Energy sensors and reproductive hypothalamo-pituitary ovarian axis (HPO) in female mammals: Role of mTOR (mammalian target of rapamycin), AMPK (AMP-activated protein kinase) and SIRT1 (Sirtuin 1)

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Abstract

In female, energy metabolism influences reproductive function by modulating the Hypothalamic Pituitary Ovarian axis including the hypothalamic GnRH neuronal network, the pituitary gonadotropin secretion and the ovarian follicle growth and steroidogenesis. Several hormones and neuropeptides or metabolites are important signals between energy balance and reproduction. These energy sensors mediate their action on reproductive cells through specific kinases or signaling pathways. This review focuses on the role of three main enzymes-specifically, mTOR, AMPK, and SIRT1 at the hypothalamic pituitary and ovarian axis in normal female fertility and then we discuss their possible involvement in some women reproductive disorders known to be associated with metabolic complications, such as polycystic ovary syndrome (PCOS) and premature ovarian failure (POF).

1. Introduction

In female animals, reproductive functions are highly dependent on successful regulation of energy metabolism. Indeed, the physiological processes including puberty, pregnancy and lactation have an important energetic costs. They are dependent on the cyclic production of sex hormones at different levels of the hypothalamus-pituitary-ovarian (HPO). This production relies on female ability in saving oxidizable fuels. Several examples in various animal species show this tight interconnection between energy metabolism and reproduction. Over 2000 years ago, Hippocrates observed that hard-working slim servant women were more fertile than their overfed and sedentary Roman employers (cited by (Franks, et al. 1996). More recently, large literature reported relationships between nutritional status and/or diet and fertility/infertility in agronomic species (sheep, cattle, chicken…), rodents and human. In females, when this physiological balance between reproduction and metabolism is disrupted reproductive disorders occur. In cattle selected for high milk production, negative energy balance in the post-partum period is associated with reduced fertility (Wathes, et al. 2007). In women, obesity and anorexia, which are an important health issue nowadays, play a significant role in reproductive disorders (Gambineri, et al. 2019, Pinheiro, et al. 2007) . They are associated with anovulation, menstrual disorders, infertility, difficulties in assisted reproduction, and adverse pregnancy outcomes. Moreover, clinical studies in women demonstrated associations between obesity, insulin resistance and polycystic ovarian syndrome (PCOS) (Shang, et al. 2020, Zeng, et al. 2020). PCOS represents the most common hormonal abnormality in reproductive-age women affecting about 10% of this population (Teede, et al. 2018b). It is a set of reproductive and metabolic symptoms associated with an imbalance of reproductive hormones. Premature ovarian failure (POF) is another reproductive disorder that can be associated with metabolic complications (Wang et al., 2014).

Energy metabolism may influence reproductive axis through different signals : hormone including Insulin-like Growth Factor-1 (IGF-1)/insulin, adipokines (leptin, adiponectin and new emerging adipokine such as chemerin…), metabolites (glucose, lipid, amino acid) and neuropeptides. These signals through modulation of various signaling pathways including mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and Sirtuin 1 (SIRT1) are able to regulate HPO axis (McIlwraith and Belsham 2020). Many studies have highlighted the importance of mTOR, AMPK and SIRT1 in a wide range of human diseases like type 2 diabetes, obesity and metabolic syndrome (Boutouja, et al. 2019, Kitada, et al. 2019, Madhavi, et al. 2019). More recently, there are a large number of researches on these three main signaling pathways related to fertility and reproductive disorders (Correia, et al. 2020, Nguyen 2019, Tatone, et al. 2018). In this review, we will summarize the physiological regulatory roles of mTOR, AMPK and SIRT 1 in female at hypothalamic, pituitary and ovarian levels to provide molecular insight of these signaling pathways in female fertility. Finally, we will discuss their potential role in women reproductive disorders including PCOS and POF.

2. mTOR

mTOR was first discovered as the cellular target of rapamycin (Brown, et al. 1994), a Streptomyce*s*–derived compound which is currently used clinically as an immunosuppressive drug. mTOR is modulated by a wide range of nutrients (especially amino acids), growth factors, hormones and stressors, with an essential role in the control of cell proliferation, growth and metabolism (Martinez de Morentin, et al. 2014, Xu, et al. 2020) (**Figure 1A**).

2.1 Structure of mTOR

The protein mTOR is a serine/threonine kinase highly conserved that belongs to the family of phosphatidylinositol-3-kinase (PI3K) -related kinase (PIKK). As recently reviewed, it is composed of several domains (Xu, et al. 2020). Moreover, the TOR system includes two structurally and functionally distinct multi-molecular complexes: mTOR complex 1 (**mTORC1**) and mTOR complex 2 (**mTORC2)** that are composed by several regulatory proteins (Figure 1A) (Xu, et al. 2020). MTORC1 is mainly involved in the regulation of cellular anabolic processes, such as protein synthesis and lipid synthesis, to promote cell metabolism and cell growth **(Figure 1A)**. Activation of this complex can be carried out in different ways: (1) by signaling pathways involving PI3K / Akt / TSC1-TSC2 (tuberous sclerosis protein 1 and 2) and Ras / Raf / Extracellular Regulated Kinase (ERK), which are often associated with tyrosine kinase receptors (e.g. IGF-1, insulin and Epidermal Growth Factor (EGF) receptors); (2) by the availability of the cell in certain amino acids, such as leucine or arginine. Conversely, an energy deficit (lack of ATP) activates the AMPK protein which phosphorylates the mTORC1 complex and inhibits it. Thus, AMPK is also a key energy sensor as described in the present review (section 3).

The mTORC2 complex **(Figure 1A**) is less well understood than mTORC1, although it is known to be activated in a PI3K-dependent fashion and it thus is also responsive to growth factors. Furthermore, mTORC2 regulates cell survival and growth (Laplante and Sabatini 2012) and it has also an indirect role in metabolism, not only in terms of mitochondrial physiology (Betz, et al. 2013) but also by being able to control glucose metabolism in AKT-dependent (Masui, et al. 2014) and FOXO-dependent (Masui, et al. 2013) manners. Finally, mTORC2 is also able to modulate the hexosamine biosynthetic pathway, thus affecting protein and lipid glycosylation, as well as glutamine metabolism both involved in anabolic processes (Moloughney, et al. 2016).

