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Cellular and Microvascular Changes of the Ovarian Follicle during Folliculogenesis: A Scanning Electron Microscopic Study*

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Summary. In order to study the three-dimensional topographic arrangement of the oocyte and zona pellucida, follicular cells and follicle microvasculature, this study applied alcali maceration methods for tissue exposure, the ruthenium red-detergent method for the extracellular matrix visualization, and the vascular corrosion cast technique to rabbits, guinea-pigs and mice ovaries at different stages of follicular development.

Macerated samples showed a gradual differentiation of the oocyte surface. This, in primordial follicles, appeared rather smooth, but, with the follicular development, displayed a gradual increase of blebs and microvilli. The latter widely covered the surface of oocytes contained in large or mature follicles.

The outer surface of the zona pellucida showed numerous fenestrations, whereas the inner one was smooth. The ruthenium red-detergent method permitted a well detailed view of the filamentous texture of the zona pellucida.

The three-dimensional distribution of the contacts between oocyte and neighbouring follicular cells was clearly evaluated in macerated samples. Follicular cells of primary follicles were characterized by their short cytoplasmic processes reaching the oocyte surface. In secondary follicles, these processes issued secondary processes. In larger follicles, the secondary processes of the corona cells were much longer and thinner, and took a tortuous course to reach the oocyte surface, which ran among the numerous oocyte microvilli. This microvillous arrangement greatly increases contact between the oocyte and corona cells, and suggests a coordinated reciprocal control of the activities of both cell types. These data also showed that the spongy and filamentous nature of the zona pellucida is closely dependent upon the temporal differentiation and enormous increase in number of follicular cell projections and their ramifications.

Maceration revealed the theca cells surface. In smaller follicles these appeared as fusiform cells which resembled fibroblasts. In larger or mature follicles, many theca cells differentiated to possess morphological features of steroidogenic cells. In addition, these cells delimited a series of intercellular communicating lacunae, continuous with wide pericapillary spaces.

The gradual differentiation of the follicle towards a structure having an endocrinial role was further emphasized in vascular corrosion casts. A simple microvascular net made of thin capillaries supplying primary follicles was seen to transform into an elaborate sinusoidal network made of thick permeable capillaries, supplying mature follicles.

The luteo-follicular complex (LFC) is the morphodynamic expression of the continuous and cyclical changes of the ovarian cells. In the last two decades, numerous scanning electron microscopic (SEM) studies of the follicular development and corpus luteum formation, including observations of microvascular casts, have been performed in different mammals (MOTTA and VAN BLERKOM, 1974, 1975; VAN BLERKOM and MOTTA, 1978; MURAKAMI et al., 1988; FAMILIARI et al., 1991; MACCHIARELLI et al., 1992).

The LFC consists of high heterogeneous population of cells whose main function is the maturation of the egg and its survival after fertilization.

In this regard, the LFC can be regarded as a peculiar kind of tissue in which the microtopographic interrelationships among its cellular elements gain a great functional meaning. It is mainly during the

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follicular growth that all the components of this tissue undergo their morphofunctional differentiation. In fact, after the recruitment, the oocyte and its investments establish new reciprocal structural relations. These, together with the developing theca elements, represent the supporting structures for the metabolic and nutritional needs of the growing oocyte. In fact, when atresia occurs, a process which results in the blockage of the oocyte morphofunctional development, an alteration of these relations at the level of the oocyte surface, the zona pellucida, (FAMILIARI et al., 1989b), the thecal cells (GREENWALD and TERRANOVA, 1988; FAMILIARI et al., 1991) and their microvasculature (KIKUTA et al., 1991) can be demonstrated.

