

Ultrastructural Characteristics of Human Granulosa Cells in a Coculture System for In Vitro Fertilization

STEFANIA A. NOTTOLA,^{1*} ROSEMARIE HEYN,¹ ALESSANDRA CAMBONI,¹ SILVIA CORRER,¹
AND GUIDO MACCHIARELLI²

¹Laboratory for Electron Microscopy "Pietro M. Motta," Department of Anatomy, University of Rome La Sapienza, Rome, Italy

²Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

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ABSTRACT The use of somatic cells for cocultures during in vitro fertilization (IVF) is currently finalized to obtain a higher number of healthy and viable embryos with a high potential of implantation. Among the different cell lines that can be used as feeder cells for cocultures, granulosa cells (GCs) are autologous cells, safe and easy to recover. The aim of the present study was to analyze the fine structure of human GCs used in a coculture system to evaluate, from a morphodynamic point of view, their role in supporting embryo development. GCs were collected during oocyte pick-up, 36 h after human chorionic gonadotropin administration, from patients undergoing IVF procedures, who had given their informed consent to be included in this protocol. After coculture, GCs were fixed and processed for light microscopy (LM) and transmission electron microscopy (TEM). By LM, GCs appeared as clusters of loosely packed cells, irregularly rounded or polyhedral in shape, varying in diameter from 18 to 25 μ m. Mitotic cells, as well as regressing elements (with pyknotic nuclei or dense cytoplasm) and cell fragments were occasionally observed. By TEM, the plasma membrane was irregular due to the presence of cytoplasmic evaginations. Linear and annular gap junctions between neighboring GCs were found. GC nuclei, rounded and eccentrically located, contained finely dispersed chromatin, one (often two) prominent nucleoli and, infrequently, peripheral patches of heterochromatin. Numerous organelles populated the GC cytoplasm, among them, mitochondria were rod-shaped or elongated, usually provided with tubular-vesicular cristae but occasionally showing atypical, longitudinally oriented cristae. Membranes of smooth endoplasmic reticulum, Golgi stacks and vesicles, secretory-like granules, cisternae of rough endoplasmic reticulum (RER), free ribosomes and polysomes, lysosomal-like bodies, microfilaments, and lipid droplets were also seen in the GC cytoplasm. In most cells, RER was scarcely represented and numerous lipid droplets filled the perinuclear space. On the contrary, some GCs contained an abundant RER and rare lipid droplets scattered in the cytoplasm. In conclusion, our data demonstrated the presence, in a coculture system, of GCs provided with ultrastructural characteristics typical of healthy, metabolically active, mostly steroidogenic cells. Protein-synthetic cells have also been detected. These data, evaluated at the light of biochemical and clinical studies, sustain the capability of human GCs cocultures to positively affect early embryo development in vitro by the secretion of steroids and proteins, putative "embryotropic" factors. *Microsc. Res. Tech.* 69:508–516, 2006. © 2006 Wiley-Liss, Inc.

INTRODUCTION

During in vitro fertilization (IVF) procedures, the coculture of human embryos with various cell types such as human and bovine oviductal cells, uterine cells, Vero kidney cells, and even ovarian cancer cells has been reported to improve embryo quality, in vitro development, and implantation ability (Carrell et al., 1999; Fabbri et al., 2000; Feng et al., 1996; Freeman et al., 1995; Menezo et al., 1992; Quinn, 1994). Coculture allows the extension of the culture in vitro for preimplantation embryos toward the blastocyst stage, thus providing a number of benefits, which include (a) the selection of the most viable embryos for the transfer, (b) a better chronological synchrony between the endometrial receptivity and the developmental stage of the embryo transferred, and (c) the availability of an adequate number of cells for blastomere biopsy and

genetic screening (Quinn, 1994; Quinn and Margalit, 1996). However, the results concerning an actual increase of pregnancy rate on a large scale after the transfer of cocultured embryos in uterus may be conflicting (Fabbri et al., 2000). In addition, if it is preferable to use cocultures or sequential culture media to obtain a larger number of viable blastocysts is currently a matter of debate (Menezo, 2004; Urman and Balaban, 2005).

*Correspondence to: Stefania A. Nottola, M.D., Ph.D.; Laboratory for Electron Microscopy "Pietro M. Motta," Department of Anatomy, Faculty of Medicine, University of Rome La Sapienza, Via Alfonso Borelli 50, Rome 00161, Italy. E-mail: stefania.nottola@uniroma1.it

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Although, quite surprisingly, the beneficial effects of the feeder cells on embryo development do not seem to be dependent on the cell type or origin, the use of human granulosa cells (GCs) for cocultures seems to be particularly advantageous, thus facilitating the routine use of cocultures. In fact, GCs are easily recovered during oocyte pick-up and readily prepared for coculture because they promptly form a confluent monolayer. In addition, they do not require subculturing or viral screening because of their autologous nature (Dirnfeld et al., 1997; Fabbri et al., 2000; Freeman et al., 1995; Plachot et al., 1993).