105

106 **2.2 Hypothalamic mTOR and female reproduction**

107

108 mTOR is highly expressed in the hypothalamus (Cota, et al. 2006). In the hypothalamic
109 arcuate nucleus (ARC), mTOR plays an important role in sensing nutrient (mainly amino
110 acids) availability and mediating the anorectic effects of leptin (Cota, et al. 2006). At the
111 reproductive level, intracerebroventricular administration of rapamycin in rats inhibits the
112 puberty onset (**Figure 2**). Indeed, in these animals, plasma LH and estradiol levels are
113 decreased, vaginal opening is delayed, and ovaries and uterus are atrophied (Roa, et al. 2009).
114 At the molecular levels, rapamycin suppressed ARC Kiss1 levels (Roa, et al. 2009)(**Figure 2**).
115 The *Kiss1* gene encodes Kisspeptins that are a family of hypothalamic peptides (Ruohonen, et
116 al. 2020). They are neuromodulators that act upstream of GnRH, and they are sensitive to sex
117 steroid feedback and metabolic cues (Ruohonen, et al. 2020). Kisspeptins are now recognized
118 as crucial regulators of the onset of puberty, the regulation of sex hormone-mediated secretion
119 of gonadotrophins, and the control of fertility (Pinilla, et al. 2012). Inactivation of mTOR was
120 also shown to diminish the positive effect of leptin on puberty onset in food-restricted females
121 demonstrating that this mTOR pathway plays a critical role in the neuroendocrine control
122 (Roa, et al. 2009).

123 Interestingly, mTOR signaling seems to regulate Kiss1 neurons indirectly, since pS6 (the
124 downstream of mTOR and S6K1) is not expressed in Kiss1 neurons (Quennell, et al. 2011).
125 Although the significant effects of rapamycin on puberty onset were determined in animal
126 experiments, patients clinically treated with rapamycin before menarche were found to have a
127 similar menarche time as patients administered rapamycin post-menarche (Sparagana, et al.
128 2017). The difference could be explained by the fact that rapamycin administered orally has
129 little effects on mTOR signaling in the brain (Zhang, et al. 2014), although rapamycin is

believed to cross the blood–brain barrier (Franz, et al. 2006).The hypothalamus is a region that integrates different signals that regulate appetite, fertility and thermogenesis. At this level, the neurons with neuropeptide Y (NPY) which intervene in the regulation of food intake but also inhibit the activity of neurons with GnRH, are sensitive to the mTOR pathway. In organotypic cultures of hypothalamic explants, inhibition of the mTOR pathway by rapamycin leads to an increase in the level of NPY transcripts (Shimizu, et al. 2010). Other neuropeptides involved in the metabolic control of the HPO including neurokinin B and the products of POMC neurons (Manfredi-Lozano, et al. 2018) could be *in vivo* regulated by mTOR signaling. It therefore seems that, in the central structures regulating the onset of puberty and / or the pulsatility of gonadotropic hormones, inhibition of mTOR leads to deregulation of the Kiss/GnRH system. In addition, recently PTEN deletion specifically in kisspeptin-expressing cells resulted in a female-specific hyperactivation of mTOR signaling in anteroventral periventricular and ARC kisspeptin neurons (Negron, et al. 2020). PI3K-mTOR hyperactivity was associated with higher hypothalamic kisspeptin protein expression and higher plasma LH levels in fasted females compared to controls. This deletion of the *Pten* gene specifically in kisspeptin-expressing cells results in a brain region-specific hypertrophy, accompanied by decreased fertility in females and reduced gonadotropin responses to gonadectomy in both sexes. PTEN acts both as a lipid and protein phosphatase and as a regulator of several signaling cascades, most notably as the direct negative regulator of phosphatidylinositol 3-kinase (PI3K). PI3K activates downstream molecules like the serine/threonine-specific protein kinase, Akt, and the mammalian target of rapamycin (mTOR) in response to insulin or leptin (Tsou and Bence 2012).

Altogether, these observations suggest that modulations of mTOR signaling in the brain are indispensable for proper functioning of the HPO axis and fertility.

2.3 Role of mTOR in pituitary and female reproduction

The mTOR protein is also expressed and active in normal pituitary cells and pituitary adenomas (Sajjad, et al. 2013), for review (Monsalves, et al. 2014). Several studies have examined the antiproliferative properties of rapamycin treatment in pituitary and pituitary adenomas cells. In normal rat pituitary cells, rapamycin inhibits basal proliferation and insulin-, cAMP-, and estradiol-induced proliferation of cells (Kawashima, et al. 2000) (**Figure 2**). Using human pituitary anterior cells in primary culture it has also demonstrated that rapamycin induces mTOR inhibition in mTOR-active GH- and ACTH cells (Sajjad, et al. 2013). Human PRL gene is expressed in the GH3 cell line and its transcription was shown to be inhibited by rapamycin (Wera, et al. 1995). In an animal model using rats that carry an inactivating germline mutation of the *TSC2* gene that results in pituitary tumor formation, rapamycin induced regression of the pituitary tumors and a concomitant decrease in the levels of phosphorylated-S6 (the target of p70S6K; (Kenerson, et al. 2005).

In vitro experiments on a mouse pituitary cell line have shown that the stimulation of protein synthesis induced by GnRH is inhibited by rapamycin (Sosnowski, et al. 2000) (**Figure 2**). Using the LβT2 gonadotrope cell line it has been demonstrated a specific role for mTORC2 in regulating membrane remodeling events (Edwards, et al. 2017). More precisely, pharmacological inhibition of mTORC2-blunted GnRH-mediated actin reorganization and similarly attenuated activation of ERK and LHβ gene expression (Edwards, et al. 2017) (**Figure 2**). Thus, mTORC2 is an important signaling intermediate in regulating membrane remodeling events and subsequent MAPK activation in gonadotrop cells.

2.4 Role of mTOR in ovary

The mTOR protein is expressed in most ovarian cells, with higher levels in the surface epithelium of the ovary, in granulosa cells and in immature oocytes. Granulosa cells are somatic cells that surround the follicle; they are steroidogenic and allow follicular growth and maturation. The outermost cells follicle, theca cells, androgens, precursors estradiol synthesized by granulosa cells. Phosphorylated mTOR (serine 2448) is strongly enriched within mitotic granulosa cells.