The introduction of new SEM techniques using chemical maceration for tissue exposure permitted a better visualization of the cell microtopography (Takahashi-Iwanaga and Fujita, 1986). In fact, these maceration methods with or without the association of enzymes and/or ultrasound microdissection allowed the selective removal of the masking extracellular matrix and permitted original studies of fine detail, mainly in those tissues where a heterogeneous population of cells showed a complicated structural arrangement (Takahashi-Iwanaga and

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**Fig. 1.** Mouse. Primordial follicle, oocyte smooth surface (O) covered by partially digested flattened follicular cells (*). ×1,500

**Fig. 2.** Mouse. Early secondary follicle. Oocyte surface (O) and follicular cell (F). ×1,250

**Fig. 3.** Mouse. Primary follicle. Follicular cell (F) possessing a short projection (arrow). O oocyte surface. ×5,500

**Fig. 4.** Mouse. Primary follicle. Follicular cell (F) with thin projections (arrow). O oocyte surface. ×5,500
In order to reveal fine details of the cellular microtopography of the developing follicle and its microvasculature, ovaries in different phases of the cycle were studied in certain rodents by means of SEM, using NaOH/KOH maceration (Takahashi-Iwanaga and Fujita, 1986; Vizza et al., 1991a), ruthenium red-saponin-triton X100 (Familiari et al., 1989a), and microvascular corrosion casts (Murakami et al., 1988; Macchiarelli et al., 1991).

MATERIALS AND METHODS

**Animals:** Rabbits, guinea-pigs and mice were used. All the animals were female, adult and virgin. In the rabbits, ovulation was induced by the injection of human chorionic gonadotropin (Macchiarelli et al., 1991). A vaginal smear was obtained and studied to evaluate the ovarian cycle phases of guinea-pigs and mice.

**Sampling:** Rabbits were anesthetized with Nembutal (50 mg/kg of body weight e.v.), guinea-pigs with ethyl-urethan (1 g/kg of body weight i.p.), and mice by ether inhalation. All animals were perfused with a washing saline solution injected through the thoracic aorta (rabbits) or the left heart ventricle (guinea-pigs and mice). Ovaries for standard SEM were also perfused with a 2.5% glutaraldheyde solution in phosphate buffer, post-fixed with osmium tetroxide, conductive-stained (Murakami, 1974), dehydrated in ethanol, critical point dried in liquid carbon dioxide, and coated with gold.

**Tissue exposure:** NaOH or KOH maceration methods were applied in rabbits, guinea-pigs and mice as described in other reports (Takahashi-Iwanaga and Fujita, 1986; Vizza et al., 1991a, b). The ruthenium red-saponin-triton X100 method was applied in mice as reported in Familiari et al. (1989a). Mercox* perfusion for vascular corrosion cast was performed in rabbits and mice as previously described (Murakami et al., 1988; Macchiarelli et al., 1991).

**Electron microscopy observations:** Observations were performed in a Cambridge Stereoscan 150, in a Hitachi FE-S 4000 SEMs operating at 5-20 kV.

RESULTS

The classification of follicles was made possible considering the following parameters: 1) location in the cortex, 2) size, 3) type of cells surrounding the oocyte (flat or polyhedral), 4) number of cell layers surrounding the oocyte, 5) the presence and differentiation of theca layers, 6) phase of the cycle.

**The oocyte-granulosa cell contacts and the zona pellucida**

In fractured samples, the ovarian cortex showed numerous follicles in early stages of development situated just below the surface epithelium. Larger follicles were found deeper in the ovarian cortex.

Alcali maceration yielded a clean oocyte surface.

**Fig. 5.** Mouse. Antral follicle, innermost surface of the zona pellucida. ×2,700

**Fig. 6.** Mouse. Antral follicle, stereo pair of filaments of the zona pellucida. ×40,000
which was visible where the detachment of follicular cells occurred. At early stages (primordial-primary follicles), the rabbit and mouse oocyte surface was smooth with rare, short microvilli (Fig. 1). Primordial follicles were characterized by the presence of few flattened follicle cells covering the oocyte (Fig. 1). Indented neighbouring cell borders were often overlapping. In primary and early secondary follicles, after maceration, one or two layers of follicular cells were present around the oocyte (Fig. 2). These cells were polyhedral (Figs. 3, 4) and presented projections reaching the oocyte surface. These projections could be thick and short (Fig. 3) or thin and long, resembling filopodia (Fig. 4). The latter were more numerous and often intermingled with each other.