Significant information concerning the morphological adaptive changes that GCs experience during the reproductive cycle have been obtained by electron microscopy on human GCs subjected to different microenvironmental situations, *in situ* as well as *in vitro* (Amsterdam and Rotmensch, 1987; Amsterdam et al., 2003; Dhar et al., 1996; Makabe et al., 2006; Motta et al., 2003; Okamura et al., 2003; Suzuki et al., 1981; Zoller, 1991). However, the behavior of human GCs in coculture has not been fully elucidated up to now through a morphofunctional approach. In this regard, we retain that an ultrastructural characterization of human GCs in this peculiar "nursing" condition could contribute to improve our knowledge about GCs morphodynamics as well as to better comprehend clinical, biochemical, and molecular patterns of the fine signaling exchange that exists between these cells and the human embryo.

Thus, the aim of the present study was to analyze the ultrastructure of human GCs used in a coculture system to evaluate, from a morphodynamic point of view, their role in sustaining the development of cocultured embryos.

METHODOLOGY

GCs were obtained from patients ($N = 14$) undergoing IVF procedures, who had given their informed consent to be included in this protocol. Patients were treated with a gonadotropin releasing hormone analogue, follicle stimulating hormone, and human chorionic gonadotropin (hCG) to achieve multiple follicular growth. GCs were collected during oocyte pick-up, 36 h after hCG administration. The cells were pelleted by centrifugation at 2,500 rpm for 10 min, mechanically dissociated, separated from red blood cells on a Percoll gradient and further centrifugated, collected by aspiration, and washed with Tyrode's salt solution. GCs so obtained were incubated with Ham's F10 medium supplemented with 20% maternal serum (MS). On day 2, after confluence, the cells were washed again with Tyrode's salt solution, reincubated with fresh Ham's F10 medium supplemented with MS, and then utilized for cocultures. On day 5 or 6, cocultured embryos were transferred into the uterus (Fabbri et al., 2000). At this time, some wells of confluent GCs were cultured for additional 2 days in a serum-free medium and finally prepared for light microscopy (LM) and transmission electron microscopy (TEM) observations.

Cultured GCs were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), diluted with culture medium (50/50). Subsequently, the cells were scraped from the culture dish and pelleted in the same fixative by centrifugation at 1,400 rpm for 5 min. After fixation for 2–5 days or longer at 4°C, the samples were washed

in phosphate buffer, postfixed in 1% osmium tetroxide in distilled water, dehydrated in ascending series of ethanol, and immersed in propylene oxide (solvent substitution). Granulosa pellets were then embedded in Epon 812 and sectioned by a Reichert–Jung Ultracut E ultramicrotome. Randomly oriented semithin sections (0.5–1 µm thick) were prepared for LM examination and stained with toluidine blue. Ultrathin sections were cut with a diamond knife, mounted on copper grids, contrasted with saturated uranyl acetate and lead citrate, and observed using a Zeiss EM 10 transmission electron microscope.

MORPHODYNAMICS OF HUMAN GCs IN SITU AND IN VITRO

As described in many mammals, including humans, GCs cyclically undergo profound morphological and biochemical changes sustained by the dynamics of the follicular–luteal complex (Amsterdam et al., 1991; Makabe et al., *in press*; Motta et al., 2003; Zoller, 1991). The progression of GCs differentiation (mature follicle) into granulosa–lutein cells (corpus luteum) triggered by luteinizing hormone (LH) induce a change in role ("luteinization") of most of the GCs, leading to cell hypertrophy, structural breakdown of the extensive gap junctional network, development of numerous surface expansions, cytoskeletal rearrangement, and multiple changes in cytoplasmic organelles (Crisp et al., 1970; Dhar et al., 1996; Guraya, 1971; Mestwerdt et al., 1977; Motta, 1969; Rotmensch et al., 1986; Wong and Adashi, 1999). Luteinization alterations, which support a steroid secretion biochemically detectable, are firstly detected in the peripheral (mural) GCs, mainly in those cells close to the basal membrane, and then they centripetally propagate to reach the inner (antral) GCs (Amsterdam and Rotmensch, 1987; Zoller, 1991). According to this gradient of propagation, cumulus-corona cells appear very scarcely or not responsive at all to the luteinization stimulus when exposed to the intraovarian milieu, acquiring ultrastructural characteristics of steroidogenic cells only after ovulation (Motta et al., 1995; Nottola et al., 1991).

Although the loss of architectural and microtopographic integrity due to plating conditions can have marked effects on the structure and metabolism of cultured GCs (Zoller, 1991), fine structural changes superimposable to those described for GCs within the follicle occur spontaneously *in vitro*, after 12 h of culture in humans (Suzuki et al., 1981) or after 48–72 h in other mammals (Balboni and Zecchi, 1981; Zoller, 1991). These changes are further enhanced when LH/hCG, cyclic adenosine 3'/5'-monophosphate (Soto et al., 1986), hCG-low density lipoproteins (Richardson et al., 1992), clomiphene citrate (Schmidt et al., 1984), or follicular fluid from large follicles (Zoller, 1991) are administered or if GCs are plated onto collagen- (Ben-Rafael et al., 1988) or matrigel-coated dishes (Hwang et al., 2000).

STRUCTURAL AND ULTRASTRUCTURAL CHARACTERISTICS OF HUMAN COCULTURED GCs

General Features

By LM we observed that granulosa pellets used for cocultures contained clusters of loosely packed cells.

