMPK has also been documented in pituitary cells, and more precisely in gonadotrope cells (Tosca et al., 2011). We showed in rat primary pituitary cells that AMPK activation in response to metformin, a biguanide insulin-sensitizing agent, inhibits both LH secretion induced by GnRH and FSH secretion induced by GnRH and activin; furthermore, AMPK activation by metformin inhibited Smad2 phosphorylation in response to activin and ERK1/2 phosphorylation in response to GnRH. Recent work shows that GnRH can rapidly activate AMPK in gonadotrope cells and that AMPK is required for the transcription of LH beta (Andrade et al., 2013).

7.2. The ovary

AMPK is expressed in diverse ovarian cells in various species (cow, goat, sheep, pig). In rodent and bovine granulosa cells, metformin or AICAR decrease steroidogenesis and MAPK3/MAPK1 phosphorylation through AMPK activation (Tosca et al., 2007, 2005). AMPK in granulosa cells can also be regulated by FSH. Indeed, in cultured granulosa cells, FSH increases mitogenesis not only by increasing the expression of cyclin D2 through an ERK-dependent pathway but also by regulating AMPK through an Akt-dependent pathway to reduce the amount of the cell cycle inhibitor protein, p27 kip (Kayampilly and Menon, 2009). AMPK also regulates the resumption of meiosis in the oocyte. AMPK triggers GVBD (Germinal Vesicle BreakDown) in denuded or

cumulus-enclosed oocytes of mice (Chen et al., 2006; LaRosa and Downs, 2007). Conversely, AMPK agonists can block GVBD in other cumulus-enclosed mammalian oocytes (in pigs and cows) (Mayes et al., 2007; Tosca et al., 2007). Two studies in cows, with near identical results, conclude that this decrease is due to an inhibition of cumulus cell proliferation (for review: (Bilodeau-Goeseels, 2011). Indeed, activation of AMPK in rat granulosa cells inhibits theirproliferation (Kayampilly and Menon, 2012). By contrast in the cow, activation of AMPK stimulates mouse oocyte maturation in vitro: AMPK is involved in the condensation of chromatin and formation of the meiotic spindle during the resumption of meiosis (Chen et al., 2006; Downs et al., 2010). In *C. elegans* (hermaphrodite), activation of AMPK by metformin increases the time of laying and reduces the average number of offspring per day, mimicking the phenotype observed in situations of food restriction (Onken and Driscoll, 2010). In *Drosophila melanogaster*, the activation of AMPK by high doses of metformin also inhibits egg laying and decreases the average number of eggs laid (Slack et al., 2012).

7.3. The testis

In males, AMPK is present in the testis and germ cells. In male pigs, AMPK regulates motility, which is essential for fertilization (Hurtado de Llera et al., 2012). Total α1AMPK deficiency in male mice affects androgen production and quality of spermatozoa, leading to a decrease in fertility (Tartarin et al., 2012). Administration of metformin to rodents (for 4 or 8 weeks) does not affect the mean sperm count or motility, or percentage of abnormal sperm. In contrast, the ingestion of resveratrol, another AMPK activator, for 28 days results in increases to all of testosterone levels, sperm count and motility (Shin et al., 2008). In rat primary Sertoli cells, AMPK activation by AICAR or adenosine increases lactate production implicating AMPK in the modulation of the nutritional function of Sertoli cells (Galardo et al., 2007 Galardo et al., 2010). LKB1 transgenic mice also have abnormal fertility; histological examination of mice lacking the short isoform of LKB1 revealed a near complete absence of mature spermatozoa. Isolated spermatids from $LKB1_s$-deficient mice were completely non-motile and displayed abnormal acrosome morphology, suggesting a cell polarity defect (Shaw, 2008).

8. Conclusions

It is undeniable that the reproductive axis displays close links to nutritional status. Nutrients (glucose, fatty acids, amino acids) and metabolic hormones including insulin/IGF-1 and adipokines are able to affect the functioning of reproductive cells not only at the central levels (hypothalamus–pituitary) but also at the gonadal axis. Several "nutrient sensing" mechanisms are operational forming complex networks that present similar functions in ovary and in testis (for example, steroid production, energy production and transfer to germ cells). A node involving mTORC and AMPK protein kinase appears to be particularly important, and indeed is conserved across a broad spectrum of species. However, most of the studies described are performed in vitro conditions and present some important limitations (immatures cells, differentiated cells induced by ovarian hyperstimulation or lineage cells that can loose some specificity). Nevertheless, there are increasing studies which take advantage of inactivation technologies (shRNA, knockout) in a cell specific manner as evoked in Tables 1 and 2. These strategies have already improved our knowledge about the molecular mechanisms involving some metabolic pathways in reproductive cells. In perspective, new challenges in future will be to use these models in a metabolic disorder context in order to elucidate the mechanisms involved, and the extent to which changes of nutrient sensors contribute to the impairment of fertility. Such work should lead to the discovery of possible medical treatments for infertility.

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Involvement of adipokines, AMPK, PI3K and the PPAR signaling pathways in ovarian follicle development and cancer

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ABSTRACT The physiological mechanisms that control energy balance are reciprocally linked to those that control reproduction, and together, these mechanisms optimize reproductive success under fluctuating metabolic conditions. Adipose tissue plays an important role in this regulation. Indeed, it releases a variety of factors, termed adipokines that regulate energy metabolism, but also reproductive functions. This article summarizes the function and regulation of some better-characterized adipokines (leptin, adiponectin, resistin, visfatin, chemerin and apelin) involved in ovarian follicle development. The follicle appears to use various "nutrient sensing" mechanisms that may form the link between nutrient status and folliculogenesis. This review examines evidence for the presence of pathways that may sense nutrient flux from within the follicle including the PI3K/Akt pathway, adenosine monophosphate-activated kinase (AMPK), and peroxisome proliferator-activated receptors (PPARs). It also reviews current information on the role of these adipokines and signalling pathways in ovarian cancers.

KEY WORDS: *metabolic sensor, ovarian tumorigenesis, follicle growth, granulosa cell, theca cell, oocyte*

Introduction

Reproductive functions are dependent on energy resources. The role of weight, body composition, fat distribution and the effect of diet have been largely investigated in women and domestic female animals where their alterations may induce abnormalities in timing of sexual maturation and fertility. However, the cellular mechanisms involved in the fine coordination of energy balance and reproduction are largely unknown. It is clear that the brain and hypothalamic structures receive endocrine and/or metabolic signals providing information on the nutritional status and the degree of fat stores. However, it is now widely accepted that there are direct nutritional effects on ovarian development and follicular atresia, which regulate production of germ cells. Thus, it suggests that there are specific energy sensing mechanisms in the follicle. Several candidates have been involved as possible link between nutritional state and the functions of the different ovarian cells. Among these energy sensors, there are factors released by the adipose tissue called adipokines (leptin, adiponectin, resistin, visfatin, chemerin…) but also various signalling pathways including AMPK, PPARs, PI3K/Akt/PTEN/Foxo that sense nutrient status in the follicle.

This paper reviews earlier and contemporary studies on the effects of these energy sensors on the growth and development of ovarian follicles and more precisely we focused on the functions of granulosa and theca cells and oocyte in different species. In the ovary, bidirectional communications between germ cells (oocytes) and somatic cells (granulosa cells) are crucial to oocyte maturation,

Abbreviations used in this paper: AMPK, adenosine monophosphate activated-kinase; APJ, apelin receptor; CMKLR, chemokine-like receptor; EOC, epithelial ovarian cancer; FAS, fatty acid synthase; Foxo, forkhead box, sub-group O; FSH, follicle-stimulating hormone; GSK, glycogen synthase kinase; GVBD, germinal vesicle breack down; IGF, insulin-like growth factor; LH, luteinizing hormone; LPA, lyposaccharide; MAPK ERK, mitogen-activated protein kinase extracellular signal-regulated kinases; mTor, mammalian target of rapamycin; Ob-R, obese gene receptor; PDK, 3-phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-biphosphate; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog Ob-R, obese gene-receptor; RARRES, retinoic acid receptor responder; TIG, tazarotene-induced gene; TZD, thiazolidinediones; TSC, tuberous sclerosis; STAT, signal transducer and activator of transcription.

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follicle growth and ovulation (Binelli and Murphy, 2010). Oocytes are derived from primodial germ cells, oogonia, which expand through mitosis in the fetal ovary. The germ cells become oocytes once they enter meiosis at birth. They become surrounded by a layer of epithelial cells (granulosa) and are now called primordial follicles. At puberty, primordial follicles resume growth and acquire many layers of granulosa cells to reach the ovulatory stage at each cycle. Ovarian follicular growth and ovulation are dependent on the growth and proliferation of granulosa cells. The period of follicular growth is characterized by mitotic activity of granulosa cells and the transformation of surrounding stroma into layers of thecal cells.

Some of these mediators of energy balance have also been described in the proliferation, migration and invasion of cancer cells. Epidemiological studies have suggested that obesity is associated with ovarian cancer. Both benign and malignant ovarian tumors can arise from each of the three ovarian cell types (germ cells, endocrine and interstitial cells, and epithelial cells (Romero and Bast, 2012). Germ cell tumors account for 3–5% of ovarian cancers, endocrine and interstitial cell tumors represent approximately 7% of all ovarian malignancies and epithelial ovarian cancers include approximately 90% of malignant ovarian tumors. Despite rapid advances in understanding ovarian cancer etiology, epithelial ovarian cancer (EOC) remains the most lethal form of gynecologic cancers. Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer-related death in women in the USA and the leading cause of gynecologic cancer death. Ovarian cancer is the sixth most common cancer and the fifth leading cause of cancer-related deaths among women in developed countries. Malignant transformation of normal ovarian epithelial cells is caused by genetic alteration that disrupts proliferation, programmed cell death and senescence. Two groups of epithelial ovarian cancers have been distinguished: type I low-grade cancers that present in early stage, grow slowly, and resist conventional chemotherapy but may respond to hormonal manipulation; and type II high-grade cancers that are generally diagnosed in advanced stage and grow aggressively but respond to chemotherapy. There has been considerable interest in the potential role of the adipokines but also in their signaling pathways such as AMPK, PPARs, and PI3K/Akt in the development of anti-cancers drugs.

Role of adipokines in the development of the ovarian follicle

The body mass index and especially the lean/fat ratio are determinants of female fertility. Adipose tissue is now hypothesized to be the largest endocrine organ in the body, secreting a large number of biologically important molecules termed adipokines. Adipose tissue acts as a store of energy and as an active endocrine organ. Adipokines modulate lipid and glucose metabolism and insulin sensitivity. In addition to their well established role in controlling adipose tissue physiology, adipokines have been shown to be involved in regulation of the reproductive functions. During the last years, roles of new adipokines have been emerging in the field of fertility and reproduction and more precisely on the ovarian functions (Campos *et al.,* 2008).

Leptin

In 1994, leptin is the first adipokine claimed to be the "missing link" between fat and reproduction. Leptin is a 16 kDa peptide hormone secreted mainly from adipose tissue which plays an integral role in the regulation of body weight and energy expenditure. Some evidence suggests that leptin is also an important signal in female reproduction, including control of ovarian function (Brann *et al.,* 2002). The leptin receptor (Ob-R), product of the diabetes (db) gene, is a member of the class I cytokine receptor superfamily, with six known isoforms. Ob-R expression has been observed in many tissues including the hypothalamus and peripheral tissues including the ovary (Brann *et al.,* 2002). Of the known isoforms, only the full-length form (Ob-Rb) contains the intracellular domains necessary to mediate signal transduction through both the signal transducer and activator of transcription 3 (STAT3), and the MAP kinase pathway.

A significant body of work has shown that Ob-R is expressed in the ovary. Leptin receptor transcripts are present in both human and rat granulosa and theca cells, as well as in cattle and porcine granulosa and theca cells. Ob-Ra and Rb levels in the ovary vary during the estrous cycle in rodent and cattle. Among the hormonal signals informing the reproductive axis about nutritional status, leptin is growing emerging as a convincing hypothesis. Plasma levels of leptin are correlated with the degree of obesity and are regulated by feeding and fasting. In the leptin deficient female ob/ob mice, treatment with leptin, increases serum levels of LH and ovarian and uterine weight compared to par-fed controls, and restores fertility. A direct ovarian action of leptin has also been demonstrated in rat ovarian granulosa cells, where leptin counteracts the synergistic effect of IGF-1 on FSH-stimulated estradiol and progesterone production. In human, leptin reduces the production of estradiol by granulosa cultured cells (Agarwal *et al.,* 1999). In bovine theca cells, leptin reduces androgen production induced by LH. The presence of Ob-R mRNA (Craig *et al.,* 2004) and protein has been detected in cattle, mouse, rat, and human oocytes, suggesting that the oocyte may be capable of responding to leptin. In porcine oocytes, Ob-R expression is dependent on the stage of follicular development and oocyte maturation. Leptin protein has been detected in the oocyte by immunofluoresence, however others have been unable to detect its mRNA transcript in the oocyte by nested RT-PCR, suggesting it may be produced elsewhere and transported into the oocyte. In the human ovary, leptin is produced by ovarian somatic cells and is present in follicular fluid at concentrations similar to serum, making leptin spatially available for the oocyte. In mice, leptin has been shown to increase the rate of meiotic resumption in preovulatory follicle-enclosed oocytes, presumably via indirect actions on the theca cells (Ryan *et al.,* 2002). In pig, it facilitates oocyte maturation through MAP kinase pathway (Craig *et al.,* 2004).

Adiponectin

Adiponectin is a novel adipocytokine that was identified in 1995 (Scherer *et al.,* 1995) and 1996. It is the most abundant protein secreted by adipose tissue (accounting for approximately 0.01% of total plasma protein at around 5-10 μg/mL in human). Its circulating concentration is inversely related to adiposity and body mass index. It plays an important role in regulating energy homeostasis, specifically lipid and glucose metabolism. Adiponectin activates AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptors alpha (PPARα). Two adiponectin receptors (AdipoR1 and AdipoR2) have been identified and found to have functional differences. The predicted structure of the two receptors is similar to the topology of G-protein coupled receptors,

but the intracellular/extracellular orientation of N-terminus and C-terminus is the opposite. The expression of all or some genes of the adiponectin system was identified in the ovary of various species including rat, pig, chicken, human and recently cattle (Maillard *et al.,* 2010, Tabandeh *et al.,* 2010). Except in bovine species, adiponectin expression is absent/low in chicken, mouse and human granulosa cells and cumulus cells. The expression of adiponectin and its receptors changes in different cells of bovine ovary during morphological and physiological development (Tabandeh *et al.,* 2010). Furthermore, adiponectin receptors have been shown to be expressed by oocytes and early embryos in porcine and mice species (Chappaz *et al.,* 2008). mRNA as well as protein for both adiponectin receptors are expressed in mouse ovulated oocytes and in *in vivo* derived preimplantation embryos at all examined stages of development. A few studies have demonstrated the relationship between adiponectin and female reproduction. Recently, some evidence suggests that adiponectin could be involved in the control of reproductive functions. Indeed, overexpression of circulating adiponectin impairs female fertility in mice, whereas the absence of adiponectin has no effect. Additionally, *in vitro* studies showed that adiponectin decreases androgen and progesterone production induced by insulin in bovine theca cells. Furthermore, our team has found in rat, chicken and human, that physiological levels of recombinant human adiponectin (5 or 10 μg/ml) are able to increase progesterone and/or estradiol secretions in response to insulin-like growth factor-I (IGF-I) in cultured granulosa cells. Several reports in different species, including humans, have indicated that adiponectin can modulate not only granulosa cell steroidogenesis but also the expression of genes associated with ovulation. There is also evidence that the functions of the two adiponectin receptors in granulosa cells may differ. In the human ovarian granulosa-like tumor cell-line (KGN), AdipoR1 seems to be involved in the cell survival whereas AdipoR2, through MAPK ERK1/2 activation could regulate steroid production (Pierre *et al.,* 2009). In bovine species, adiponectin decreased insulin-induced steroidogenesis and increased IGF-1-induced proliferation of cultured granulosa cell through a potential involvement of ERK1/2 MAPK pathway, whereas it did not modify oocyte maturation and embryo development *in vitro* (Maillard *et al.,* 2010). In porcine species, adiponectin stimulates oocyte meiotic maturation and *in vitro* embryo development (Chappaz *et al.,* 2008). Also, in mice, the addition of adiponectin to mouse cumulus-oocyte-complex improves oocyte quality as assessed by fertilization and early stages of embryo growth. Thus, addition of adiponectin to *in vitro* maturation media could improve the developmental competence of *in vitro* matured oocytes in human infertility care. Of clinical relevance, adiponectin levels are reduced in women with polycystic ovarian syndrome compared with fertile women, which is possibly linked with elevated androgens and obesity, and altered adipose tissue functions.

Resistin

Resistin was discovered in 2001 as a Thiazolidinediones-downregulated gene in mouse adipocytes (Steppan *et al.,* 2001). Resistin is an adipocyte-derived cytokine that plays an important role in the development of insulin resistance and obesity in rodents. Resistin is a cysteine-rich protein of around 12kDa that belongs to a family of polypeptides named resistin-like molecules. In mice, adipocytes may be the major source of resistin, whereas in humans, resistin mainly come from monocytes and macrophages. Despite much research on resistin's action, the receptor(s) mediating its biological effects has not yet been identified, and little is known on the intracellular signaling pathways activated by this protein. In cultured human theca cells, 17α-hydroxylase activity is increased by resistin in the presence of forskolin or forskolin±insulin, suggesting a role of resistin in stimulation of androgen production by theca cells (Munir *et al.,* 2005). Some studies have shown elevated concentrations of serum resistin in women with polycystic ovary syndrome, which is known to be associated with insulin resistance, hyperinsulinemia and hyperandrogenism. In cow and rat, resistin mRNA and protein are present in various ovarian structures such as theca cells and oocyte. However, resistin expression is species-dependent in granulosa cells (Maillard *et al.,* 2011). In both species, recombinant resistin can modulate steroidogenesis and proliferation in basal state or in response to IGF1 *in vitro* (Maillard *et al.,* 2011). In bovine species, resistin inhibits *in vitro* steroidogenesis of undifferentiated (small follicles) granulosa cells and inhibits mitogenesis of differentiated (large follicle) granulosa cells suggesting that the ovarian response to resistin is altered during follicular development (Spicer *et al.,* 2011). However, in this latter species, it had no effect on IGF-I- or insulin-induced progesterone and androstenedione production by theca cells (Spicer *et al.,* 2011). The role of resistin in oocyte is still unknown.

Visfatin

Visfatin (52 kDa), discovered in 2005, also known as nicotinamide phosphoribosyltranferase (nampt) or pre B cell colony enhancing factor (PBEF) is a multifunctional protein predominantly expressed in the visceral adipose tissue (Dahl *et al.,* 2012). Visfatin concentration in follicular fluid is positively correlated with the number of follicles retrieved from women undergoing *in vitro* fertilization, suggesting that visfatin may act in ovarian function, like follicles growth up, cells differentiation, oocyte maturation or ovulation. A recent study showed that visfatin mRNA is expressed in human granulosa cells and a hCG treatment for 24h increased considerably visfatin mRNA expression suggesting a role of visfatin in final follicle maturation or ovulation (Shen *et al.,* 2010). In our laboratory, we have showed that visfatin increases IGF-1-induced steroid production in primary human granulosa cells and in the human granulosa KGN cell-line.

Chemerin

Chemerin also known as RARRES2 or TIG2, was discovered in 1997. It is a secreted 18-kDa inactive pro-protein and cleaved into a 16-kDa active form by extracellular serine protease cleavage, which is present in plasma and serum (Goralski *et al.,* 2007). The plasma chemerin levels are significantly higher in obese subjects than those with normal weight. It was also recently reported that circulating chemerin levels increased in PCOS non obese patients versus control. Chemerin and its receptor, CMKLR1 are present in human ovarian follicles (mainly in granulosa and theca cells) and in KGN cells. In primary human granulosa cells, chemerin decreases IGF-1-induced thymidine incorporation, progesterone and oestradiol production through a decrease in the phosphorylation of IGF-1R beta subunit and MAPK ERK1/2 signaling pathways (Reverchon *et al.,* 2012).

Apelin

In 1998, Tatemoto *et al.,* (1998) isolated a novel endogenous apelin peptide from the bovine stomach, which was found to be

a ligand of the apelin receptor (also known as the APJ receptor) (Tatemoto *et al.,* 1998). APJ receptor is an orphan G protein-coupled receptor. Apelin is produced through processing from the C-terminal portion in the pre-proprotein consisting of 77 amino acid residues and exists in multiple molecular forms. APJ receptor most closely resembles the angiotensin type 1 receptor; however, the APJ receptor does not bind to angiotensin II. Apelin peptide is a potent angiogenic factor inducing endothelial cell (EC) proliferation, EC migration, and the development of blood vessels *in vivo*. The apelin levels produced by adipocytes, vascular stromal cells, and the heart, are increased with high insulin levels, obesity or insulin-resistance. The APJ receptor is expressed in granulosa cells, and both apelin and the APJ receptor are expressed in the theca cell layer of follicles in bovine ovaries (Shimizu *et al.,* 2009). In theca cells, apelin and APJ expression is induced by LH. In granulosa cells, the increase in expression of APJ receptor mRNA is associated with the follicular atresia (Shimizu *et al.,* 2009). The expression of apelin and APJ in mature follicles suggests that the apelin/APJ system could play an important role during follicle selection and dominance in the cow (Schilffarth *et al.,* 2009).

Thus, various *in vitro* studies have shown that adipokines are able to affect directly the ovarian functions. Some of these effects are summarized in the Fig. 1.

Role of adipokines in ovarian cancer

There is some evidence that adipokines are involved in the development and the progression of several cancers including breast, prostate and colon. More specifically, in patients with these cancers, the plasma adiponectin levels are significantly reduced. The roles of adipokines in ovarian cancers have been much less studied. Leptin is suggested to be associated with cancer development and progression in many epithelial cancers including epithelial ovarian cancer (EOC) (Mor *et al.,* 2005). *In vitro* studies consistently show that leptin has angiogenic and proliferative potential in cancer (Choi *et al.,* 2005). There is some evidence that plasma adiponectin concentrations are significantly lower in serum of patients with early-stage ovarian cancer compared with healthy women. Adiponectin has been shown to exhibit anti-tumorigenic properties, such as suppression of tumor growth, cellular proliferation in addition to inducing cell growth arrest and apoptosis. In leghorn chicken ovarian tumors, adipoR1 mRNA expression is significantly decreased as compared to non-tumorous chicken ovaries (Ocon-Grove *et al.,* 2008). The authors of this latter study speculate that adiponectin signaling in the ovary is likely to limit ovarian tumor growth, possibly by suppressing tumor cell proliferation and inducing apoptosis. Visfatin protein expression is significantly increased in ovarian serous adenocarcinomas as compared to benign ovarian tissue (Shackelford *et al.,* 2010). So far, no data (plasma or change of expression) about chemerin, resistin and apelin and ovarian cancers are known.

