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Mitochondrial morphology in human fetal and adult female germ cells

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The aim of this study has been to observe, by electron microscopy, the morphological changes affecting mitochondria and associated organelles in the human female germ cell during oogenesis, maturation and fertilization. In the primordial germ cell (PGC), rounded mitochondria with a pale matrix and small vesicular cristae are disposed near the nucleus and significantly increase in number during PGC migration and settlement in the gonadal ridge, where they differentiate into oogonia. In these early stages of mammalian oogenesis, aggregates of mitochondria are typically clustered around or in close relationship with the nuage. In oocytes at early prophase stage, mitochondria proliferate while aligned along the outer surface of the nuclear membrane, contain a more dense matrix than before, and have lamellar cristae. Oocytes of primordial and primary follicles mostly contain round or irregular mitochondria whose matrix has become very light. These mitochondria show typical parallel, arched cristae, and are clustered near the nucleus with other organelles forming the Balbiani's vitelline body. When follicles grow, the mitochondria of the oocytes become even more numerous and are dispersed in the ooplasm. Both paranuclear accumulation and subsequent dispersion of mitochondria in the cytoplasm are likely to be regulated by microtubules. By ovulation, mitochondria are the most prominent organelles in the ooplasm. They form voluminous aggregates with smooth endoplasmic reticulum (SER) tubules and vesicles. These mitochondrial–SER aggregates (M–SER) and the mitochondrial–vesicle complexes (MV) could be involved in the production of a reservoir of substances or membranes anticipating subsequent fertilization and early embryogenesis. Just after fertilization, the mitochondria of the oocyte undergo a further substantial change in size, shape, and microtopography. In the pronuclear zygote, mitochondria concentrate around the pronuclei. During the first embryonic cleavage divisions, round or oval mitochondria with a dense matrix and few arched cristae are gradually replaced by elongated ones with a less dense matrix and numerous transverse cristae. A progressive reduction in size and number of M–SER aggregates and MV complexes also occurs. In summary, oocyte mitochondria show dynamic morphological

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changes as they increase in number and populate different cell domains within the oocyte. They form complex relationships with other cell organelles, according to the different energetic –metabolic needs of the cell during differentiation, maturation, and fertilization, and are ultimately inherited by the developing embryo, where they eventually assume a more typical somatic cell form.

Key words: embryo/germ cell/mitochondria/oocyte/ultrastructure

Introduction

Since their discovery in the late 19th century, mitochondria have been considered as cell 'intruders', i.e. they are presumed to have originally been free-living bacteria with their own oxidative metabolism prior to their incorporation into primitive cells (for review, see Jansen and de Boer, 1998). The assumption that mitochondria are semi-autonomous cell organelles has received support from electron microscopic and biochemical studies that have revealed that mitochondria contain their own DNA (mtDNA), ribosomes and RNA (Smith and Alcivar, 1993). This extra-chromosomal genetic system is involved not only in the synthesis of mitochondrial proteins but is apparently required for mitochondrial proliferation and regeneration. Inheritance of the mitochondrial genome does not follow Mendelian rules, but is transmitted, possibly in haploid form (Jansen and de Boer, 1998), from one generation to the next by way of the oocyte cytoplasm, known as cytoplasmic inheritance. Thus, in contrast to nuclear inheritance, cytoplasmic inheritance is mostly along the maternal line, although a role for the fertilizing sperm-derived centrosome in organizing mitotic spindles in early human embryos has recently been established (Sathananthan *et al.*, 1996; Palermo *et al.*, 1997).

Oogenesis includes many important events, among which are the formation and proliferation of female germ cells, the initiation of meiosis, and the establishment of a maternal store of materials to support fertilization and early embryo development (Makabe *et al.*, 1991; Gosden, 1995; Motta *et al.*, 1995). As with most of the fine structural changes that accompany early mammalian embryogenesis, differentiation of maternally-derived mitochondria follows a sequential pattern (Van Blerkom and Motta, 1979). Moreover, the morphology of mitochondria changes substantially during oogenesis and during embryonic cleavage (Smith and Alcivar, 1993).

This article follows the dynamics of ultrastructural morphological changes among the mitochondria and associated organelles in the human female from the stage of the primordial germ cell, through oogenesis to maturation, fertilization, and early embryonic cleavage.

