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Research

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Abstract

The extracellular matrix (ECM) of porcine mature oocytes was revealed by transmission electron microscopy (TEM) after treatment with tannic acid and ruthenium red. Present in the perivitelline space (PVS) and on the surface of the zona pellucida (ZP), it appeared to be composed of thin filaments and granules at the interconnections of the filaments, which were interpreted respectively as hyaluronic acid chains and bound proteoglycans. In order to determine whether this material is produced by the corona cells (the same ECM was found also on the surface of the zona pellucida and between cumulus cells) or by the oocyte itself, the synthesis of glycoproteins and glycosaminoglycans was checked by autoradiography on semi-thin and thin sections observed by light and electron microscopy. Immature oocytes within or without cumulus cells, were incubated with L [3H-] fucose or L [3H-] glucosamine – precursors respectively of glycoproteins and hyaluronic acid or hyaluronan (HA) bound to proteoglycans – for various times (with or without chase) and at different stages during *in vitro* maturation. In the first case, incorporation was found in both cumulus cells and ooplasm (notably in the Golgi area for 3H-fucose) and labeled material accumulated in the ECM of the PVS and of the ZP surface. Labeling in the PVS with both precursors was maximum between metaphase I (MI) and metaphase II (MII) and was partially extracted by hyaluronidase but not by neuraminidase. Tunicamycin, an inhibitor of glycoprotein synthesis, significantly decreased the amount of ^{3}H-fucose labeled molecules in the PVS and increased the incidence of polyspermic penetration during subsequent *in vivo* fertilization. Since cumulus-free oocytes also secreted ^{3}H-glucosamine containing compounds, both oocyte and cumulus cells probably contribute to the production of the ECM found in the PVS of mature oocytes. ECM and particularly its HA moiety present on both sides of the ZP may constitute a favourable factor for sperm penetration.

Background

Using ruthenium red and tannic acid to improve the contrast of glycoproteins and/or proteoglycans in TEM, we could observe material in the PVS of the oocytes that can be interpreted as ECM. Similar observations were previously made in other eutherian species [1-3]. In fact the

concept of oocyte ECM was first introduced in a preliminary histochemical study of mature rabbit and pig oocytes [4]. In the present study, the structure of the ECM deposited on both sides of the ZP was found indistinguishable from that distributed in the extracellular space of the cumulus oophorus. Again, this was already observed in a marsupial oocyte [5] and was first indicated by a fuzzy aspect of the ZP surface penetrated by cumulus matrix and a reticulated structure in the PVS of the mature rabbit oocyte [6].

The diverse origin of egg envelopes was reviewed by Wilson [7] who classified them according to their origin as primary (from oocyte), secondary (from follicle cells), and tertiary (from oviduct). In the present work, we investigated the origin of the oocyte ECM, e.g. is it synthesized during resumption of meiosis by the cumulus oophorus, by the oocyte, or by both, which seems to be the case.

Materials and Methods

Collection of oocytes

We used prepubertal gilts of miniature pigs (crossbread of Minnesota and Göttingen strains) and cyclic sows of the Large White breed. Ten prepubertal, 5 – 6 month old gilts, were stimulated by 1000 IU pregnant mare serum gonadotropin (PMSG) (Antex, Leo, Copenhagen, DK) and 72 hour later by 1500 IU human chorionic gonadotropin (hCG) (Praedyn, Spofa, Prague, CZ). Injection of hCG represented time 0 for subsequent *in vivo* or *in vitro* meiotic maturation. The nuclear stages found at successive intervals after hCG administration were: GV until 16 hour, late diakinesis (LD)-MI around 20 hour, and MII around 40 hour. Untreated cyclic sows were monitored daily for the onset of estrus with a boar. Ovulated oocytes were recovered by flushing oviducts with phosphate buffered saline at the expected time of ovulation. Procedures relative to animal care were in accordance with the rules established by the French Ministry of Agriculture (guidelines of 04. 19. 1988).

Electron microscope morphological and cytochemical studies

TEM was carried out according to Reima et al [8]. Glycoproteins and /or proteoglycans were preserved in preovulatory oocytes, oocytes cultured *in vitro* and ovulated oocytes with 0.5% ruthenium red [9] or 1.0% tannic acid [10] during osmium tetroxide postfixation.

Culture

Oocyte-cumulus complexes (OCCs) were mechanically isolated at 0 or 16–20 hour after hCG from excised ovaries after rupture of the follicular wall of the large preovulatory follicles. OCCs or oocytes deprived of cumulus cells were cultured, under paraffin oil at 38°C in an atmosphere of 5% CO2 in air, in 0.1 ml of the following medium: 72 ml of isotonic TC 199 medium (Usol, Prague, CZ) supplemented with 18 ml of 1.45% NaHCO3, 0.002% phenol red, 10 ml of 5.5% (v/v) glucose solution, 4 mg sodium pyruvate, 30 mg/ml freeze-dried calf serum growth proteins (Usol), 50 IU penicillin and 50 IU/ml streptomycin. In these conditions, germinal vesicle breakdown (GVBD) occurred 16–20 hour after hCG, as *in vivo*.

Incubation with labeled precursors

The culture medium was enriched with 3.7 MBq/ml of L-[^{3}H]-fucose (Radiochemical-Centre, Amersham, specific activity 1073 GBq/mM) or with 3.7 MBq/ml of L-[^{3}H]-glucosamine, (same origin; specific activity 140.6 GBq/mM) in order to study incorporation in glycoproteins and glycosaminoglycans. Incubation periods varied from 1 to 40 hour, starting from 0 hour after hCG or from the GVBD stage followed or not by culture without precursor (chase) for 3 to 4 hours. Ten microgram/ml Tunicanmycin (Calbiochem, San Diego, CA, USA) added with the precursors was used to inhibit N-linked glycosylation of asparagine residues by blocking formation of dolichol-diphospho-N-acetylglucosamine [11]. Although inhibiting essentially glycoprotein synthesis, Tunicamycin may also affect N-linked oligosaccharide biosynthesis of proteoglycans [12].

Oocytes with or without cumulus cells were incubated with labeled sugars as reported in Tables 1a and 1b. After incorporation, oocytes in all groups were washed in medium containing unlabeled sugars; they were either submitted to bovine hyaluronidase (Koch-Light Laboratories, Coinbrook, England) treatment (200 IU/ml, 30 minutes at 38°C) or not. Most oocytes were processed for TEM and autoradiography. Part of the OCCs treated with Tunicamycin or not but not with hyaluronidase were transferred *in vivo*.