In secondary follicles, the zona pellucida appeared as a continuous layer surrounding the oocyte. By standard SEM the outer surface of the zona pellucida was clearly smooth. However, the material of the zona pellucida, due to both the presence of a large amount of surrounding extracellular amorphous material and the hydrophilic peculiarity of its components, is particularly hard to define by SEM observations without appropriate techniques which can unmask and stabilize its components. When the ruthenium-red-saponin-triton X100 method was applied, the amorphous material masking the zona pellucida was removed by the detergent action of the saponin and of triton X100, and the zona pellucida structural glycoproteins were stabilized with the cationic dye ruthenium red (FAMILIARI et al., 1989a). By this method, growing follicles in the mouse displayed a zona pellucida made of a network of thin filaments (Fig. 5). These filaments were arranged as beads aligned to form strings and were connected by globular structures (Fig. 6).

In the larger secondary or tertiary follicles, the
Fig. 8. Mouse. Secondary follicle, stereo pair of oocyte-cumulus complex. ×400

Fig. 9. Mouse. Secondary follicle, stereo pair of the flask shaped and cylindric follicular cells. ×2,000

Fig. 10. Mouse. Secondary follicle, follicular cells (F) are radially arranged around the oocyte (O) and send numerous projections towards the oolemma. ×700

Fig. 11. Mouse. Secondary follicle. A follicular cell (F) with thin cytoplasmic projections (arrow). ×5,000
follicular cells, delimited by a thin theca layer, were arranged in a few layers around the oocyte (Fig. 7). These follicular cells were smooth surfaced and showed various shapes (Fig. 8). The cells of the innermost layer were mainly cylindric. The cells of the second or outer layers were either cylindric, rounded, or of various polyhedral shapes. Some of these showed a typical flask shape and presented a basal ovoid body and an elongated apical pole (Fig. 9). Cytoplasmic projections were mostly seen merging from the apical pole of the inner follicular cells and reaching the oocyte surface. The follicular cells, radially arranged around the oocyte (Fig. 10), presented numerous thin projections (Fig. 11) or, in some cases, 3 to 4 thick projections arising from the apical pole (Figs. 12-13). These projections could vary in length, ranging from 3-5 μm. The top of the thicker projections displayed a flat surface from which several thinner and shorter projections—similar to spines—arose (Fig. 13). These spines reached the oocyte surface either perpendicularly or tangentially. (Figs. 14-15).

Fig. 12. Mouse. Secondary follicle. In fractured follicles the apical surface of follicular cells can be seen. ×1,200

Fig. 13. Mouse. Secondary follicle. The apical pole of a follicular cell presents some primary processes (P plus arrow) from which branch thinner secondary processes (S plus arrow). ×8,200

Fig. 14. Mouse. Small pre-antral follicle. The relation between a follicular cell (F) and the oocyte surface (O) is shown. ×4,000

Fig. 15. Mouse. Pre-antral follicle. A thick follicular cell projection (arrow) reaches the oocyte surface (O). ×8,000
Larger maturing follicles were always characterized by an increasing number of follicle cells, now called granulosa cells, stratified around the oocyte (Fig. 16). This mass of radially arranged follicle cells ultimately led in the antral follicle to the formation of the cumulus oophorus. The follicular cells of the granulosa layers were usually rounded or polygonal. In macerated samples, the follicle cells close to the oocyte displayed numerous thin cytoplasmic extensions, similar to long microvilli, which reached the oocyte surface rich in short microvilli (Fig. 17). These projections were highly intermingled with each other (Fig. 18). In fact, the intermingling of these projections was so developed that a network of corona cell and oolemma microvilli completely covered the oocyte. Corona cell projections reached the oocyte surface either perpendicularly or tangentially (Figs. 17, 19) and at the level of the contact with the oolemma, often showing a dilatation or a ramification (Fig. 19).