Materials and ultrastructural methodology

Tissues from human embryos and fetuses have been taken from cases of spontaneous abortion or surgical delivery (Motta and Makabe, 1982, 1986a,b; Makabe and Motta, 1989; Makabe *et al.*, 1989, 1991; Hoang-Ngoc Minh *et al.*, 1993; Motta *et al.*, 1997a,b). Gestational age was estimated through a comparison of various data, including crown–heel and crown–rump measurements and determination of the first day of the last maternal menstrual cycle, and has been converted to an estimated post-fertilization time. In addition, ovarian biopsies were sampled during laparoscopy or abdominal surgery (Makabe *et al.*, 1992; Motta *et al.*, 1994). Finally, human oocytes either unfertilized after in-vitro insemination or fertilized, from pronuclear to morula stages, were obtained during IVF protocols (Motta *et al.*, 1988, 1995, 1996; Nottola *et al.*, 1993; Makabe *et al.*, 1997). All the samples were

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processed for electron microscopy after the informed consent of patients.

Samples were fixed by immersion in 0.5–2.5% glutaraldehyde in phosphate or sodium cacodylate buffer and processed for light, transmission and scanning electron microscopy (LM, TEM and SEM) as reported elsewhere (Motta and Makabe, 1982, 1986a,b; Motta *et al.*, 1988, 1994). Specimens prepared by conventional SEM procedures were compared with those prepared following the osmium dymethylsulphoxide osmium (ODO) maceration method (Tanaka and Naguro, 1981). The advantage of this technique is to extract the soluble cytoplasmic matrix from the freeze-cracked cell surface, thus preserving cellular scaffolding and allowing the clear demonstration of the oocyte's organelles by three-dimensional (3-D) microtopography (Makabe *et al.*, 1992, 1997; Motta *et al.*, 1994). Specimens were then observed by high-resolution field-emission SEM.

Pre-meiotic germ cells and the onset of oogenesis

The germinal component of the gonads (including the ovaries) originates from a small number of primordial germ cells (PGCs) that are firstly detected in human embryos at the third week post-fertilization in the yolk sac, near the developing allantois (Witschi, 1948). The main cytoplasmic feature of PGCs is the extreme paucity of organelles, including mitochondria (Figures 1 and 2). Cisternae of rough endoplasmic reticulum, ribosomes and polysomes, a single Golgi complex, occasional lipid droplets, bundles of microfilaments and microtubules are all present, but only glycogen is to be found in conspicuous amounts, accumulating especially at the beginning of the PGCs' migration (Fukuda, 1976; Fujimoto *et al.*, 1977; Makabe and Motta, 1989; Makabe *et al.*, 1989, 1991; Hoang-Ngoc Minh *et al.*, 1993). The mitochondria are large and round, with vesicular cristae, and are located near the voluminous, vesicular PGC nucleus (Figures 2 and 16). The mitochondria are larger and more globular than those of somatic cells (De Pol *et al.*, 1997; Viebahn *et al.*, 1998), and significantly increase in number during migration out of the yolk sac (weeks 4–5 post-fertilization) and during settlement in the gonadal ridge (weeks 5–6 post-fertilization) (Fukuda, 1976; Hoang-Ngoc Minh *et al.*, 1993; Jansen and de Boer, 1998). Interestingly, the primordial germ cell antibody 2 (PG-2) has been reported to be specific for PGCs during rabbit embryonic development and its epitope is located in close vicinity to the outer mitochondrial membrane or perimitochondrial cytoplasm (Viebahn *et al.*, 1998). The possible use of similar mitochondrial markers in identifying human PGCs in their earliest stages of differentiation from the somatic cell line has, however, not been investigated yet.

PGCs begin to differentiate into oogonia at the ninth week post-fertilization. Cytoplasmic structures are diffusely dispersed in the cytoplasm and the mean number of oogonial mitochondria has doubled in comparison with those found in PGCs. Now mitochondria reach the size of 0.8–1.0 μm, with a spherical to ovoid shape. A qualitative change in the form of the mitochondrial cristae from a tubulo-vesicular pattern (circular profiles, Figure 3) to a sparse, lamellar configuration starts at this stage of oogenesis (Figure 16) (Jansen and de Boer, 1998). A close spatial relationship sometimes occurs between mitochondria and smooth endoplasmic reticulum (SER) membranes, an observation that will become typical of the subsequent meiotic period of intra-ovarian differentiation of the oocytes (Baker and Franchi, 1967; Gondos *et al.*, 1971; Dvorák and Tesarík, 1980; Hoang-Ngoc Minh *et al.*, 1993).