In vivo transfer of cultured oocytes

Prepubertal gilts treated with PMSG and hCG were ovariectomized unilaterally after incision in the linea alba at 0, 16 and 20 hour after hCG injection. These animals were then inseminated twice with freshly collected semen, at 24 and 32 hour after hCG. Cultured OCCs were transferred surgically to the ampullae on the ovariectomized side around 40 hour after hCG. The recipient gilts were slaughtered 16–18 hour later, the eggs were flushed from oviducts and prepared for autoradiography.

The ovulated oocytes of the control side were mounted on slides, fixed in acetic acid/alcohol fixative (1:3 v/v) for 24 hours, stained with 1% orcein and examined by phase-contrast microscopy for fertilization.

Autoradiography

Oocytes and fertilized eggs were washed in cold medium containing 1% glucosamine or fucose and fixed as for

Table 1: The EFFECT of TUNICAMYCIN on ^{3}H-FUCOSE INCORPORATION in cumulus, ooplasm and PVS, as observed on semi-thin sections

Oocyte stage at onset of experiment	Duration of culture (hr)	Culture medium (1)	Labeling (2) Cumulus cells	Labeling (2) ooplasm	Labeling (2) PVS
GV (0 hr post hCG)	8		+++	+	++
	8	T	+++		+
	36			++	+++
	36	T		+	+
LD (20 hr post hCG)	8		+++	++	++
	8	T	+++	+	+
	20			++	++
	20	T		+	+

(1) The modified TC 199 was supplemented with ^{3}H-Fucose in the presence (T) or absence of Tunicamycin; (2) To evaluate labeling on semi-thin sections the following scale was used : ++++ very intense, +++intense, ++medium,+ just over background

morphological evaluation. Semi-thin sections on glass slides were coated with liquid nuclear emulsion Ilford K5 (Ilford Ltd, Mobberley, UK), exposed for 30 days at 4°C and developed in Kodak D 19 (Eastman Kodak, Rochester, NY, USA). The autoradiograms were stained with 1% toluidine blue (Gurr, High Wycombe, UK) pH 4.2. An arbitrary scale of labeling intensity was used (see Table 1).

Results

Cytochemical characterization of the extracellular matrix

Oocytes matured *in vitro* or *in vivo* showed material contrasted with tannic acid and ruthenium red in the intercellular space of the cumulus oophorus when present, on the surface of the ZP and in the PVS. Tannic acid stained only filaments (Fig. 1a), whereas ruthenium red stained filaments and beads aligned on them (Fig. 1b). In the PVS, these granules were also present along the inner side of ZP (Fig. 1b) and sometimes on the surface of the oolemma.

Incorporation of labeled precursors

Thin sections: localization of labeling

Cumulus-enclosed oocytes incubated in ^{3}H-fucose for 24 hours (from 0 hour post hCG to MI) showed heavy accumulation of labeling in the ooplasm, in the PVS, and in corona cells. Silver grains were also associated with profiles of Golgi vesicles (Fig. 2a). Isolated oocytes were also labeled, although less intensely (no "dense foci"), in ooplasm and PVS (Fig. 2b).

Cumulus-enclosed oocytes incubated in the same conditions with ^{3}H-glucosamine were heavily labeled in ooplasm and PVS, as well as in corona cells and their projections in the ZP (Fig. 3a,3b). Cumulus-deprived oocytes showed cytoplasmic labeling and PVS labeling (Fig. 3c,3d). Similar labeling on consecutive sections indicated that the autoradiographic emulsion was homogeneously distributed and that the background was low.

Semi-thin sections: quantitative results on cumulus enclosed oocytes

The results of several experiments of incorporation are summarized in Tables 1 and 2. Table 1 indicates that ^{3}H-fucose incorporation was very high in cumulus cells after 8 hour of incubation starting at GV or LD stages, and that ooplasmic incorporation was higher for a 36 hours incubation starting at the GV stage and 8 or 20 hour of incubation starting at LD stage. Accumulation of labeled material in the PVS was visible after 8 or 20 hour of incubation at LD stage and maximum after 36 hour of incubation at GV stage. Tunicamycin reduced labeling in the PVS and induced low uniform labeling in the ooplasm in most cases (Table 1).

Short incubations with ^{3}H-glucosamine already resulted in high labeling of cumulus cells but low labeling of the PVS, except at LD stage and after chase (Table 2). Long incubations (≥20 hours) in ^{3}H-glucosamine, with or without chase, induced heavy labeling in the perivitelline space. Figure 4 shows that a short incubation after GVBD *in vivo* or *in vitro* followed by chase until MII also gave an accumulation of labeling in the PVS. After GVBD *in vivo* or *in vitro* and long incubation, Tunicamycin did not inhibit, but increased, ^{3}H-glucosamine labeling. Until MII, hyaluronidase removed almost all incorporated ^{3}H-glucosamine particularly in the PVS, in oocytes with or without Tunicamycin treatment (Fig. 5).

Effect of Tunicamycin on maturation and fertilization

Tunicamycin did not prevent GVBD and the extrusion of the first polar body *in vitro*. Oocytes transferred *in vivo* after 36 hour culture *in vitro* in the presence of ^{3}H-glucosamine with or without Tunicamycin were similarly