These appeared irregularly rounded or polyhedral in shape, varying in diameter from 18 to 25 μm . Mitotically active cells were sometimes encountered inside the clusters. GCs generally appeared well preserved, although regressing elements with piknotic nuclei or dense cytoplasm, as well as cell fragments, were occasionally detected. GCs usually showed large, eccentrically located, rounded nuclei, with one or more nucleoli. Numerous organelles, including densely staining lipid droplets, filled the cytoplasm of most cells (Fig. 1). The variability in size shown by GCs may depend on the presence of different subpopulations of GCs in the follicular aspirate (Amsterdam and Rotmensch, 1987; Rotmensch et al., 1986; Zoller, 1991). Regressing cells and cell remnants occasionally observed could be considered as the morphologic expression of apoptotic changes affecting a percentage of GCs during the culture period. Apoptotic cell death is in fact morphologically characterized by nuclear condensation, cytoskeletal disarray, membrane blebbing, cell shrinkage, and disruption into small membrane-enclosed fragments called apoptotic bodies (Amsterdam et al., 1997; 2003; Makino et al., 2005). Phagocytosis by neighboring cells may complete the degradation of the apoptotic GCs (Billig et al., 1996).

Surface Characteristics and Intercellular Junctions

When observed by TEM, the free plasma membranes of cocultured GCs created a network of intercellular spaces, irregularly shaped due to the protrusion of pleomorphic cytoplasmic evaginations (microvilli or blebs of varying size) (Fig. 2). Development of microvilli, pseudopodia, and cytoplasmic protrusions have been traditionally associated to cell expansion, considered as indirect signs of GCs luteinization both *in vivo* and *in vitro* (Gulyas, 1984; Suzuki et al., 1981) and, as far as the microvilli are specifically concerned, with the expression of LH/hCG receptors on the GC surface (Amsterdam and Rotmensch, 1987; Makabe et al., 1982). On the other hand, it should not be excluded that a high development of surface evaginations *in vitro* may in part represent an unspecific adaptation to the culture conditions of cells that, in this way, develop a greater surface of exchange with the medium (Balboni and Zecchi, 1981).

In other areas, GCs associated in small groups appeared joined together in some points by small linear gap junctions. Annular nexuses were also occasionally seen in the GC cytoplasm (Figs. 3 and 4). The molecular nature and permeability of these kinds of junctions have been assessed by Furger et al. (1996), who documented functional gap junction formation among human GCs during culture. The junctional elements described may also represent the remnants of a well-developed junctional complex, formed by a tandem of gap and adherence junctions, that join neighboring

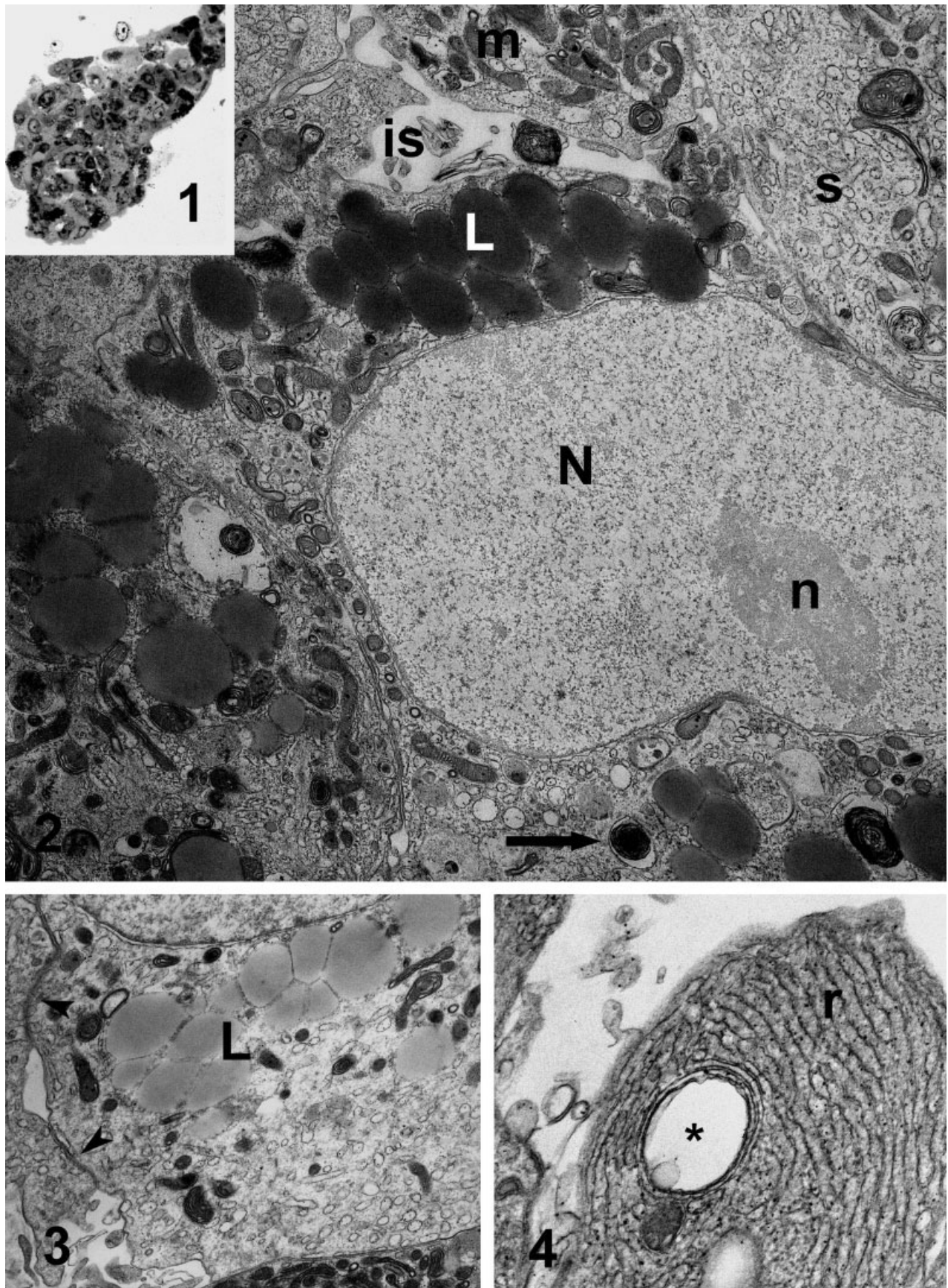
GCs during differentiation inside the follicle (Amsterdam and Rotmensch, 1987; Rotmensch et al., 1986; Spaniel-Borowski and Sterzik, 1987). Granulosa cell maturation and luteinization are in fact accompanied by a decrease in gap junctions and an increase in annular nexuses, the latter considered residual pre-existing linear gap junctions are internalized by endocytosis (Amsterdam and Rotmensch, 1987; Schmidt et al., 1984). Conversely, for other authors (Suzuki et al., 1981), annular gaps should be interpreted as actual (interdigitating) junctions.