In some cases the oogonial cytoplasm shows the presence of a particular structure formed by clusters of mitochondria in close

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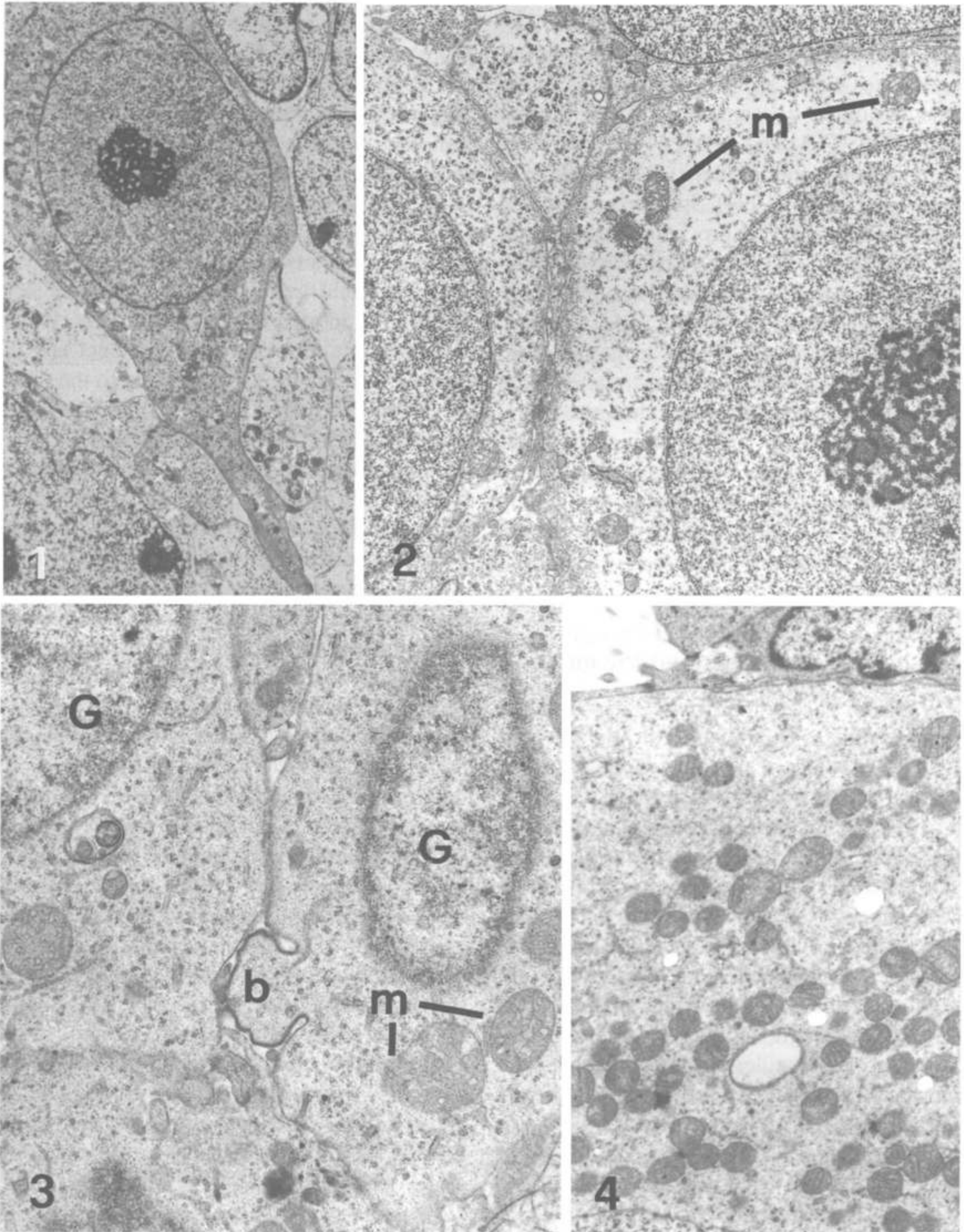

Figures 1–4.

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association with an electron dense, granular substance containing RNA and/or protein. This substance, morphologically similar to the material of the nucleolus, has been variably termed a nucleolar-like body, nucleolar granules, intermitochondrial substance or, most commonly, nuage. It may be considered to be a cytoplasmic marker of 'germ plasm', or the earliest differentiation of the germ-cell lineage in mammals (Figure 16) (Weakley, 1971; Kellokumpu-Lehtinen and Söderström, 1978; Van Blerkom and Motta, 1979; Dvorák and Tesarík, 1980; Eddy, 1996) and could be related to germ granules described in many lower animals (Strome *et al.*, 1994). When the nuage is evident in both the male and the female germ cells of a species, it appears earlier and in a higher number in oogonia (Kellokumpu-Lehtinen and Söderström, 1978). A similar structure has been also observed in certain cells of the early rabbit embryo (Motta and Van Blerkom, 1974). In addition, nuage-like material is not only present in germ cells and embryos, but has been revealed in a variety of other cells, such as neurons and tumour cells, which have in common high protein-synthesis activity (Hindelang-Gertner *et al.*, 1974; Motta and Van Blerkom, 1974); in this regard, it has been postulated that the close association of mitochondria and RNA found in the nuage could be directly concerned with the production of new mitochondria (Weakley, 1976), an observation that could possibly link female germplasm development to the needs of mitochondrial inheritance.