Most of these adipokines (leptin, adiponectin, resistin) mediate their effect through the same signaling pathways named AMPK, PI3K, MAPK and PPAR that are also involved in the ovarian follicle development and cancer.

Role of adenosine monophosphate-activated protein kinase (AMPK) in ovarian follicle development

AMPK is a serine/threonine kinase and is highly conserved throughout eukaryotes. It is a heterotrimeric protein consisting of a catalytic alpha - and regulatory beta - and gamma -subunits. It is known as the fuel gauge of the cell because it mediates a nutrient signaling pathway that senses cellular energy status. AMPK is an important cellular energy sensor that is activated in response to deficits in ATP and acts by shutting down energy consumption and turning on energy-generating pathways (Hardie, 2003). It is activated during physiopathological situations (food restriction, exercise, stress), by hormones (leptin, adiponectin..) or by pharmacological agents (5-aminoimidazole-4-carboxamide-1-β-D-riboside [AICAR], metformin, thiazolidinediones). The potential involvement of AMPK in reproduction and more specifically in ovarian function is recent.

In various animal species (human, rodents, cow and chicken), AMPK has been identified in different cell types of the follicle (oocyte, cumulus cells, granulosa and theca) and in the corpus luteum (Tosca *et al.,* 2005). In human, AMPK activation is decreased in PCOS thecal cells, and metformin treatment of these cells increases AMPK activity. Thus, activation of AMPK in the ovary itself could be a reason why patients with PCOS who are treated with metformin experience amelioration of ovulatory dysfunction.

AMPK has been extensively studied *in vitro* steroidogenesis of granulosa cells of rat, chicken and bovine species and oocyte maturation in the mouse, cow and pig (Downs *et al.,* 2002, Mayes *et al.,* 2007, Tosca *et al.,* 2007a). In rat and cattle, AMPK inhibits the secretion of progesterone and / or that of oestradiol in response to the AICAR or metformin (Tosca *et al.,* 2007a, Tosca *et al.,* 2005). This decrease is partly explained by an inhibition of the expression

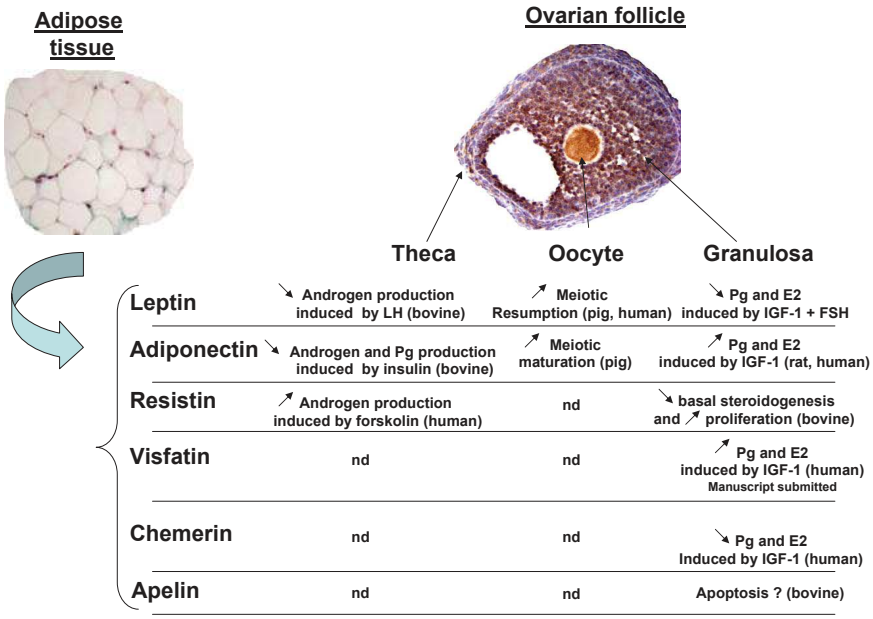

Fig. 1. *In vitro* effects of some adipokines on the functions of granulosa and theca cells and oocytes in different species. *ND, Not yet determined.*

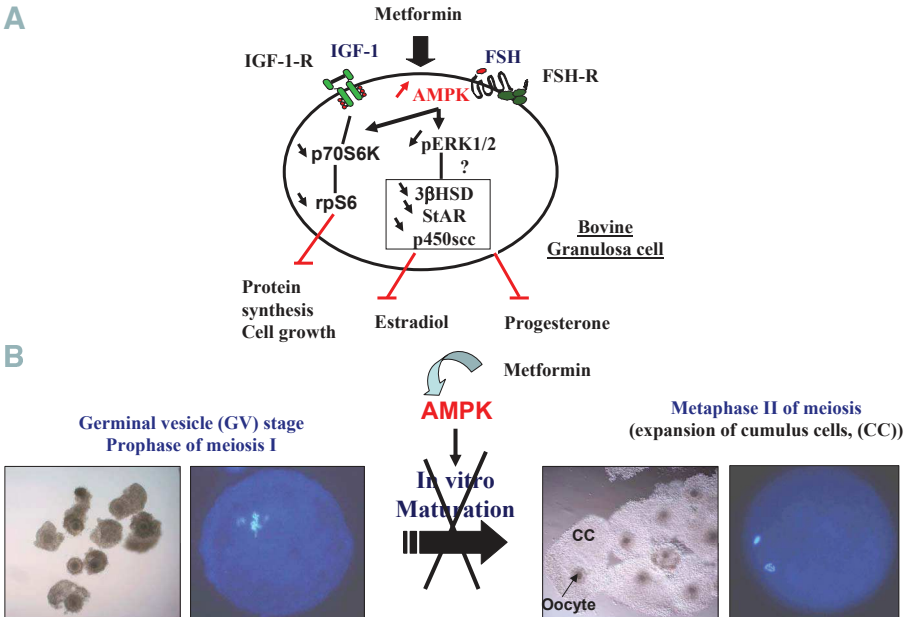

Fig. 2. *In vitro* effects of metformin induced-AMPK activation in bovine granulosa cells and oocyte. (A) *Metformin induced AMPK activation reduces IGF-1 and FSH-induced estradiol and progesterone secretion through inhibition of MAPK ERK1/2 phosphorylation (Tosca* et al.*, 2007). It also reduces IGF-1-induced protein synthesis and cell growth through an inhibition of p70S6K and ribosomal S6 protein phosphorylation (Tosca* et al.*, 2010).* **(B)** *AMPK activation in response to metformin blocks meiotic progression at the GV stage (adapted from Tosca* et al.*, 2007).*

of some factors of steroidogenesis (3βHSD [3β-hydroxysteroid dehydrogenase-], P450scc [p450 side chain cleavage] or StAR [Steroidogenic acute regulatory protein]) and by inhibition of MAPK / ERK (mitogen-activated protein kinase / extracellular-regulated kinase). A schema is represented in Fig. 2A to summarize the effects of metformin-induced AMPK activation in bovine granulosa cell steroidogenesis. In chickens, the activation of AMPK decreases in granulosa cells during folliculogenesis terminal, suggesting a role of the kinase in the pre-ovulatory period (period of the rapid growth of the follicle) in this species. AlphaAMPK is also involved in the regulation of ovarian cell proliferation and apoptosis. In cultured bovine granulosa cells, metformin inhibits proliferation and protein synthesis in response to IGF-1 through an AMPK-dependent mechanism as indicated in the Fig. 2A (Tosca *et al.,* 2010). Furthermore, in primary rat granulosa cells, FSH promotes granulosa cell proliferation by increasing cyclin D2 mRNA expression and by reducing p27 kip expression by inhibiting AMPK activation through an Akt-dependent pathway (Kayampilly and Menon, 2009).

Some studies have also established a link between AMPK and meiotic maturation of the oocyte. In mice, AMPK activation enhances the resumption of meiosis (acceleration of rupture of the nuclear membrane or GVBD stage (germinal vesicle breack down), whereas in pigs or cattle, a pharmacological activation of the AMPK blocks the nuclear maturation of the oocyte (blocked in prophase of first meiotic division stage or germinal vesicle (GV) as indicated in Fig. 2B (Bilodeau-Goeseels 2011, Mayes *et al.,* 2007, Tosca *et al.,* 2007b). Although there are differences between species, AMPK appears to be important in the transition GV-GVBD during oocyte nuclear maturation. The role of AMPK in nuclear maturation could therefore condition early embryonic development. Finally, AMPK was also found in the corpus luteum in rat. The corpus luteum is characterized by steroid production. AMPK could control at this level the process of luteinization, for example by modulating some components of the cholesterol production pathways. AMPK and PPARs (peroxisome proliferator-activated receptors) are tightly linked, some drugs activating AMPK activate also PPAR-gamma and the activation of PPAR in cow increases the production of progesterone by the corpus luteum. Thus, AMPK could control steroidogenesis in ovarian cells (granulosa, theca cells and corpus luteum cells) and germ cell maturation.

AMPK in ovarian cancer

Several data suggest that AMPK is associated with the development of some cancers. First, one of the AMPK kinase called LKB1 is known to be a tumor suppressor (mutation of its locus has been suspected in the development of granulosa cell tumours). Then, AMPK regulates two proteins of the PI3K pathways, TSC2 (a second tumor suppressor) and Raptor (a protein associated to mTOR in the mTORC1 complex), involved in the protein synthesis and cell growth. Early studies have shown that AMPK activated by stress or pharmacological activators inhibits protein synthesis via regulation of mTOR/S6K and translation elongation factor 2. Second, AMPK inhibits expression and/or activity of enzymes, Fatty Acid Synthase and mTOR that are increased in cancers. Third, *in vitro* AMPK activation inhibits the growth of some cancer cells. Recent studies introduced AMPK as an important regulatory factor in cell migration through the transcriptional up-regulation of integrins (Kim *et al.,* 2011). It could be one possible mechanism by which metformin exerts its potential anti-neoplastic properties. In ovarian cancer cells, AMPK is an important regulatory factor for cell migration through LPA_2-mediated cytoskeleton reorganization and tumor metastasis *in vivo*. Thus, AMPK may be a key therapeutic target for the control of ovarian cancer progression (Kim *et al.,* 2011).

Role of PI3K signaling in the ovarian follicle and cancer

The phosphoinositide-3 kinase (PI3K) pathway plays an important role in many biological functions including metabolic control, immunity, and cancer (Katso *et al.,* 2001). Although the basic framework of PI3K signaling is well understood, much remains to be learned about its role in reproductive biology. PI3K is activated by IGF-1 and FSH, which normally regulates granulosa cell differentiation and follicular development after antrum formation but also by LH, which is essential for ovulation.

Mammalian phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases able to phosphorylate the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). The phosphoinositol-3-kinase family is divided into three different classes: Class I, Class II, and Class III, based on their substrate

preferences, primary structure and regulation. The class I PI3K is the best studied to regulate the ovarian follicle. There are two subgroups of class I PI3Ks, class IA and class IB. Class IA PI3Ks are activated by growth factors receptors including insulin and IGF-1 receptors that play a key role in the follicle development. Class IB PI3Ks are activated by G-protein coupled receptors (GPCRs) including FSH and LH receptors that control differentiation and secretion of ovarian follicular cells.

Once activated, PI3K phosphorylates the membrane phospholipid, phosphatidylinositol 4,5-biphosphate (PIP2), thereby producing phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn acts as a second messenger to activate PDK1 (3-phosphoinositide-dependent kinase-1). PDK1 phosphorylates many important kinases such as Akt/PKB and p70 S6 kinase in their respective activation loops. Activated Akt directly phosphorylates various substrates including GSK3 (glycogen synthase kinase-3), Bad, the proapoptotic forkhead transcription factors (FOXO1, FOXO3a and FOXO4) and the tuberous sclerosis 2 (TSC2). The inactivation of GSK3 leads to the dephosphorylation and activation of glycogen synthase and hence to an acceleration of glycogen synthesis. The phosphorylation of FOXOs by Akt in response to growth factors leads to the exclusion of FOXOs from the nucleus and consequently prevents their actions. Akt also exerts anti-apoptotic effects by phosphorylating Bad. In parallel, Akt activates the mTORC complex (mTOR, mammalian target of rapamycin and its regulatory proteins associated) by phosphorylation of TSC2 and destabilization of the TSC1/TSC2 complex. mTORC may then increase phosphorylation of the initiation factor 4E-binding protein (4E-BP1) and S6K (S6 kinase) promoting protein synthesis, ribosome biogenesis and autophagy. The biological effects of the PI3K pathway can be negatively regulated at the level of PIP3 by phospholipid phosphatases, including the phosphatase and tensin homolog (PTEN) and SH2-containing 5′-phophatase-2 (SHIP2), which dephosphorylate and inactivate PIP3, as indicated in Fig. 3.

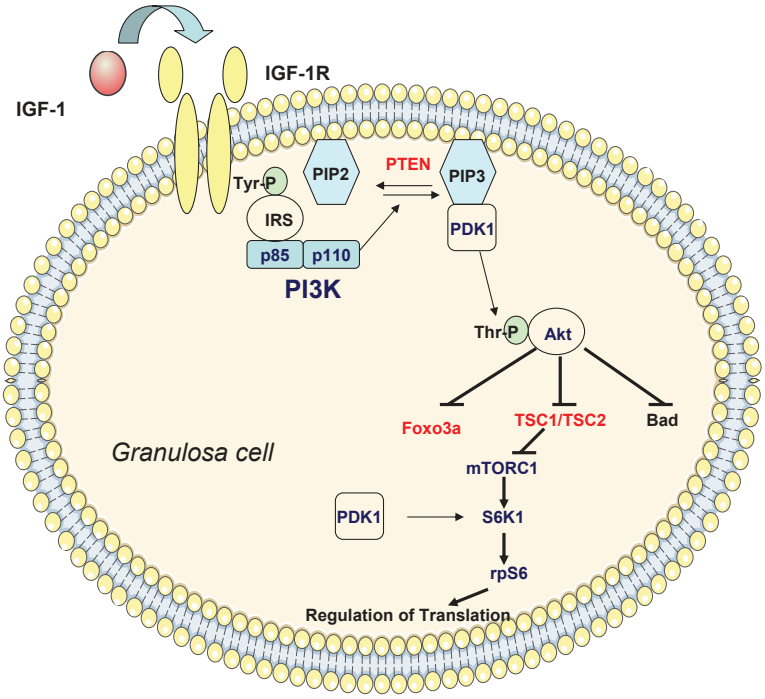

Fig. 3. Illustration of the class IA PI3K signaling pathway activated in response to IGF-1. *As revealed by genetically modified mouse models, signaling molecules that maintain the quiescence of primordial follicles are PTEN, Foxo3a, and the TSC1/TSC2 complex (red), and signaling molecules that maintain the survival of primordial follicles are PI3K, PDK1, mTORC1, S6K1, and rpS6 (blue).*

PI3K signaling and follicle growth

Several roles of the PI3K pathway in the ovarian follicle have been recently elucidated by using conditional knockout mouse models as described in the recent review written by Zhen *et al.*, 2012 (Zheng *et al.,* 2012). PTEN is the most studied in the mammalian ovary. The phosphatidylinositol 3-kinase (PI3K) pathway, known to be essential for the regulation of cell proliferation, survival, migration, and metabolism in different tissues, is also likely to play a role in the regulation of the activation of primordial follicles in the mice ovary. Indeed, deletion of *Pten* (leading to activation of PI3K) specifically in oocytes promptly activates follicular development in mice very early in life (Reddy *et al.,* 2008). This early follicular activation resulting from heightened PI3K-Akt signaling in oocytes lacking PTEN suggests that signaling from oocytes to follicles is a contributing factor in regulating follicular activities. Interestingly, another group found that PI3K-Akt–dependent hyperphosphorylation of the transcription factor Foxo3 also in oocytes induces premature oocyte activation suggesting that Foxo3 acts downstream of PTEN to negatively regulate follicular activation (Adhikari *et al.,* 2009). This irreversible premature oocyte activation leads to rapid depletion of follicle reserve, leading to a demise of ovarian function similar to premature ovarian failure in humans. Although these studies highlight roles of oocyte-specific PTEN in regulating follicular function, another recent study shows that granulosa cell–specific deletion of *Pten* leads to repressed structural luteolysis and enhanced proliferation of granulosa cells resulting to increased follicle growth, ovulation rate and litter size and prolonged corpora luteal lifespan. It has been shown that the PI3K/PDK1, PI3K/AKT/S6K1, PI3/rpS6 cascade in primary oocytes controls ovarian aging by regulating the survival of primordial follicles. Furthermore, deletion of Tsc1 or Tsc2 specifically from oocytes leads to a global activation of all primordial follicles around the time of puberty, ending up with follicular depletion in early adulthood and causing premature ovarian failure (POF) in mice (Adhikari *et al.,* 2009, Adhikari *et al.,* 2010). Furthermore, Tanaka *et al.,* 2012 have recently showed that conditional deletion of TSC1 by a knock-in allele of the anti-Müllerian hormone type 2 receptor (*Amhr2*) driving Cre expression and subsequent activation of mTOR in granulosa cells and in oviductal and uterine stromal cells affects fertility in female mice. Heterodimeric complex of tuberous sclerosis complex 1 (TSC1 or hamartin) and TSC2 (or tuberin) is one of the most important sensors involved in the regulation of mTORC1 activity. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth and proliferation in response to diverse signals. mTOR is part of two distinct multiprotein complexes: rapamycin sensitive mTOR complex 1 (mTORC1) and rapamycin insensitive mTORC2. The effects of rapamycin treatment of mice *in vivo* are consistent with

effects on follicle growth to ovulatory stages. mTOR inhibition appears to slow the growth of the follicle in two ways: increased cell cycle arrest in the G1 stage of the cell cycle (as previously seen in several different cell types), and via the induction of mitotic anomalies in granulosa cells (Yaba *et al.,* 2008). mTOR is also involved in the meiotic maturation of the oocyte. Indeed, rapamycin inhibits spindle migration and asymmetric division during mouse oocyte maturation via mTOR-mediated small GTPase signaling pathways.

Thus, in the oocyte, the PI3K signaling pathway is essential for the survival (through PDK1, Akt, mTORC1, S6K1 and RPS6) and to maintain the quiescence (through PTEN, Foxo3 and TSC1/TSC2) of primordial follicles as indicated in Fig. 3. In granulosa cells it regulates the proliferation and differentiation of granulosa cells in response to gonadotropins suggesting a role in the cyclic follicle recruitment. In the oocyte, PI3K seems to not be determinant for meiotic resumption.

PI3K signaling and ovarian cancer

In ovarian cancer, the PI3Kinase/Akt/mTOR activity is frequently elevated, leading to make the cancer more aggressive and facilitating to spread to other organs. Abnormalities of phosphatidylinositol 3 kinase (PI3K) signaling have been detected in both type I and type II ovarian cancers (for review (Bast and Mills, 2012)). Cells having increased PI-3 kinase activity possess mutated PTEN and have elevated levels of PKB and PDK1 activity, which in turn activates several protein serine/threonine kinases, including PKC, ribosomal S6 kinase, SGK, Rho kinase and PAK1, all of which are associated with increased invasion and metastasis. Moreover, Akt, the most extensively studied downstream target of PDK1, has also been shown to be overexpressed in ovarian carcinomas (Arboleda *et al.,* 2003). The first indication of aberrations targeting the PI3K pathway in ovarian cancer was the demonstration of AKT2 amplification. In culture of ovarian cancer cells, rapamycin (inhibitor of TORC1) decreased proliferation and activation of TORC1 targets such as inhibition of the phosphorylation of S6 (protein involved in protein synthesis). Hence, the use of mTOR inhibitors (rapamycin and its derivatives) is considered to be a good target for ovarian cancer therapy. Neverthess, rapamycin presents some undesirable properties including poor water-solubility, that's why several analogues have been developed such as tensirolimus and everolimus, two synthetic ester analog of rapamycin promising target for anticancer therapy. Theses agents have shown remarkable inhibition of cell proliferation, tumor angiogenesis, and abnormal cell metabolism against ovarian cancers *in vitro* and *in vivo*. Indeed, everolimus delayed ovarian tumor development in a transgenic mouse model developing spontaneous epithelial ovarian cancer. Hence, even if all untreated transgenic mice developed advanced ovarian carcinoma, only 30% of mice administrated with everolimus developed early ovarian carcinoma. Both tensirolimus and everolimus inhibit downstream signalling of mTOR and lead to cell cycle arrest and apoptosis.

Thus, strong associations of deregulated PI3K signaling pathways with ovarian carcinoma have also been well established. To maintain optimal activities of PI3K signaling within the ovary seems to be crucial for maintaining normal development and physiology of the ovary.

Role of peroxisome proliferator-activated receptors in ovarian follicle and cancer

The peroxisome proliferator-activated receptors (PPARs) are other metabolic sensors that are largely involved in the follicle development. They are transcription factors and members of the nuclear receptor superfamily (Feige *et al.,* 2006). The three closely related PPAR isoforms identified as PPARα, PPARβ and PPARγ are encoded by separate genes and fulfill specific functions. As transcription factors requiring activation, the PPARs modulate the expression of target genes in the cytoplasm or in the nucleus in response to ligand binding. Physiological ligands of PPARs include fatty acids and their derivatives, leukotrienes and prostaglandins.