Proliferation

In rat, the activated mTOR pathway stimulates proliferation (FSH-induced) granulosa cells (Kayampilly and Menon 2007, Yu, et al. 2011) and that of theca cells (induced by LH and insulin) (Palaniappan and Menon 2012) (**Figure 2**). Inhibition of mTORC1 in theca primary cells induces a decrease in proliferation markers expression, such as PCNA (proliferating cell nuclear antigen) or cyclin D3, despite the induction of proliferation by hCG (human chorionic gonadotropin) which mimics the effect of the LH (Palaniappan and Menon 2010). In addition, intraperitoneal injection of rapamycin in mice increases abnormal mitosis of granulosa cells preantral follicles (Yu, et al. 2011). The abnomal proliferation of these cells leads to a proportional decrease at the dose used of ovulated follicles. However, these follicles do not show any functional abnormalities *in vitro* ; oocytes can be fertilized and develop up to the blastocyst stage (Yu, et al. 2011). Pharmacological study in rats showed that while causing an accumulation of primordial follicles, long-term rapamycin treatment also resulted in reduced antral follicles and ovulation rates. In mice, rapamycin-sensitive mTORC1 signaling is also involved in physiological primordial follicle activation in mouse ovary (Tong, et al. 2013). Short-term rapamycin treatment increases ovarian lifespan in young and middle-aged female mice (Dou, et al. 2017). Culture of human ovarian cortex is also sensitive to

rapamycin (McLaughlin, et al. 2011). Indeed, the addition rapamycin during the six days of culture inhibits follicle growth and increases the number of small or even oocyte-free follicles (McLaughlin, et al. 2011). This inhibition is not correlated with an increase apoptosis, but a decrease in cell proliferation. Thus, mTOR regulates cell growth in ovarian cells.

Steroidogenesis

The mTOR signaling also participates in the regulation of ovarian steroidogenesis. In 2004, Alam et al. demonstrated that differentiation granulosa cells, induced by FSH involves the PI3K / mTOR pathway (Alam, et al. 2004). Indeed, the addition rapamycin to primary cell cultures of granulosa from prepubertal rats inhibits expression of proteins involved in granulosa cell differentiation: inhibin alpha, VEGF (vascular endothelial growth factor) and the LH receptor. Subsequently, numerous works completed this study demonstrating the involvement of the mTOR pathway in steroidogenesis (induced by the combination FSH +TGFbeta1) of granulosa cells from cultured rats *in vitro*. However, in other study, mTOR is not involved in the synthesis of estradiol triggered by simple stimulation by FSH. In women as in rodents, Cure et al. does show no change in synthesis estradiol following treatment with sirolimus and tacrolimus (Cure, et al. 2004).

The use of transgenic animal models has improved the understanding of the mTOR role *in vivo* in ovary (**Table 1**). Indeed, specific deletion of Rictor (rapamycin-insensitive companion of mTOR, a key component of mTORC2) in oocyte of primordial follicles leads to massive follicular death, excessive loss of functional ovarian follicles and abnormal gonadal hormone secretion (Chen, et al. 2015). Furthermore, deletion of TSC1 or TSC2 (tumor suppressor tuberous sclerosis complex 1), negative regulators of mTORC1 activity, promotes

the growth of all primordial follicles in neonatal animals, leading to the exhaustion of the entire follicle pool, followed by a premature ovarian failure phenotype (Adhikari, et al. 2009, Adhikari, et al. 2010). Specific disruption of TSC1 in granulosa cells of secondary follicles improves the follicle growth, leading to increased ovulatory capacity and delivery of more pups followed by a premature ovarian failure phenotype (Huang, et al. 2013). At the molecular level, a recent paper identified miR-92b-3p as a novel regulator of primordial follicle assembly by negatively regulating TSC1 in mTOR/Rps6 signaling (Li, et al. 2019). Also, the caseinolytic peptidase P (Clpp) could be a negative regulator of mTOR signaling in the ovary since Clpp −/− mice ovaries show accelerated depletion of follicular reserve, associated with mTOR pathway activation (Wang, et al. 2018).

3. AMPK

As is the case for mTOR, other cellular energy-sensing systems participate in the control of the female reproduction through the HPO axis. For example, AMPK, a member of the metabolite-sensing protein kinase family, also contributes to the metabolic gating of fertility (Bertoldo, et al. 2015a, Tosca, et al. 2008). In addition, it is well known that AMPK is able to interfere with mTOR activation (Gwinn, et al. 2008, Xu, et al. 2012). Thus, it is somewhat predictable that the above described phenomena concerning mTOR could involve also changes in AMPK signaling.

3.1 Stucture of AMPK

AMPK is a highly conserved heterotrimeric serine/threonine kinase(Carling, et al. 2012). (**Figure 1B**). AMPK is regarded as the genuine (and indispensable) cellular energy sensor. In fact, in contrast to other putative metabolic gauges, AMPK is able to directly sense changes in availability of the real energy sources of the cells, namely, adenine nucleotides. Any changes

253 that affect intracellular ATP levels, such as glucose deprivation, hypoxia, ischemia, and
254 oxidative stresses, will activate AMPK. A number of hormones that regulate energy
255 homeostasis (leptin, ghrelin, adiponectin, and resistin) have also been shown to exhibit tissue-
256 specific stimulation or inhibition of AMPK kinase (for review (Kahn, et al. 2005)) (**Figure**
257 **1B**).

258 **3.2 Hypothalamic/brain AMPK and female reproduction**

259 Besides this ubiquitous role at the cellular level, and as described earlier for the mTOR
260 pathway, brain AMPK signaling plays also a relevant role in the central control energy
261 balance and food intake. As expected for a signaling system that becomes activated in
262 conditions of energy shortage, activation of AMPK stimulates appetite (Kahn, et al. 2005), as
263 a mean to increase calorie incorporation. In keeping with such function, leptin suppresses
264 hypothalamic AMPK activity, while ghrelin stimulates it (Kahn, et al. 2005). In fact, AMPK
265 and mTOR are mutually regulated; for instance, AMPK can inhibit mTOR signaling in
266 different cell systems (Inoki, et al. 2003). Hence, these two metabolic cell sensors have been
267 proposed to act as functional antagonists, so that they reciprocally cooperate in the central
268 control of energy homeostasis. Considering the reciprocal regulation and function of mTOR
269 and AMPK in brain centers to regulate metabolic homeostasis, it is tempting to hypothesize
270 that this interaction may also be relevant for the dynamic modulation of the HPO related to
271 fertility.