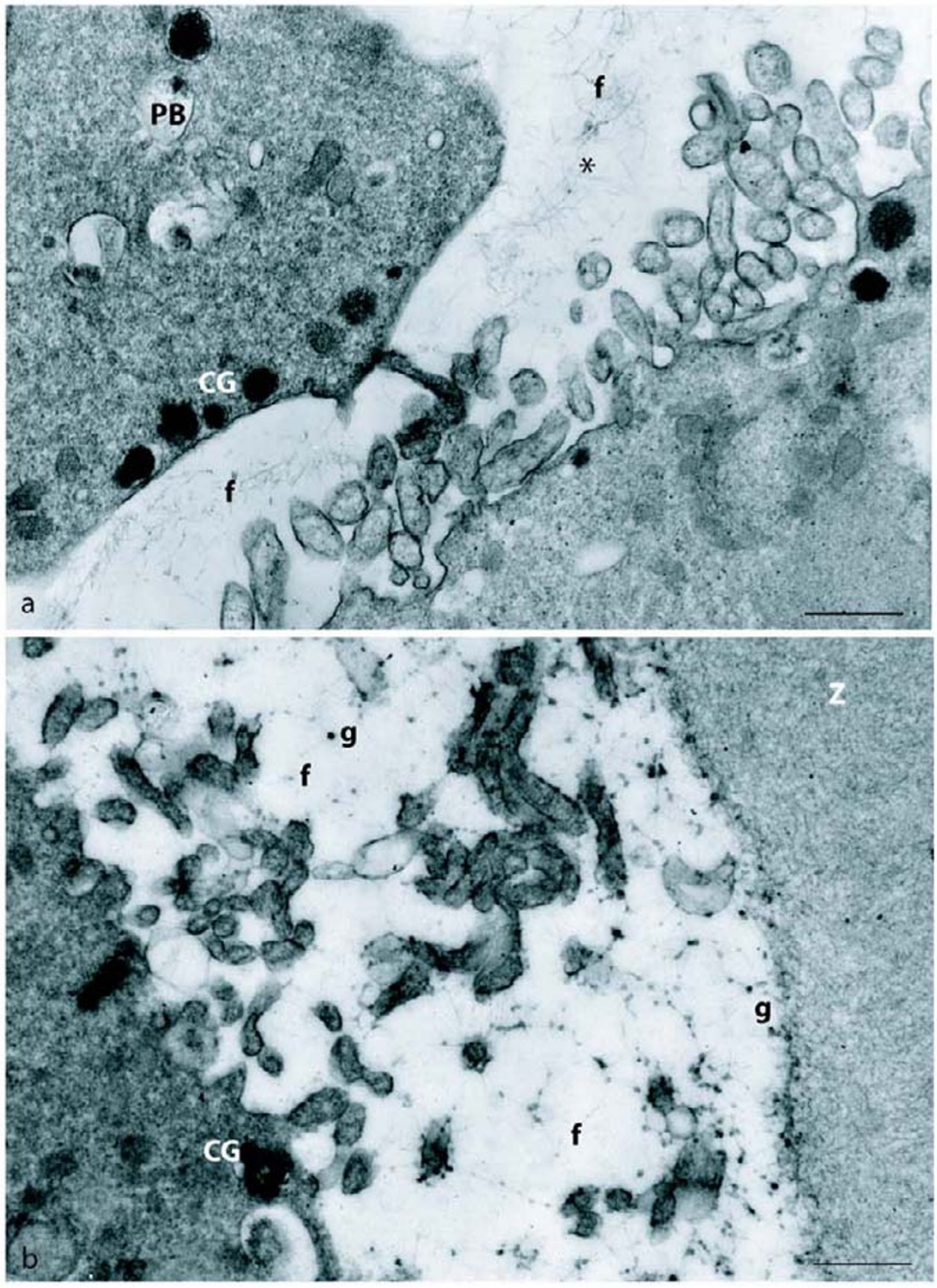

Figure 1
TEM of the PVS of pig oocytes matured *in vitro* until MII. In the PVS, the ECM appears as a meshwork of fibrils (f) after tannic acid treatment (a) or as a meshwork of fibrils (f)and attached granules (g) after ruthenium red treatment (b). In the latter case, granules are also visible on the inner side of the ZP (Z). Part of the first polar body (PB) containing cortical granules is visible in (a); these granules (CG) are contrasted in both cases. *bar = 0.5 μm*