Nuclear and Cytoplasmic Organization

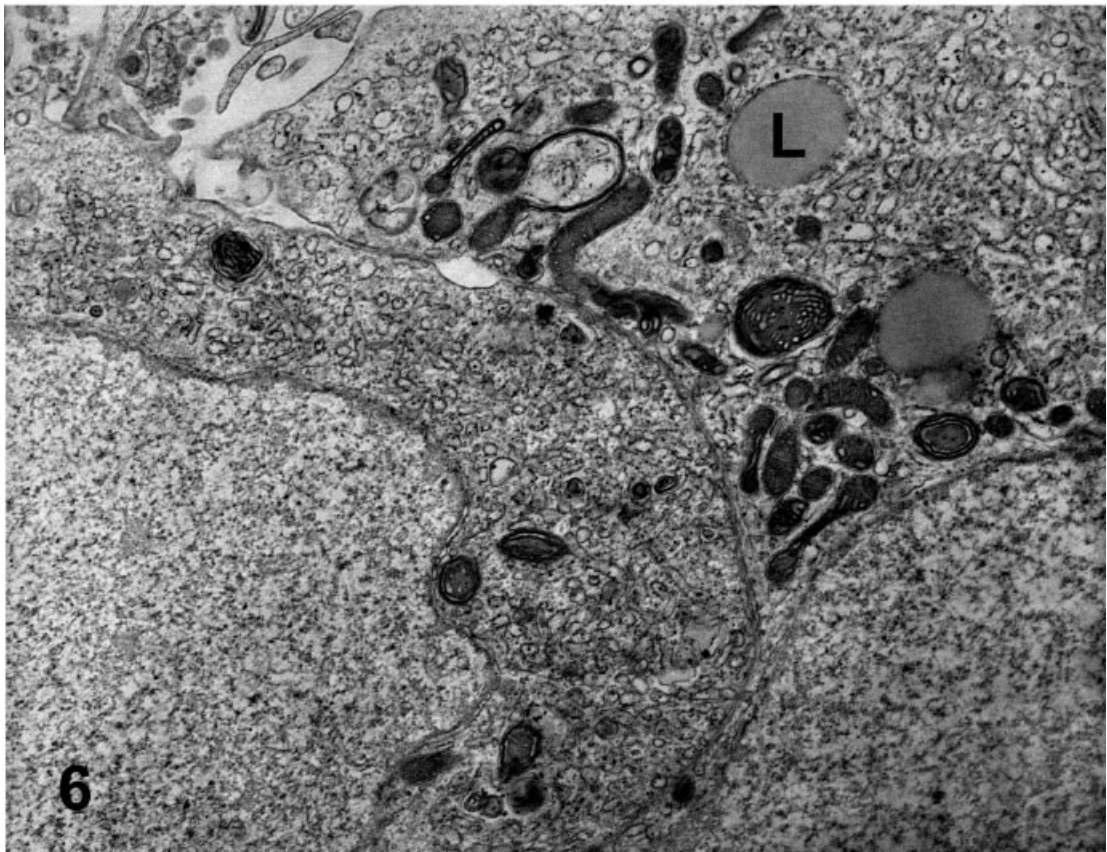
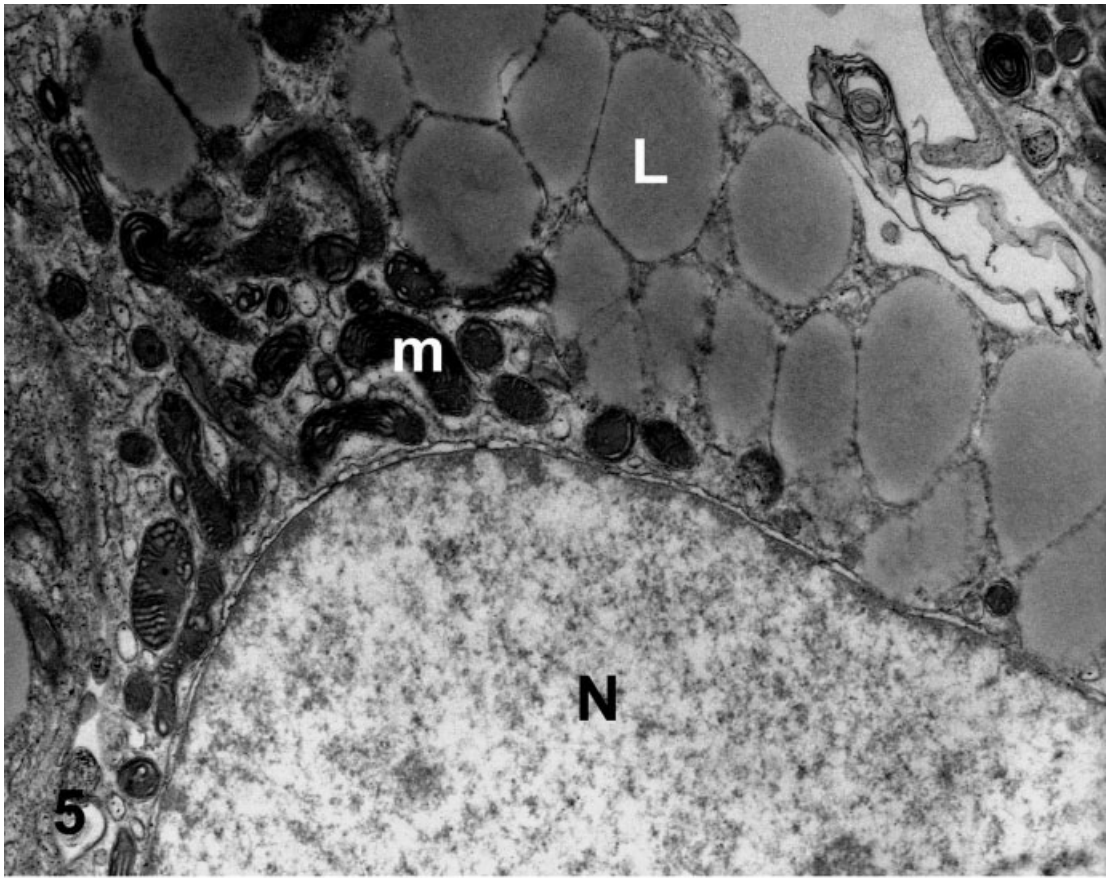
Granulosa cell nuclei were found to be large, round, often eccentric, by both LM and TEM. Using TEM they appeared surrounded by a nuclear membrane sometimes indented. The nucleoplasm contained finely dispersed chromatin and one or two prominent nucleoli (Figs. 2, 5, and 6). Peripheral patches of heterochromatin were occasionally found close to the inner leaflet of the nuclear membrane. These features are typical of metabolically active cells (Rotmensch et al., 1986; Suzuki et al., 1981). In general, by a stereological approach, nuclear parameters appear quite constant in a given GC population, even if GCs belong to a heterogeneous group (Dhar et al., 1996; Zoller, 1991).