272 Thus, activation of AMPK has been shown to inhibit GnRH secretion in the mouse
273 hypothalamic GnRH neuronal cell line (GT1-7 cells) *in vitro*, where it may act as transducer
274 of the effects of globular adiponectin (Coyral-Castel, et al. 2008) (Wen, et al. 2008) (**Figure**
275 **2**). In addition, adiponectin has been shown to inhibit *Kiss1* gene expression in GT1-7 cells
276 apparently via activation of AMPK (Wen, et al. 2012). In addition, activation of hypothalamic

AMPK *in vivo* after AICAR intracerebro-ventricular injection has been shown to alter estrous cyclicity in adult female rats, thus resulting in the lowering of the time interval between consecutive estrous cycles (Coyral-Castel, et al. 2008). More recently, Roa et al., 2018 showed that hypothalamic AMPK signaling plays a key role in the metabolic control of puberty acting via a repressive modulation of ARC Kiss1 neurons in conditions of negative energy balance in female rodents (Roa, et al. 2018) (**Table 1**). Other studies show that malnutrition-induced AMPK activation of ependymocytes of the lower brainstem might be involved in suppression of GnRH/LH release and then gonadal activities (Minabe, et al. 2015). In addition, a recent study shows that hindbrain AMPK mediates short-term food deprivation inhibition of the GnRH/LH axis (Shakya, et al. 2018).

Thus, these findings are suggestive of a role of brain AMPK in the metabolic control of reproduction, and are compatible with a predominant inhibitory role of AMPK pathways in the central regulation of the HPO axis. Such inhibitory action is in agreement with the role of AMPK as sensor of energy insufficiency and functional antagonist of mTOR.

3.3 AMPK and pituitary

In rat, the different subunits of AMPK are expressed in pituitary. In particular, AMPK alpha 1 is detected mainly in gonadotrophs and thyrotrophs, is less abundant in lactotrophs and somatotrophs, and is undetectable in corticotrophs (Tosca, et al. 2011). In rat pituitary cells, metformin-induced AMPK activation decreases gonadotropin secretion and MAPK ERK1/2 phosphorylation induced by GnRH and FSH release, FSHbeta subunit expression, and SMAD2 phosphorylation induced by activin (Tosca, et al. 2011) (**Figure 2**). In LbetaT2 gonadotropes, adiponectin-induced AMPK activation also decreases LH secretion (Lu, et al. 2008). In a good agreement with a suppressive effect of AMPK activition in gonadotrop cells,

300 a recent study shows that AMPK activation reduces the transcriptional activity of the murine
301 luteinizing hormone β-subunit gene (Moriyama, et al. 2020) (**Figure 2**).

302 **3.4 AMPK and ovarian functions**

303 AMPK protein exists in different mammalian ovarian cells, including oocytes, granulosa
304 cells, theca cells, and corpus luteum (Tosca, et al. 2007, Tosca, et al. 2005). Several data show
305 that AMPK is involved in the regulation of primordial follicle activation (Lu, et al. 2017) and
306 also in different biological functions of ovarian cells. In situ ovarian intrabursal administration
307 of AMPK inhibitor Compound C in mice, primordial follicle activation and stimulation of
308 follicle growth were observed, and thus more healthy pups were delivered (Lu, et al. 2017).
309 AMPK inhibition by Compound C promotes the growth of cultured ovary in vitro by
310 activating mTOR and increasing the expression of Ctgf (connective tissue growth factor) in
311 the YAP (hippo signaling Yes-Associated Protein) signaling pathway (Lu, et al. 2017).

312 ***Proliferation/apoptosis***

313 AMPK participates in regulating balance between ovarian cells proliferation and
314 apoptosis (**Figure 2**). As showed metformin inhibited proliferation by AMPK in response to
315 IGF-1 in bovine granulosa cells (Tosca, et al. 2010). Whereas, in rat granulosa cells FSH
316 stimulated proliferation by reducing p27 via AMPK inhibition (Kayampilly and Menon
317 2009). Additionally, authors observed that dihydrotestosterone decreased mitogenesis by
318 AMPK activation. Furthermore, inhibition of AMPK by compound C reversed the DHT-
319 mediated reduction in positive cell cycle regulator, cyclin D2, and 5-bromo-2'-deoxyuridine
320 incorporation (Kayampilly and Menon 2012). On the other hand, Tosca et al. (2005) did not
321 show essential effect of AMPK on rat granulosa cell proliferation (Tosca, et al. 2005). Some
322 experiment about AMPK involvement in metformin effect on cell proliferation was done also

in rat theca interna cells and showed that metformin inhibited insulin-induced proliferation and the up-regulation of the cell cycle regulatory proteins, cyclin D3 and CDK4 by activation AMPK. Effect was reversed with the addition of AMPK inhibitor, compound C (Will, et al. 2012). Thus, most of the studies show that modulation of AMPK activation is involved in the regulation of granulosa and theca cell proliferation in response to various factors.

Steroidogenesis

AMPK action on ovarian steroidogenesis was described by several papers (**Figure 2**). Literature showed that AMPK inhibits progesterone (P4) production by decreasing 3β-Hydroxysteroid dehydrogenase (HSD3B) mRNA and protein expression, with no effect on oestradiol (E2) secretion in rat granulosa cells (Tosca, et al. 2005). Beside metformin-inducted P4 inhibition and decreasing in HSD3B, CYP11A1 (Cytochrome P450 Family 11 Subfamily A Member 1) and STAR (steroidogenic acute regulatory protein) were mediated by AMPK activation in primary rat granulosa cells (Tosca, et al. 2006). Moreover, P4 and E2 production by bovine granulosa cells was decreased in response to metformin via AMPK pathway (Tosca, et al. 2007). Additionally, AMPK activation could be involved in the negative effects of one adipokine called apelinon steroidogenesis in porcine ovarian follicles (Rak, et al. 2017) and those of vitamin D3 in cultured granulosa cells of dehydroepiandrosterone-induced PCOS mice (Bakhshalizadeh, et al. 2018). Thus, in most of species, AMPK activation is associated to a negative effect on the *in vitro* ovarian steroidogenesis.