The presence of the nuage in some instances can be spatially associated with the nuclear pores and with the narrow cytoplasmic bridges that often still join neighboring dividing oogonia (Figure 2) (Ruby *et al.*, 1970; Mackay *et al.*, 1989). The occasional finding of mitochondria and other organelles within the intercellular bridges has led some authors (Ruby *et al.*, 1970; Dvorák and Tesarík, 1980; Makabe *et al.*, 1989; Hoang-Ngoc Minh *et al.*, 1993) to suggest that there is cytoplasmic flow taking place between still connected dividing germ cells.

Germ cells entering meiosis: primary oocyte formation and growth

After a series of mitotic divisions, proliferating oogonia begin to enter meiosis from the 12th week post-fertilization (Gondos *et al.*, 1986). The cells, now termed primary oocytes, progress through prophase of the first meiotic division before halting at the late diplotene (dictyate) stage. Oocytes lose their intercellular bridges and, from the 16th week post-fertilization, single oocytes come to be surrounded by flattened somatic cells (prefollicular cells), representing the first formation

Figure 1. Transmission electron micrograph of migrating primordial germ cell. Note the elongated ('amoeboid') form of the cell and the paucity of organelles in the cytoplasm. 4-week embryo. Original magnification ×2500.

Figure 2. Transmission electron micrograph of genital ridge primordial germ cell cytoplasm. Round/oval mitochondria (m) with vesicular cristae, randomly located in the cytoplasm. 6-week embryo. Original magnification ×6500 (reproduced from Makabe *et al.*, 1989, with permission).

Figure 3. Transmission electron micrograph of oogonia (G) in the developing ovary connected by an intercellular bridge (b) after mitosis. Rounded mitochondria with tubulo/vesicular profiles (m) are present in the cytoplasm. 11-week old embryo. Original magnification ×12 000 (reproduced from Hoang-Ngoc Minh *et al.*, 1993, with permission).

Figure 4. Transmission electron micrograph of leptotene oocyte in developing ovary. Increasing numbers of mitochondria are randomly distributed in the cytoplasm. Mitochondrial cristae appear lamellar in shape. The mitochondrial matrix is slightly denser compared with earlier stages. 23–24-week embryo. Original magnification ×7500 (reproduced from Hoang-Ngoc Minh *et al.*, 1993, with permission).

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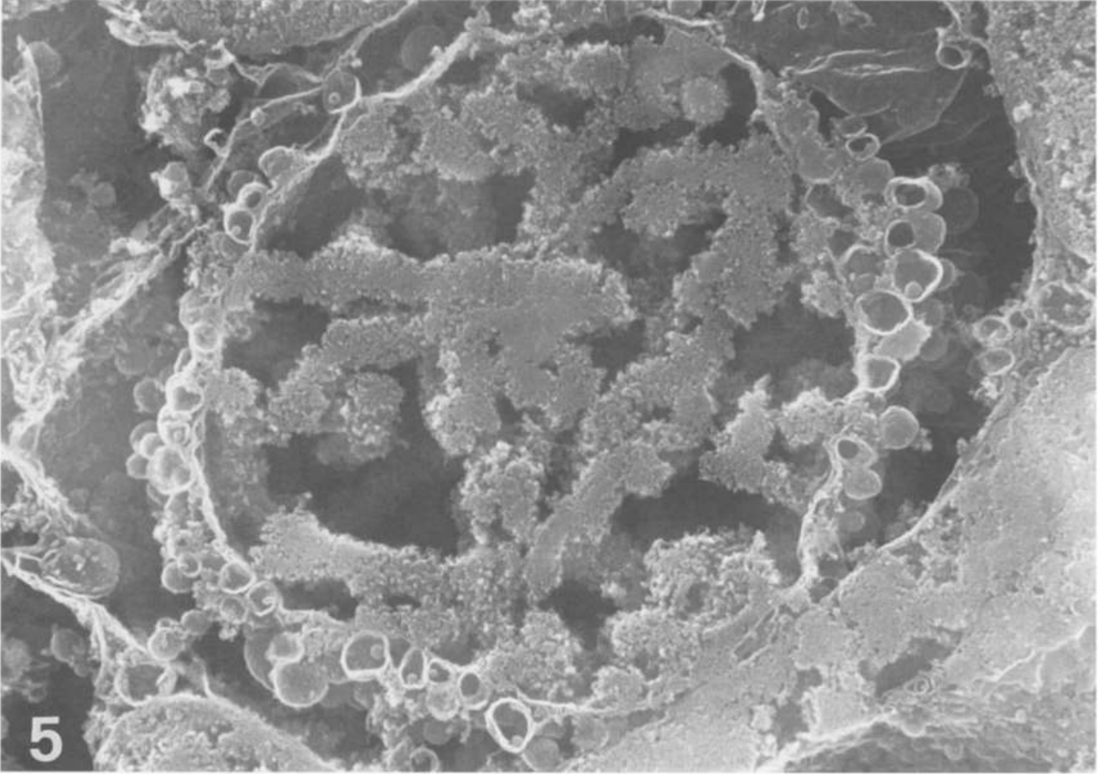