In several species including rat, all three PPAR isotypes are detected in the ovary. PPARγ, which has been studied more extensively than the other two isotypes, is detected in the mouse, rat, pig, sheep, cow, bull and human ovary (reviewed in (Komar, 2005)). PPARγ is expressed strongly in the granulosa cells of rat, mouse, and sheep, as well as in oocytes from cattle, zebrafish, *Xenopus*, and human. PPARγ is detected in different classes of follicles (primary/secondary to preovulatory follicles) and its expression increases with the development of follicles. After the LH surge, PPARγ mRNA expression is downregulated. Results from a study by Cui *et al.,* (2002) indicated that PPARγ plays an important role in normal ovarian function. Indeed, using *cre/loxP* technology, the expression of PPARγ was disrupted in the ovary, rendering 1/3 of the females sterile, and the remaining females sub-fertile (Cui *et al.,* 2002). Activation of PPARγ by natural and synthetic ligands (rosiglitazone) in the granulosa cells appears to regulate the

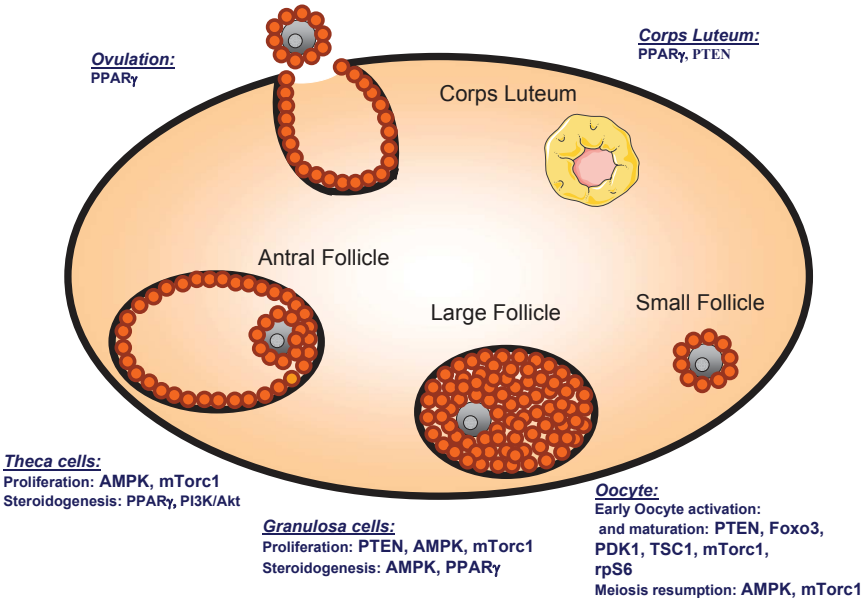

Fig. 4. Some examples of the involvement of AMPK, PI3K/Akt and PPARγ signaling pathways in the different ovarian functions and cell compartments.

synthesis of steroid hormones (Froment *et al.,* 2003). Thus, PPARγ may be indirectly involved in oocyte maturation via the granulosa cells. PPARs may also limit the synthesis of estradiol by reducing production of androgenic precursors by theca cells due to the expression of PPARs in these cells. Indeed, disruption of PPARγ gene in the ovary using *cre/loxP* technology led to female subfertility (Cui *et al.,* 2002). On the other hand, PPARs may be directly involved in oocyte maturation and ovulation. Indeed, it has been reported that rosiglitazone, a synthetic PPARγ ligand, at 100μM stimulates AMP-activated protein kinase (AMPK) and enhances the meiotic resumption of mouse oocytes. Furthermore, granulosa cell-specific deletion of PPAR gamma in mice results in marked impairment of ovulation due to defective follicular rupture (Kim *et al.,* 2008). PPARγ is also involved in processes that are critical to normal ovarian function such as angiogenesis, inflammation, and cell cycle control, indicating that PPARγ may be an important player regulating ovarian gene expression.

Peroxisome proliferator-activated receptors and ovarian cancer

In cancer biology, PPARγ is the most intensively studied PPAR isoform. It is expressed in high levels in different cancer including colon, breast, bladder, prostate, head and neck, cervical and endometrial cancer. PPARγ protein is significantly increased in malignant ovarian tumours (grade 1, 2 and 3) compared to benign tumours and normal ovaries (Zhang *et al.,* 2005). These findings suggest an involvement of PPARγ in the onset and development of ovarian carcinoma. PPARγ heterozygous knockout mice (PPARγ+/-) have an increased susceptibility to develop mammary, ovarian and skin cancer after administration of a carcinogen compared to wild-type mice, suggesting that PPAR-γ might also provide a protective effect against ovarian cancer development. PPARγ agonists have been studied for their potential use as cancer therapeutic agents. Ciglitazone and troglitazone cause a decrease in proliferation of three human epithelial ovarian cancer cell lines (Ovcar3, CaOv3, and Skov3), and two human ovarian carcinoma cell lines (ES-2 and PA-1) mainly through cell cycle arrest. In the ovarian carcinoma cells, ligand-mediated PPARγ activation suppress COX-2 expression via the NFκB pathway suggesting that high expression of PPARγ and low expression of COX-2 might play an important role in inhibiting ovarian carcinogenesis. Treatment with CA (clofibric acid), a ligand for PPARα or pioglitazone, a ligand for PPARγ significantly induces apoptosis in ovarian cancer cells suggesting that these ligands could be promising agents towards a novel therapeutic strategy against ovarian cancer (Shigeto *et al.,* 2007). Despite, PPARβ protein is expressed in normal ovaries and ovarian cancer, a higher expression of PPARβ may be related with the tumor stage and metastasis of epithelial ovarian carcinoma.

Conclusions

The adipokines, leptin, adiponectin, resistin, but also visfatin, chemerine and apelin produced by adipose tissue and altered with obesity, clearly influence energy homeostasis but also undoubtedly affect female fertility. More precisely, several *in vivo* and *in vitro* studies showed the presence of these adipokines and/or their receptors in the different ovarian cells and suggest that they could directly affect the processes of ovarian steroidogenesis and ovulation. These adipokines mediate their actions by activating different signaling pathways including AMPK, PI3K/Akt and PPARs that are already known to play a crucial role in the ovarian development and follicular atresia, which regulate production of germ cells (Fig. 4). Some of these signaling pathways and adipokines are deregulated in many tumors, including ovarian ones. Thus, they could be considered as attractive targets to be investigated in the development of new therapeutic strategies for the ovarian cancers but also for reproductive pathologies such SOPK.

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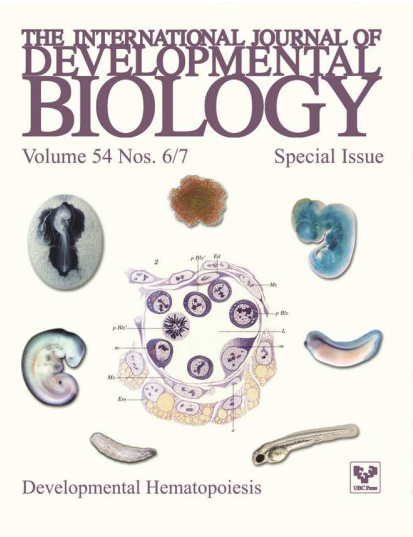

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The effect of nutrition and metabolic status on the development of follicles, oocytes and embryos in ruminants

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The impact of nutrition and energy reserves on the fertility of ruminants has been extensively described. However, the metabolic factors and the molecular mechanisms involved in the interactions between nutrition and ovarian function are still poorly understood. These factors could be hormonal (either reproductive and/or metabolic) and/or dietary and metabolic (glucose, amino acids and fatty acids). In this review, we briefly summarize the impact of those nutrients (fatty acids, glucose and amino acids) and metabolic hormones (insulin/IGF-I, growth hormone, T3/4, ghrelin, apelin and the adipokines (leptin, adiponectin and resistin)) implicated in the development of ovarian follicles, oocytes and embryos in ruminants. We then discuss the current hypotheses on the mechanisms of action of these factors on ovarian function. We particularly describe the role of some energy sensors including adenosine monophosphate-activated kinase and peroxisome proliferator-activated receptors in the ovarian cells.

Keywords: nutrients, metabolic hormones, ovary, mechanism of action, ruminant

Implications

In pursuit of sustainable and economically viable livestock production systems that meet consumer demands, farmers are under increasing pressure to maintain or increase fertility of their herds. Nutritional management both before and after calving has a great impact on fertility. Nutrition influences ovarian follicle development in ruminants possibly through changes in metabolic hormones and also through direct effects of nutrients on the ovary. These interactions can be manipulated to improve reproductive performance. Here, we summarize the impact of nutrients and metabolic hormones implicated in the development of ovarian follicles, oocytes and embryos in ruminants.

Introduction

In ruminants as in other mammals, alterations of energy and protein metabolism related to variations in the diet can influence reproductive functions at different levels of the gonadotropic axis. For example, in dairy cows, *postpartum* energy balance affects the number of follicles, their rate of growth and development, and the size of the ovulatory follicle (Lucy *et al.*, 1991a; Boland *et al.*, 2001). In this species, negative energy balance (NEB) can also attenuate LH pulse frequency (Butler, 2003). However, there is evidence that indicates that nutritional effects on the recruitment and growth of follicles are mediated mainly by direct metabolic signals to the ovary that vary with metabolic status (Lucy, 2003; Webb *et al.*, 2004). In sheep, an increase in the energy content of the diet for a few days before reproduction during the late luteal phase improves the ovulation rate (Downing and Scaramuzzi, 1991). Many inter-related factors are involved in the nutritional effects on fertility in ruminants. These factors are the nutrients themselves (fatty acids, glucose and amino acids) and the hormonal signals from various peripheral tissues (insulin (pancreas), IGF-I (liver), growth hormone (GH) (pituitary), thyroid hormones (T3/4) and adipokines (adipose tissue) such as leptin, adiponectin and resistin)). In the present study, we will describe the effects of these factors on the ovary (follicles and oocytes) and embryos in ruminants.

Effect of fatty acids

In this part of the review, we will detail the effects of non-esterified fatty acids (NEFAs) and also the effects of dietary supplementation with fat on ovarian function.

NEFAs

In cattle, NEB is an important risk factor for delayed *postpartum* cyclicity (Beam and Butler, 1999). Follicles emerging

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before an NEB nadir have reduced growth and produce less oestradiol, and therefore require more time and a larger size to achieve a secretion rate of oestradiol capable of triggering the LH surge and ovulation (Beam and Butler, 1999). It has been suggested that the harmful effects of NEB on ovarian function are exerted through decreased LH-pulsatility and reduced circulating concentrations of IGF-I, insulin and glucose (Beam and Butler, 1999). However, metabolic alterations associated with NEB may also affect ovarian function by acting directly on the ovary. Increased lipolysis of body fat also occurs during periods of NEB, causing an elevation in the blood concentrations of NEFAs (Rukkwamsuk *et al.*, 2000). For example, during periods of NEB, total serum concentrations of palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids are increased (Rukkwamsuk *et al.*, 2000). Although there was no significant uptake of NEFAs by the bovine ovary in one study (Rabiee *et al.*, 1997), others have reported that in cattle increased concentrations of NEFAs in blood were reflected in the ovarian follicular microenvironment (Leroy *et al.*, 2004 and 2005). Thus, on balance, NEFAs may affect follicular growth and fertility by acting directly on the different ovarian cells, including the oocyte within the growing follicle, and may also affect the development of the fertilized oocyte, morula and early blastocyst.

Several reports show that elevated concentrations of plasma NEFAs and in the range of concentrations frequently observed in the follicular fluid of the dominant follicle in dairy cows were associated with reduced *in vitro* developmental competence of the oocyte (Jorritsma *et al.*, 2004, Leroy *et al.*, 2005, Aardema *et al.*, 2011) and compromised the viability of the bovine granulosa cells (Vanholder *et al.*, 2005), but with a positive effect on the production of oestradiol by granulosa cells (Vanholder *et al.*, 2005). A recent study has shown that exposure of the maturing oocytes to elevated concentrations of NEFAs has a negative impact on fertility not only through a reduction in the developmental capacity of the oocyte but also through compromised quality, viability and metabolic capacity of the early embryos (Van Hoeck *et al.*, 2011). The same laboratory suggests that these negative effects of NEFAs on the embryo could be a consequence of modified energy metabolism and particularly, mitochondrial β-oxidation of fatty acids in the oocyte (Van Hoeck *et al.*, 2013). A recent study has shown that bovine cumulus cells are able to protect maturing oocytes from potential harmful effects of increased local concentrations of fatty acids by intracellular storage of lipids (Aardema *et al.*, 2013).

In conclusion, the current state of knowledge suggests that nutritional states that result in high-circulating concentrations of NEFAs can, and often do, compromise development of the dominant follicle and also that of the early embryo, and thus diets or metabolic states that favour high concentrations of NEFAs should be avoided during the cycle of conception and the early postconception period.

Supplementation with dietary fat

Several reviews have reported that dietary supplementation with fat influences reproduction in cows through effects on follicle growth and steroid production, oocyte maturation and embryo development (Mattos *et al.*, 2000; Wathes *et al.*, 2007; Santos *et al.*, 2008; Thatcher *et al.*, 2011). The precise composition of the lipid supplement is crucial to these effects. For example, concerning the polyunsaturated fatty acids (PUFAs), the ratio of n-6 to n-3 may play an important role in reproduction in sheep and cattle (for review, Gulliver *et al.*, 2012).

Effects on the follicle development. In the dairy cow, a diet enriched with long-chain fatty acids increases the number of medium follicles (6 to 9 mm) and the diameter of the preovulatory follicle (Lucy *et al.*, 1991b). These positive effects of long-chain fatty acids on the size of the dominant follicle have been confirmed in several studies (Mattos *et al.*, 2000; Robinson *et al.*, 2002; Ambrose *et al.*, 2006; Bilby *et al.*, 2006). In addition, different effects were observed on the follicular growth between monounsaturated fatty acids (MUFA) and polyunsaturated (PUFA) fatty acids: PUFAs promoted follicular growth to a greater extent than did MUFAs (Bilby *et al.*, 2006).

Several studies have suggested that supplementation of cattle with PUFAs increased the plasma concentrations of steroid by stimulating ovarian production of steroid hormones through various mechanisms, including increased availability of lipoprotein–cholesterol (Hawkins *et al.*, 1995; Lammoglia *et al.*, 1996), modulation of prostaglandin synthesis (Knickerbocker *et al.*, 1986), and the direct stimulation of ovarian steroidogenesis (Staples *et al.*, 1998). In another study on cows, it was shown that diets enriched in n-6 PUFAs increased the secretion of progesterone by the granulosa cells (Wehrman *et al.*, 1991). Although progesterone production appears *in vivo* to be lower with n-3 and higher with n-6, the exact mechanism through which n-3 and n-6 modulate progesterone and oestradiol is unclear (for review, Gulliver *et al.*, 2012). In dairy cows, diets enriched with fatty acids (both MUFAs and PUFAs) significantly increased the concentration of cholesterol in plasma, follicular fluid and the corpus luteum (Staples *et al.*, 1998). It is well known that cholesterol is the precursor for the synthesis of progesterone by the ovarian cells. Thus, higher fertility in cattle fed diets enriched with PUFAs may be associated with increased circulating concentrations of progesterone in the luteal phases, both before and after artificial insemination. Prostaglandins are important regulators of parturition, and in ruminants they are responsible for the regression of the corpus luteum, leading to a new oestrous cycle. In cows, oestradiol had stimulatory effects on uterine secretion of prostaglandin F2α (PGF2α) (Knickerbocker *et al.*, 1986), and consequently may increase the sensitivity of the corpus luteum to PGF2α, which would promote regression of the corpus luteum. Thus, decreased plasma concentrations of oestradiol could prevent premature regression of the corpus luteum and therefore early embryonic mortality. Furthermore, the type of PUFA will also affect the synthesis of PGF2α. Indeed, dietary PUFA intake inhibits PGF2α production in bovine endometrial explants, with a more pronounced effect

following n-6 rather than n-3 supplementation (for review, Wathes *et al.*, 2007).

Effects on the cumulus–oocyte complex (COC) and the embryo. Compared with rodents, the oocytes and embryos of farm animals including cattle contain relatively high levels of intracellular lipids, with triglycerides being the major component. For example, the mouse oocyte contains 4 ng of triglyceride, whereas the cow oocyte contains 63 ng, and the sheep oocyte contains 89 ng (McEvoy *et al.*, 2000; Sturmey *et al.*, 2009). Fatty acids are an endogenous source of energy during oocyte maturation; several lines of evidence support the idea of fatty-acid oxidation as a source of essential ATP for oocyte maturation and for early development of the embryo. For example, the triglyceride content of the bovine oocytes decreased during *in vitro* maturation (IVM), as well as after fertilization (Ferguson and Leese, 1999). In addition, lipase activity was increased in the bovine oocytes following IVM (Cetica *et al.*, 2002). In the oocytes from mice, inhibition of β-oxidation during the IVM of oocytes prevented the AMP-activated, protein kinase-mediated resumption of meiosis in the mouse (Downs *et al.*, 2009), and in cattle it impaired the developmental competence of oocytes (Dunning *et al.*, 2010). Furthermore, Paczkowski *et al.* (2013) have shown that the oxidation of fatty acids is essential for nuclear maturation of the oocytes in mice, cattle and pigs (Paczkowski *et al.*, 2013).

Dietary fatty acids altered the composition of fatty acids in the cumulus cells, granulosa cells and oocytes that may also have consequences on oocyte quality (Kim *et al.*, 2001). For example, in ewes, the number and quality of oocytes was increased when animals were fed a diet enriched in PUFAs (saponified fish oil) 74.3% *v.* 57%; (Zeron *et al.*, 2002). Moreover, in these animals, there was an increase in the proportion of long-chain fatty acids in the plasma and cumulus cells (Zeron *et al.*, 2002). In dairy cows, another study has shown that a diet enriched in a mixture of saturated and unsaturated fats affected oocyte development and maturation (Fouladi-Nashta *et al.*, 2007). The two fatty acids linoleic acid (LA) and linolenic acid (ALA) are both found in the plasma and follicular fluid of cows (Childs *et al.*, 2008), and it has been reported that supplementation of bovine oocytes with ALA during IVM resulted in an increased maturation rate, a higher yield of blastocysts and the production of better-quality blastocysts (Marei *et al.*, 2009). Opposing results have been observed for supplementation with LA by the same group (Marei *et al.*, 2009). Indeed, they reported that treatment of bovine COCs with physiological concentrations of LA affected the molecular mechanisms that control nuclear maturation of oocytes, leading to a decreased proportion of oocytes reaching the MII stage after 24 h in culture, and the inhibition of subsequent early embryo development (Marei *et al.*, 2010). Further studies from this group revealed that LA induced alterations in the distribution of mitochondria and their activity, associated with increased concentrations of reactive oxygen species (Marei *et al.*, 2012).

Thus, the current state of knowledge suggests that PUFAs could promote the growth of follicle and could increase granulosa cell steroidogenesis in cattle. However, the ratio of n-6 to n-3 in ruminant diets is particularly important in determining the relative availability of the precursors for eicosanoid formation. The effects of PUFA on the oocyte and embryo development are unclear and need more investigations.

Effect of glucose

The published evidence suggests that the adverse effects of systemic treatment with insulin on reproductive function do not occur if the circulating concentrations of glucose are maintained at physiological levels (Downing *et al.*, 1999), and concentrations of glucose that are either above or below the normal physiological range can have deleterious effects on fertility. For example, one study (Downie and Gelman, 1976) suggested that low-circulating glucose may be responsible for infertility in cows, and in another study pregnancy rate was higher in cows with high-circulating concentrations of glucose compared with cows with low-circulating concentrations of glucose; however, there was a trend for pregnancy rate to decline in cows with very high concentrations of circulating glucose (Pehrson *et al.*, 1992). Finally, Selvaraju *et al.* (2002) reported that insulin and glucose concentrations were higher in cows that subsequently became pregnant than in non-pregnant animals (Selvaraju *et al.*, 2002). All of this evidence suggests that fertility of cows is strongly influenced by the insulin glucose system.

Downing *et al.* (1999) stated that there is a synergism between insulin and glucose at the ovarian level, and it is likely that the effects of short-term nutrition on ovulation rate in ewes may be mediated by direct ovarian actions of insulin and glucose (Downing *et al.*, 1999). Mammals primarily rely on intracellular oxidation of glucose and fatty acids to provide the energy necessary to support most physiological processes. Although peripheral tissues may utilize both glucose and fatty acids, the ovary uses glucose as its principal source of energy (Rabiee *et al.*, 1999; Scaramuzzi *et al.*, 2010). Glucose is available for cellular oxidation and energy production by entering the cells passively when its circulating concentrations are high and also actively at low concentrations, with the latter implying a role for insulin (Wade and Schneider, 1992). It seems that the effect of glucose on fertility is primarily related to its properties as a metabolic fuel.

Within the follicle, glucose can be metabolized by four different pathways: (i) by glycolysis to produce ATP and pyruvate or lactate; (ii) by the pentose phosphate pathway to provide precursors for the synthesis of purine nucleotides and NADPH for various biosynthetic pathways including antioxidant defence; (iii) by the hexosamine biosynthetic pathway to provide substrates for the glycosylation of proteins and the synthesis of the hyaluronic acid required for cumulus expansion; and (iv) by the polyol pathway producing sorbitol and fructose whose roles in follicular function remains largely unknown (for a review see Collado-Fernandez *et al.* (2012)). In cattle, the role of these four pathways for the utilization of glucose during the growth and development of follicles and

their oocytes is not clearly known. Nowadays, it is believed that the follicle has well-developed systems to sense glucose and nutritional status (Webb *et al*., 2004; Garnsworthy *et al*., 2008; Scaramuzzi *et al*., 2010). Different glucose transporters, including the GLUT family, are expressed in the oocyte, the somatic cells of the follicle and in the early embryo, and the expression of some of them is controlled by steroids and insulin (Purcell and Moley, 2009). This system allows the follicle to regulate its growth and development, mainly by altering FSH-induced effects on the synthesis of oestradiol by the granulosa cells, in accordance with the availability of glucose (Webb *et al*., 2004; Scaramuzzi *et al*., 2010).

The role of glucose is essential in determining the quality of the oocyte (Sutton-McDowall *et al*., 2010) because it is its main energy source. In sheep, glucogenic treatments improve oocyte quality, evaluated by the kinetics of their *in vitro* development and by the production of blastocysts (Berlinguer *et al*., 2012). However, even if glucose is the preferred energy substrate for the cumulus cells (Sutton-McDowall *et al*., 2010), the oocytes of most mammalian species consume little glucose, with pyruvate being the usual energy substrate (Eppig, 1976; Rieger and Loskutoff, 1994). In general, oocytes take up pyruvate efficiently and have a lower capacity for glucose transport, as well as limited expression and activity of some glycolytic enzymes (reviewed by Purcell and Moley, 2009; Sutton-McDowall *et al*., 2010). Thus, oocytes rely on the uptake of CC-derived oxidizable substrates, principally pyruvate for the synthesis of ATP. However, ATP and glucose can also be directly transferred to oocytes from GC/CC through gap junctions (Wang *et al*., 2012).

Effect of amino acids

In contrast with fatty acids and glucose, there are few studies reporting the effects of amino acids on ovarian functions in ruminants. Downing *et al.* (1995) intravenously infused a mixture of branched-chain amino acids in sheep and observed an increased ovulation rate, whereas Garnsworthy *et al.* (2008) reported that diets with a high content of leucine increased plasma concentrations of insulin in dairy cows, but did not alter their follicular dynamics (Garnsworthy *et al*., 2008). In dairy heifers, an association was observed between the total concentration of free amino acids in the plasma and oocyte cleavage, suggesting that dietary amino acids could mediate the oocyte quality (Rooke *et al*., 2009); however, Sinclair, *et al.* (2000) also showed that in heifers excessive levels of protein in the diet negatively affected the subsequent development of oocytes *in vitro* (Sinclair *et al*., 2000)

In comparison with carbohydrate metabolism, and despite their important roles, there is little information about amino-acid uptake and metabolism by follicles and oocytes, especially during the earlier stages of folliculogenesis in cattle. Studies measuring amino acids in follicular fluid and the reproductive tract in mice have provided valuable information on the availability of substrates, reporting significantly higher levels of some amino acids in the follicular fluid compared with the reproductive tract (Harris *et al*., 2005). In cattle, LH has been shown to increase glutamine oxidative metabolism by oocytes and COCs (Zuelke and Brackett, 1993). Furthermore, the addition of glutamine to bovine IVM media promoted the nuclear maturation of oocytes (Bilodeau-Goeseels, 2006), although supplementation of defined IVM media with non-essential and essential amino acids increased the levels of maternal mRNA in the oocytes and enhanced embryo development (Watson *et al*., 2000). Finally, Hemmings *et al.* (2012) quantified the amino-acid profiles (i.e. rates of depletion and accumulation) of bovine oocytes at MII, following IVM (Hemmings *et al*., 2012). Glutamine, arginine and asparagine were all depleted at the highest rates, whereas alanine and glycine accumulated in the media (Hemmings *et al*., 2012). Similar to studies carried out on embryos, oocytes with a higher potential for development after IVF and those of lesser potential had different amino-acid profiles (Hemmings *et al*., 2012). Overall, the quality of oocytes and embryos was related to amino-acid turnover; those with the highest turnover of amino acids had the poorest quality. For example, oocytes that failed to cleave depleted more glutamine, released more alanine and had a greater depletion of total amino acids compared with oocytes that cleaved (Hemmings *et al*., 2012). Furthermore, these data were used to predict fertilization and cleavage potential (Hemmings *et al*., 2012). These studies provide further evidence of the importance of amino-acid metabolism to the developmental competence of oocytes.