Oocyte maturation

Some evidence indicated a key role of AMPK action in oocyte maturation (**Figure 2**). AMPK activation by AICAR induced oocyte maturation in mice (Chen, et al. 2006). In

346 contrast, AMPK activation by metformin or AICAR caused oocyte arrest in germinal vesicle
347 stage in porcine and bovine cumulus-oocyte complexes (COCs), but only in the presence of
348 cumulus cells (Mayes, et al. 2007). Cumulus cells are necessary for promoting the inhibitory
349 effect of AMPK activation on the recovery of swine and bovine oocyte meiosis. At the
350 opposite, in mice, phospho-AMPK provides a meiotic maturation signal in mouse COCs and
351 induces oocyte maturation. This discrepancy shows that the role of AMPK in follicular
352 development is species dependent. Thus, the species difference in AMPK regulation in oocyte
353 maturation is worth in-depth studies.

354 ## 4. SIRT1

355 Several reports have suggested that AMPK interacted with the NAD+-dependent
356 deacetylase, SIRT1, another enzyme crucial in the regulation of glucose metabolism (Canto,
357 et al. 2009, Silvestre, et al. 2014). AMPK and SIRT1 share many target molecules and
358 regulate each other, which suggests that both enzymes cooperate in a cycle (Ruderman, et al.
359 2010). AMPKα elevates the expression of SIRT1 by up-regulating the intracellular levels of
360 its co-substrate NAD+ or the activity of nicotinamide (Canto, et al. 2009). Similarly, SIRT1
361 can activate AMPK via deacetylation of LKB1, which promotes LKB1 translocation from the
362 nucleus to the cytosol, where it is activated and phosphorylates and activates AMPK
363 (Rogacka, et al. 2018). Under stress conditions, SIRT1 cooperates with AMPK in order to
364 restore energy balance or promoting cell death (Fulco and Sartorelli 2008). Concerning the
365 reproductive process, our laboratory showed that AMPK/SIRT1 signalling pathway was
366 involved in the regulation of visfatin expression in reponse to metformin (Reverchon, et al.
367 2013).

368

369 ### 4.1 Structure of SIRT1

Sirtuins are part of the family of class 3 histone deacetylases (HDACs), which, unlike class 1 and 2 HDACs, use the cofactor NAD + (nicotinamide adenine dinucleotide) as substrate. In mammals, the sirtuins family includes seven isoforms (SIRT1 to 7) (Haigis and Sinclair 2010). Among the seven sirtuins, SIRT1 is the most studied and well-characterized sirtuin with respect to its physiological functions. Several studies have suggested that resveratrol is an agonist of sirtuin type 1 (SIRT1) and exerts a wide variety of beneficial effects in a SIRT1-dependent manner to prevent the development of many illnesses, through, e.g., antidiabetic, antioxidant and antiinflammatory approaches (**Figure 1 C**). SIRT1 is ubiquitously expressed and has many important functions in peripheral metabolic tissues, including adipose tissue, liver, and muscle, that underlie its role in sensing energy balance (Boutant and Canto 2016). Indeed, SIRT1 is activated during fasting or caloric restriction to increase fatty acid oxidation and gluconeogenesis and suppress insulin secretion, insulin action, and adipogenesis (**Figure 1 C**). Like AMPK and mTOR, SIRT1 functions as a cellular energy sensor. In addition to its well-characterized effects in peripheral tissues, evidence suggests that it plays a role in the interactions between metabolism and reproductive functions.

4.2 SIRT1 and hypothalamic function in reproduction

SIRT1 is expressed in different neurons involved in the metabolism regulation in hypothalamus (Yamamoto and Takahashi 2018). Concerning the reproductive function, SIRT1 knockout mice exhibit a diminished hypothalamic GnRH expression and in turn reduced serum LH and FSH levels and spermatogenesis arrest, suggesting an important role of SIRT1 in the HPG axis (Kolthur-Seetharam, et al. 2009). SIRT1 is expressed in hypothalamic Kiss1 neurons and suppresses Kiss1 expression (Vazquez, et al. 2018). Thus, in

394 female, it acts as a molecule that restrains female puberty via epigenetic repression of the
395 puberty-activating gene, Kiss1 (Vazquez, et al. 2018). Using an inducible, conditional
396 deletion of the SIRT1 deacetylase domain or conditional overexpression of wild-type SIRT1
397 in astrocytes, Choi et al recently showed that SIRT1 regulates female reproductive functions
398 (Choi, et al. 2019). Indeed, astrocyte SIRT1 promotes and lack of SIRT1 impairs estrus
399 cycles. SIRT1 inactivation reduces ovary size and number of corpora lutea and a greater
400 proportion of SIRT1 overexpressing mice showed LH surges. Regarding potential central
401 actions of SIRT1 in the control of the reproductive axis, mice with congenital Sirt1 deficiency
402 displayed central hypogonadism due to defective GnRH neuronal migration to the
403 hypothalamus (Di Sante, et al. 2015). Thus, hypothalamic SIRT1 seems to play an important
404 role in the regulation of female fertility.

405

406 **4.3 SIRT1 and gonadotropin expression and secretion**

407

408 SIRT1 KO mice have smaller pituitary than wild-type mice (Lemieux, et al. 2005). In
409 normal animals, the distribution of SIRT1 among the hormone-secreting pituitary cells in
410 mice and humans demonstrates that SIRT1 is abundantly localized in TSH-positive cells but
411 is barely detectable in the ACTH-, FSH-, LH/GH-, or prolactin-positive cells (Akieda-Asai, et
412 al. 2010). GnRH treatment decreases SIRT1 level via the miR-132/212 induction in the
413 pituitary. This results in the downregulation of SIRT1-dependent FOXO1 deacetylation and a
414 decrease in the FOXO1-mediated inhibition of Fshβ transcription that ultimately increases the
415 Fshβ expression in rat primary pituitary cells and LβT2 cell line (Lannes, et al. 2015).

416

417 **4.4 SIRT1 and ovarian functions**

SIRT1 is involved in control of basic ovarian functions (see (Tatone, et al. 2015) for review). In ovary, SIRT1 is expressed in stroma, large ovarian follicles, particularly in the oocyte and granulosa cells (Morita, et al. 2012), and loss of SIRT1 causes small ovaries with early-stage follicular development but no evidence of ovulation (Tatone, et al. 2015). Indeed, SIRT 1 overexpression is associated with promotion of mice ovarian follicullogenesis and fecundity (Long, et al. 2019), while its deletion has an opposite effect (Tatone, et al. 2018). In human follicle, SIRT1 is detected in follicular fluid, and its concentration is independent of its serum levels. In addition, ovarian hyperstimulation resulted in significantly higher serum SIRT1 levels in pregnant women compared with non-pregnant women suggesting a role of ovarian SIRT1 in the reproductive success (Bodis, et al. 2019).