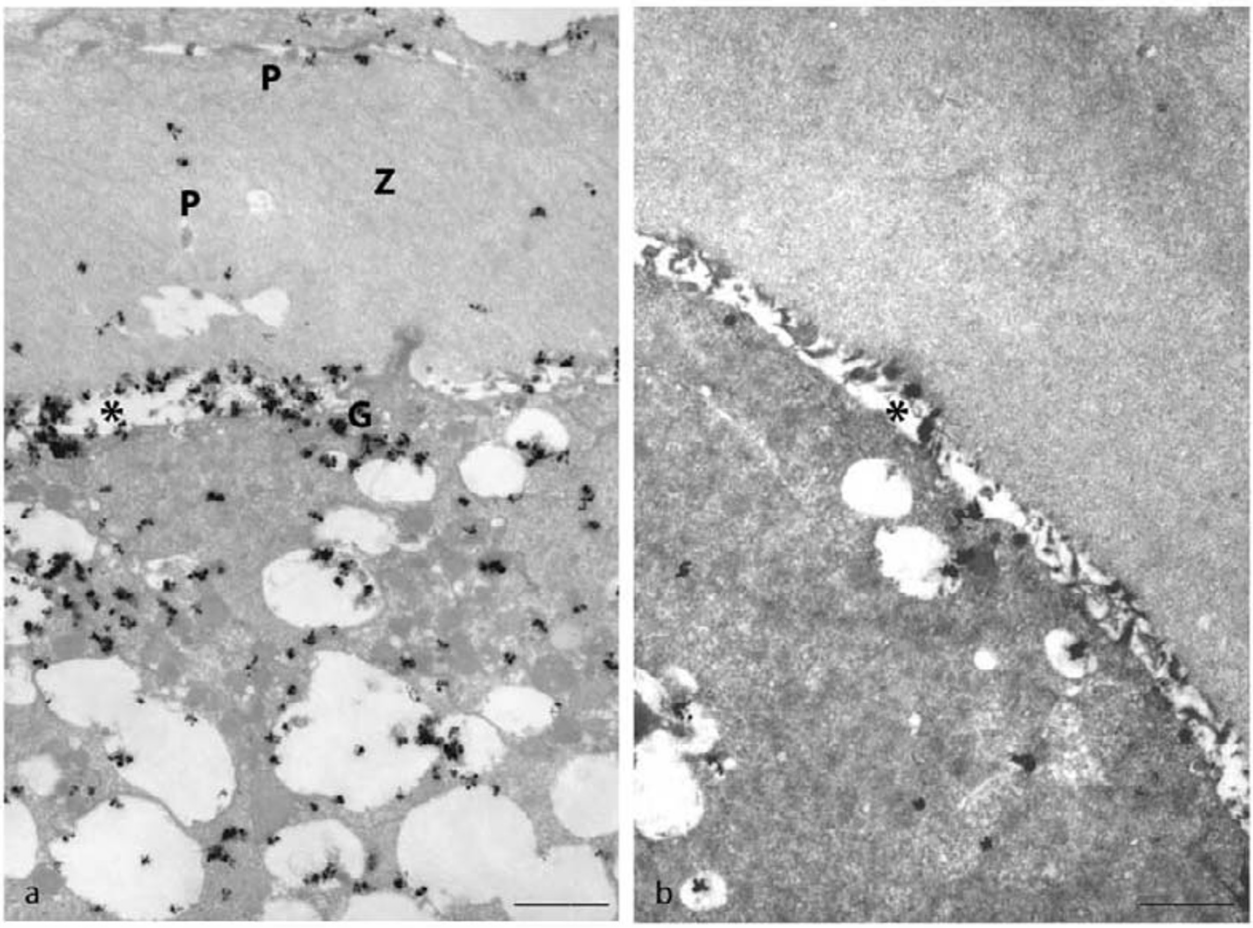

Figure 2
TEM autoradiography of thin sections of oocytes cultured from 0 hr post hCG until MI in presence of ^{3}H-fucose, with (a) or without (b) cumulus cells. *bar = 1 µm* a : general heavy labeling of the ZP (Z) surface, of corona cell projections (P), and particularly of the PVS (*) and Golgi vesicles (G).b : low labeling in PVS (*) and ooplasm.

labeled as control non transferred oocytes, although the intensity may decrease in cytoplasm while not in PVS after around 20 hour chase *in vivo* (Fig. 6). Transferred oocytes were all fertilized and both pronuclei generally developed, although the incidence of polyspermy was relatively high with Tunicamycin, at least in oocytes incubated *in vitro* during the second half of maturation (Table 3).

Discussion

We will essentially discuss the composition and origin of the ECM in the mature pig oocyte. It seems also necessary to question the role of the oocyte ECM components.

Demonstration of the extracellular matrix

Our first observations on perivitelline ECM [4] showed that the ZP of rabbit and pig ovulated oocytes was surrounded by two layers (external and internal) of much more negatively charged (acidic) material. The components of this ECM, revealed by deposition of colloidal iron hydroxide *in toto* or on thin sections of hydrophilic resin, were partly removed by treatment with hyaluronidase, but not with neuraminidase, as compared to controls.

These results showed the fine topographical localisation of hyaluronan or its acidic form hyaluronic acid (HA), which has been found associated with both outer and

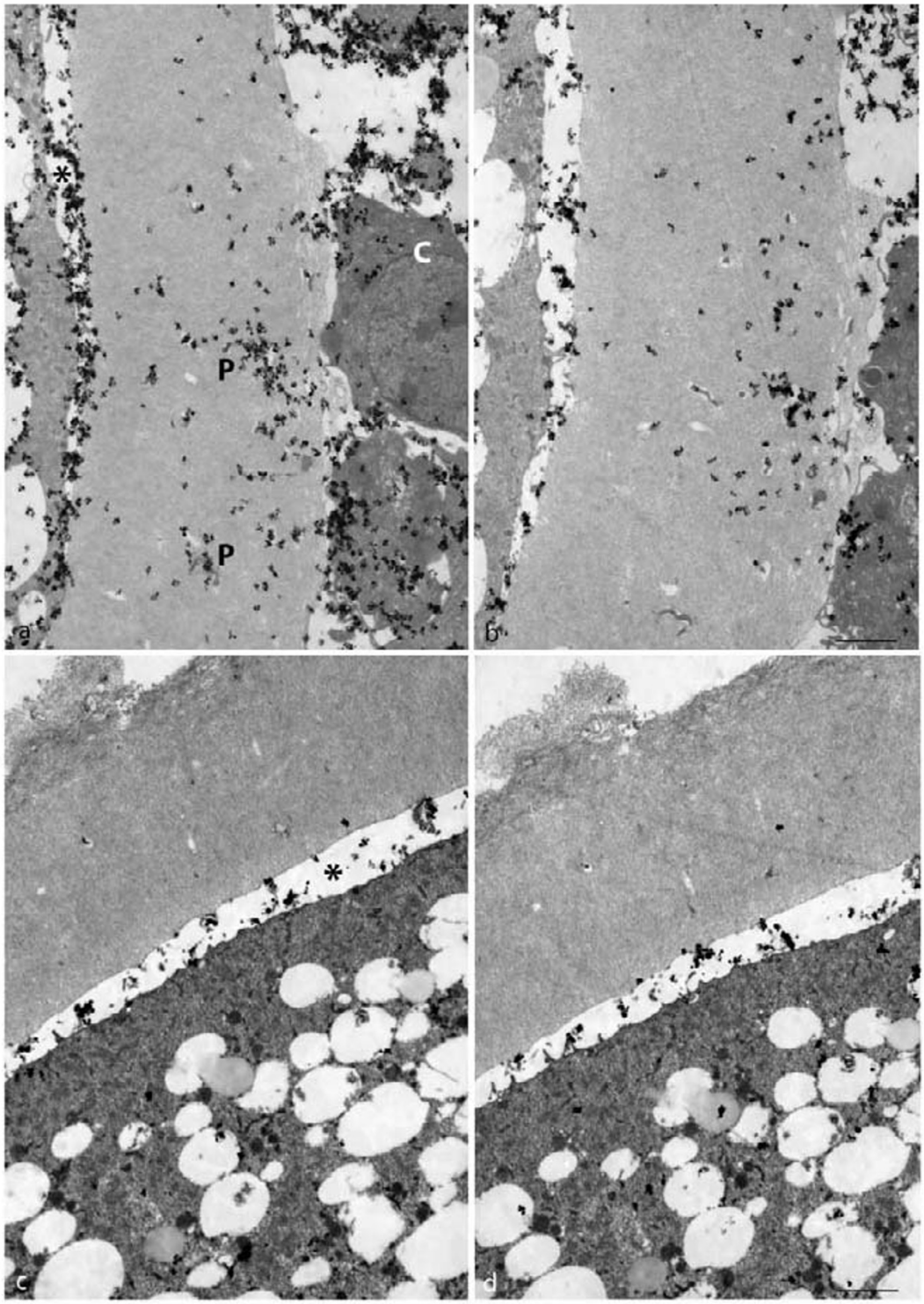

Figure 3
TEM of serial sections of oocytes cultured from 0 hr post hCG until MI in presence of ^{3}H-glucosamine, with (a, b) or without (c, d) cumulus cells. *bar* = 2 μm a, b : heavy labeling of corona cells (C) and their projections (P) in the ZP, of the PVS (*) and of the ooplasm.c, d : light ooplasm and PVS (*) labeling.