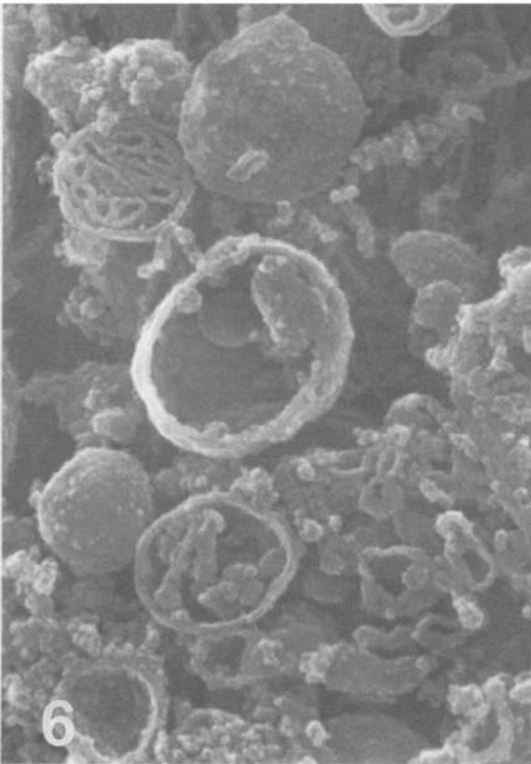

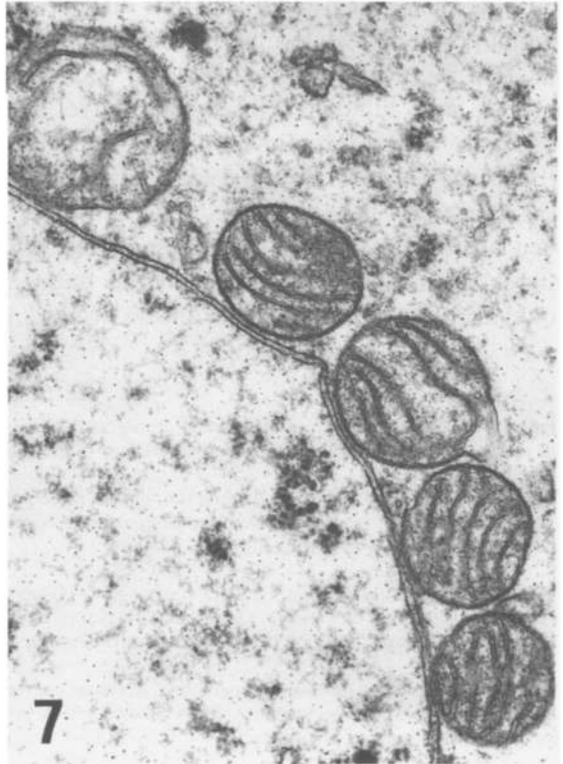

Figures 5–7.

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of primordial follicles, each of which thus contains a quiescent oocyte, blocked in prophase of its first meiotic division (Francavilla *et al.*, 1990; Makabe *et al.*, 1991; Hoang-Ngoc Minh *et al.*, 1993; Motta *et al.*, 1994).

Oocytes are larger than oogonia and have undergone genetic recombination of maternally and paternally derived nuclear DNA. During oocyte growth, cytoplasmic organelles greatly increase in number and show morphological changes (Picton and Gosden, 1999). Two mitochondrial redistributions have been reported during the progression of the oocyte through the first meiotic prophase. First, there is a change from a random cytoplasmic distribution during leptotene (Figure 4) to a perinuclear location during zygotene, where they are arranged in a single (or sometimes double) row around the nuclear membrane (Figures 5, 6, 7, 16). Second, there is a change from this perinuclear position as the mitochondria migrate during diplotene toward one pole of the cell, where they form a crescent-shaped mass to one side of and close to the nucleus (Dvorák and Tesarík, 1980; Pozo *et al.*, 1990). This structure which, besides mitochondria, includes various other organelles such as the Golgi complex, membranes of SER, vacuoles, compound aggregates (secondary lysosomes, actually autolysosomes), lipid droplets and annulate lamellae (Baca and Zamboni, 1967; Hertig and Adams, 1967; Hertig, 1968; Dvorák and Tesarík, 1980) condense to form Balbiani's vitelline body (Figures 8 and 16). The persistence and gradual increased complexity of the mitochondria–nuclear membrane associations in the human primary oocyte are features also observed in other mammals (Weakley, 1976; Van Blerkom and Motta, 1979; Tokura *et al.*, 1993; Calarco, 1995), and similar mitochondrial movement and accumulation has been described in the developing frog oocyte (Callen *et al.*, 1980). Such a ubiquitous phenomenon could reflect the level of energy required by the nucleus during the progress of the first meiotic prophase up to the meiotic arrest and/or an adaptation for the future needs of the mitochondria in the fertilized oocyte. Microtubules, present throughout the oocyte cytoplasm, are most prevalent around the circumference of the nuclear envelope (Dvorák and Tesarík, 1980) and were clearly demonstrated in our studies using ODO macerated samples (Makabe *et al.*, 1992; Motta *et al.*, 1994). Mitochondria are still observed at this time clustered among nuage.