The cytoplasm appeared densely populated by a large variety of organelles (Figs. 2, 5, and 6). Mitochondria were numerous, rod-shaped, or elongated, usually provided with tubular-vesicular cristae (Figs. 7 and 8). Mitochondria showing atypical, longitudinally oriented cristae, lying parallel to the long axis of the mitochondrion, were also occasionally observed. This transformation appeared complete (Fig. 9) or incomplete. In this latter instance, mitochondrial poles still contained transversely oriented cristae (Fig. 10). Dense granules were often found in the mitochondrial matrix (Figs. 7–9). Pleomorphic mitochondria varying in size and shape and mostly showing tubular-vesicular cristae have been commonly described in luteinizing GCs (Amsterdam and Rotmensch, 1987) and became more numerous in human cultured GCs by increasing the time of culture (Suzuki et al., 1981). In IVF cycles, GCs sampled from follicles containing fertilizable oocytes possess mitochondria with the same features (Rotmensch et al., 1986). The presence of enlarged mitochondria with pale matrix and reduced mitochondrial membrane potential in GCs have been instead associated with poor IVF outcome (Makino et al., 2005). In humans, enzymes involved in progesterone secretion are usually found associated with mitochondria showing tubular-vesicular cristae (Amsterdam and Rotmensch, 1987; Hsueh et al., 1984; Zoller, 1991). Unexpectedly, in our study, peculiar mitochondria provided with longitudinally oriented cristae have been detected among the aforementioned conventional mitochondrial subpopulation. This kind of mitochondria has been pre-

Fig. 1: Panoramic view of human GCs. Note the presence of a cluster of loosely packed cells. LM: $\times 250$. **Fig. 2:** Human granulosa cell. The nucleoplasm (N) contains finely dispersed chromatin and a nucleolus (n). Numerous well preserved organelles are seen in the cytoplasm. m, mitochondria; s, smooth endoplasmic reticulum; L, lipid droplets; arrow, dense body. A network of irregularly shaped intercellular spaces (is) is seen. TEM: $\times 10,000$. **Fig. 3:** Human GCs joined together by small linear gap junctions (arrowheads). L, lipid droplets. TEM: $\times 10,000$. **Fig. 4:** An annular nexus (*) and numerous parallel stacks of RER (r) are seen in the cytoplasm of a granulosa cell. TEM: $\times 18,000$.