Proliferation/apoptosis

The amount of SIRTs in ovarian cells is associated with their state and health. Atresia of porcine ovarian follicles was associated with a decrease in SIRT1 expression (Zhao, et al. 2014). Zhang et al. reported that the expression levels of SIRT1 were decreased in the ovaries of aged mice and mice treated with chemotherapy, but increased in calorie-restricted mice (Zhang, et al. 2016). The transfection-induced overexpression of SIRT1 in cultured porcine ovarian granulosa cells reduces their proliferation, increases insulin-like growth factor I (IGF-I) release and modified the response of granulosa cells to exogenous follicle-stimulating hormone (Pavlova, et al. 2013, Sirotkin, et al. 2014). In human luteinized granulosa cells, SIRT1 also play an anti-apoptotic role (Han, et al. 2017). Recently, Luo et al showed that overexpression of miR-23a inhibits the expression of SIRT1 and increases apoptosis in human granulosa cells (Luo, et al. 2019).

Steroidogenesis

SIRT1 plays a role in the activation of steroidogenesis associated with luteinization and the terminal differentiation of rat granulosa cells (Morita, et al. 2012). Thus, stimulation of SIRT1 by resveratrol could potentially be beneficial in the treatment of luteal phase deficiency. Studies in human luteinized granulosa cells and KGN cells showed that SIRT1 signalling is involved in the response of ovarian cells to the insulin sensitizer metformin (Reverchon, et al. 2013). In cultured bovine granulosa cells, visfatin improves basal and IGF1-induced steroidogenesis and IGF1 receptor signaling through SIRT1 (Reverchon, et al. 2016).

Oocyte and Oxydative stress

SIRT 1 is expressed in mouse ovulated metaphase II (MII) oocytes, and its expression gradually decreases upon fertilization until the blastocyst stage suggesting that SIRT1 is supposed to be relevant to oogenesis rather than fertilization and early embryo development. SIRT1 mRNA levels are significant decreased in MII oocytes aged *in vivo* or *in vitro*, when compared to fresh MII oocytes (Ma, et al. 2015). More recently, Ma et al., 2018 showed that a SIRT1/Nrf2 (Nuclear factor erythroid 2-related factor 2) pathway is involved in oocyte aging by regulating Cyclin B1 (Ma, et al. 2018). In mouse GV and MII oocytes, SIRT1 expression has been associated with changes in chromatin configuration (Manosalva and Gonzalez 2010), oxidative stress response, reproductive aging, and postovulatory aging (Di Emidio, et al. 2014). In mouse oocyte, resveratrol treatment through SIRT1 increases resistance of mouse oocytes to postovulatory aging *in vivo* (Liang, et al. 2018). Experimental evidence in mouse oocytes demonstrated the role of redox signalling in the regulation of SIRT1 (Di Emidio, et al. 2014). Indeed in oocytes arrested at GV stage the gene transcript encoding SIRT1 is induced in response to *in vitro* exposure to H2O2. By changing its intracellular localization, activating GV chromatin rearrangement, and modulating antioxidant enzymatic response, SIRT1 has

been shown to orchestrate the adaptive response to oxidative stress in mouse oocytes (Di Emidio, et al. 2014). More recently, it was shown that continuous overexpression of SIRT1 in oocytes by suppressing mTOR enhances reproductive capacity, preserves ovarian reserve and extends ovary lifespan in mice (Long, et al. 2019). These results are in good agreement with data showing that SIRT1 activator (SRT1720) improves the follicle reserve and prolongs the ovarian lifespan of diet-induced obesity in female mice via activating SIRT1 and suppressing mTOR signaling (Zhou, et al. 2014). In addition, activation or overexpression of SIRT1 not only partly prevents the deficient phenotypes of aged oocytes but also ameliorates the meiotic anomalies and oxidative stress in NMNAT2-depleted oocytes (Wu, et al. 2019). NMNAT2 belongs to the NMNAT family members that can catalyze the synthesis of NAD+ both in the de novo pathway and recycling pathway.

5. Energy sensors involved in ovarian pathological disorders

As a consequence of physiological roles of mTOR, AMPK and SIRT1, changes in their activity have been observed in some reproductive pathologies, such as PCOS (Tatone, et al. 2018) and POF.

5.1 PCOS

PCOS is a reproductive disorder affecting about 10% of women of reproductive age (Azziz, et al. 2016). Its diagnosis requires the presence of at least two of the following features; menstrual irregularities (oligo-/anovulation), signs of androgen excess and polycystic ovaries on ultrasound (corresponding to a follicle number per ovary ≥ 20 and/or an ovarian volume ≥ 10 mL in either ovary) (Teede, et al. 2018a). PCOS is typically associated with metabolic features like insulin resistance, abdominal obesity and an increased risk of developing type 2 diabetes obesity. At the reproductive neuroendocrine level, women with

PCOS exhibit reduced sensitivity to the inhibitory effects of sex steroids on gonadotropin secretion (Christman, et al. 1991, Eagleson, et al. 2000) and increased pituitary responsiveness to GnRH (Patel, et al. 2004), with both contributing to LH hypersecretion.

Some data have suggested that the mTOR signaling system could be an important pathophysiological basis of PCOS. Indeed, mTOR expression and phosphorylation, Rictor expression and its downstream effectors are increased in DHEA-treated PCOS mouse model (Yaba and Demir 2012). Moreover, a recent study showed that mTOR expression and phosphorylation in granulosa cells were higher in PCOS than non PCOS patients (Kuang, et al. 2020). Since DHEA increases mTOR expression in proliferative and differentiating cells (premature luteinization of granulosa cells), the mTOR signal pathway in DHEA metabolism could be important in the PCOS mouse ovary, possibly impairing selection of the dominant follicle and leading to abnormal follicular development (Yaba and Demir 2012). However, another study observed that the mTOR protein amount in luteal granulosa cells was similar in PCOS and healthy patients but less mTOR protein expression was found in luteal granulosa cells with PCOS compared to that in healthy patients upon stimulation with insulin (Song, et al. 2018). Moreover, the level of mTOR mRNA in granulosa cells from PCOS patients are decreased in response to berberine that is an isoquinoline alkaloid used in Chinese medicine (Kuang, et al. 2020). In humans, the lipid-lowering and insulin-resistance improving actions of berberine have clearly been demonstrated in numerous randomized clinical trials (Imenshahidi and Hosseinzadeh 2019). Thus, mTOR could be linked to PCOS through its involvement in the regulation of insulin sensitivity.