The conversion of primordial follicle into primary follicle is marked by little if any real follicular growth (Motta *et al.*, 1994). The growing oocyte is instead very active in the synthesis of proteins from nuclear and mitochondrial transcripts. Some of these proteins are required for the differentiation of the oocyte itself, others are for interactions with the surrounding somatic follicular cells, and yet others will be important for enzyme production, formation of the zona pellucida (ZP), fertilization ability, and early embryonic development (Picton and Gosden, 1999).

As the follicle emerges from its resting stage and oocyte growth proceeds, organelles disperse toward the periphery of the oocyte

Figure 5. Scanning electron micrograph (SEM) of zygotene oocyte. Mitochondria are located around the nucleus. 14-week embryo. SEM of osmium dimethyl sulphoxide (ODO)-treated sample (see text), original magnification ×7500.

Figure 6. Scanning electron micrograph (SEM) of zygotene oocyte. High magnification of mitochondria associated with the outer leaflet of the nuclear envelope, showing spherical form and distribution of cristae. 14-week embryo. SEM of ODO-treated sample, original magnification ×37 000.

Figure 7. Transmission electron micrograph of pachytene oocyte. Mitochondria are closely applied to the nuclear membrane. Cristae are lamellar and oriented parallel to the nuclear membrane. 18-week embryo. Original magnification ×28 000.

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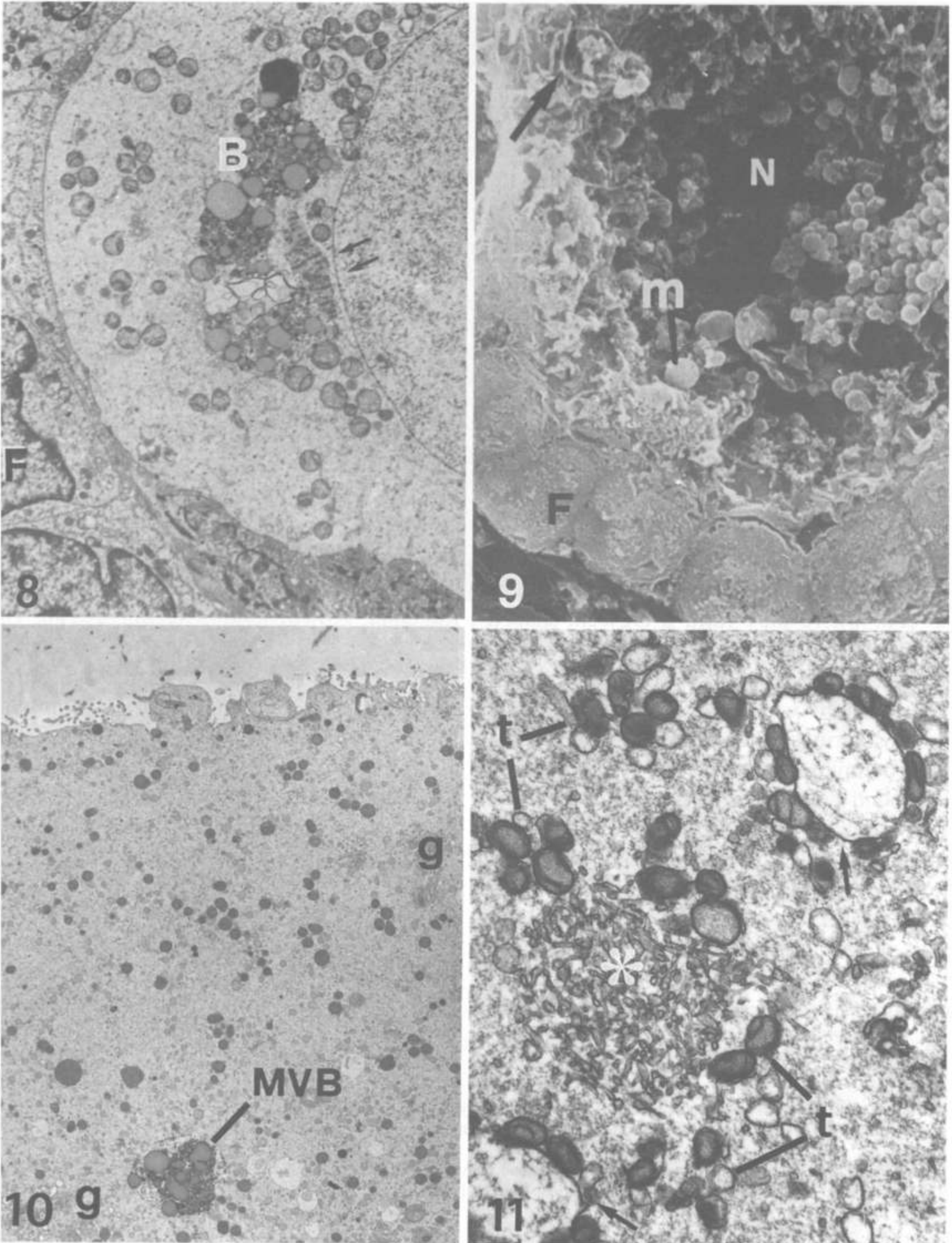