Figs. 1-4.



Figs. 5–6.

viously found in human hemopoietic cells, in human ovarian carcinoma, and in other organs (lung, liver, and ureter) of different mammals. It seems possible that this feature is generated and enhanced by high oxygen concentrations, by administration of cytostatic drugs and antibiotics, or by ageing (Barastegui and Ruano-Gil, 1984; Gadhially, 1982; Romert and Matthiessen, 1986; Skinnider and Ghadhially, 1976), but the origin of this mutation is mostly unknown. The correlation between this morphological transformation and mitochondrial enzyme content also needs to be further elucidated. Thus, we hypothesize that the presence of these unusual mitochondrial forms may be possibly related not only to the great polymorphism usually shown by the mitochondrial population in luteinizing GCs but also to the composition of microenvironment in which GCs are maintained during culture. However, the actual functional status and significance of these mitochondria should be further investigated using integrated biomolecular/morphological approaches. In this regard, the role of prohibitin, an evolutionary conserved protein, in modulating mitochondria structure and function during GC growth and differentiation could also be explored in humans (Thompson et al., 2001).

In our study, membranes of smooth endoplasmic reticulum (SER), Golgi stacks and vesicles, secretory-like granules, cisternae of rough endoplasmic reticulum (RER), free ribosomes and polysomes, dense lysosomal-like bodies, microfilaments, and lipid droplets were also seen in the cytoplasm of cocultured GCs (Figs. 2, 3, 7, and 10). In most cells, RER was scarcely represented and numerous, clustered lipid droplets filled the perinuclear space, often in close association with mitochondria and SER membranes (Figs. 5, 7, and 8). The presence of a rich network of SER tubules and of a well-developed Golgi apparatus in the GCs confirm that luteinization is in progress (Amsterdam and Rotmensch, 1987; Bjersing, 1967; Schmidt et al., 1984; Suzuki et al., 1981). In this regard, isolated RER cisternae and ribosomes in luteinizing GCs may contribute to luteinization by synthesizing the enzymatic pattern involved in the steroid production (Crisp et al., 1970). Lysosomes may be involved in steroidogenesis as well, by processing low density lipoproteins and ultimately providing an adequate substrate in the form of cholesterol for progesterone synthesis (Amsterdam and Rotmensch, 1987). Microfilaments and other cytoskeletal components have been considered essential in driving GCs toward luteal differentiation (Motta et al., 2003) or death (Amsterdam et al., 1997).

The most representative organelles contained in the observed cocultured GCs were undoubtedly the lipid droplets. Accumulation of lipids is one of the early signs of luteinization (Amsterdam and Aharoni, 1994; Crisp et al., 1970; Gulyas, 1984). Clusters of non-extractable, electrondense lipid droplets are numerous in GCs, both *in vivo*, especially in mural cells (Zoller, 1991) and *in vitro*, after culture (Schmidt et al., 1984). Lipid inclusions that increase in volume after the LH

surge (or after LH/hCG administration in culture) contain precursors (cholesterol) for progesterone synthesis (Amsterdam and Rotmensch, 1987; Crisp et al., 1970; Dhar et al., 1996; Delforge et al., 1972; Gulyas, 1984; Van Voorhis, 1999). In these cells, lipids could move to and accumulate in the perinuclear region, thanks to a specific cytoskeletal activity occurring when the luteinization process is in progress (Amsterdam and Aharoni, 1994; Amsterdam and Rotmensch 1987; Soto et al., 1986). Numerous lipid droplets were also found filling the cytoplasm of human GCs from IVF cycles sampled from follicles containing mature, fertilizable oocytes (Rotmensch et al., 1986). Thus, accumulation of lipid droplets in GC cytoplasm seems correlated with a profitable steroidogenesis in healthy, metabolically active GCs.

However, according to an old theory, scarcely supported nowadays, such a lipid accumulation might not be a physiological sign, GCs filled with lipids being not functional, metabolically blocked cells (Bjersing, 1967). In other terms, a high number of lipid droplets could reflect the storage of unreleased steroids, thus preluding to an incipient degeneration (Hyttel et al., 1986). In spite of this, the morphological finding of an increase in the amount of lipid inclusions in luteinizing GCs, well-documented in the literature (Amsterdam and Rotmensch, 1987; Zoller, 1991), lead us to suggest that the lipid amount may be associated with maturative (luteinizing) rather than involutive changes.

A third, complex hypothesis, which dynamically includes the above two, should not be actually disregarded. According to this hypothesis, GCs filled with lipid droplets may be still active cells otherwise destined to apoptosis. In fact, enhanced steroidogenesis can be maintained during the initial steps of apoptosis as long as the steroidogenic apparatus, including mitochondria, remains intact (Amsterdam et al., 1997; 2003). GCs' "programmed cell death" (apoptosis) is an event that plays a crucial role in the ovary by limiting the number of follicles that can ovulate. Thus it may be not surprising if a given population of GCs retains its capability to respond in culture to a predetermined ovarian mandate. However, as previously reported, the samples we observed only occasionally showed obvious regressive changes.