**Differentiation and microvasculature of the theca**

The theca layer started differentiation in early secondary follicles. In rabbits, the vascular supply of smaller follicles (primordial-primary) was directly derived from the microvasculature of the interstitial tissue. The capillaries supplying interstitial tissue were situated in the outer part of the ovarian cortex. In the smaller species (rats and mice), the interstitial microvasculature was very poor in respect to that observed in the rabbit.

Secondary pre-antral follicles were characterized by the onset of theca layer differentiation. At this stage, the inner theca comprised a few layers of fusiform cells which resembled fibroblasts. In macerated samples, the follicles were well isolated from the surrounding tissues, and the surface of the theca interna cells could be well visualized (Fig. 20). These cells were irregular fusiform, and often intermingled...
with each other (Fig. 21). The surface of these theca cells—in some areas still meshed by residual collagen fibers—showed blebs and small, thin microvilli. Larger, mature follicles showed a thick theca layer (theca interna) surrounding the granulosa compartment (Fig. 22). In the samples where the maceration removed most of the interstitial connective tissue, the theca cells of the inner layers appeared fusiform, star-shaped or polyhedral (Fig. 23). The former were probably fibroblast-like cells or transitional forms, and the latter corresponded to mature endocrine cells. These cells presented a surface characterized by numerous blebs and microvilli. In mouse antral follicles (Fig. 24), adjacent thecal cells delimited intercellular lacunae into which microvilli, blebs and other cellular extensions were projected (Fig. 25). In some areas, these lacunae were continuous with wide intercellular and perivascular spaces. As corroborated by parallel transmission electron microscopic observations, some among the most external fusiform or star-shaped theca cells likely correspond to differentiating myoid elements or typical smooth muscle cells (FAMILIARI et al., 1989; VIZZA et al., 1991b).

In vascular corrosion casts, the preantral follicles displayed thin microvessels arranged to form baskets of 80–200 μm in diameter (Fig. 26). These capillary baskets were supplied by one or two twigs of the cortical arteries and were drained by one or two efferents for the cortical veins. The capillary network was organized in a single layer (theca interna) and surrounded a large avascular space (granulosa-oocyte areas). The antral and ovulatory follicles were characterized by voluminous vascular baskets. The avascular area was visible in fractured samples (Fig. 27). In rabbits, these baskets clearly presented a multilayered wall. In fact, the largest follicles displayed: an outer, coarse capillary net; a medial layer formed by a few capillaries and larger vessels of venular and arteriolar nature; and an inner, very rich capillary layer. The inner layer was made of thick capillaries with a sinusoidal aspect and forming rounded meshes. Blind ends were often seen stemming from these sinusoids (Fig. 28). At ovulation, both in mice and rabbits, the capillary baskets showed a large apical avascular area related to the stigma. In addition, the sinusoids further enlarged

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Fig. 17. Rabbit. Antral follicle. Note the numerous thin and long corona cell projections which reach the oocyte surface (O) very rich in short microvilli. ×2,500

Fig. 18. Mouse. Antral follicle. The dense net of microvilli of the corona cells is seen from the oocyte side. ×3,000

Fig. 19. Rabbit. Antral follicle. The corona cell cytoplasmic projections present dilatations (D plus arrow) and ramifications (R plus arrow) when join the oocyte surface covered by very numerous and short microvilli (m). ×15,000
and showed many resin blebs due to the passage of cast medium among newly formed intercellular spaces (Fig. 27).

**DISCUSSION**

During follicular development observations showed a process of maturation mainly consisting in the differentiation of structures serving for regulating the oocyte functions.