Figures 8–11.

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(Figure 9), and the nuage gradually diminishes and finally disappears (Dvorák and Tesarík, 1980). In maturing oocytes the nucleus ultimately acquires a vesicular appearance and contains a dense nucleolus with associated chromatin (the germinal vesicle stage) (Sathananthan *et al.*, 1991, 1993). The Golgi apparatus enlarges and transforms from a few flattened sacs into numerous units in the cortex of the cell (Figure 10), where it assembles ZP glycoproteins and cortical granules, which appear as dense, membrane-bound structures (Sathananthan *et al.*, 1985; Picton and Gosden, 1999). Thereafter, the extensive SER becomes progressively more cortically located; compound aggregates disperse in the ooplasm. The cortical cytoplasm now contains multivesicular bodies (MVB) (Figure 10), which are heterolysosomes in nature. Signs of pinocytotic activity are also observable. Further, the number of ribosomes increases but polyribosomes are rare. Annulate lamellae are now also rare, in comparison with the primordial stage (Baca and Zamboni, 1967; Dvorák and Tesarík, 1980).

Mitochondria become more numerous and leave their juxta-nuclear position, being dispersed in the ooplasm in the company of most other organelles (Figures 9 and 10) (Dvorák and Tesarík, 1980; Familiari *et al.*, 1989; Makabe *et al.*, 1991). As previously reported for nuclear polarization, such a morphodynamic redistribution and dislocation of organelles around the cytoplasm is likely to be regulated by microtubular activity, which is demonstrable with parallel TEM and high resolution 3-D SEM observations done on macerated samples using the ODO method (Figure 16) (Makabe *et al.*, 1992; Motta *et al.*, 1994). There is a temporal, spatial, and developmental relationship between the location of microtubule organizing centres and the progressive translocation of mitochondria to and from the nuclear region (Van Blerkom, 1991; Makabe *et al.*, 1992; Barnett *et al.*, 1996). Microtubule organizing centres are foci for mitochondrial aggregation and are also likely to be involved in some of the morphodynamic events occurring during granulosa cell differentiation in the maturing follicle, as well as during their further transformation to corpus luteum cells (Motta and DiDio, 1974). In addition, it has recently been pointed out that in somatic cells mitochondria exist in the cytoplasm as a dynamic tubular network with projections that move, break, and reseal in response to local environmental changes, modulated by the action of a dynamin-related protein (Smirnova *et al.*, 1998).

During oocyte formation and growth, mito-

Figure 8. Transmission electron micrograph of adult diplotene oocyte in primordial follicle. Note the presence of numerous, rounded mitochondria with parallel/arched cristae and a pale matrix, associated with the periphery of Balbiani's vitelline body (B). Double arrows = stacks of annulate lamellae; F = follicular cells. Original magnification ×4500 (reproduced from Motta *et al.*, 1994, with permission).

Figure 9. Scanning electron micrograph (SEM) of adult ovary, primary follicle. Oocyte mitochondria (m), together with other organelles, are migrating toward the periphery of the ooplasm. The nucleus has been removed through maceration, leaving an empty area in the centre of the cell (N). F = follicular cells; arrow = intraoocytic microvilli from follicular cells. SEM of ODO treated sample, original magnification ×5400 (reproduced from Motta *et al.*, 1994, with permission).

Figure 10. Transmission electron micrograph of germinal vesicle-stage, late primary oocyte. Numerous round mitochondria with a dense matrix are scattered in the ooplasm. g = Golgi membranes. MVB = multivesicular body. Original magnification ×2500.