On the basis of the data so far reported and discussed, cocultured GCs generally showed ultrastructural characteristics typical for metabolically active cells provided with steroidogenic capability. However, among the GC population, we also observed some cells containing an abundant RER, often found arranged in parallel stacks (Fig. 4), and rare, isolated lipid droplets, scattered in the cytoplasm (Fig. 6). Therefore, two sub-populations have been found among cocultured GCs, differing in their content and distribution of RER and lipid droplets. This difference should be explained considering the GCs heterogeneity inside a given GCs population (at the follicular aspirate); in this regard it should be emphasized that the lipid composition of GCs

Fig. 5: Numerous lipid droplets (L) showing a moderate electron density are clustered near the nucleus (N) of a granulosa cell, in close association with pleomorphic mitochondria (m) and membranes of smooth endoplasmic reticulum. TEM: $\times 18,000$. **Fig. 6:** Lipid droplets (L) are very scarce and scattered in the cytoplasm of these GCs. TEM: $\times 15,000$.

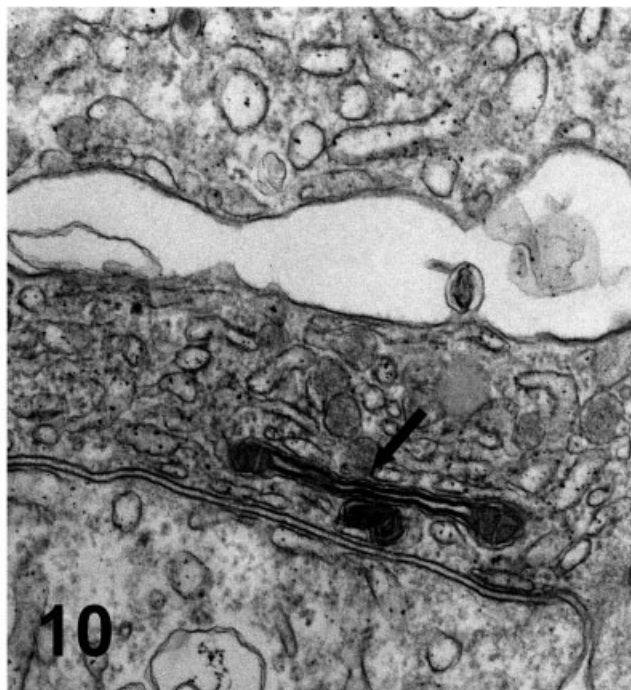
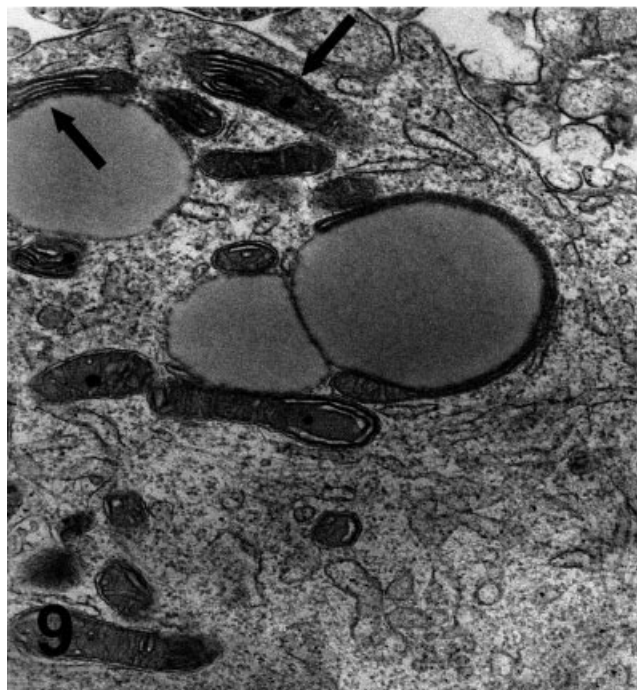
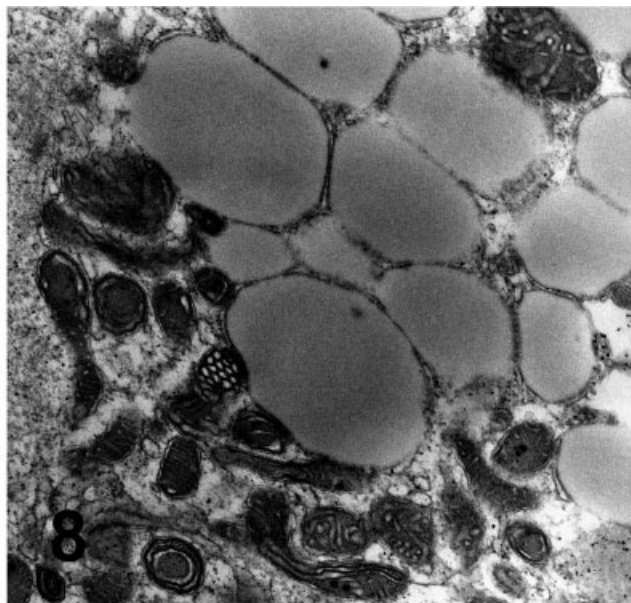
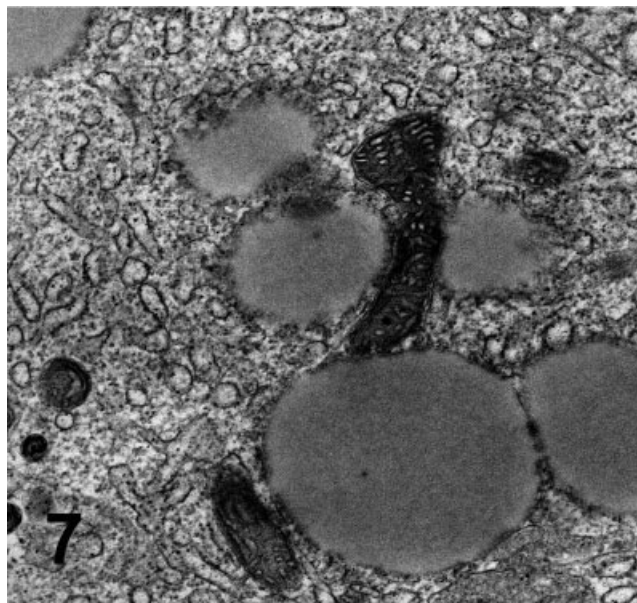