By these SEM techniques, the oocyte maturation in particular was characterized not only by its increasing size but also by a drastically increasing number of microvilli. These rapid morphodynamic events are clearly designed to establish and maintain a close physical and metabolic relationship with the follicular cells (Phillips et al., 1978; Brower et al., 1982). Such intercellular relations have their morphological expression in the "gap-junctions" demonstrated between oocyte and follicular cells (Burghardt and Anderson, 1981), and have a crucial functional meaning throughout the follicular development, from follicular recruitment to ovulation (Motta et al., 1971; Gilula et al., 1978; Tesarik and Dvorak, 1982).
The ruthenium-red/saponin/triton X100 method showed the zona pellucida structures as three-dimensional lattices of intermingled filaments anchored to rounded structures. These structures were evidenced by ruthenium red, owing to their glycoprotein nature (FAMILIARI et al., 1989a). These data are consistent with those of GREEVE and WASSARMAN (1985), who suggested that the zona pellucida is a highly organized extracellular coat in which glycoproteins are arranged into filaments characterized by a structural repeat. Such a type of organization for the zona pellucida was observed in mature follicles. Therefore, the maturation of the zona pellucida not only gives rise to a protective barrier surrounding the oocyte, but when fully differentiated might function as a “sieving structure” able to control the sperm-oocyte binding at the moment of fertilization (LEVEILLE et al., 1987; FAMILIARI et al., 1988). In addition, our observations demonstrated how the microtopographical arrangement and 3-D structure of the follicular cells gradually changed during follicular development. These changes were particularly evident in the innermost layers of follicular cells, i.e. those cells which directly contact the oocyte. Studies on these contacts have been mainly performed on freeze-fracture replicae and serial thin-sections by electron microscopy (LARSEN et al., 1991), with only a few observations made by means of SEM (MOTTA and VAN BLERKOM, 1974; PHILLIPS et al., 1978; MAKABE et al., 1990). This limitation is mainly due to the fact that, when the follicular cell surface is studied by standard SEM, it is usually masked by extracellular material. The application of alcali maceration methods allowed the demonstration of the microtopographic arrangement of the cytoplasmic extensions of the follicular cells and their distribution on the oocyte surface, during follicular growth. These data showed that in the early stages the follicular cell extensions are usually poorly ramified. As the follicle grows, these projections gradually develop numerous branches in form of short spines or very long microvilli similar to filopodia. This phenomenon is likely related to the increased needs of the oocyte to establish contacts with the follicular cells (BROWER and SCHULTZ, 1982). In addition, our data clearly showed that the follicle cell projections mainly develop when the zona pellucida appears and becomes finely texturized. Therefore, it seems that the characteristic filamentous and spongy structure of the zona pellucida might be related to the progressive morphologic development of the follicular cell projections (WOLGEMUTH et al., 1984). It is likely that the primary and thicker extensions of the follicular cells (Figs. 13-14) enter into the fenestrations of the outer part of the zona pellucida, and the secondary or thinner projections intertwine among the filamentous net of the zona.
Fig. 26. Rabbit. Vascular corrosion cast of a pre-antral follicle. ×100

Fig. 27. Rabbit. Fractured vascular corrosion cast of an ovulated follicle. Note the inner rich sinusoidal net and the numerous blebs (B plus arrow) of resin extravasation due to the capillary permeabilization and vessels rupture following ovulation. ×40