Table 2: The EFFECT of TUNICAMYCIN on 3H-GLUCOSAMINE INCORPORATION in cumulus, ooplasm and PVS, as observed on semi-thin sections

Oocyte stage at onset of experiment	Duration of culture (hr)	Culture medium (1)	Labeling (2) Cumulus cells	Labeling (2) ooplasm	Labeling (2) PVS
GV (0 hr post hCG)	1		++	+	+
	1+3CH		++	+	+
	20		++++	++	+++
	20+24CH		+++	+	+++
	40			++	+++
	40 (H)			+	+
	40	T		++	++++
	40 (H)	T		+	+
LD (20 hrs post hCG)	1		++	+	+
	1+3CH		++	+	++
	24			++	+++
	24 (H)			+	+
	24	T		++	++++
	24 (H)	T		+	+

(1) The modified TC 199 was supplemented with ^{3}H-glucosamine in the presence (T) or absence of Tunicamycin (2) Same scale of labeling as in Table 1 CH = chase (H) = hyaluronidase treatment at the end of culture

inner sides of the ZP and in the PVS of mammalian oocytes in former light microscope studies reviewed in [1]. Two HA rich layers surrounding and impregnating the two sides of the ZP may partially explain some descriptions of the ZP as composed of several layers differently stained [1]. HA was more recently localized on the ZP surface in hamster oocytes with fluorescent cartilage proteoglycan HA binding domain [13] and in the oocyte PVS of the same species with a hyaluronidase gold complex on thin sections [14].

As classical techniques for TEM do not reveal the ECM well, the use of tannic acid and ruthenium red allowed to confirm the presence of an ECM of similar appearance (fibrils and granules) between the cumulus cells, on the outer and on the inner sides of the ZP in mature pig oocytes, as well as in other eutherian species [1,3]. Scanning electron microscopy (SEM) observations also detected the ECM in the PVS: a sponge-like structure was observed on the inner side of the ZP of mature pig oocytes [15] and an ECM composed of filaments and granules was also found on the inner side of the zona of human oocytes after fixation in the presence of ruthenium red [16].

As hyaluronidase was shown to remove the filaments and trypsin partly digested the granules of hamster [17], opossum [5] and human oocyte ECM [3], these authors concluded that the filaments were composed of HA and the granules of proteins. The granules effectively result from the condensation of proteoglycans by ruthenium red [18]. Similarly, in the cumulus oophorus ECM of mouse maturing oocytes, the filaments (and incidentally the associated granules) revealed by dialyzed iron-equivalent to colloidal iron hydroxide used by ourselves [4] – were sensitive to hyaluronidase [19]. The above ultrastructural observations are in agreement with the long chain structure of the HA molecule; in fact, schematically aggregates of proteoglycans and HA appear like bottle-brush structures [20].

The appearance of an ECM in the PVS of oocytes around maturation may not be restricted to mammals, since an analogous 'secretion' was observed in other groups, e.g. in amphibians [21].

Synthesis of the extracellular matrix

Labeling target

The ZP is synthesized during the oocyte growth phase and by the oocyte itself, at least in the mouse [22-24]. In pig, transcripts of ZP1 were found only in growing oocytes [25] and no labelling was observed at the TEM level in follicular cells using three monoclonal antibodies to ZP proteins [26]. The glycoproteins of the cortical granules are synthesized during oogenesis and at least before GVBD in most species. In pig, the cortical granule density increases in the oocyte cortex during maturation by centrifugal migration rather than by new synthesis [27]. Indeed, there was no silver grain condensation over these granules in our autoradiograms. So the observed accumulation of labeled material on both sides of the ZP, in the transverse