Fig. 7 and 8: Clusters of lipid droplets are found in close association with numerous elongated or irregularly shaped mitochondria provided with tubular-vesicular cristae. Dense granules can be observed in the mitochondrial matrix. Tubules of smooth endoplasmic reticulum are also seen. TEM: $\times 20,000$ (Fig. 7); $\times 18,000$ (Fig. 8). **Fig. 9 and 10:** Elongated mitochondria showing atypical, longitudinally oriented cristae lying parallel to the long axis of the mitochondrion

are occasionally observable among the highly pleomorphic mitochondrial population. This transformation appears almost complete in the mitochondria shown in Figure 9 (arrows) whereas a mitochondrion with both poles still showing transverse cristae is seen in Figure 10 (arrow). Dense granules are present in the mitochondrial matrix (Fig. 9). TEM: $\times 20,000$.

cells shows quantitatively, by a stereological approach, the greatest individual variability if compared with that of other organelles (Dhar et al., 1996). Protein release in culture by GCs has been revealed by electrophoretic analysis (Fabbri et al., 2000). GCs seem also capable of producing free amino acids, peptides, glycoproteins, and growth factors (Ying and Zhang, 1999).

Functional and Clinical Considerations

The presence of healthy GCs from follicular aspirates has been positively correlated with IVF outcome in different protocols. In fact, as mentioned earlier, success of oocyte fertilization has been associated with morphological evidence of LH/hCG responsiveness and luteinization in GCs (Rotmensch et al., 1986). In addition, it

has been reported that incidence of apoptotic bodies in GCs is low in patients from whom many oocytes have been retrieved for IVF (Seifer et al., 1996) and in pregnant with respect to non-pregnant IVF patients (Nakahara et al., 1997). However, further studies are needed to definitely demonstrate a prognostic role for apoptosis in GCs of women undergoing IVF (Benifla et al., 2002).

The way of action of GCs in coculture is not completely known. It has been proposed that two mechanisms may exist, which, in combination, may enhance the efficacy of the coculture on early embryo development. GCs may control the composition of the culture medium by stabilizing the pH and by detoxifying the medium removing undesirable factors, such as oxidative agents and hypoxanthine (negative conditioning). GCs may also secrete some substances, which may have beneficial effects on the embryo (positive conditioning) (Dirnfeld et al., 1997; Fabbri et al., 2000; Freeman et al., 1995). In this regard, it has been suggested that local production of steroids with autocrine/paracrine effect may positively affect the microenvironment in which the early embryo develops (Fabbri et al., 2000; Makrigiannakis et al., 2000; Nottola et al., 1991). Cocultured embryos may be capable of steroid uptake and metabolism and could release factors modulating GC steroidogenesis as well (Seifer et al., 1996). The release of a specific protein of 90 kDa in the culture medium has been also positively associated with the percentage of good-quality embryos, which was significantly higher in the group of patients showing the presence of the 90-kDa band (Fabbri et al., 2000).

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our morphological ultrastructural study provides additional information on GC behavior during coculture, reinforcing the concept of positive conditioning of the medium by their secretions. In particular, the data hereby shown demonstrated that in a coculture system, GCs belong to a rather heterogeneous population of cells. The major number of cells showed ultrastructural characteristics of healthy, metabolically active and mostly steroidogenic cells, features that well-associate with those described by Suzuki et al. (1981) in *in vitro* spontaneously luteinizing GCs and with those shown by Rotmensch et al. (1986) in GCs derived from follicles containing fertilizable oocytes. However, cells not fully differentiated into steroid elements, presumably still protein synthetic in nature, have also been detected. These data, also evaluated at the light of biochemical and clinical reports, revealed that human GCs in coculture are capable of positively affecting early embryo development *in vitro* by producing small amounts of steroids and proteins that alone or together with other substances (nutrients, growth factors?) might act as "embryotrophic" factors.

Further parallel morphological, biomolecular, and clinical studies on GCs cocultured in the presence of extracellular matrix (Heng et al., 2004; Hwang et al., 2000) or other components provided exogenously to the medium (Taniguchi et al., 2004) are necessary to better define and, possibly, enhance the beneficial effects of

the GC cocultures, thus improving the efficiency of specific IVF procedures.

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