Nutrient sensors

Metabolic hormones

The effects of acute changes in the dietary intake on ovarian activity have been correlated with changes in the circulating concentrations of metabolic hormones, including insulin, IGF-I, GH, the thyroid hormones, triiodothyronine (T3), and thyroxine (T4), ghrelin, apelin and the adipokines (leptin, adiponectin, resistin), and there is an extensive literature describing the effects of diet on the circulating concentrations of these metabolic hormones. Here, we have focused more on the action of the metabolic hormones on ovarian functions in ruminants.

Insulin (pancreas) and IGF-I (liver). Insulin and IGF-I are produced by the pancreas and liver, respectively, and are often proposed as signalling molecules linking metabolism to fertility (Diskin *et al*., 2003). They interact with the reproductive axis at the level of the central nervous system as well as in the follicle itself. Here, we only described some effects of insulin and IGF-I on the ruminant ovary.

Components of the insulin and IGF systems in the ovary. The insulin system consists of insulin itself and the insulin receptor (IR), which mediates the action of insulin. The IR has a high degree of homology with the IGF-I receptor (IGF-IR), and IGF-IR and IR hybrid receptors have been reported (Dupont and LeRoith, 2001). The IGF system is composed of

several members, including IGF-I and IGF-II, two receptors (IGFR-1 and IGFR-2) and at least six binding proteins (insulin-like growth factor binding proteins: IGFBP-1, -2, -3, -4, -5 and -6) (Monget *et al.*, 2002). The essential components of both the insulin and IGF systems are all present in the follicle in ruminants (Table 1). The IGFBPs are present in biological fluids and act by inhibiting or potentiating the action of the two IGFs (IGF-I and IGF-II) in target cells (Monget *et al.*, 2002). The regulation of the bioavailability of IGF-I by IGFBPs is necessary for the co-ordinated development *in vitro*, of the bovine oocyte and follicle (Thomas *et al.*, 2007). The receptors for insulin and IGF-I belong to the same subfamily of receptor tyrosine kinases with two extracellular α-subunits and two trans-membrane β-subunits. They have structures that are highly homologous and they induce a number of similar intracellular signalling pathways (Dupont and LeRoith, 2001). Upon activation by ligand binding, both IR and IGF-1R, which contain endogenous tyrosine kinase activity, phosphorylate several intracellular substrates such as the insulin receptor substrate (IRS) proteins (IRS-1 through -6) and Shc, leading to the activation of at least two signalling pathways, PI3K/AKT and MAPK. In ruminants, IRs and IGF-1Rs are widely distributed throughout all ovarian compartments, including the follicles (Spicer *et al.*, 1994; Shimizu *et al.*, 2008) and luteal cells (Einspanier *et al.*, 1990). In bovine granulosa and theca cells, the expression of the mRNA for IR changes considerably during development from the preantral to the pre-ovulatory stage of folliculogenesis (Shimizu *et al.*, 2008). Similarly, the expression of IGF-1R in bovine granulosa and theca cells is increased during the final stages of follicular development and decreased at the onset of atresia (Armstrong *et al.*, 2000). Because IGF-I and/or insulin have an essential role in the final stage of follicle development, it has been suggested that abnormal concentrations of these metabolic hormones lead to follicular dysfunction, resulting in excessive atresia or the formation of follicular cysts (Braw-Tal *et al.*, 2009).

The effects of insulin on follicular function. Numerous studies have shown the importance of insulin as a signal mediating the effects of acute changes in nutrient intake on follicle dynamics in cattle (Webb *et al.*, 2004). The infusion of insulin into beef heifers increased the diameter of the dominant follicle (Simpson *et al.*, 1994). *In vitro* research has shown dose-dependent stimulatory effects of insulin on the proliferation and steroid synthesis of the bovine granulosa cells and theca cells; these effects can be exerted via their direct stimulatory actions on the follicle as well as by increased local responsiveness of the follicle to FSH and LH (Spicer *et al.*, 1994).

The effects of IGFs on follicular function. IGF-I is a hormone that modulates the maturation of the dominant follicle during the first follicular wave *postpartum* (Beam and Butler, 1998), and circulating IGF-I in ovulatory cows at the first follicular wave *postpartum* is higher than that in anovulatory cows, regardless of parity (Beam and Butler, 1998). As for insulin, *in vitro* research has shown dose-dependent stimulatory effects of IGF-I on the proliferation and steroid synthesis of bovine granulosa cells and theca cells; these effects can be exerted via their direct stimulatory actions on the follicle as well as by increased local responsiveness of the follicle to FSH and LH (Spicer and Echternkamp, 1995). In the bovine granulosa cells, IGF-I induced the upregulation of steroidogenic and apoptotic regulatory genes via the activation of phosphatidylinositol-dependent kinase/AKT (Mani *et al.*, 2010). The addition of IGF-I to the culture medium improved the *in vitro* development of caprine preantral follicles (Magalhaes-Padilha *et al.*, 2012).

The effects of insulin and IGF on the oocyte and embryo. Both IGF-1R and IR are also present in the bovine oocytes and embryos (Nuttinck *et al.*, 2004). Several studies have observed that COCs treated with IGF-I, alone or in combination with either epidermal growth factor or angiotensin II, showed increased cumulus expansion, improved rates of nuclear maturation and enhanced metabolism of pyruvate (Lorenzo *et al.*, 1994; Stefanello *et al.*, 2006). The intra-ovarian administration of IGF-I was able to improve the developmental capacity of oocytes from pre-pubertal cattle (Oropeza *et al.*, 2004). A short *in vivo* exposure of oocytes to a supra-physiological IGF-I microenvironment increased the *in vitro* proliferation of the inner cell mass cell during the transition from morula to blastocyst (Velazquez *et al.*, 2012). In bovine blastocysts, insulin acted as a survival factor, blocking apoptosis, whereas IGF-I stimulated the development of blastocyst and also total cell number (Byrne *et al.*, 2002).

Collectively, these data indicate that maintaining the concentrations of IGF-I and insulin within a normal physiological range is required for proper ovarian follicular function and embryo development.

GH (anterior pituitary gland). GH produced by the adenohypophysis exerts effects on almost every organ of the body, including the ovary, either directly after binding to specific GH receptors (GHR) or indirectly after binding to hepatic GHR, and stimulating the production of hepatic IGF-I (Webb *et al.*, 2004). Treatment of cattle with exogenous GH has significant effects on follicular development (Gong *et al.*, 1993) and the function of the corpus luteum (Lucy *et al.*, 1999). GH can selectively stimulate particular populations of follicles; for example, in heifers, it inhibited the development of the preovulatory follicle but stimulated the growth of the second-largest follicles (Lucy *et al.*, 1994). The possibility that GH can act at ovarian sites is suggested by the detection of GH-binding activity, GHR immunoreactivity and expression of the mRNA encoding GHR in the ovarian tissue in cows (Kolle *et al.*, 1998). GH receptors have been detected in the granulosa cells, thecal cells and luteal cells (Kolle *et al.*, 1998) (Table 1), and the abundance of the mRNA encoding GHR in the bovine ovary varied in a cell-specific way during the ovarian cycle (Kolle *et al.*, 1998). However, the involvement of GH in the physiological mechanisms underlying nutritional influence on ovarian function in the cattle can be attributed to the stimulatory action of GH

Table 1 *The expression of various metabolic factors in the ovary and embryo of ruminants*

	Follicle			Corpus luteum	Embryo	References
Metabolic hormone	Theca	Granulosa	Oocyte			
Insulin/IGF-I receptors	+	+	+	+	+	Armstrong *et al.* (2000), Shimizu *et al.* (2008)
GH receptor	+	+	nr (ruminants) + (human)	+	nr (ruminants) + (human)	Kolle *et al.*, (1998), de Prada and VandeVoort (2008)
Thyroid hormone receptor (TR)	nr	nr (ruminants) + (human and mouse)	nr (ruminants) + (human and mouse)	nr (ruminants) + (human and mouse)	nr	Zhang *et al.* (1997)
Ghrelin and GHS-R	+	+	+	+	+	Miller *et al.* (2005), Du *et al.*, (2009 and 2010)
Leptin and Ob-R	+	+	+	+	+	Spicer and Francisco (1997), Boelhauve *et al.* (2005), Dayi *et al.* (2005), Paula-Lopes *et al.* (2007)
Adiponectin, AdipoR1 and R2	+	+	+	+	+	Maillard *et al.* (2010), Tabandeh *et al.* (2010)
Resistin	+	+	+	+	nr	Maillard *et al.* (2011), Spicer *et al.* (2011)
Apelin and APJ	+	+	nr	+	nr	Shirasuna *et al.* (2008), Shimizu *et al.* (2009)
PPARγ	−/+	+	+	+	+	Lohrke *et al.* (1998), Mohan *et al.* (2002), Froment *et al.* (2003)
AMPK	+	+	+	+	+	Tosca *et al.* (2007a and 2007b), Pikiou *et al.* (2013)

GH = growth hormone; GHS-R = growth hormone secretagogue receptor; nr = not reported in the literature; PPAR = peroxisome proliferator-activated receptors.

on the hepatic synthesis of IGF-I, and a direct action of GH on the follicle remains to be demonstrated convincingly (Webb *et al.*, 2004).

T3 and T4 (thyroid gland). In cattle, greater nutrient intake after calving increased the concentrations of T4 in the plasma (Ciccioli *et al.*, 2003), similar to the response in non-lactating cows (Delavaud *et al.*, 2002). Follicular fluid from the bovine ovaries contains free fractions of thyroid hormones that suggests a possible role for these hormones in the regulation of follicular function. In super-ovulated Brahman cows, induced hypothyroidism improved weight gain and body score condition, increased ovarian response to FSH, and affected the ovulation, fertility and secretion of progesterone (Bernal *et al.*, 1999). In cows, the physiological status of the bovine antral follicles (i.e. dominant *v.* subordinate) may impinge on the accumulation of T4 in the follicular fluid (Ashkar *et al.*, 2010), and hormonally induced ovarian hyperstimulation increased the circulating levels of free T4 and the follicular fluid content of total T4 (Mutinati *et al.*, 2010). Furthermore, the thyroid hormones (T3 and T4) directly altered *in vitro* steroidogenesis in the bovine granulosa and theca cells (Spicer *et al.*, 2001). A recent study has shown that the supplementation of IVM media with T3 can have a beneficial effect on the kinetics of embryo development (Costa *et al.*, 2013). In the ovaries of human and mice, thyroid hormone receptors (TRα-1, TRα-2 and TRβ-2) are present in the oocytes, cumulus, granulosa and luteal cells (Zhang *et al.*, 1997) (Table 1). Thus, thyroid hormones may have direct stimulatory effects on the ovarian function and embryo development in cattle.

Ghrelin (stomach). Ghrelin is primarily secreted from the X/A-like cells of the oxyntic gland in the stomach. The X/A-like cells constitute a distinct population of endocrine cells in the oxyntic mucosa that make up ~20% of all endocrine cells in the oxyntic gland. Ghrelin is also present in several other tissues, including the reproductive tissues of sheep and cattle (Miller *et al.*, 2005; Deaver *et al.*, 2013). The functional ghrelin receptor, GH secretagogue receptor 1 A (GHS-R1A), belongs to a large family of rhodopsin-like, G-protein coupled, 7-transmembrane domain receptors (Howard *et al.*, 1996). Ghrelin is an acute regulator of feed intake; circulating ghrelin concentrations increase during fasting or NEB, and exogenous administration of ghrelin stimulates feed intake in rats and cattle (Bradford and Allen, 2008). In these species, Ghrelin also influences energy metabolism and increases metabolic efficiency. Thus, it appears that ghrelin is a key metabolic component involved in the physiological response to energy deprivation.

Ghrelin and its receptor are present in the ovary of the adult and foetal sheep (Miller *et al.*, 2005) (Table 1). In the sheep ovary, ghrelin and its mRNA are present throughout the oestrous cycle with the highest levels observed during the luteal phase (Du *et al.*, 2009). More precisely, detectable concentrations of ghrelin are found in the oocytes and thecal cells at all stages of follicular development, the granulosa cells of antral follicles and in all developmental stages of the

corpora lutea (Du *et al*., 2009) (Table 1). In the sheep, ghrelin promotes *in vitro* oocyte maturation via the ERK1/2 pathway (Bai *et al*., 2012) and *in vitro* formation of blastocysts (Wang *et al*., 2013). In the Holstein heifers, the expression of ghrelin was limited to the granulosa cells in the ovarian follicles, suggesting that the follicular distribution of ghrelin is species dependent (Deaver *et al*., 2013). In the dairy heifers, ghrelin and GHS-R1A and their mRNAs are also present in the ampulla and isthmus of the oviducts and the uterus (Deaver *et al*., 2013).

However, despite the presence of ghrelin and its receptor in various parts of the reproductive tract, its effects, if any, *in vivo* on fertility in ruminants are not known.

Adipokines (adipose tissue). The traditional view of the adipose tissue as a passive store of dormant triglycerides, which are mobilized during NEB, has been challenged by recent research that has shown that the adipose tissue also secretes a variety of hormones collectively known as the adipokines (Tersigni *et al*., 2011). Leptin, adiponectin and resistin are the three best studied adipokines in relation to ovarian function in ruminants. These hormones are significant mediators of energy metabolism and they have all been implicated as mediators of nutritional influences on reproduction (Tersigni *et al*., 2011).

Leptin. A positive association between nutrient intake and concentrations of leptin in the plasma has been reported in sheep (Delavaud *et al*., 2000) and cattle (Delavaud *et al*., 2000 and 2002). The effects of leptin can be divided into two main categories: the first is a central effect in the brain after binding to its long-form receptor (Ob Rb), and the second a peripheral effect (including the ovary). The Ob Rb is the primary mediator of the action of leptin because it is the only isoform that is capable, after ligand binding, of relaying full downstream signalling along the signal transduction pathways. There are at least six isoforms of Ob-R that are expressed in several organs including the ovary, where leptin can directly exert regulatory actions.

Leptin and its receptor are present in the follicle; the concentration of leptin in the follicular fluid is related to atresia in the small follicles (Dayi *et al*., 2005) (Table 1). In the ovarian follicles, the highest coexpression of the leptin/Ob-R system was observed in the theca and in the interstitial and granulosa cells of small follicles with E2 concentration <0.5 ng/ml (Sarkar *et al*., 2010). The actions of leptin on ovarian and follicular function appear to be inhibitory; it inhibits in a dose-dependent manner, insulin-induced production of progesterone and oestradiol by cultured granulosa cells from small and large bovine follicles (Spicer and Francisco, 1997). There is also evidence for this inhibitory effect of leptin *in vivo* on oestradiol in sheep during the follicular phase of the cycle (Kendall *et al*., 2004). Similarly, leptin inhibited the insulin-induced production of progesterone and androstenedione by the thecal cells *in vitro* (Spicer and Francisco, 1998). Leptin has also a weak inhibitory effect on gonadotropin- and/or IGF-I-induced steroidogenesis of the bovine thecal and granulosa cells (Spicer *et al*., 2000). In the bovine species, leptin and its receptor transcripts are present in the corpus luteum and their expression decrease during luteal regression (Sarkar *et al*., 2010). Leptin increased *in vitro* IGF-I-induced luteal progesterone production (Nicklin et al., 2007).

Leptin enhanced bovine oocyte maturation and improved the ability of the bovine oocyte to sustain embryonic development. Indeed, the addition of leptin to the IVM medium enhanced meiotic maturation and embryo development of calf oocytes, and improved the quality of embryos derived from these oocytes (Jia *et al*., 2012). van Tol *et al*. (2008) confirmed that leptin enhanced meiotic maturation of bovine oocytes (van Tol *et al*., 2008). Furthermore, they showed that this effect was cumulus cell-mediated (van Tol *et al*., 2008), whereas Paula-Lopes *et al*. (2007) observed that leptin enhanced both oocyte maturation and their developmental capacity by mechanisms that were both cumulus cell-independent and -dependent (Paula-Lopes *et al*., 2007). In contrast, Cordova *et al*. (2011) observed that the addition of leptin to the IVM medium used to mature oocytes from prepubertal cows did not increase the development potential of the oocytes (Cordova *et al*., 2011).

In conclusion, it seems that elevated circulating concentrations of leptin that are a result of hunger and poor nutrition are associated with a pattern of inhibition of the ovarian function but, paradoxically, with improved oocyte quality. However, the positive effects of leptin on oocyte quality were obtained *in vitro*, and the relationship between the circulating concentrations of leptin and oocyte quality *in vivo* are not known for ruminants. In mice, leptin is essential for normal preimplantation and/or implantation processes, but it is not required for pregnancy and parturition once implantation is established (Malik *et al*., 2001).

Adiponectin. Adiponectin is a protein produced mainly by the white adipose tissue. In humans, the level of expression of its mRNA varies depending on the location of the adipose tissue, and it is lower in the visceral adipose tissue than in the subcutaneous adipose tissue (for review, Kadowaki and Yamauchi (2005). The expression of adiponectin is higher in thin than in obese subjects (for review, Kadowaki and Yamauchi (2005). Adiponectin is involved in lipid and carbohydrate metabolism and seems to have an important role in the pathophysiology of obesity and type 2 diabetes (for review, Kadowaki and Yamauchi (2005). In healthy humans and cattle, the blood concentrations of adiponectin are of the order of 5 to 30 mg/l, which represents 0.01% of the total plasma proteins (for review, Kadowaki and Yamauchi (2005); Giesy *et al*. (2012). In humans and rodents, low concentrations of plasma adiponectin are predictive of insulin resistance and type 2 diabetes. In these species, dietary factors can modulate circulating adiponectin (Reis *et al*., 2010). In this latter review, it was concluded that diets rich in saturated fat reduced the concentration of adiponectin, whereas diets rich in PUFAs and supplementation with n-3 PUFAs increased both gene expression of adiponectin and its concentration in the circulation. However, an effect of dietary components on the

circulating concentration of adiponectin has not yet been reported in ruminants. In the plasma from humans, mice and cattle, adiponectin circulates in a variety of high molecular weight (HMW) dimeric and trimeric forms. In dairy cows during late pregnancy, adiponectin circulated almost exclusively as HMW complexes, and this pattern was unaffected by early lactation (Giesy *et al.*, 2012). Decreasing plasma concentrations of adiponectin with advancing lactation are in good agreement with decreased insulin sensitivity that also occurs with advancing lactation. These are the expected adaptive responses to the increased glucose requirements of the lactating mammary gland, which preferentially diverts glucose from the adipose tissue, skeletal muscle and other tissues to the mammary gland (Mielenz *et al.*, 2013). In humans and rodents, exogenous adiponectin improved insulin sensitivity by the activation to its receptors AdipoR1 and AdipoR2 (for review, Kadowaki and Yamauchi (2005); they are receptors with seven transmembrane domains and they have an inverted topology compared with normal G-protein-coupled receptors. Once activated, AdipoR binds an adapter protein (APPL), which in turn activates adenosine monophosphate-activated kinase (AMPK) (Kadowaki and Yamauchi, 2005). AMPK is a major component of the signalling pathway that regulates the metabolic effects of adiponectin (Kadowaki and Yamauchi, 2005).

Some evidence suggests that adiponectin could regulate ovarian function and affect the embryo at very early stages of pregnancy during the pre-implantation period (Palin *et al.*, 2012). In the bovine species, adiponectin and its receptors are present in different follicular cells (oocytes, theca, granulosa and cumulus cells) and luteal cells (Maillard *et al.*, 2010) (Table 1). Moreover, it has been shown that the physiological status of the ovary is related to the pattern of expression of adiponectin and its receptors in follicular and luteal cells from the bovine ovary (Tabandeh *et al.*, 2010). Indeed, the expression of adiponectin, AdipoR1 and AdipoR2 was higher in the theca and cumulus cells and the oocytes of dominant follicles compared with those of atretic follicles during both the follicular and luteal phases (Tabandeh *et al.*, 2012). In our laboratory, we have shown that adiponectin decreased insulin-induced steroidogenesis and increased IGF-I-induced proliferation of the bovine granulosa cells in culture possibly by a mechanism that involved the ERK1/2 MAPK pathway (Maillard *et al.*, 2010). Lagaly *et al.* (2008), have also observed an inhibitory effect of adiponectin on bovine steroidogenesis but only in the theca cells. Concerning the oocyte and embryo, we have shown that adiponectin did not modify either oocyte maturation or embryo development *in vitro* in bovine species.

The available data on the physiological role of adiponectin in the follicle suggest that it has no direct actions and that its action appears to be the modulation of ovarian actions of insulin and IGF-I. However, more research is required to confirm or refute these suggestions. The role of adiponectin *in vivo* in the early embryonic development of cattle remains to be investigated.

Resistin. Resistin is a protein of 108 amino acids in humans and 114 amino acids in mice belonging to the family of 'resistin-like molecules' or 'FIZZ' (found in the inflammatory zone) (Schwartz and Lazar, 2011). It consists of two homodimers linked by disulphide bridges (Schwartz and Lazar, 2011). In mice, resistin is produced by adipocytes, whereas in humans it is produced by macrophages in the bone marrow and transported to adipocytes (Kaser *et al.*, 2003; Patel *et al.*, 2003). Very little information is currently available on the mode of action of resistin. No receptor has been clearly identified, and the signalling pathway used by resistin remains obscure. Recent tudies suggest that resistin could bind to a receptor tyrosine kinase known as receptor tyrosine kinase-like orphan receptor (ROR1) in murine pre-3T3-L1 adipocytes or to the receptor known as TLR4 (Toll-like receptor 4) in the mouse hypothalamus (cited in Reverchon *et al.*, 2013).

In cattle, resistin has not been extensively studied. It is produced by the adipose tissue and in greater amounts in lactating compared with non-lactating cattle (Komatsu *et al.*, 2003). Resistin is also present in the bovine ovaries (Table 1). Furthermore, human recombinant resistin decreases basal but not IGF-I-induced progesterone and oestradiol production by the bovine granulosa cells (Maillard *et al.*, 2011). Spicer *et al.* (2011), have also observed that resistin preferentially inhibited steroidogenesis in the granulosa cells from small follicles and inhibited proliferation of the granulosa cells from large follicles, suggesting that the ovarian response to resistin is altered during follicular development. The little data available suggest that resistin may be involved in the nutritional regulation of ovarian function and fertility in ruminants and on this basis is worthy of further investigation.