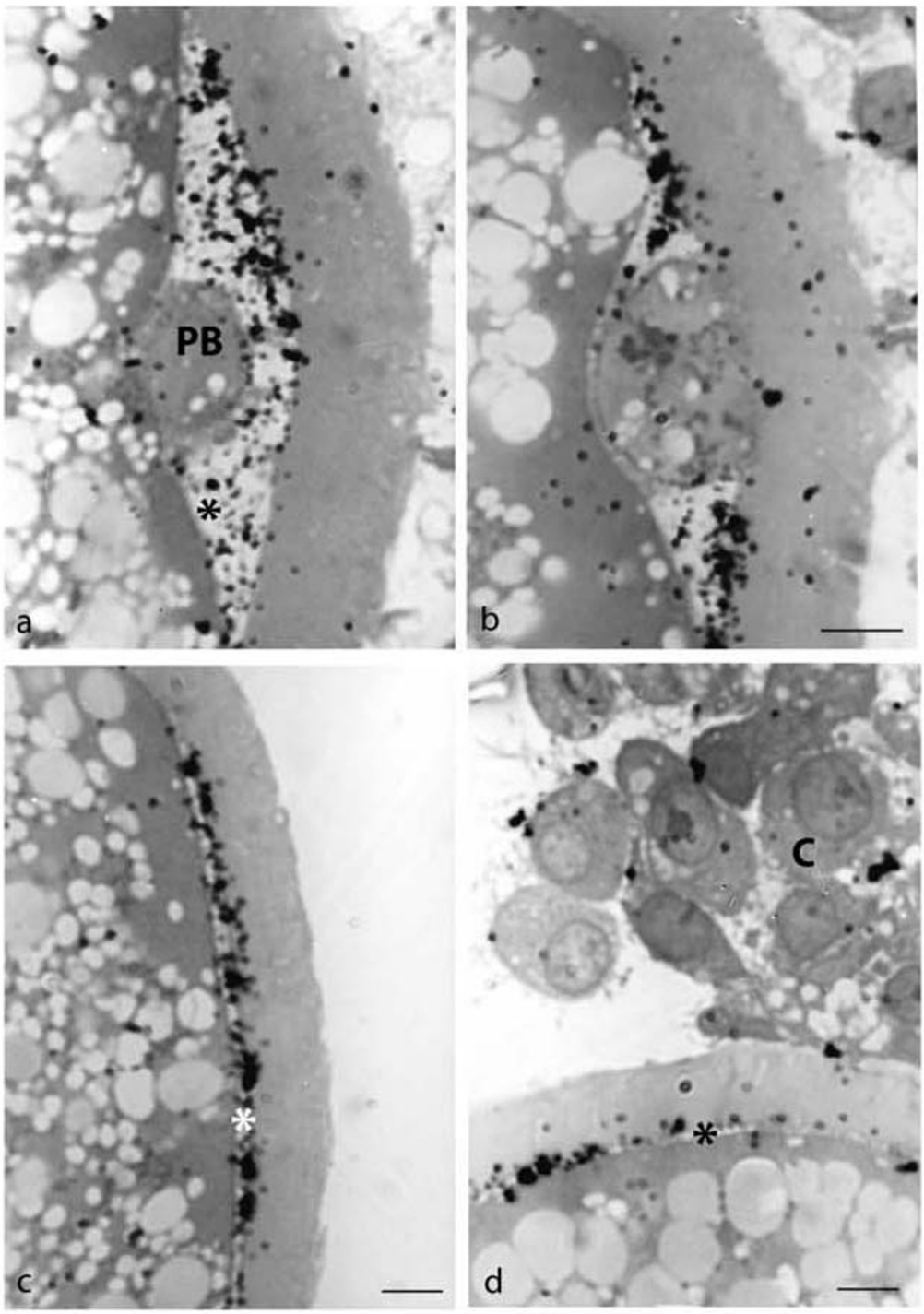

Figure 4
Light microscope autoradiography of oocytes incubated after GVBD for 3 hrs in the presence of ^{3}H-glucosamine and chased until MII after initiation of maturation *in vivo* (a, b, c) or *in vitro* (d). *bar = 5 μm*. a and b : successive semi-thin sections show similar label accumulation in the PVS (*) around the first polar body (PB).c : accumulation in the PVS (*) away from the polar body.d : after complete *in vitro* maturation, the PVS (*) is also heavily labeled. Some grains are found around remaining cumulus cells (C).

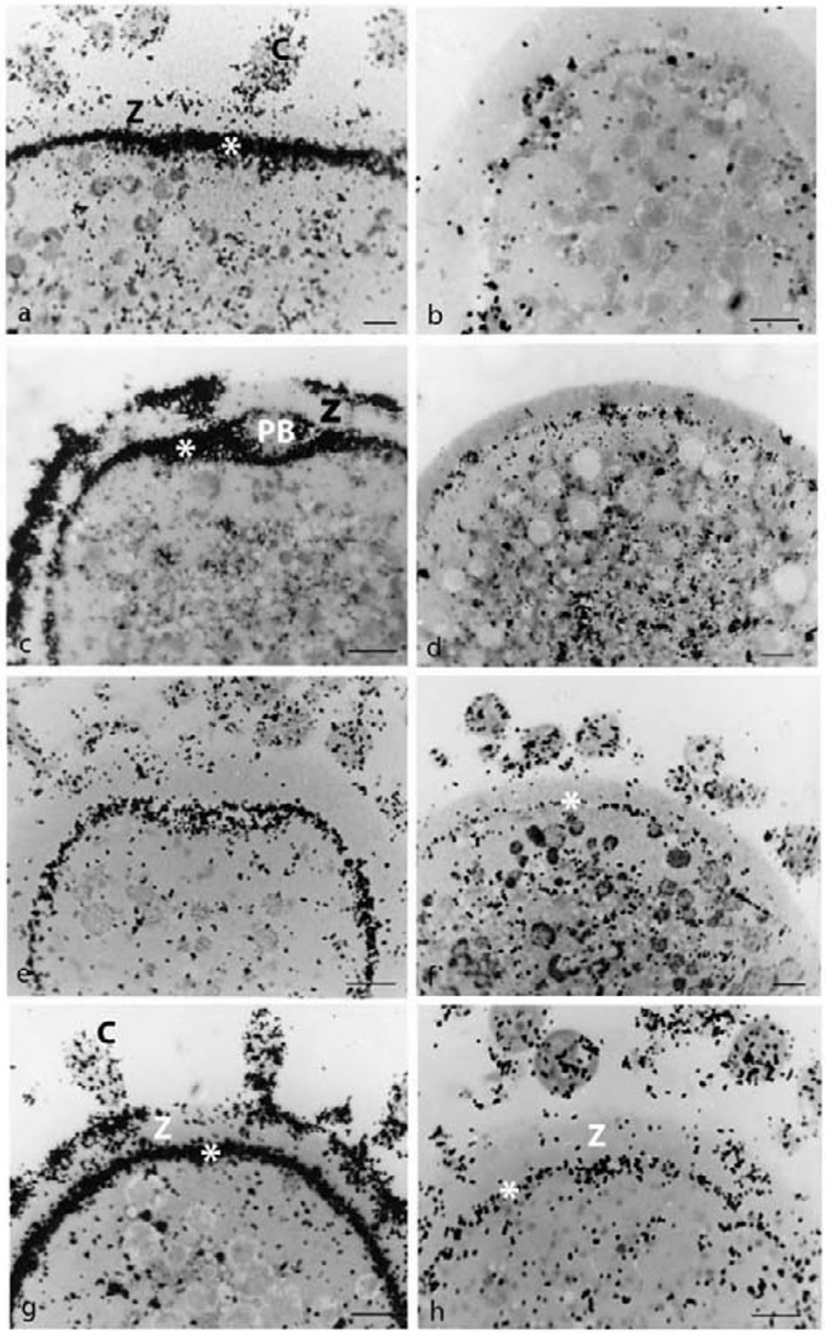

Figure 5
Effect of Tunicamycin and hyaluronidase on continuous incorporation of ^{3}H-glucosamine from GVBD stage until MII after initiation of maturation *in vivo* (a, b, c, d) or *in vitro* (e, f, g, h). *bar = 10 μm* a : labeling of corona cells (C), of the surface of the ZP (Z), of the PVS (*) (accumulation) and of the ooplasm in a control oocyte.b : hyaluronidase treatment removes most of the labeling.c : after Tunicamycin treatment, labeling is accumulated in the PVS (*), around the polar body (PB) and on the surface of the ZP (Z).d : after Tunicamycin and hyaluronidase treatment, labeling is reduced.e : labeling of control oocyte, similar to : a.f : after hyaluronidase treatment, labeling in PVS (*) is reduced.g : heavy labeling specially of corona cells (C), ZP surface (Z), PVS (*) after Tunicamycin treatment.h : after Tunicamycin and hyaluronidase treatment, labeling of PVS (*) and ZP (Z) surface is reduced.