Fig. 28. Rabbit. Vascular corrosion cast of an antral follicle. Note the multi-layered arrangement of the basket wall. The inner-sinusoidal capillary net is evidenced by the artificial yellow staining. ×500
When these cytoplasmic extensions reach the oocyte, they thus establish both "gap" and "intermediate" junctions with the oolemma (LARSEN et al., 1991) and are even able to penetrate within deep oolemma invaginations. In human developing follicles, TEM occasionally showed long microvilli of follicular cells placed deep in the oocyte (ZAMBONI, 1971). In addition, as seen by high-resolution SEM of osmium-DMSO-osmium macerated samples, these microvilli running within the ooplasm appeared surrounded by several vesicles of the Golgi complex and by membranes belonging to the endoplasmic reticulum, and were often seen to end close to the nucleus (MAKABE et al., 1990). These data, therefore, likely suggest that the numerous oocyte-follicle cell contacts serve as devices controlling the oocyte growth, supplying nutrients to oocyte and, especially inducing a metabolic modulation of the block and resumption of the oocyte meiosis (MOTTA et al., 1971; GILULA et al., 1978; BURGHARDT and ANDERSON, 1981; TESARIK and DVORAK, 1982). In particular, the looseness of gap junctions between cumulus-corona cells and oocyte was related to the resumption of oocyte meiosis (LARSEN et al., 1991).

When the theca is not yet developed, the follicular unit is not independent from the surrounding tissue. This is even evidenced by that pre-thecal follicles receive their blood supply by the interstitial tissue microvasculature (MACCHIARELLI et al., 1991). When the oocyte and its somatic cell investments mature, they require a differentiation of the follicle towards the formation of a device specialized for accomplishing their nutritional and functional needs. This results in the formation of a structure, the theca, which appears to play three main roles: 1) Supporting the functional endocrine differentiation of the follicle; 2) supplying an adequate blood supply to the entire follicular unit; and 3) isolating each maturing follicular unit from the rest of the ovarian tissue.

In the macerated samples we studied, many theca cells gradually developed some ultrastructural features such as surface blebs and rounded microvilli which, together with a cytoplasm provided with lipid droplets, rounded mitochondria with tubular or vesicular cristae, and membranes of SER, are typical of steroid-producing cells (FAMILIARI et al., 1991). In addition, these cells delimited intercellular lacunae often opening into interstitial and pericapillary spaces. This network of communicating interstitial spaces was arranged in a fashion similar to that usually observed in other typical endocrine glands. The similarity is even more evident if the reference is done with steroidogenic tissues, i.e., Leydig cells of the testis (MOTTA et al., 1973), adrenal cortex (MOTTA et al., 1979), or corpus luteum (MOTTA et al., 1969). The large amount of blebs and microvilli and the presence of intercellular lacunae suggest that fluids containing secretory products of theca cells (androgens to be aromatized by granulosa cells) may be stored in this intercellular compartment and/or directed towards the granulosa layer through its selective basal membrane, depending upon the functional demand of the whole follicular unit (ISHIMURA and FUJITA, 1991; FAMILIARI et al., 1991). In vascular corrosion casts the occurrence of changes related to the adaptation of the microvasculature to the above functional needs of developing follicles has been clearly demonstrated (MACCHIARELLI et al., 1992). In fact, follicular microvasculature first becomes independent from the interstitial tissue vascularization with the formation of capillary plexuses arranged in characteristic baskets. Following this, peculiar changes in such capillary plexuses gradually take place. These changes consist of the occurrence of many sinusoids, neo-angiogenesis (proved by the presence of numerous capillary blind ends) (MURAKAMI et al., 1988; KIKUTA et al., 1991), the formation of an avascular area in the apex of the preovulatory follicle (the stigma), capillary dilatation and increased vascular permeability (OKUDA et al., 1983; MACCHIARELLI et al., 1991, 1992). Actually, the capillaries gradually adapt their structure and distribution not only to the incoming ovulation (formation of a stigma, and capillary dilatation), but also to the developing thecal steroidogenic capability (sinusoid formation and increasing capillary permeability). These morphodynamic alterations reflect the transformation of a capillary net originally supplying a simple epithelium (primary follicle) in a typical sinusoidal network supplying an endocrine gland (thecal gland of mature follicles and then corpora lutea) (MACCHIARELLI et al., 1992).

REFERENCES