Other adipokines including chemerin and visfatin, known to regulate insulin sensitivity, have been sequenced and are present in the cattle ovaries. However, the physiological effects, if any, of these newer adipokines in ovarian function remains to be determined.

Apelin (stomach). Apelin is a peptide isolated from extracts of the bovine stomach (Tatemoto *et al.*, 1998). It has been identified as the endogenous ligand of the human orphan, APJ receptor (Tatemoto *et al.*, 1998). Apelin is derived from a larger precursor, preproapelin, the cDNA for which has been cloned in humans, cattle, rats and mice (Tatemoto *et al.*, 1998). Plasma apelin is upregulated in rodents and humans during obesity, there is a strong relationship between adipocyte-secreted apelin and insulin levels (Boucher *et al.*, 2005). In goats, feeding caused a slight increase in plasma apelin concentrations, and this increase was enhanced by water deprivation (Sato *et al.*, 2012).

In the bovine ovary, the apelin/APJ system is involved in mechanisms regulating angiogenesis during follicular maturation and luteal development (Schilffarth *et al.*, 2009). Apelin and APJ are expressed in the bovine granulosa cells, and progesterone increases the expression of APJ in these cells (Shimizu *et al.*, 2009) (Table 1). Theca cells also

have both apelin and APJ and their expression is increased by exogenous LH (Shimizu *et al.*, 2009). These latter authors suggest that the apelin/APJ system could play a role during follicle selection and dominance in cows. The apelin–APJ system is also present in the bovine corpus luteum (Shirasuna *et al.*, 2008) where it could be involved in the maturation of corpus luteum and the luteolytic cascade as a regulator of intra-luteal arterioles (Shirasuna *et al.*, 2008).

If similarly to the other gut hormones, such as ghrelin, apelin is nutritionally regulated, then it could be another potential regulator of nutritional influences on ovarian function in ruminants.

AMPK

Most of the metabolic hormones discussed above modulate the phosphorylation of AMPK (Tosca *et al.*, 2008). AMPK is a metabolic sensor of the energy state of a cell: it has a key role in the regulation of lipid, carbohydrate and protein metabolism in the peripheral and central tissues (Tosca *et al.*, 2008). It is a heterotrimeric serine/threonine kinase that is composed of a catalytic α subunit and two regulatory subunits β and γ subunits, each encoded by a different gene and for each of which, there are two or three isoforms (Hardie and Carling, 1997). These different isoforms allow the possible formation of 12 $\alpha\beta\gamma$ complexes. The balance of these complexes depends on the tissue (Hardie and Carling, 1997). The activity of AMPK is regulated allosterically by the binding of AMP or ATP onto the γ regulatory subunit by phosphorylation of the α subunit at threonine 172 by an AMPK kinase (either LKB1 (serine/threonine kinase 11) or CaMKKβ (calmodulin-dependent kinase kinase-β)) and its subsequent dephosphorylation by a phosphatase (protein phosphatase-1, protein phosphatase 2A or protein phosphatase 2C) (Hardie and Carling, 1997). The main mechanism for the activation of AMPK is a decrease in the intracellular ratio of ATP to AMP. AMPK can be activated by certain physiological conditions (exercise, stress) by the action of metabolic hormones (leptin, adiponectin, ghrelin) and by the pharmacological agents, 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR), metformin and the thiazolidinediones (Hardie and Carling, 1997). It regulates energy homeostasis maintaining a constant concentration of intracellular ATP by stimulating catabolic pathways and inhibiting anabolic pathways (Hardie and Carling, 1997). In the ovary, AMPK controls cellular proliferation and survival and also reproductive functions such as ovarian steroidogenesis and oocyte maturation (Dupont *et al.*, 2008).

In cattle, AMPK has been identified in different cell types of ovarian follicles (oocyte, cumulus cells, granulosa and theca) and in the corpus luteum (Tosca *et al.*, 2007a and 2007b; Gallet *et al.*, 2011) (Table 1). In cultured bovine granulosa cells, AMPK inhibited the secretion of progesterone and/or oestradiol in response to AICAR or metformin (Tosca *et al.*, 2007a). This decrease can be explained by the inhibition of the steroidogenic enzymes, 3β-hydroxysteroid-dehydrogenase (3βHSD) and P450scc (p450 side-chain cleavage) and of steroidogenic acute regulatory protein (StAR) and also by inhibition of the MAPK/ERK pathway (mitogen-activated protein kinase/extracellular-regulated kinase) (Tosca *et al.*, 2007a). In these cells, the activation of AMPK in response to metformin also reduced cell growth and protein synthesis, MAPK ERK1/2 signalling and P90RSK phosphorylation in response to IGF-I (Tosca *et al.*, 2010). *In vivo*, we showed that the infusion of glucose (Gallet *et al.*, 2011) or feeding lupins (Zouaidi, unpublished data) inhibited AMPK in sheep follicles, suggesting that good nutrition or glucose can block the inhibitory actions of AMPK in the follicle.

Some studies have also established a link between AMPK and meiotic maturation of the oocyte (Downs and Chen, 2006). In cattle, similar to the pig, pharmacological activation of AMPK blocked nuclear maturation of the oocyte (at prophase of the first meiotic division or at the germinal vesicle stage (Bilodeau-Goeseels *et al.*, 2007; Tosca *et al.*, 2007b), whereas in mice AMPK improved the resumption of meiosis by accelerating rupture of the nuclear membrane (Bilodeau-Goeseels, 2011) or breakdown of the germinal vesicle (Downs and Chen, 2006). Although there are some differences among species, AMPK appears to be important for the breakdown of the germinal vesicle during nuclear maturation of the oocyte. Recently, Pikiou *et al.* (2013) suggested that activation of AMPK in response to metformin could decrease the ability of bovine oocytes to cleave following *in vitro* fertilization (Pikiou *et al.*, 2013). Although the role of AMPK in nuclear maturation could be to promote early embryonic development, it is unclear whether AMPK is involved in cytoplasmic maturation of oocytes. In cows, AMPK is also found in the corpus luteum. In cultured bovine luteal cells, the activation of AMPK regulated LH-induced progesterone secretion (Hou *et al.*, 2010).

Thus, AMPK could control ovarian steroidogenesis in the granulosa cells, oocyte maturation and embryo development, and it could also be involved in the function of the corpus luteum. An improved understanding of the role of AMPK during meiosis in the oocytes will facilitate the control of this process *in vitro*, resulting in increased developmental competence and increased efficiency of procedures for the *in vitro* embryo production.

Peroxisome proliferator-activated receptors (PPARs)

PPARs are nuclear transcription factors that are classified as members of the steroid hormone receptor superfamily. To date, three related PPAR isotypes have been described: PPARα, PPARβ/δ and PPARγ. The three isotypes share a high degree of homology but differ in tissue distribution and ligand specificity (Berger and Moller, 2002). The PPAR family (α, β/δ and γ) integrates energy regulation with lipid and glucose metabolism and affects insulin sensitivity (Kota *et al.*, 2005). Furthermore, PPARγ expression in the adipose tissue is nutritionally regulated (Vidal-Puig *et al.*, 1996).

There are a variety of natural and synthetic agents that activate PPARs. Among its natural ligands, there are long-chain fatty acids, especially PUFAs, including linoleic and arachidonic acids, and some derivatives including 15-deoxy-delta 12, 14-prostaglandin J2 (PGJ2), eicosapentaenoic acid and acid

9- and 13-hydroxyoctadecadienoic acid (Tontonoz *et al*., 1995; Kliewer *et al*., 1997). Prostaglandins activate all members of the PPAR family, with a preferential activation of PPARβ by PGJ2 (Forman *et al*., 1997). Various synthetic ligands, such as industrial plasticizers (phthalates), nonsteroidal anti-inflammatory drugs, fibrates (a class of drugs used to treat hyperlipidemia), and thiazolidinediones, activate these receptors (Forman *et al*., 1997). However, there is a ligand specificity for each form of PPAR. For example, fibrates (WY-14 643, clofibrate) show a strong affinity for PPARα, but at higher concentration they can also activate PPARγ. Thiazolidinediones (troglitazone, ciglitazone, pioglitazone and rosiglitazone) activate PPARγ.

The PPARs form and function as heterodimers with retinoid-X-receptor (RXR). Once the ligand binds (e.g. long-chain fatty acids, fibrates, thiazolidinediones) to the ligand-binding domain, it produces a covalent modification of the PPAR structure (Waku *et al*., 2009) activating the nuclear receptor. The activated PPAR/RXR binds to a specific DNA sequence (PPAR research response element) in the promoter region of specific target genes inducing or repressing their expression.

The three PPAR isoforms have been detected in the ovary and PPARγ is the one most extensively studied in the ovarian tissue (Table 1). This is because, in addition to the high expression level of PPARγ in the follicle of various species (for review see Froment *et al*., 2003; Dupont *et al*., 2008), its synthetic ligands and widely used as drugs treat polycystic ovary syndrome, one of the commonest ovarian dysfunctions causing infertility in women. In ruminants, PPARγ was detected in the ovine and bovine ovary (Sundvold *et al*., 1997; Lohrke *et al*., 1998; Froment *et al*., 2003). In the bovine species, transcripts for RXRα, RXRβ and PPARγ were detected at all stages of early pregnancy beginning from the unfertilized oocyte through to the hatched blastocyst (Mohan *et al*., 2002) (Table 1). In cyclic ewes, the expression of PPARγ is mainly limited to the granulosa cells of antral follicles and corpora lutea (Froment *et al*., 2003). In cows, the concentration of PPARγ in the corpus luteum increased after ovulation, and then decreased during regression of the corpus luteum (Lohrke *et al*., 1998; Viergutz *et al*., 2000). Furthermore, in mice, the conditional inactivation of PPARγ in the ovary leads to reduced fertility (Cui *et al*., 2002). This decrease is not because of an alteration in ovarian folliculogenesis, but a drop in the number of embryos implanted and probably because of decreased progesterone secretion by the corpora lutea (Cui *et al*., 2002). In bovine large lutein cells, PPARγ plays a role in the arrest of the cell cycle to maintain a differentiated state (Viergutz *et al*., 2000). Furthermore, synthetic ligands of PPARγ increased progesterone secretion by bovine lutein cells (Lohrke *et al*., 1998).

Thus, it seems that PPARγ is essential *in vivo* for the formation and maintenance of a functional corpus luteum capable of secretion of progesterone compatible with embryo implantation. One hypothesis is that PPARγ is involved in the beneficial effects of some PUFAs on the fertility of cattle.

Conclusions

In ruminants, fertility is a key parameter for the profitability of the farmers, and a positive nutritional status and good metabolic health are both associated with successful reproduction and help ensure high fertility. Some nutritional strategies could reduce the risk of metabolic disturbances and, consequently, not only improve herd health but also enhance fertility. The data presented in this review highlight the importance of specific nutrients (glucose, saturated and PUFAs, and certain amino acids) on ovarian function in ruminants. Nutrition influences ovarian follicular development in ruminants possibly through changes in metabolic hormones and also by the direct effects of particular nutrients on the ovary. Metabolic hormones that appear to have important established functions in these processes include insulin, leptin and the IGFs. Other metabolic hormones that may have important roles include GH, T3/4, ghrelin, apelin, and some novel adipokines produced by the adipose tissue, including adiponectin and resistin. The importance of these metabolic hormones is either debatable or unknown and they are interesting areas for future research. The adipokines, in particular, have emerged as potentially important regulatory factors in the fields of fertility and reproduction in human and possibly ruminants. These adipokines and their receptors are expressed in cattle ovary; however, further studies are necessary to determine whether their plasma concentrations are nutritionally regulated in ruminants. There is emerging evidence that dietary long-chain n-3 PUFAs can act as specific regulators of some reproductive processes. The molecular mechanisms of action of these PUFAs is still unknown in ruminants. However, PUFAs could mediate their action through the activation of PPARγ or/and AMPK by modulating plasma concentration of some adipokines such as adiponectin or resistin, as has been described in humans and rodents. AMPK appears to be a key signal regulating the amount of energy required for the growth of follicles, oocytes and embryos

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Expression and Regulation of INTELECTIN1 in Human Granulosa-Lutein Cells: Role in IGF-1-Induced Steroidogenesis Through NAMPT[1]

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ABSTRACT

?2 **INTELECTIN (ITLN) is an adipokine involved in the regulation of insulin sensitivity and inflammatory and immunity responses. Serum ITLN levels are lower in obese, diabetic, and polycystic ovary syndrome (PCOS) women than in control subjects. ITLN has never been studied in ovarian cells. Here, we identified ITLN1 in human ovarian follicles and investigated the molecular mechanisms involved in the regulation of its expression in response to the insulin sensitizers metformin and rosiglitazone, in human granulosa-lutein cells (hGLCs) and in a human ovarian granulosa-like tumor cell line (KGN). We also studied the effects of human recombinant ITLN1 (hRom1) on steroid production and on the activation of various signaling pathways. Using RT-PCR, immunoblotting, and immunohistochemistry, we found that INTL1 is present in human follicular cells. Using ELISA, we showed that INTL levels are similar in plasma and follicular fluid (FF) in control patients, whereas they are higher in FF than in plasma in PCOS patients. In KGN cells and hGLCs, insulin (10^{-8} M), insulin-like growth factor-1 (IGF-1; 10^{-8} M), and metformin (10^{-2} M or 10^{-3} M) increased INTL1 expression (mRNA and protein) after 12 and 24 h of stimulation. For metformin, this effect was mediated by adenosine monophosphate-activated kinase (PRKA). Furthermore, hRom1 increased nicotinamide phosphoribosyltransferase (NAMPT) expression in KGN and hGLCs. We also showed that hRom1 increased IGF-1-induced progesterone and estradiol secretion and this was associated with an increase in the STAR and CYP19A1 protein levels and an increase in IGF-1R signaling. Furthermore, all these data were abolished when NAMPT was knocked down in KGN cells, suggesting that INTL1 improves IGF-1-induced steroidogenesis** ?3 **through induction of NAMPT in hGLCs.**

adipokine, fertility, IGF-1, NAMPT, ovary, PCOS, signaling pathways

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INTRODUCTION

INTELECTIN1 (INTL1, also named OMENTIN1, endothelial lectin HL-1, and intestinal lactoferrin receptor) is an adipokine produced predominantly by visceral adipose tissue in humans and rhesus monkeys [1–4]. Mature INTL is a secretory glycoprotein consisting of 295 amino acids and N-linked oligosaccharides. It is a 120-kDa homotrimer in which 40-kDa polypeptides are bridged by disulfide bonds. A homolog of INTL1, referred to as INTL2, shares 83% amino acid identity with INTL1 [1]. The *INTL1* and *INTL2* genes are localized adjacent to each other in the human 1q22-q23 chromosomal region, which has been previously linked to type 2 diabetes mellitus in several populations. INTL1 is the major circulating form in human plasma [5]. It has been described as a calcium-dependent lectin with affinity for galactofuranosyl residues that are constituents of pathogens and dominant immunogens [6]. Thus, one of the INTL1 functions is the specific recognition of pathogens and bacterial components. Furthermore, some evidence shows that INTL1 expression is altered by inflammatory states and obesity [5, 7, 8]. INTL1 enhances insulin action [2]. It is inversely related to obesity [5] and is increased after weight loss [9], and is downregulated by insulin and glucose [10].

INTL1 has also been linked to metabolic syndrome [11], and polycystic ovary syndrome (PCOS) [10]. PCOS is one of the most common causes of female infertility, affecting 6%–10% of reproductive age women. It is characterized by irregular menses, hyperandrogenism, anovulation, and polycystic ovaries [12, 13]. Most PCOS patients are obese. Plasma INTL1 levels are higher in women than in men [5] and are decreased in women with PCOS compared with those in body mass index (BMI)-matched control subjects [10, 14]. Treatment with metformin for 3 or 6 months significantly increased serum INTL1 levels as well as the INTL1-to-insulin ratio in PCOS patients [15, 16]. INTL1 plasma levels in regular menstrual cycles are different from those in irregular menstrual cycles in PCOS patients, suggesting that INTL1 could affect the secretion of hormones that regulate ovarian and menstrual functions or affect receptors for these hormones [17]. INTL1 is expressed in adipose tissue but also in reproductive tissues including the placenta and ovary in human, monkey, and sheep [2, 6, 18]. However, the physiological roles of INTL1 in these organs, a receptor, or relevant signal transduction pathways have still to be determined.

New functions of adipokines have been described in the reproductive field and more precisely in ovarian functions [19]. Furthermore, increasing evidence shows that the dysregulated expression of adipokines plays an important role in the pathology of PCOS. Leptin and its receptor are involved in folliculogenesis, ovarian steroidogenesis, and development of dominant follicles and oocytes. Increased serum leptin concentrations have been observed in women with PCOS in comparison to those in weight-matched controls in certain studies [20–23]. Some evidence supports the fact that adiponectin (ADIPOQ) and its receptors ADIPOR1 and ADIPOR2 can modulate not only follicle growth but also embryo development in mice and humans [24]. A meta-analysis revealed that serum ADIPOQ levels are lower in women with PCOS than in BMI-matched healthy controls [25]. Resistin (RE-TN), RARRES2, and nicotinamide phosphoribosyltransferase (NAMPT) are also expressed in ovarian cells, and several studies have shown that these adipokines can modulate ovarian follicular development and steroidogenesis [26–32].

The objectives of our work were to identify INTL1 in the human follicle and to study the regulation of its expression in human granulosa-lutein cells (hGLCs; luteal granulosa cells from women undergoing in vitro fertilization, and KGN cells) in response to follicle-stimulating hormone (FSH), insulin, insulin-like growth factor-1 (IGF-1), and two insulin sensitizers, metformin and rosiglitazone. We also investigated the effects and the molecular mechanism of human recombinant INTL1 on steroid production in these cells.

MATERIALS AND METHODS

Patients

Sixty infertile nonobese women (33 ± 4 years old, with BMI <30) were recruited at the Service de médecine et Biologie de la Reproduction, CHRU de Tours, in 2011 for mechanical or male factor infertility without any known endocrinopathy (PCOS, hyperprolactinemia, hypo- and hypertrophism). Fifteen infertile nonobese women with PCOS (31 ± 4 years old, with BMI <30) were also studied. The diagnosis of PCOS was based on the criteria proposed by the ?4 2003 ASRM/ESHRE Rotterdam consensus. This study was approved by the Hospital Ethical Committee (CHRU Bretonneau, France), and the patients gave their consent and did not receive any monetary compensation for participating in the study. Only one cycle was studied from each patient. Visceral (ATv) and subcutaneous (ATsc) adipose tissues from adult women (nonobese and nondiabetic), used as the positive control, were obtained from Tebu-Bio (Le Perray-en-Yvelines, France).

Collection and Processing of Follicular Fluid Samples

Follicular fluid (FF) was collected in sterile tubes preheated to 37°C, which contained no culture medium. The entire content of the follicles with a mean diameter of ≥15 mm (between 15 and 22 mm) was aspirated. The FF samples of each follicle from individual patients were pooled. FF was collected from 30 infertile women, 15 with male and/or female tubal factor of infertility (control group) and 15 with PCOS (PCOS group). For the control group, 7 ± 2 follicles were aspirated per patient, whereas in the PCOS group, approximately 13 ± 4 follicles were aspirated per patient. Only FF free of blood contamination upon visual inspection was used. The FF samples were centrifuged at 300 × *g* for 7 min to separate cell remnants, and the supernatant was stored at −80°C in 2 aliquots for later use.

KGN Cell Culture

The human ovarian granulosa-like tumor cell line KGN was cultured in Dulbecco's minimal essential medium (DMEM)-F12 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin [Sigma]) in a 5% CO_2 atmosphere at 37°C. KGN cells were obtained in 2002 from Drs. Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan [33]. The cells are undifferentiated and maintain the physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of CYP19A1 [33].

TABLE 1. Primers used for q-RT-PCR.

Gene	Direction	Primer
NAMPT	Forward	5′-AAGAGACTGCTGGCATAGGA-3′
NAMPT	Reverse	5′-ACCACAGATACAGGCACTGA-3′
ACTB	Forward	5′-ACGGAACCACAGTTTATCATC-3′
ACTB	Reverse	5′-GTCCCAGTCTTCAACTATACC-3′
RPL19	Forward	5′-AATCGCCAATGCCAACTC-3′
RPL19	Reverse	5′-CCCTTTCGCTTACCTATACC-3′
PPIA	Forward	5′-GCATACAGGTCCTGGCATCT-3′
PPIA	Reverse	5′-TGTCCACAGTCAGCAATGGT-3′
ITLN1	Forward	5′-ACTACGACATCCAGGCCAAG-3′
ITLN1	Reverse	5′-GCAGTGGTGCTCAGTGTTAC-3′
RETN	Forward	5′-TGGTGTCTAGCAAGACCCTG-3′
RETN	Reverse	5′-GCAGTGACATGTGGTCTCGG-3′
RARRES2	Forward	5′-AGACAAGCTGCCGGAAGAGG-3′
RARRES2	Reverse	5′-TGGAGAAGGCGAACTGTCCA-3′

Hormones and Reagents

Purified ovine FSH-20 (oFSH; lot no.AFP-7028D, 4453 IU/mg, FSH activity = 175 times the activity of oFSH-S1) was a gift from National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone Pituitary Program (Bethesda, MD). Recombinant human insulin-like growth factor-1 (IGF-1), insulin, and luteinizing hormone (LH) from human pituitary used for culture treatment were from Sigma. Recombinant human INTL1 was from R&D (Lille, France). Metformin was obtained from Sigma, and rosiglitazone was a gift from Dr. B. Staels (Lille, France).