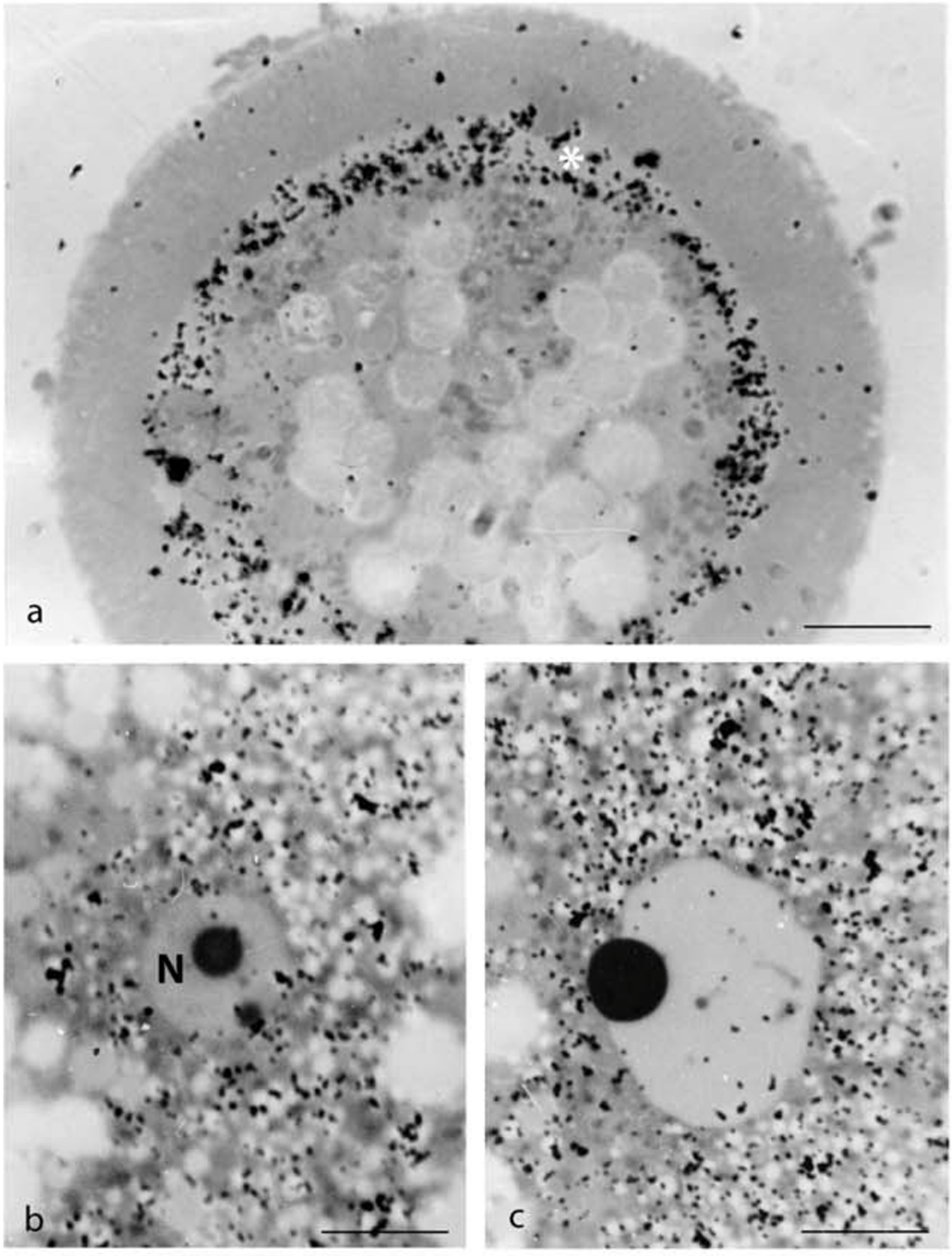

Figure 6
Labeling of fertilized eggs incubated in presence of ^{3}H-glucosamine during the whole *in vitro* maturation and collected 20 hr after transfer *in vivo* in an inseminated gilt. *bar = 10 μm* a : heavy PVS (*) labeling still visible.b : ooplasm labeling around a pronucleus (N).c : similar labeling in an oocyte treated with Tunicamycin during maturation.

Table 3: *in vitro* MATURATION and *in vivo* FERTILIZATION of PIG OOCYTES CULTURED in MEDIUM with TUNICAMYCIN and ^{3}H-GLUCOSAMINE

Time of culture for *in vitro* maturation	Culture conditions (1)		No of oocytes used	No of oocytes (2) in			Oocytes (3) fertilized showing	
	^{3}H-glucosamine	Tunicamycin		GV	MI	MII	syngamy	3–4 pronuclei
0 – 40 hr (GV → MII)		T	55	2 [>5]	10 [18]	43 [78]		
	G		8				6 [75]	2 [25]
	G	T	10				8 [80]	2 [20]
16 or 20 – 40 hr (LD → MII)		T	48	0 [0]	4 [8]	44 [>91]		
	G		10				9 [90]	1 [10]
	G	T	12				6 [50]	6 [50]

(1) Pig oocytes isolated 72 hr after PMSG injection (0 hr) or 16–20 hr after hCG injection were cultured in the modified TC 199 supplemented with (G) or without ^{3}H-glucosamine in presence (T) or absence of Tunicamycin. (2) At the end of the culture, cumulus cells were removed, oocytes were mounted on slides, fixed and stained with aceto-orcein. (3) In four experiments, the cultured oocytes were transferred into the oviducts of mated unilaterally ovariectomized gilts and flushed out 16–18 hr after transfer. All eggs were evaluated on semi-thin sections. [] = percent

canaliculi, and possibly on the oocyte surface (resolution limited by silver grain size), is interpreted essentially as synthesis of ECM and accessorily of transmembrane or membrane-bound components.