In addition, as described previously, PCOS patients have high levels of LH and blockade of central mTOR signaling by rapamycin causes decreased LH secretion in rodents (Roa, et al. 2009). Thus, rapamycin treatment is expected to be used to eliminate metabolic syndrome with PCOS and reduce LH secretion for preventing or treating anovulatory PCOS

517 despite potential adverse effects on fertility and short-term metabolism. Additional studies are
518 needed to explore the role of mTOR in the PCOS syndrome.

519 The interest in the involvement of AMPK and SIRT1 in PCOS development and
520 progression has been increasing (Tatone, et al. 2018). AMPK inhibitor Compound C
521 significantly reduced the effect of metformin and pioglitazone combination therapy on
522 restoring ovarian follicular development in PCOS rats (Wu, et al. 2018). In PCOS patients
523 with hyperinsulinemia, treatment with metformin enhances endometrial AMPKα expression
524 (Carvajal, et al. 2013). In addition, compared with non-PCOS patients, AMPKα
525 phosphorylation of the endometrium in PCOS patients is reduced (Li, et al. 2015). AMPK
526 activation is also observed in human endometrial cells exposed to a PCOS environment in
527 response to myo-inositol that is an insulin-sensitizing compound (Cabrera-Cruz, et al. 2020).
528 In KGN human granulosa cells, AMPK signaling pathways is activated in response to
529 berberine that is known to improve insulin sensitivity and ovulation function in PCOS
530 patients (Li, et al. 2020). Recently, Zhang et al showed that AMPK pathway is involved in the
531 reduction of the embryo loss rate in response to adiponectin treatment in PCOS mice (Zhang,
532 et al. 2020). In the well-established DHEA-induced PCOS mouse model, expression of SIRT1
533 and activation of AMPK are largely enhanced in ovary suggesting that SIRT1 through AMPK
534 activation could modulate autophagy in PCOS ovaries (Di Emidio, et al. 2020). The
535 AMPKα-SIRT1 pathway has also been demonstrated to be involved in the beneficial effects
536 of metformin and exenatide (a GLP-1 receptor agonist) on the reproductive and endocrine
537 functions of rats with PCOS (Tao, et al. 2019). In rats PCOS, combined treatment with
538 metformin and resveratrol improves ovarian follicular cell architecture by inducing
539 antioxidant and antiinflammatory systems via SIRT1 and AMPK activation (Furat Rencber, et
540 al. 2018). The SIRT1 agonist, resveratrol, can reduce oxidative stress in rats with
541 hyperandrogen-induced polycystic ovary syndrome (PCOS) through inhibiting p66Shc

(Wang, et al. 2020). Taken together, these results emphasized the significance of the AMPK/SIRT1 pathway in the treatment of PCOS. In addition, in PCOS human granulosa cells, SIRT1 expression is enhanced to inhibit excessive mitophagy in response to melatonin treatment suggesting a protective role of SIRT1 (Yi, et al. 2020).

5.2 POF

POF, is a challenging reproductive issue causing loss of ovarian function in women younger than 40 years of age (Torrealday, et al. 2017). POF is characterized by hypoestrogenism; increased serum gonadotropin levels; and, most important, amenorrhea (Nelson 2009). Ovaries of patients with POF are characterized by loss of secondary follicles and arrested folliculogenesis, which leads to a decrease or elimination of estrogen production and infertility. The roles of mTOR signaling in follicular development have been largely investigated (cf **Table 1**); abnormalities in this process lead to a series of pathologies such as POF and infertility (Liu, et al. 2018). Concerning the role of AMPK in POF, oocyte-specific deletion of LKB1 (an AMPK Kinase) from the primordial follicle stage leads to severe female fertility defects due to defective follicular development (Jiang, et al. 2016). At the molecular level, the loss of LKB1 in oocytes leads to enhanced mTORC1-S6K-rpS6 signaling with reduced AMPK activity (Jiang, et al. 2016). Thus, the AMPK/mTOR signaling may play a key role in the POF in women. However, it remains to be demonstrated in human since most of the experiments have been performed in mice. The role of SIRT1 remains to be deepened in human reproductive HPO axis (Zbroch, et al. 2018). Indeed, a recent study shows that loss of SIRT1 specifically in mouse oocyte induces premature ageing (Iljas, et al. 2020). In addition, Ma et al suggested a role of SIRT1 in the protection of premature ovarian insufficiency in mice in response to melatonin (Ma, et al. 2017).

6 Conclusions

Mammalian TOR, AMPK and SIRT1 are three energy sensors expressed in the reproductive cells of the HPO axis. It is now clear that a modulation of their expression by using conditional mutagenesis in mouse models or a modulation of their activation by using pharmacological approaches can impact the function of cells from HPO and consequently affect female fertility. However, if the activation level of mTOR and AMPK is frequently determined in the studies, those of SIRT1 is rarely evaluated. In addition, the molecular mechanisms of these energy sensor in the reproductive cells of the HPO axis remain to be *in vivo* investigated. Indeed, most of the reported effects have been obtained *in vitro* by using sometimes pharmacological or chemicals (rapamycin for mTOR, metformin or AICAR for AMPK and resveratrol for SIRT1). Indeed, only few specific deletion in mouse of these energy sensors mainly in oocytes have been performed in reproductive cells. For example no animals models with specific deletion for AMPK in gonadotrop cells (where AMPKα1 is strongly expressed) have been yet reported. Thus, some tools like genome editing could be used to make targeted genetic modifications of the mTOR, AMPK or SIRT1 in order to address their role in HPO axis. In rodents, some evidence shows that mTOR, AMPK and SIRT1 are involved and could be proposed as therapeutic target for reproductive pathologies such as PCOS (Liu, et al. 2016, Nejabati, et al. 2020, Tao, et al. 2017). We described here the involvement of mTOR, AMPK and SIRT1 in HPO axis but it is important to precise that these components also play a crucial role in the fertilization events, embryo development and uterine implantation processes in normal and pathological conditions (Correia, et al. 2020, Griffiths, et al. 2020, Martin-Hidalgo, et al. 2018, Ochiai and Kuroda 2020, Tatone, et al. 2018).