Antibodies

Sheep anti-human INTL1 polyclonal antibody was purchased from R&D. Affinity-purified rabbit anti-human NAMPT (code BL2122) polyclonal antibody was purchased from Bethyl Laboratories, Inc., (Montgomery, TX). Rabbit polyclonal antibodies to IGF-1R-beta subunit (C20), phospho-MAPK3/1 (Thr202/Tyr204), phospho-MAPK14 (Thr180/Tyr182), phospho-AKT (Ser 473), and phospho-PRKA alpha Thr172 were obtained from New England Biolabs, Inc. (Beverly, MA). Rabbit polyclonal antibodies to PRKAA and IRS-1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal antibody to MAPK1 (C14) and MAPK14 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CYP11A1, STAR, and HSD3B antibodies were a gift from Dr. Dale Buchanan Hales (University of Illinois, Chicago, IL) and Dr. Van Luu-The (CHUL Research Center and Laval University, Quebec, Canada), respectively. Mouse monoclonal antibodies to vinculin and CYP19A1 were purchased from Sigma and Serotec (Varilhes, France), respectively. PY20 antibodies were obtained from BD Biosciences (Le Pont de Claix, France). All antibodies were diluted 1/1000 in Western blotting. ?5

Isolation and Culture of Human Granulosa-Lutein Cells

The hLGCs were collected from preovulatory follicles during oocyte retrieval for in vitro fertilization (IVF). The ovarian stimulation protocol and ?6 IVF and ICSI procedures used have already been reported [28, 34]. After isolation of cumulus oocyte complexes (used for IVF), FF samples were pooled ?7 and then centrifuged (400 × *g*, 10 min). To remove most of the red blood cells, the pellet was centrifuged (400 × *g*, 20 min) on a two-layer discontinuous Percoll gradient (40%, 60% in Ham's medium, Gibco-BRL; Life Technologies, Cergy Pontoise, France). The 40% fraction was collected and treated with hemolytic medium (NH_4Cl 10 mmol/l in Tris HCl, pH 7.5 [Sigma]) to remove the remaining red blood cells. Following centrifugation, the pellet was washed with fresh medium (Ham's F12), cells were counted in a hemocytometer, and cell viability was determined using Trypan Blue dye exclusion. Cells were ?8 cultured in McCoy 5A medium supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l, 0.1% bovine serum albumin, 0.1 μmol/l androstenedione, 5 mg/l transferrin, 20 μg/l selenium) and 5% fetal bovine serum. The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures were kept under a water-saturated atmosphere of 95% air/5% CO_2 at 37°C.

RNA Extraction and RT-PCR

Total RNA from hGLCs, KGN cells, and ATv and ATsc was extracted with Trizol reagent according to the manufacturer's procedure (Life Technologies

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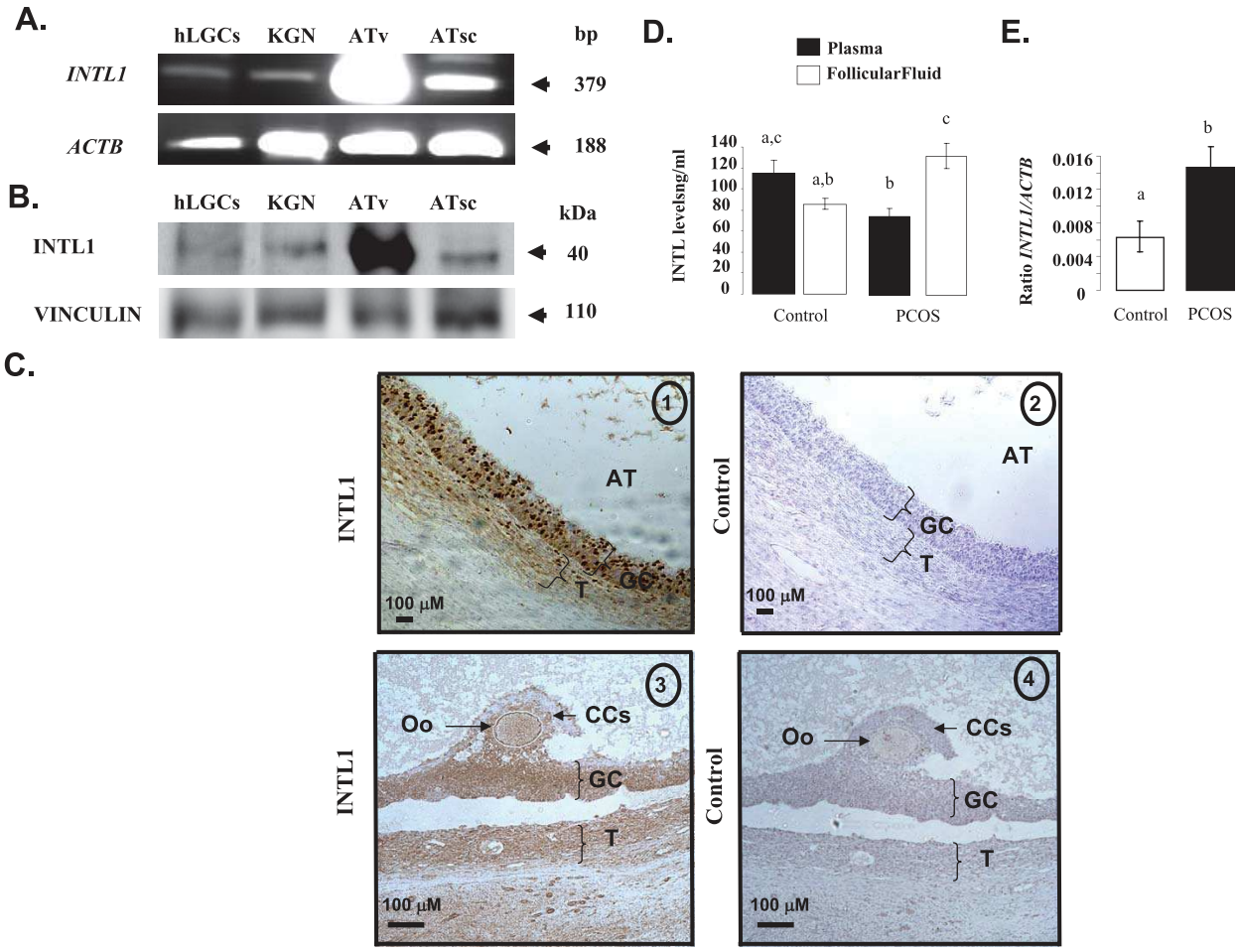

FIG. 1. Expression of *INTL1* in hGLCs and human ovarian follicles. **A**) RT-PCR assays from total RNA of hGLCs, ovarian granulosa cell lines (KGN), human visceral (ATvis), and subcutaneous (ATsc) adipose tissues were performed with primers designed to amplify one fragment of *INTL1* (379 bp) and one fragment of *ACTB* (188 bp) as the housekeeping gene (35 cycles of PCR were required to produce the signal shown). Human visceral and subcutaneous adipose tissues were used as positive controls for *INTL1* expression (mRNA [**A**] and protein [**B**], see below)). The expected sizes of the different products of RT-PCR amplification are indicated on the right. The data are representative of 4 experiments with hGLCs from 4 patients (non-PCOS nonobese) in each experiment and KGN cells from 4 different passages. **B**) Protein extracts (50 µg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with specific antibodies against INTL1. Equal protein loading was verified by a reprobing the membrane with an anti-vinculin antibody. Human granulosa-lutein cells from four different patients were used. The data are from 1 representative experiment with only 1 sample of hGLCs from 2 different patients. **C**) INTL1 localization is shown in a human ovarian follicle by immunochemistry. DAB-immunoperoxidase staining was performed with paraffin-embedded human ovary tissue by using antibodies against INTL1 (1, 3) or no primary antibodies but sheep IgG (2) or an INTL1 blocking peptide (4). Immunospecific staining is brown. Sections were counterstained with hematoxylin. INTL1 was detected in granulosa cells (GC), cumulus cells (CCs), theca cells (T), and oocytes (Oo) of ovarian follicles. AT, antrum. Bars = 100 µm. Immunohistochemistry was performed with 2 different human ovary slides from each of 4 patients. **D**) Measurement of the concentrations of INTL in plasma and follicular fluid (FF) of control and PCOS women treated with IVF. FF (□) and plasma (■) INTL levels are shown after collecting blood and FF of infertile women undergoing IVF (control n = 15 and PCOS n = 15) on the day of oocyte retrieval. Each concentration was determined by ELISA as described in *Materials and Methods*. The mean of these data is presented. Different letters indicate significant differences at $P < 0.05$. **E**) The *INTL1* gene expression was measured by quantitative real-time-PCR in hGLCs from PCOS (n = 8) and control (n = 10) patients. *ACTB* was used as the reference gene. The mean of these data are presented. Different letters indicate significant differences at $P < 0.05$.

SAS, Saint Aubin, France). Reverse transcription PCR (RT-PCR) was used to detect *NAMPT* expression in hGLCs, KGN cells, and in ATv and ATsc. Reverse transcription of total RNA (1 µg) was denatured and retrotranscripted with the reverse transcriptase Moloney murine leukemia virus (MMLV) reverse transcriptase (15 U) in a 20-µl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ), 50 pmol of oligo(dT)15 and 5 U of ribonuclease inhibitor. All samples were incubated at 37°C for 1 h. cDNAs were amplified in a 50-µl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 U of *Taq* polymerase and 2 µl of the RT mixture. PCR was performed with specific primer pairs for human *INTL1* (forward, 5′-ACTACGACATCCAGGCCAAG-3′ and reverse, 5′-GCAGTGGTGCTCAGTGTTAC-3′) and *actb* (forward, 5′-ACGGAACCA-CAGTTTATCATC-3′, and reverse, 5′-GTCCCAGTCTTCAACTATACC-3′). First, the samples were denatured at 94°C for 5 min, then 35 PCR cycles were processed (95°C, for 1 min; 58°C for 1 min; 72°C for 1 min), with a final extension step at 72°C for 10 min. PCR products were migrated on 1.5% agarose gel stained with ethidium bromide and the specificity of the amplified products was assessed by sequencing (Genome Express Co., Meylan, France). MMLV and RNase inhibitor (RNasin; were purchased from Promega, Madison, WI), and RT-PCR consumables were from Sigma.

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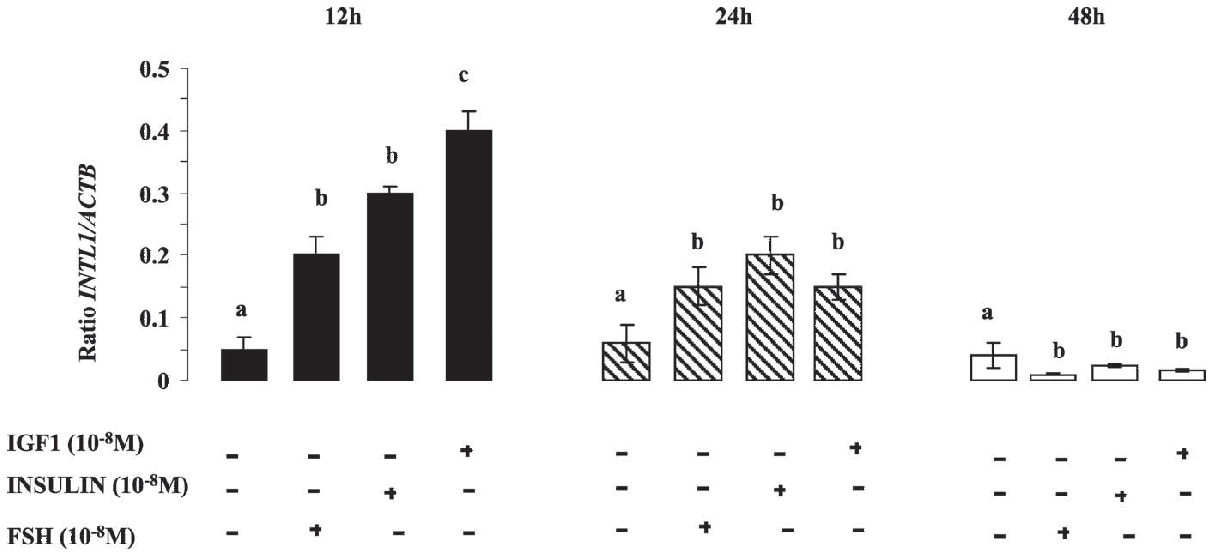

FIG. 2. Effects of IGF-1, insulin, and FSH on *INTL1* expression in human granulosa-lutein cells. *INTL1* gene expression was measured by quantitative real-time PCR in hGCs after 12, 24, and 48 h of stimulation with or without IGF-1 (10^{-8}M), insulin (10^{-8} M) and FSH (10^{-8} M). *ACTB* was used as the reference gene. The data are representative of six experiments. Each experiment used hGLCs from four patients (non-PCOS nonobese). Different letters indicate significant differences at $P < 0.05$.

Real-Time Quantitative PCR

Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers as indicated in Table 1 in a total volume of 20 μl in a MyiQ Cycle device (Bio-Rad). Samples were tested in duplicate on the same plate, and PCR amplification with water instead of cDNA was done systematically as a negative control. After incubation for 2 min at 50°C and a denaturation step of 10 min at 95°C, samples were subjected to 40 cycles (30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C), followed by the acquisition of the melting curve. Primers' efficiency (E) was performed from serial dilutions of a pool of obtained cDNA and ranged from 1.8 to 2. Three reference genes were used, ?9 *PPIA*, *RPL19*, and *ACTB*. For each gene, expression was calculated according to primer efficiency and Cq, where expression = E^{-Cq}. Then, relative expression of the *NAMPT* reference gene was analyzed. Only one reference gene is represented in the Figures.

Stable NAMPT Knockdown Using Lentiviral Vectors

Short hairpin RNA (shRNA) was designed according to rules described previously [35]. The sequence of shRNA against *NAMPT*, called NAMPT1, was 5′-GAGTGTTACTGGCTTACAA-3′, and a second shRNA, called NAMPT2 (5′-GAGTGTTACGGGGTTCCAG-3′), was from published reports [36]. Scrambled shRNA sequence was created by mutating critical bases on shRNA against *NAMPT*, namely, 5′-GAGTGTTACGGGGTTCCAG-3′. All shRNAs were subcloned into the pSicoR lentiviral vector [37], in which shRNA is driven by a U6 promoter, and a reporter green fluorescent protein (GFP) expression cassette is driven by an immediate early promoter of cytomegalovirus (CMV). We produced G protein of vesicular stomatitis virus (VSV-G) pseudotyped shRNA lentiviral vectors. Lentiviral transductions were conducted at a multiplicity of infection of 6. Two days after transduction, cells were treated with puromycin (2 μg/ml), and the stable cell line (KGN) was established.

Adenoviruses and Infection of Human Granulosa-Lutein Cells

Dominant negative PRKA adenovirus (AdN AMPK) was constructed from PRKAA1 carrying the Asp-157 to Ala (D157A) mutation as described previously [38]. Recombinant adenovirus was propagated in HEK293 cells, purified by cesium chloride density centrifugation, and stored as described previously [38]. hCGLs were infected with 20 plaque-forming units (PFU) /cell adenovirus (AdN AMPK and AdN GFP adenovirus [AdN GFP as the control]) in serum-starved McCoy 5A as previously described [39]. After 2 h, 1 additional volume of serum-starved McCoy 5A was added, and the cells were cultured for 24 h in the presence or absence of metformin (10^{-2} and 10^{-3} M). Preliminary studies revealed that within 24 h of infection (20 PFU/cell) with a GFP-expressing virus, the majority of hGLCs (>90%) expressed GFP.

Protein Extraction and Western Blotting

hGLCs purified on a Percoll gradient, KGN cells, and ATv and ATsc were homogenized in buffer A (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40) containing various protease inhibitors (2 mm PMSF, 10 μg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate) as previously described [40, 41]. Lysates were incubated on ice for 30 min and then centrifuged at 12 000 × *g* for 20 min at 4°C. The protein concentration in the resulting supernatants was then determined using the BCA protein assay. After denaturation, the samples were subjected to electrophoresis on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France). The membrane was blocked for 30 min in TBS-Tween-milk 5% and incubated for 16 h with appropriate primary antibodies at a 1/1000 final dilution. Finally the blots were incubated for 1 h and 30 min at room temperature with horseradish-peroxidase (HRP)-conjugated anti-rabbit, anti-mouse, or anti-sheep immunoglobulin G (IgG; dilution 1/5000). Proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using a G:Box SynGene (Ozyme) with GeneSnap software (release 7.09.17; Chicago, IL). Signals detected were quantified with the GeneTools software (release 4.01.02; Synegene, Fredrick, MD). The results are expressed as the intensity signal in arbitrary units after normalization as indicated in the Figure legends.

Immunoprecipitation

After normalization for protein concentration (250 μg) of KGN cell lysates, IGF-1R or IRS1 was immunoprecipitated from the supernatants by using 5 μg of appropriate antibodies at 4°C overnight. The immunocomplexes were precipitated with 40 μl of protein A-agarose for 1 h at 4°C. After two sequential washes using buffer A with a 1/2 dilution, the resulting pellets were boiled for 4 min in reducing Laemmli buffer containing 80 mM dithiothreitol. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked and probed with the various antibodies as indicated in the Figure legends.

Progesterone and Estradiol Radioimmunoassay

The concentration of progesterone and estradiol in the serum-free culture ?10 medium of KGN (scrambled, NAMPT-/-1, NAMPT-/-2, or parental KGN cells)

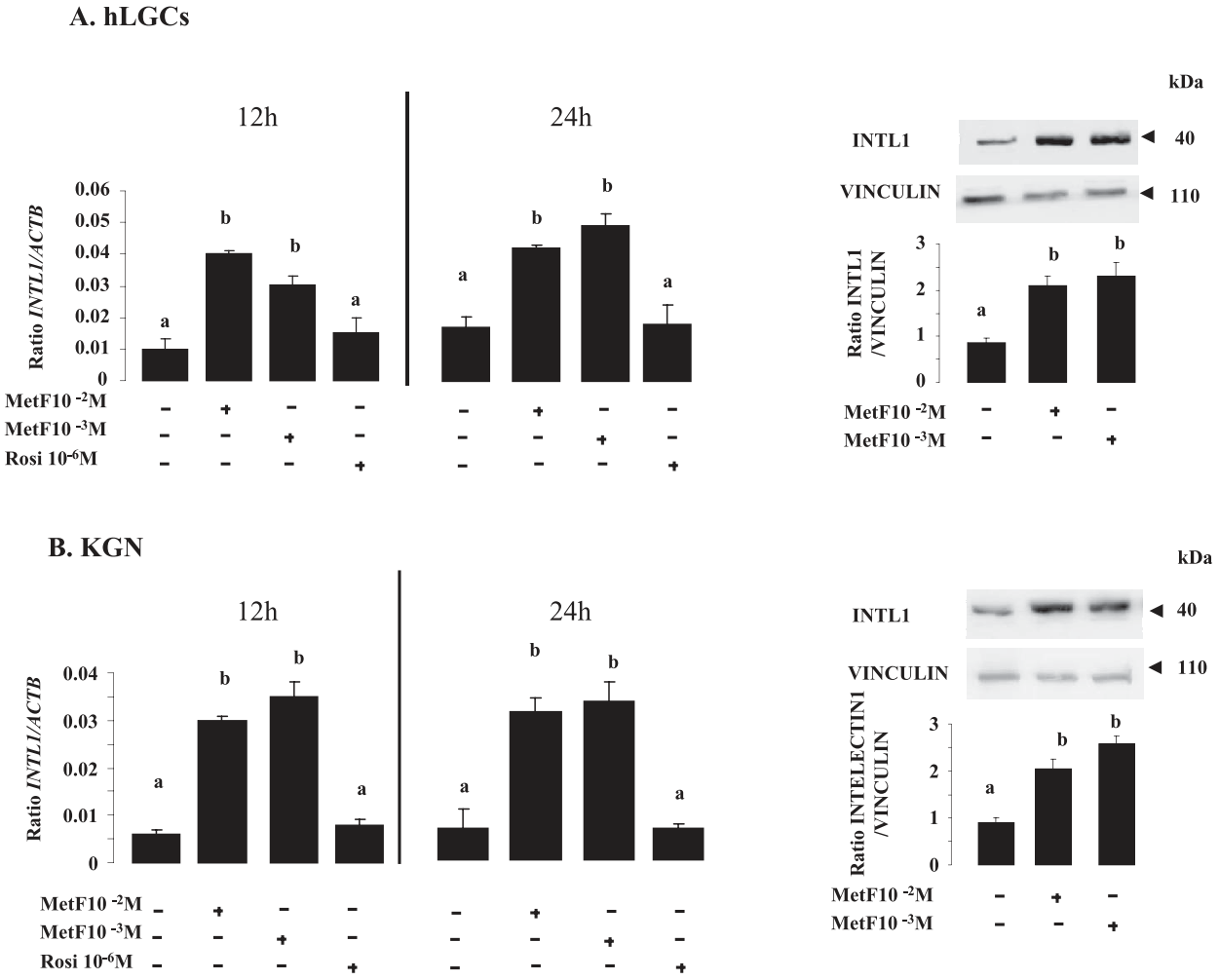

FIG. 3. Effect of metformin (MetF) and rosiglitzone (Rosi) on *INTL1* expression at the mRNA (left panel) and protein (right panel) level in hGLCs (**A**) and in KGN (**B**) cells. Left panel) *INTL1* gene expression was measured by quantitative real-time PCR in hGLCs (**A**) and KGN cells (**B**) after 12 and 24 h of stimulation with or without different doses of MetF (10^{-2} and 10^{-3} M) or Rosi (10^{-6} M). *ACTB* was used as the reference gene. The data are representative of six experiments in hGLCs and KGN cells. Each experiment used hGLCs from four different patients (non-PCOS nonobese) and KGN cells from different passages. Different letters indicate significant differences at $P < 0.05$. Right Panel) Protein levels of INTL1 were analyzed by Western blotting in hGLCs (**A**) and KGN (**B**) cells after 24 h of stimulation with or without MetF (10^{-2} and 10^{-3} M) or Rosi (10^{-6} M). Vinculin was used as the loading control. The data are representative of six experiments in hGLCs and KGN cells. Each experiment used hGLCs from four different patients (non-PCOS nonobese) and KGN cells from different passages. In each experiment, each treatment (in the presence or absence of metformin or rosiglitazone) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at $P < 0.05$.

and hGLCs was measured after 48 h of incubation with INTL1 with or without IGF-1, FSH, or LH by a radioimmunoassay protocol as previously described [40, 41]. The limit of detection of progesterone was 12 pg/tube (60 pg/well) and the intra- and interassay coefficients of variation were less than 10% and 11%, respectively. The limit of detection of estradiol was 1.5 pg/tube (7.5 pg/well), and the intra- and interassay coefficients of variation were less than 7% and 9%, respectively. Results are expressed as the concentration of steroids/cell protein concentration/well.

Immunohistochemistry

Ovary sections from women (age 34 ± 2 years, nonobese non-PCOS) obtained from Euromedex (Souffelweyersheim, France) were deparaffinized, hydrated, and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France) and then allowed to cool to room temperature. After being washed in a phosphate-buffered saline (PBS) bath for 5 min, sections were immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). After 2 washes in a PBS bath for 5 min, nonspecific background was eliminated by blocking with 5% lamb serum in PBS for 20 min, followed by incubation overnight at 4°C with PBS containing sheep primary antibody raised against INTL1 (1/100). Sections were washed twice for 5 min each time in a PBS bath and were incubated for 30 min at room temperature with a ''ready-to-use'' labeled polymer-HRP anti-sheep (Envision Plus HRP system; Dako Cytomation). The sections were then washed twice in PBS, and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (liquid DAB+substrate chromogen system; DakoCytomation). Negative controls involved replacing primary antibodies with sheep IgG or by using INTL1 blocking peptide (Antibodies-online GmbH, France).

INTL ELISA Assay

INTL levels in plasma and FF were measured using a commercially available ELISA (Biovendor, Heidelberg, Germany), according to the manufacturer's protocol, with an intra-assay coefficient of variation of <6%.