Precursor specificity

Both glycoproteins and proteoglycans were probably synthesized since we used precursors specific for these molecules. Fucose is a better precursor for glycoproteins [28], as confirmed by Golgi labeling in both oocyte and cumulus cells, whereas glucosamine is a direct precursor of N-acetyl-glucosamine-glucuronic acid chains constitutive of HA forming aggregates with proteoglycans. In fact, ^{3}H-glucosamine was incorporated essentially in the 'mucopolysaccharide' fraction by granulosa cells of pig small follicles [29], in HA of OCC of bovine small follicles [30] and in hyaluronidase digested glycosaminoglycans of pig OCC [31]. ^{3}H-glucosamine was also used to study the retention of synthesized HA in expanding pig OCC [32]. Similarly, in our experiments, the conditions were adequate for accumulation of HA in the ECM since incorporation occurred in the presence of serum [33]; effectively serum provides inter-α-inhibitor proteins binding HA, whereas the cumulus cells express the tumor necrosis factor stimulated gene-6 protein (binding to HA and interacting with inter-α-inhibitor) and link protein (binding HA to proteoglycans); all these components form stable complexes as reviewed by Salustri [34] and confirmed by Sun et al. [35].

The experiments including a hyaluronidase treatment confirmed that labeling with ^{3}H-glucosamine was largely found in HA bound proteoglycans, whereas Tunicamycin effectively inhibited ^{3}H-fucose incorporation into glycoproteins. Tunicamycin block of the glycoprotein pathway may indirectly increase glycosaminoglycan synthesis (Table 3).

Chronology of the synthesis

In mouse, ^{3}H-glucosamine-labelled compounds accumulated *in vivo* in oocytes of large follicles, on both sides of the ZP, 4 and 8 h after injection of hCG [36]. In human oocyte cumulus complexes of late preovulatory follicles, ^{3}H-fucose and ^{3}H-glucosamine were incorporated *in vitro* by oocyte and cumulus cells; after chase, labeling accumulated also on both sides and in the canaliculi of the ZP [37]. These experiments do not give the complete chronological profile of ECM synthesis during maturation. In pig oocytes, the incorporation of ^{3}H-fucose and ^{3}H-glucosamine was generally intense after long incubations starting either at GV or LD stage. The process may be relatively slow since a 1 hr incubation did not result in important labeling at any stage, except after GVBD and with chase. The moderate effect of Tunicamycin on ^{3}H-fucose labeling around LD may suggest that the synthesis of glycoproteins is maximal at this period; it may decline later [38]. Labeling in the PVS was very heavy after long incorporation of ^{3}H-glucosamine at both GV and LD stages. It means that maximum accumulation of ECM in the PVS occurs only at the end of maturation and that accumulated material is stable as further shown by labelling of fertilized eggs. The timing of oocyte ECM secretion fits with the centripetal cumulus mucification [39].

Origin of the oocyte ECM

Labeling found in the cytoplasm of corona cells, including cytoplasmic projections in the ZP, and of the oocytes, indicates that both cumulus cells and oocytes contribute to the synthesis of ECM. HA is built on the inner side of

the plasma membrane by hyaluronate synthase as a huge chain (ten thousand dimers) and translocated to the outer side or released free (review by Lee and Spicer [40]); different hyaluronate synthases were found during maturation in pig cumulus cells and oocyte, respectively [41]. Of course, it is well known that cumulus cells produce their own ECM during expansion [30,34]. These latter and our own studies show that this ECM 'contaminates' the ZP surface. Moreover, through their trans-zona projections, cumulus cells may also contribute to the perivitelline ECM as long as these cell processes are not retracted. However, oocytes deprived of cumulus did produce part of the perivitelline ECM (at least proteoglycans). The oocyte may also indirectly contribute to OCC mucification; it is supposed that the cumulus expansion-enabling factor involved is growth differentiation factor-9 produced by the oocyte which can upregulate hyaluronan synthase and downregulate urokinase plasminogen activator of the cumulus cells, thereby promoting mucification [34]. In pig oocytes, two different factors may contribute to HA production and retention [32].

Effect of Tunicamycin on fertilizability

Tunicamycin did not prevent *in vitro* maturation and *in vivo* fertilization of pig oocytes versus control oocytes after incubation in ^{3}H-glucosamine which did not appear inhibitory per se. An increase of polyspermy may be explained by a change in the composition (proportion of glycoproteins/glycosaminoglycans) in the ECM of the PVS. This emphasizes the potential role of ECM in the PVS for a normal fertilization.

Roles of the oocyte ECM

The roles of the cumulus oophorus was reviewed by Tanghe et al. [42]. We will focus below on the ECM located on both sides of the ZP in mature oocytes. Among the various components found in the cumulus ECM [34,43,44], only HA has been well characterized in the oocyte ECM, we will discuss only the roles of this compound.

Role of HA on the surface of the ZP

It is possible that the cumulus may just facilitate fertilization *in vivo*, since after its removal *in vitro* fertilization can succeed, more or less according to the species [42]. There are species such as cow and sheep where the cumulus and corona cells are rapidly removed after ovulation, possibly before fertilization [45]. In the pig, penetration occurs as soon as 1–2 hr after ovulation [46]. Therefore if acrosomal hyaluronidase has a role [2], it may be to remove HA bound to the surface of the ZP at the site of sperm attachment.

Role of HA in the PVS

Inside the PVS, HA may also play a positive role during fertilization, offering a fluid (hydrated) medium for actively swimming rabbit spermatozoa (Thibault, 2003, personal communication) and facilitating the last flagellar movements of hamster spermatozoa before [47] and after contact with the oolemma [48]. Finally, HA and eventually other glycosaminoglycans may enhance the viability of oocytes and their further development *in vitro* [49].

Conclusion

The ZP of maturing oocytes is surrounded on both sides by ECM, partly produced by the expanding cumulus and partly by the oocyte (material in the PVS). The oocyte ECM is constituted of glycoproteins and mainly proteoglycans associated with HA, the latter probably playing multiple adjuvant roles during and after fertilization.

It is now the task of proteomic technologies to draw up the list of the glycoproteins and proteoglycans of the PVS and of the oolemma synthesized during resumption of maturation and to define their precise roles.

Authors' contributions

A. P and J. M collected the oocytes and did the surgical operations; V. K executed the incorporation and autoradiography experiments; J. P prepared the specimens for electron microscopy; J-E. F took electron micrographs, made the final interpretations and wrote the manuscript; J. D prepared the plates.

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