Figure legends

Figure 1 : Role of mTOR (A), AMPK (B) and SIRT1 (C) in the cellular homeostasis.

A. mTOR system includes two structurally and functionally distinct multi-molecular complexes: mTOR complex 1 (mTORC1**) and mTOR complex 2 (**mTORC2)A. The mTORC1 complex is composed of mTOR protein and several regulatory proteins: raptor, mLST8 (mammalian lethal with sec13 protein8), deptor (DEP domain-containing mTOR-inte-racting protein) and PRAS40 (proline-rich Akt substrate of 40-kDa). The mTORC2 complex consists of the proteins mTOR, rictor, Sin1 (stress-activated map kinase-interacting protein 1), protor (protein observed with rictor) and mLST8. **B. AMPK is composed of one catalytic α-subunit, and two regulatory subunits, β and γ.** It is activated by phosphorylation at Thr-172 of the α-subunit, which is promoted by either increased AMP/ATP and ADP/ATP ratios or by the serine/threonine kinase 11 (STK11/LKB1), calcium/calmodulin-dependent protein kinase kinase (CAMKK), and also, the transforming growth factor-β-activated kinase (TAK1). Upon AMPK phosphorylation, ATP-producing catabolic processes such as fatty acid oxidation, glycolysis, and autophagy by regulating downstream factors, including mTOR signaling are activated, while ATP-consuming anabolic phenomena such as protein synthesis, glycogenolysis, and lipogenesis are inhibited, thereby restoring the AMP/ATP balance. **C**. **SIRT1 is a NAD+-dependent deacetylase.** It is activated by phosphorylation and sumoylation and inhibited by methylation and nitrosylation. SIRT1 inhibits apoptosis, oxidant stress, hypoxia, and inflammation whereas it protects against metabolic stress and improves lifetime.

Figure 2 : Role of mTOR, AMPK and SIRT1 activation in the female reproductive functions at the Hypothalamic Pituitary and Ovary axis level. Involvement of mTOR has been mainly demonstrated by using the mTOR inhibitor, Rapamycin.

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1075 **Table 1 : The reproductive consequences of targeted disruption or overexpression of mTOR, AMPK and SIRT1 or associated components in mice HPG axis.**

Component modified	Targeted cell	Genetic transformation	Physiological and biochemical consequences	References
Raptor (regulatory-associated protein of mTORC1)	Oocyte (of primordial follicles)	Specific deletion	Follicular development and fertility not affected, loss of mTORC1 signalling in oocytes triggered compensatory activation of the PI3K signalling.	(Gorre, et al. 2014)
Rictor (rapamycin-insensitive companion of mTOR, a key component of mTORC2)	Oocyte (of primordial follicles)	Specific deletion	POF phenotype, massive follicular death, abnormal gonadal hormone secretion	(Chen, et al. 2015)
TSC1 or TSC2 (tumor suppressor tuberous sclerosis complex 1), negative regulators of mTORC1 activity	Oocyte	Specific deletion	Promotes the growth of all primordial follicles in neonatal animals, leading to the exhaustion of the entire follicle pool, followed by a POF phenotype	(Adhikari, et al. 2009, Adhikari, et al. 2010)
double deletion of TSC1 and PTEN	oocyte	Specific deletion	synergistic enhancement of oocyte growth and follicle activation when compared with singly mutated mice indicating that mTORC1 activation and increased AKT signaling synergistically stimulate the growth of primordial follicles	(Adhikari, et al. 2010)
Disruption of TSC1	granulosa cells of secondary follicles	Specific deletion	Enhancement of follicle growth, leading to increased ovulatory capacity and delivery of more pups followed by a POF phenotype	(Huang, et al. 2013)
Conditional knockout of mTOR	primordial or growing oocytes	Specific deletion	MTOR-dependent pathways in primordial or growing oocytes differentially affected downstream processes including follicular development, sex-specific identity of early granulosa cells, maintenance of oocyte genome integrity, oocyte gene expression, meiosis, and preimplantation developmental competence	(Guo, et al. 2018)
Disruption of LKB1	Oocyte from the primordial follicle stage	Specific deletion	Mice were severely subfertile with significantly enlarged ovaries. The entire primordial follicle pool was activated but failed to mature and ovulate, subsequently causing POF	(Jiang, et al. 2016)
Disruption of AMPK (α1AMPK)	Oocyte	Specific deletion	a decrease of 27% in litter size was observed. Absence of AMPK, modifies oocyte quality through energy processes and oocyte/somatic cell communication	(Bertoldo, et al. 2015b)
Disruption of AMPKα2	Kiss1 neurons	Specific deletion	females are fertile and have normal litter size.	(Torsoni, et al. 2016)
Disruption of AMPKα2	Kiss1 neurons	Specific deletion	conditional ablation of the AMPKα1 subunit in Kiss1 cells, largely prevented the delay in puberty onset caused by chronic subnutrition	(Roa, et al. 2018)
Disruption of both AMPK α1 and α2	Gonadotropes, and peri-ovulatory follicles	Specific deletion	normal estrous cyclicity, normal steroidogenesis during pregnancy, and no ovulatory defect but a delay in the timing of embryo implantation	(Griffiths, et al. 2020, McCallum, et al. 2018)
SIRT1	Whole body	Knockout	Mice are infertile, small ovaries, lack of ovulation, they have decreased levels of the gonadotropic hormones indicating a defect in the hypothalamic-pituitary-gonadal (HPG) axis	(Kolthur-Seetharam, et al. 2009, McBurney, et al. 2003)
SIRT1	Oocytes	Specific deletion	no effect in young females (no impact on ovarian reserve, oocyte chromosome segregation, mitochondrial function, antioxidant defence or litter sizes) but 50% of females lacking oocyte-Sirt1 become prematurely sterile between 9 and 11 months of age. Thus, loss of Sirt1 induces premature ageing.	(Iljas, et al. 2020)
SIRT1 H355Y	Whole body	Mutation of the catalytic domain	Animals have lower postnatal mortality than Sirt1−/− animals. Unlike Sirt1−/− females, homozygosity for the H355Y mutation did not affect female fertility	(Seifert, et al. 2012)
SIRT1	Oocytes	Specific over-expression	Sustains the ovarian follicular pool and/or oocyte quality during ageing. Thus, increasing Sirt1 function appears to combat deleterious effects of reproductive ageing in vivo	(Long, et al. 2019)

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