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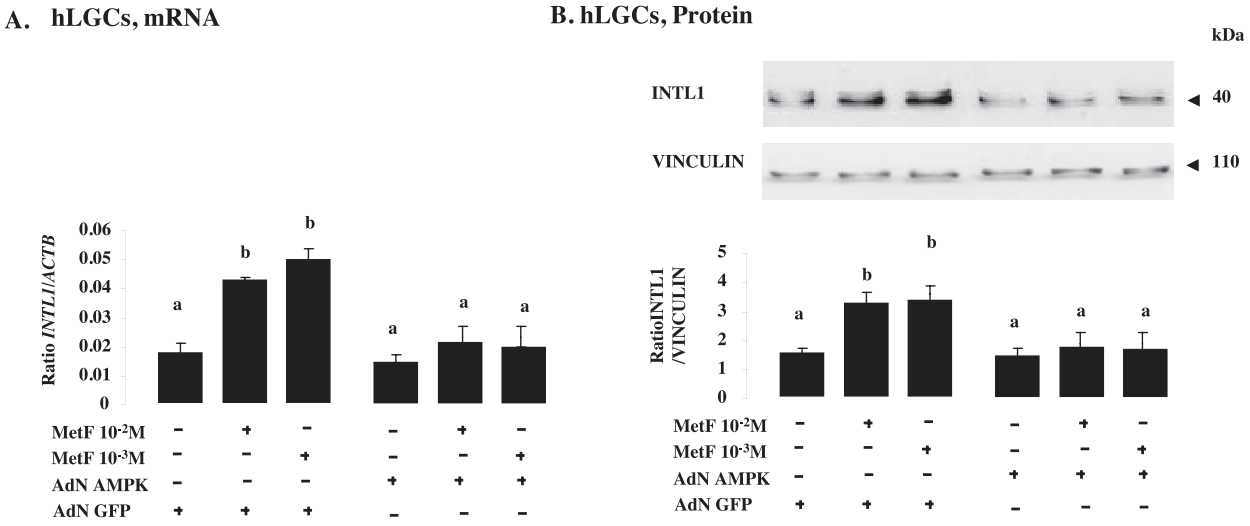

FIG. 4. Effects of the overexpression of dominant negative PRKA (AdN AMPK) on metformin-induced *INTL1* expression in human granulosa-lutein cells. Expression of *INTL1* mRNA (**A**) and protein (**B**) by human granulosa-lutein cells producing the mutant dominant negative PRKA or GFP protein in the presence or absence of metformin (10^{-2} and 10^{-3} M). Granulosa cells were infected with 100 Pfu/cell of either AdN GFP or AdN AMPK for 16 h and were then stimulated, or not, for 24 h with metformin. *INTL1* gene and protein expression levels were measured by quantitative real-time PCR and Western blotting, respectively, as described in the legend to Figure 3. The data are means ± SEM from five independent experiments. Each experiment used hGLCs from four different patients (non-PCOS nonobese) and KGN cells from different passages. Bars with different letters are significantly different ($P < 0.05$).

Statistical Analysis

All experimental results are expressed as means ± SEM. Statistical analyses were carried out using a *t*-test or one-way analysis of variance (ANOVA; for comparison of various means), and if ANOVA revealed significant effects, it was supplemented with the Fisher test. A *P* value of <0.05 was considered significant.

RESULTS

INTL1 Is Expressed in Human Ovarian Cells

We determined the expression of INTL1 in hLGCs and in the human granulosa tumor cell line KGN. As shown in Figure 1A, using RT-PCR, we amplified 1 cDNA fragment of 379 bp, corresponding to *INTL1*. We found that the transcript of *INTL1* is expressed in hGLCs and, as expected, is strongly in the visceral adipose tissue (Fig. 1A). Immunoblotting of protein extracts revealed the presence of INTL1 (40 kDa) in hGLCs and KGN cells (Fig. 1B). Immunohistochemistry with human ovarian follicle sections showed that INTL1 is present in granulosa cells but also in cumulus cells, oocytes, and theca cells (Fig. 1C). We also measured the concentration of INTL in plasma and FF of infertile control and PCOS women. As shown in Figure 1D, and as expected [14], women with PCOS had significantly lower plasma INTL levels than those of BMI-matched control subjects. Furthermore, the INTL levels were similar in plasma and FF in infertile control subjects, whereas they were was higher in FF than in plasma in PCOS patients ($P < 0.05$). We also observed that INTL1 mRNA expression was two-fold higher in hGLCs from PCOS than those from control patients (Fig. 1E). Thus, INTL1 is expressed in human ovarian follicles and more particularly in granulosa cells.

INTL1 Expression Is Regulated by Insulin, IGF-1, and FSH in Granulosa-Lutein Cells

We also determined whether insulin, IGF-1, and FSH, hormones involved in the ovarian functions, were able to regulate *INTL1* expression. Cells starved overnight in culture medium without serum were incubated for 12, 24, or 48 h with insulin (10^{-8} M), IGF-1 (10^{-8} M), or FSH (10^{-8} M). We showed that after 12 or 24 h of stimulation, these three hormones increased *INTL1* mRNA expression, whereas after 48 h of stimulation, they inhibited it (Fig. 2). We also confirmed these results at the INTL1 protein levels (data not shown). Similar results were observed in KGN cells (data not shown).

INTL1 mRNA and Protein Expression Is Increased by Metformin Through PRKA Signaling Pathways in Granulosa-Lutein Cells

We next investigated the effect of two insulin sensitizers, metformin and rosiglitazone, on *INTL1* expression in primary hGLCs (Fig. 3A) and KGN (Fig. 3B) cells. It has been reported that these two insulin sensitizers improve secondary pathologies that are frequently associated with insulin resistance such as PCOS. Overnight-starved cells in culture medium without serum were incubated for different times (12 and 24 h) with different concentrations of metformin (10^{-3} and 10^{-2} M) or rosiglitazone (10^{-6} M). These doses of metformin and rosiglitazone are known to affect human granulosa cell steroidogenesis [27]. We showed that after 12 or 24 h of stimulation, metformin increased *INTL1* mRNA expression in a dose-independent manner, whereas rosiglitazone (10^{-6} M) had no effect (Fig. 3). We next examined whether these effects of metformin on *INTL1* mRNA were also observed at the protein level. As shown in Figure 3 (right panel), metformin treatment (10^{-3} and 10^{-2} M) for 24 h resulted in a two-fold increase in the level of INTL1 protein in hGLCs (Fig. 3A) and KGN cells (Fig. 3B). Rosiglitazone treatment (10^{-6} M, 24 h) did not affect the amount of INTL1 protein (data not shown). We have previously shown that PRKA is important in the molecular mechanism of metformin action in granulosa cells [42, 43].

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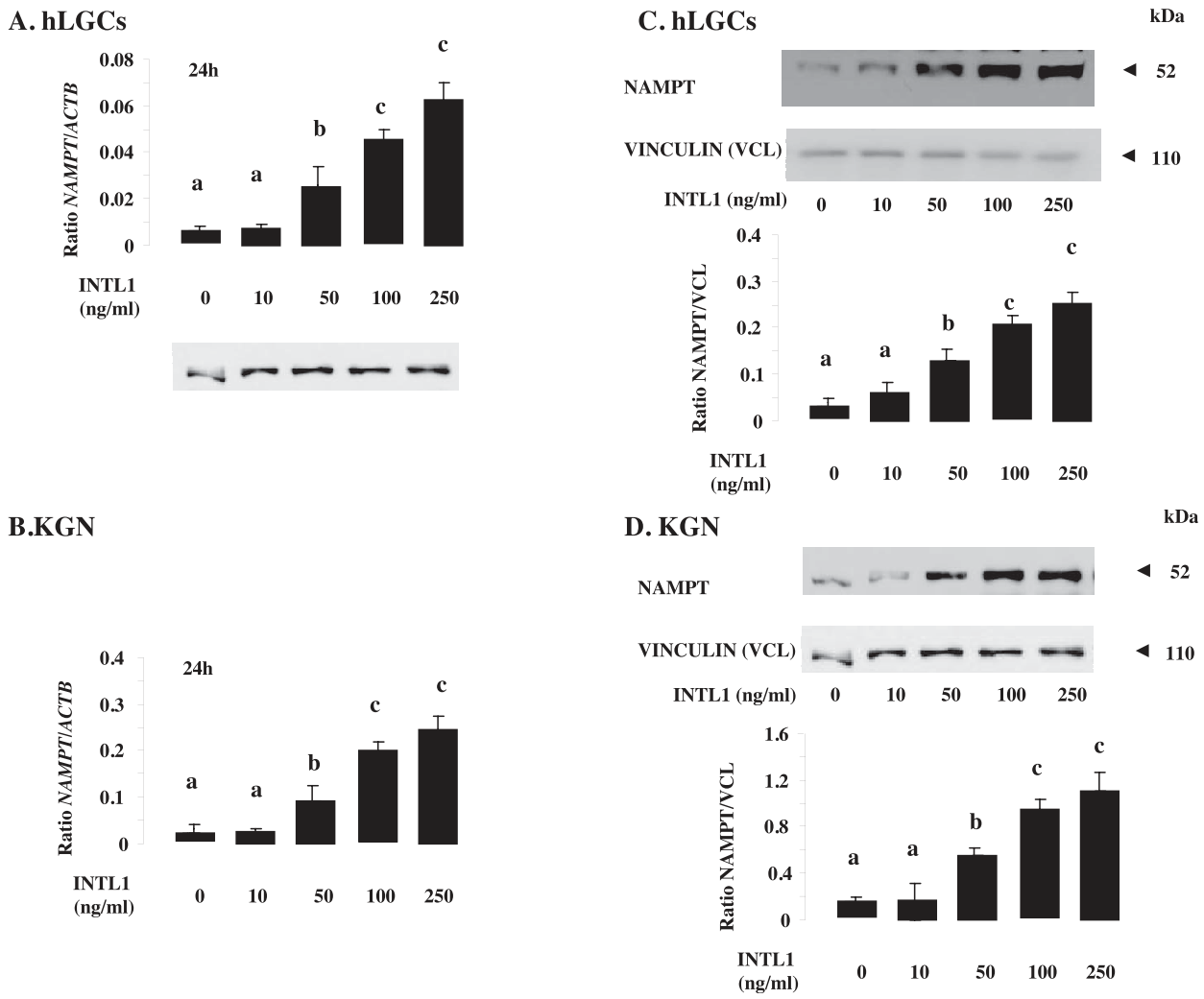

FIG. 5. Effect of human recombinant INTL1 on NAMPT expression at the mRNA (**A**, **B**) and protein (**C**, **D**) levels in human granulosa-lutein cells (**A**, **C**) and KGN (**B**, **D**) cells.

A and **B**) *INTL1* gene expression was measured by quantitative real-time PCR in hGLCs (**A**) and KGN (**B**) cells after 24 h of stimulation without or with different concentrations of human recombinant INTL1 (10, 50, 100, and 250 ng/ml). *ACTB* was used as the reference gene. **C** and **D**) Protein levels of NAMPT were analyzed by Western blotting in hGLCs (**C**) and KGN (**D**) cells after 24 h of stimulation with or without different concentrations of human recombinant INTL1 (10, 50, 100, and 250 ng/ml). Vinculin was used as the loading control. The data are means ± SEM from five independent experiments for hGLCs and KGN cells. For hGLCs, each experiment was from four different patients (non-PCOS nonobese). In each experiment, each treatment (in the presence or absence of different doses or INTL1) was applied in duplicate. Different letters indicate significant differences at $P < 0.05$.

To determine whether PRKA activation through its phosphorylation is involved in the regulation of *INTL1* expression in response to metformin in hGLCs, we used an adenoviral vector to overexpress a dominant-negative PRKA (AdN AMPK) or a GFP (AdN GFP) adenoviral vector as the control. As shown in Figure 4A, expression of AdN AMPK in hGLCs strongly reduced the metformin-induced increase in mRNA and protein *INTL1* expression (Figs. 4, A and B). The expression of AdN GFP under the same conditions did not affect the stimulatory effects of metformin. Similar results were observed in KGN cells (data not shown).

Human Recombinant INTELECTIN1 Increases NAMPT Expression in hGLCs and KGN Cells

We next examined whether human recombinant INTL1 could affect the expression of other adipokines already described in human granulosa cells, such as NAMPT, RARRES2, and RE-TN, (ADIPOQ is not expressed in granulosa-lutein cells) [28, 29, 40]. Overnight-starved hGLCs were incubated for 24 h with different concentrations of human recombinant INTL1 (0, 10, 50, 100, and 250 ng/ml). We showed that after 24 h of stimulation, human recombinant INTL1 increased *NAMPT* mRNA and protein expression in a dose-dependent manner (Figs. 5, A and B), whereas it did not significantly affect the expression of *RETN* and *RARRES2* (data not shown). Similar results were observed in KGN cells

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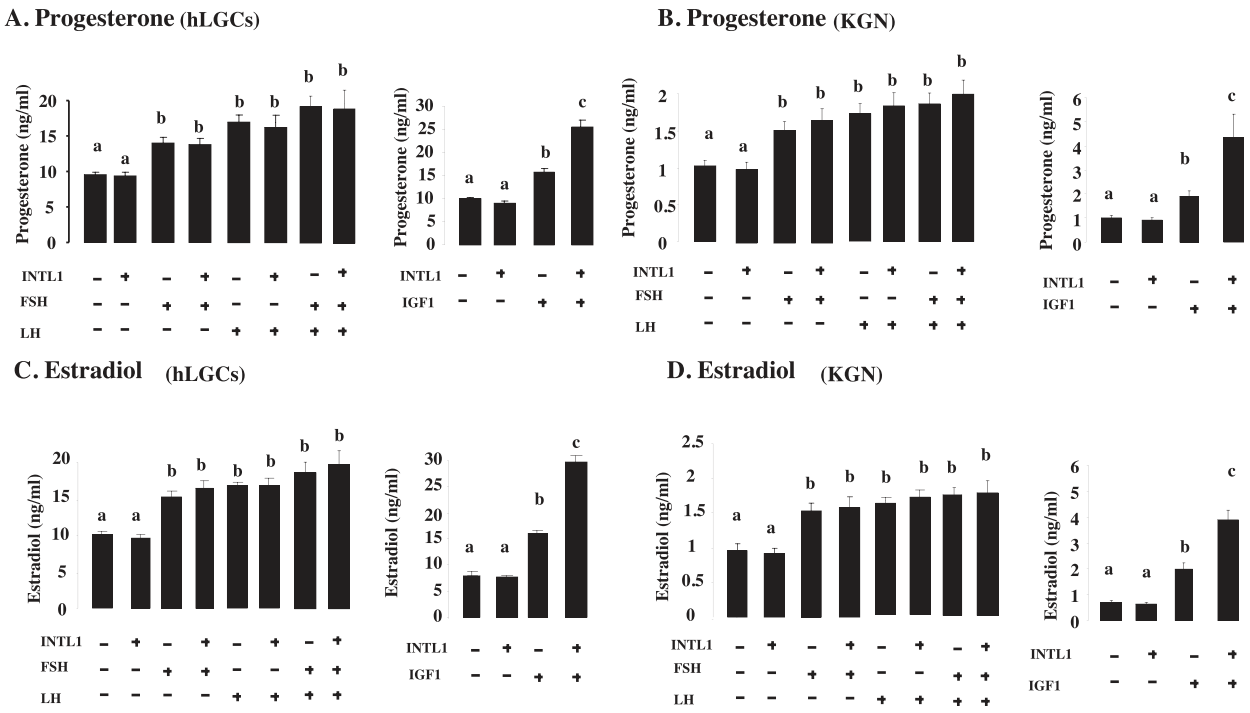

FIG. 6. Effects of recombinant human INTL1 on basal and IGF-1, FSH, or LH-induced progesterone (**A** and **B**) and estradiol (**C** and **D**) secretion by hGLCs (**A** and **C**) and KGN (**B** and **D**) cells. **A** and **C**) KGN and hGLCs were cultured in a medium with serum and then in serum-free medium in the absence or presence of INTL1 (250 ng/ml) with or without IGF-1 (10^{-8} M), FSH (10^{-8} M), and/or LH (10^{-8} M) for 48 h as described in *Materials and Methods*. Culture medium was collected and levels of progesterone (**A** and **B**) and estradiol (**C** and **D**) production were measured by RIA, and data are represented as the progesterone or estradiol concentration (ng/ml)/cellular protein concentration/well. Results are means ± SEM of the six cultures of hGLCs (we used hGLCs from 4 different patients [non-PCOS nonobese] for each culture) and 4 independent experiments (at 4 different passages) for KGN cells. In each culture, each treatment (INTL1 in the presence or absence of IGF-1 [10^{-8} M], FSH [10^{-8} M], and/or LH [10^{-8} M]) was applied in quadruplicate. Bars with different letters indicate significant differences ($P < 0.05$).

(Figs. 5, C and D), suggesting that KGN cells are a good model for understanding the molecular mechanisms involved in the relationship between INTL1 and NAMPT in human granulosa cells.

Effect of Human Recombinant INTELECTIN1 on Basal and IGF-1-, FSH-, or LH-Induced Progesterone and Estradiol Production by hGLCs and KGN Cells

We next investigated the effects of INTL1 treatment on steroidogenesis in hGLCs and KGN cells (Fig. 6). Cells were incubated in serum-free medium with human recombinant INTL1 (250 ng/ml) for 48 h in the presence or absence of IGF-1 (10^{-8} M), FSH (10^{-8}M), and/or LH (10^{-8} M). As expected, IGF-1, FSH, or LH treatment alone increased progesterone and estradiol secretion in primary hGLCs (Figs. 6, A and C [40]) and KGN cells (Figs. 6, B and D [41]). In primary granulosa-lutein cells, in the presence of IGF-1, INTL1 treatment (250 ng/ml) produced a two-fold increase in secretion of progesterone (Fig. 6A, $P < 0.001$) and estradiol (Fig. 6C, $P < 0.001$), whereas no effect of INTL1 was observed in the absence or presence of FSH and/or LH. In KGN cells, similar effects were observed (Figs. 6, B and D).

INTL1 Increases IGF-1-Induced Steroid Production and STAR and CYP19A1 Protein Levels Through NAMPT in KGN Cells

We determined whether the effect of INTL1 on steroidogenesis induced by IGF-1 was mediated by NAMPT. We used the shRNA technology to specifically knock down the expression of NAMPT in KGN cells. Two days after the lentiviral infection, the NAMPT-/-1 and NAMPT-/-2 cells showed almost 100% knockdown of NAMPT mRNA, protein (Figs. 7, A and B) and activity (data not shown). The scrambled cells produce a shRNA targeting no known mRNAs. They did not differ significantly from the parental KGN cells in any of the experiments performed (data not shown). The NAMPT-/-1, NAMPT-/-2, and scrambled cells were incubated in serum-free medium with human recombinant INTL1 (250 ng/ml) for 48 h in the presence or absence of IGF-1 (10^{-8} M). As shown in Figures 7, C and D, INTL1 treatment increased IGF-1-induced progesterone and estradiol secretion in scrambled cells but not in NAMPT-/-1 and NAMPT-/-2.

We next examined whether this stimulatory effect of INTL1 mediated by NAMPT on progesterone and estradiol production was due to effects on the three key enzymes of steroidogenesis (HSD3B, CYP11A1, and CYP19A1) and/or of STAR, an important cholesterol carrier. INTL1 treatment (250 ng/ml, 48 h) in the presence of IGF-1 increased by two-fold and 1.5-fold the production of STAR (Fig. 8A) and CYP19A1 (Fig. 8B) proteins, respectively, in scrambled KGN cells, whereas these

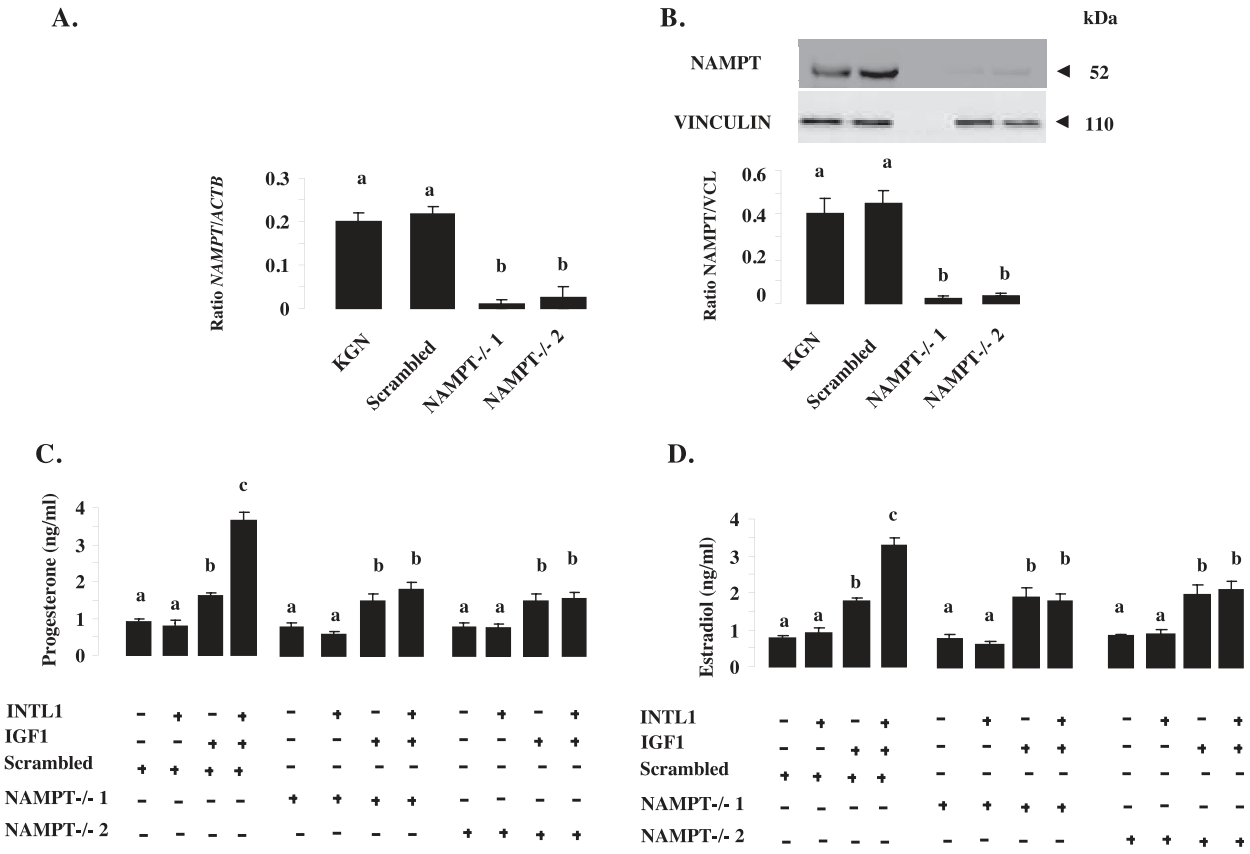

FIG. 7. Effect of human recombinant INTL1 on IGF-1-induced progesterone (**C**) and estradiol (**D**) in NAMPT-knockdown KGN cells mRNA (**A**) and protein (**B**) expression levels of NAMPT in scrambled, NAMPT-/-1, NAMPT-/-2, and parental KGN cells. KGN cells at 50% of confluence were infected with lenti-NAMPT1-GFP virus (NAMPT-/-1 cells), lenti-NAMPT2-GFP virus (NAMPT-/-2 cells), or lenti-control cells (scrambled). After six passages, total RNA and protein from scrambled, NAMPT-/-1, NAMPT-/-2, and parental KGN cells were prepared. *NAMPT* gene expression was measured by quantitative real-time PCR. Protein lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with specific antibodies against human NAMPT. Equal protein loading was verified by reprobing membranes with an anti-vinculin antibody. Results are representative of at least three independent experiments. Bars with different letter indicate significant differences ($P < 0.05$). **C** and **D**) Effects of human recombinant INTL1 (250 ng/ml) on IGF-1-induced progesterone (**C**) and estradiol (**D**) production in scrambled and NAMPT-/-1 and NAMPT-/-2 cells. Cells were cultured for 48 h in DMEM-F12 without serum in the absence or presence INTL1 with or without IGF-1 (10^{-8} M). Culture medium was collected, and progesterone (**C**) and estradiol (**D**) production levels were measured by radioimmunoassay. Results are representative of at least three independent experiments. Results are expressed as means ± SEM. Different letters indicate significant differences ($P < 0.05$).

effects were not observed in NAMPT-/-1 (Figs. 8, A and B) or NAMPT-/-2 (data not shown). However, INTL1 treatment did not affect IGF-1-induced HSD3B and CYP11A1 in scrambled or NAMPT-/-1 and NAMPT-/-2 cells (data not shown).

Effect of Human Recombinant INTL1 on IGF-1-Induced IGF-1R-beta, IRS1, and MAPK3/1 phosphorylation in KGN cells

We next investigated the molecular mechanisms involved in the effect of INTL1 on steroid production in response to IGF-1 in KGN cells. More precisely, we determined whether INTL1 treatment could modulate IGF-1R signaling through NAMPT. Scrambled and NAMPT-/-1 KGN cells were incubated in serum-free medium supplemented with recombinant human INTL1 (250 ng/mL) for 48 h (conditions used to measure progesterone and estradiol production) in the absence or presence of IGF-1 (10^{-8} M) treatment for 10 min. As shown in Figure 9, IGF-1 treatment alone increased by approximately 2-fold ($P < 0.05$) tyrosine phosphorylation of IGF-1R receptor β subunit and IRS-1 and phosphorylation levels of MAPK3/1 in scrambled and NAMPT-/-1 cells. Treatment with only recombinant human INTL1 did not affect basal phosphorylation of IGF-1Rβ, IRS1, and MAPK3/1 in both cell types, whereas it increased by approximately two-fold IGF-1-induced tyrosine phosphorylation of IGF-1Rβ subunit and IRS-1 and phosphorylation of MAPK3/1 in scrambled cells but not in NAMPT-/-1 (Figure 9). We observed the same results with NAMPT-/-2 as those with NAMPT-/-1 (data not shown).

DISCUSSION

In this study. we report for the first time that INTL1 is expressed in human ovarian cells. In hGLCs and KGN cells, we showed that *INTL1* expression is regulated by insulin, IGF-1, FSH, and metformin. Metformin-induced is mediated through PRKA. We have also shown that INTL1 increased ?11
NAMPT expression in a dose-dependent manner. It enhanced IGF-1-induced steroid production, and this was associated with increased STAR and CYP19A1 protein levels and IGF-1R

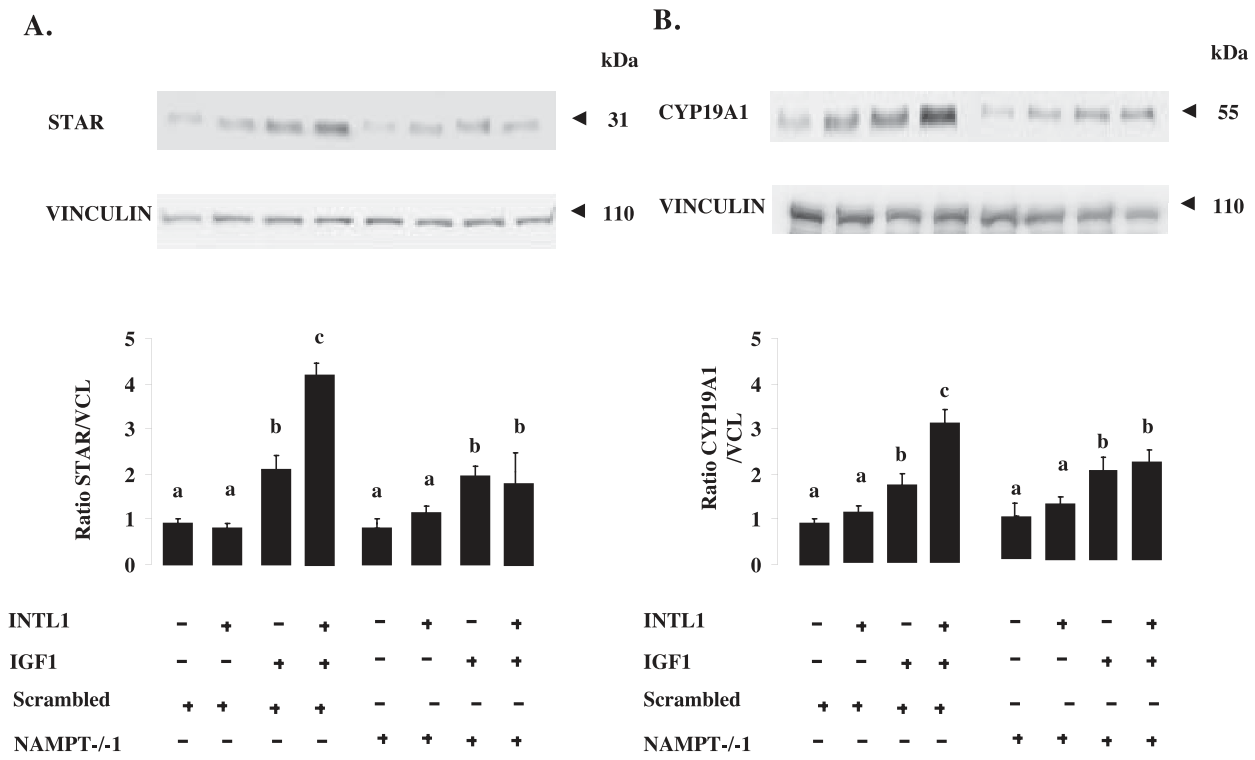

FIG. 8. Effects of human recombinant INTL1 on STAR (**A**) and CYP19A1 (**B**) protein levels in response to IGF-1 in NAMPT-knockdown KGN cells. Scrambled and NAMPT-/-1 cells were cultured for 48 h in DMEM-F12 without serum in the absence or presence of INTL1 with or without IGF-1 (10^{-8} M). Lysates from scrambled and NAMPT-/-1 cells from the different conditions were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-STAR, anti-CYP19A1, and vinculin antibodies. Densitometric analyses of bands were carried out, and the STAR:vinculin and CYP19A1:vinculin ratios are shown. Results are expressed as means ± SEM. Different letters indicate significant differences ($P < 0.05$).

signaling in both hGLCs and KGN cells (Fig. 10). Furthermore, *NAMPT* knockdown by shRNA abolished these effects in KGN cells.

Here we have shown that plasma and FF INTL concentrations were not significantly different in control women, whereas in PCOS patients, INTL concentrations were almost twice as high in FF compared to that in plasma. These results suggest an increase in the production of INTL in ovarian cells from PCOS patients compared with control subjects. However, it has been reported that in addition to decreased plasma INTL1 levels in women with PCOS, compared with control patients, there were significantly lower levels of INTL1 mRNA and protein in omental adipose tissue of women with PCOS [10]. In our study, we confirmed the lower INTL plasma levels in PCOS patients. Furthermore we showed that the FF and hGLCs mRNA expression INTL levels were increased in PCOS patients. However, we also observed that INTL1 mRNA expression was insulin-responsive and INTL1 was a positive regulator of CYP19A1 in control hGLCs. However, PCOS ovarian follicles are generally insulin-resistant [44, 45] and CYP19A1 deficient [46]. Thus, our results suggest that the potential link between INTL1 and PCOS is unclear at the present time and needs to be clarified.

Here we have shown that *INTL1* gene expression was significantly increased after 24 h of stimulation by insulin or metformin in vitro in cultured granulosa-lutein cells. In omental adipose tissue explants, insulin dose-dependently decreased *INTL1* mRNA expression, protein levels, and secretion after 24 h of stimulation into conditioned medium [10], suggesting a different regulation of *INTL1* expression by insulin according to various tissues. We also observed that the insulin sensitizer metformin strongly increased *INTL1* gene and protein expression through PRKA in granulosa-lutein cells. Metformin, a derivative of biguanide, is an insulin-sensitizing agent used to treat type 2 diabetes mellitus and PCOS [47]. In women with PCOS, metformin treatment restores the cyclic nature of menstruation and increases ovulation (by improving follicular growth), fertilization, and pregnancy rates [48]. These improvements have been attributed to decreases in the level of insulin, subsequently attenuating a hyperandrogenic status. In the human KGN granulosa-cell line, metformin enhances insulin action and more precisely glucose uptake and thereby could facilitate follicle growth [49]. In our study, we have shown that metformin increases *INTL1* expression and INTL1 enhances IGF-1R signaling through NAMPT. IGF-1 and insulin have commons signaling pathways and functions. Thus, the insulin sensitizing effects of metformin could be mediated by INTL1 in human granulosa cells.

In granulosa-lutein cells (hGLCs and KGN cells), we observed that INTL1 increased NAMPT expression in a dose-dependent manner, which is essential for the stimulatory effect of INTL1 on IGF-1-induced steroidogenesis. NAMPT is a rate-limiting enzyme in the biosynthetic pathway of nicotinamide adenine dinucleotide [50] and is ubiquitously expressed in many tissues including human granulosa cells [27, 30]. NAMPT is a homodimeric protein that exhibits both an intracellular enzymatic activity leading to NAD synthesis and an extracellular form as a cytokine function via binding to its

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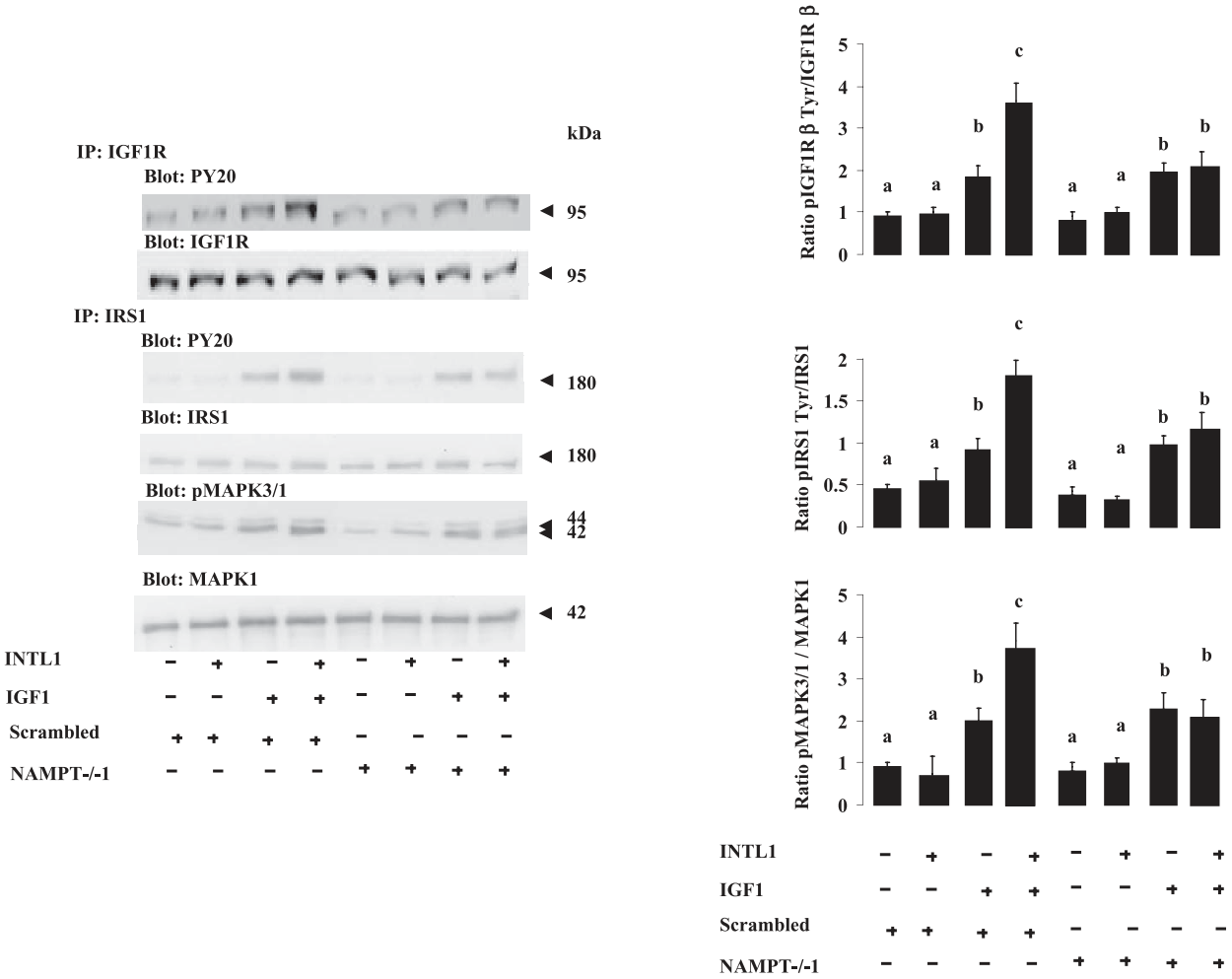

FIG. 9. Effect of human recombinant INTL1 on IGF-1-induced tyrosine phosphorylation of IGF-1R-beta subunit and IRS-1 and phosphorylation of MAPK3/1 in NAMPT-knockdown KGN cells. Lysates were prepared from scrambled and NAMPT-/-1 cells preincubated with INTL1 (250 ng/ml) for 48 h and then stimulated with IGF-1 (10^{-8} M) for 10 min. Left panel) IGF-1R -beta subunit and IRS-1 were immunoprecipitated from whole cell lysates, and samples were subjected to Western blotting with antibodies against PY20. Membranes were then reprobed with IGF-1R-beta subunit or IRS-1 antibodies to evaluate IGF-1R-beta subunit or IRS-1 levels in each lane. To determine MAPK3/1 phosphorylation, whole lysates were resolved by SDS-PAGE and then transferred to nitrocellulose membranes, and phosphorylation of MAPK3/1 was analyzed with polyclonal antibodies raised against phosphor-MAPK3/1. To determine the level of phosphorylation, blots were stripped and reprobed with antibodies against MAPK1. Right panel) The phosphorylation:total protein ratio was determined and plotted. Values are means ± SEM from three independent experiments. Different letters indicate significant differences ($P < 0.05$).

hypothetical receptor [51]. The question is how NAMPT in response to INTL1 can increase IGF-1R signaling in human granulosa cells. NAMPT was initially reported to mimic the effects of insulin by binding to the insulin receptor (IR) and by stimulating the phosphorylation of downstream components [52]. However, the original work describing NAMPT binding to IR has been retracted [52]. Therefore, IR is no longer considered a NAMPT receptor, although there is evidence that regulation of insulin signaling pathways at the level of IRSs, Akt, and MAPK3/1 is implicated in producing the pathophysiological effects of NAMPT. NAMPT activity leads to NAD release, a cofactor for NAD-consuming enzymes such as sirtuins. Sirtuins (SIRTs) are NAD-dependent histone deacetylases that regulate gene expression, differentiation, and development. SIRT1 is expressed in human ovary and in granulosa-lutein cells [27, 53]. SIRT1 activation ameliorates insulin sensitivity and in Leydig cells, SIRT1 promotes steroidogenesis [54]. Interestingly, SIRT1 modulates insulin/IGF-1 signaling through various mechanisms. It upregulates IGF-1 level by depressing IGFBP1 [55] and enhances insulin/IGF-1 signaling by deacetylation of IRS2, which increases downstream signaling [56]. Thus, INTL1 through NAMPT expression could increase IGF-1-induced steroidogenesis through these mechanisms.

INTL1 and NAMPT are two adipokines that increase insulin sensitivity. In the ovary, insulin and IGF-1 act synergistically with FSH to augment estrogen production by granulosa cells and with LH to augment androgen production by thecal stromal cells. PCOS is often associated with hyperinsulinemia and peripheral insulin resistance. Whether the ovary is resistant to insulin is a matter of controversy. Some studies showed that insulin resistance occurs in the ovary in PCOS [57]. However,

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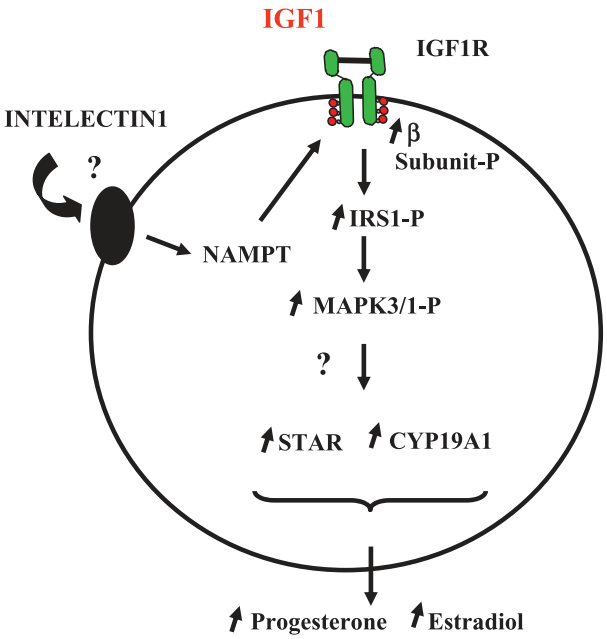

FIG. 10. Schematic representation of human recombinant INTL1 effects on IGF-1R signaling and steroidogenesis in human granulosa-lutein cells.

little is known about the biological significance of this metabolic phenotype to ovary dysfunction. In granulosa-lutein cells isolated from ovaries of women with classic PCOS, insulin action on glucose metabolism is significantly decreased, whereas insulin action on steroidogenesis is unchanged compared with granulosa-lutein cells from control women [58, 59]. This result suggests that in PCOS, there is selective insulin resistance in the ovary. Insulin resistance has also a direct negative effects on oocyte quality (for review see [60]). NAMPT is expressed in human granulosa cells but also in human oocytes [27]. Taken together, INTL1 and NAMPT, by modulating insulin sensitivity in granulosa cells, could affect ovarian functions. However, whether these adipokines affect insulin sensitivity in vivo in human ovary it remains to be determined.

We have shown that INTL1 through NAMPT expression increases IGF-1-induced steroid production in hGLCs and KGN cells. We have previously shown that ADIPOQ [40] and NAMPT [27] also increase IGF-1-induced progesterone and estradiol secretions, whereas RARRES2 [28] and RE-TN [29] decrease them in hGLCs. Here, we observed that INTL1, through NAMPT expression, increased IGF-1-induced STAR and CYP19A1 protein level (HSD3B and CYP11A1 protein levels were not affected). These results could explain the increase in progesterone and estradiol secretion in response to IGF-1. In the present study, we have also shown that INTL1, through NAMPT, increased IGF-1-induced MAPK3/1 phosphorylation, whereas we did not observe an effect on Akt signaling pathway (data not shown). The potential involvement of MAPK3/1 in the regulation of steroidogenesis is still a matter of debate [61, 62]. Thus, the involvement of MAPK3/1 in the INTL1 effects on human granulosa-lutein cell steroidogenesis remains to be determined.

In conclusion, INTL1 is expressed in human ovarian cells. In contrast to those in control patients, INTL levels are higher in FF than in plasma in PCOS patients. In granulosa-lutein cells, *INTL1* expression is increased in the short term (12 or 24 h) by various hormones involved in follicle development and the insulin sensitizer metformin. Furthermore, INTL1, through induction of NAMPT expression, ameliorates IGF-1-induced steroidogenesis and IGF-1R signaling. Thus, INTL1 could be involved in ovarian functions. Further investigations are required to understand its role in the pathophysiology of PCOS.

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Maxime REVERCHON

Identification et rôle *in vitro* de la chemerine, résistine et visfatine dans l'ovaire humain et bovin

Résumé

Les adipocytokines (adipo), produites par le tissu adipeux jouent un rôle clé dans la régulation des fonctions métaboliques mais qu'en est-il pour les fonctions de reproduction ? Nous montrons que la chemerine et ses récepteurs, la visfatine et la résistine sont présents dans les cellules ovariennes humaines et bovines. *In vitro* nous observons que la chemerine et la résistine diminuent la stéroïdogenèse des cellules de la granulosa (CG) humaine induite par IGF-1 alors que la visfatine l'augmente. Des résultats similaires sont observés chez la vache pour la chemerine et la visfatine. Dans les deux espèces, les adipo influencent la prolifération des CG, et les voies de signalisation AKT, MAPK-ERK1/2 et P38 ou l'AMPK. Chez le bovin, la chemerine bloque la maturation ovocytaire *in vitro*. Nous observons aussi dans cette espèce que la concentration plasmatique de résistine et son expression dans les adipocytes est augmentée après vêlage lorsque la lipomobilisation est élevée. Ces travaux confirment le rôle de la résistine dans la régulation métabolique chez la vache et montrent l'importance des adipo dans les cellules ovariennes humaine et bovine. Il reste à élargir leur rôle au niveau central dans les fonctions de reproduction.

Mots clés : Adipocytokines, cellules de la granulosa, stéroïdogenèse, maturation ovocytaire

Abstract

The adipokines (adipo), produced by the adipose tissue play a key role in the regulation of metabolic functions, but what about for reproductive functions? We show that chemerin and its receptors, visfatin and resistin are present in human and bovine ovary cells. *In vitro* we observe that chemerin and resistin decrease steroidogenesis in granulosa cells (GC) in response to IGF-1 while visfatin increases it. Similar results are observed for chemerin and visfatin in cows. In both species, chemerin, visfatin and resistin affect the proliferation of CG and signaling pathways including AKT, MAPK-ERK1 / 2 and P38 or AMPK. In cattle, chemerin blocks *in vitro* oocyte maturation. In this species, we also observe that the plasma resistin and its expression in adipocytes are increased after calving when the fatty acid mobilization is high. This work confirms the role of resistin in the metabolic regulations in cow and shows the importance of adipo in the human and bovine ovary cells. It remains to investigate their role at the central level in the reproductive functions.

Keywords: Adipokines, granulosa cells, steroidogenesis, oocyte maturation