Figure 11. Transmission electron micrograph of secondary (pre-ovulatory) oocyte. *Typical mitochondrial–smooth endoplasmic reticulum (M–SER) aggregates (see text) and multivesicular (MV) complexes (arrows) in the ooplasm. Mitochondria are rounded, with a dense matrix and sparse, peripheral arched cristae. Note also the presence of transitional forms (t) between M–SER aggregates and MV complexes. Original magnification ×10 500.

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chondria retain their spherical profile, but change the pattern of their cristae. Mitochondrial dimensions increase from the dividing oogonia to the oocytes of primordial and primary follicles, reaching a diameter of 1–1.5 μm. Mitochondrial diameter then undergoes a slight reduction (to 0.5–0.7 μm) through follicular development (Figure 16) (Dvorák and Tesarík, 1980; Pozo *et al.*, 1990). There is also a change in the morphology of inner mitochondrial membranes (Figure 6), or cristae, from the tubulo–vesicular profile seen in PGCs (Figure 2) and oogonia (Figure 3) (and still sporadically observable during early leptotene) to a lamellar pattern observed during late leptotene (Figure 4), zygotene (Figure 5) and pachytene (Figure 7; and Figure 3 (Jansen, 2000), by which stage mitochondria are proliferating close to the nucleus and the cristae are orientated parallel to the nuclear membrane. In diplotene oocytes, cristae adopt an arch-like pattern, representing a looser arrangement of the inner mitochondrial membranes, or are disposed parallel to the outer mitochondrial membrane (Figure 8). In the adult growing oocyte, as follicular development commences, the peripheral arched mitochondrial cristae can show a particularly irregular configuration (Dvorák and Tesarík, 1980).

The density of the mitochondrial matrix also changes during progression of the first meiotic prophase. During zygotene and pachytene the density is high, whereas during diplotene in the quiescent oocyte mitochondria show a lighter matrix (Figure 8) (Gondos *et al.*, 1971; Pozo *et al.*, 1990). In growing oocytes, in turn, mitochondria are mostly characterized by an increasingly dense matrix (Figure 10). Granular inclusions in the mitochondrial matrix may be seen during oocyte growth (Dvorák and Tesarík, 1980).

Defective oocytes of both primordial and growing follicles undergoing atresia can contain normal or abnormal mitochondria (Hubbard and Oxberry, 1991; Hoang-Ngoc Minh *et al.*, 1993; De Pol *et al.*, 1997). Among the latter, mitochondria scattered in the ooplasm appear very irregularly shaped and show few swollen cristae, often associated with vacuolization and/or higher electron density of their matrix (Stanková and Cech, 1983; Familiari *et al.*, 1993; Hoang-Ngoc Minh *et al.*, 1993).

Resumption of meiosis and preovulatory development of the oocyte

The fully mature secondary oocyte, competent for ovulation, reveals profound nuclear and cytoplasmic changes presumably representing the acquisition of fertilization ability. The ultrastructural appearances are unique. The first meiotic division has been completed, with germinal vesicle breakdown followed by the extrusion of the first polar body into the perivitelline space. The second meiotic division follows immediately and by metaphase II chromosomes can be seen in a cortical region of the ooplasm devoid of organelles (Sathananthan *et al.*, 1993). Resumption of meiosis seems associated, at least in mice, to rapid mitochondrial translocation (in this case towards the nucleus) (Van Blerkom and Runner, 1984).

In human preovulatory secondary oocytes, cortical granules are stratified in two or three rows in subplasmalemmal areas (Baca and Zamboni, 1967; Motta *et al.*, 1988; Makabe *et al.*, 1991; Sathananthan *et al.*, 1993). Mitochondria are rounded and provided with arched cristae, and characteristically associate with SER membranes, forming numerous and often voluminous structures scattered in the cortical areas of the ooplasm. These structures comprise: (i) tubular membranes of SER anastomosed with each other and closely intermingling with mitochondria (M-SER aggregates); and (ii) large vesicles filled with flocculent, slightly electron-dense material, surrounded by a rim

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of mitochondria (MV complexes) (Figure 11) (Sundstrom *et al.*, 1985a; Szollosi *et al.*, 1986; Motta *et al.*, 1988; Sathananthan *et al.*, 1993). Aggregation of SER membranes and tubules is apparently the first phenomenon to occur. SER membranes will be subsequently surrounded by mitochondria. These M–SER aggregates probably represent the precursor of MV complexes, which develop later, during the maturational phase, in which conventional SER membranes are rare and most of the mitochondria are associated with the above structures (Sundstrom *et al.*, 1985b). Thus, both the above associations between mitochondria and cytoplasmic membranes of endoplasmic reticulum are likely to belong to the same functional apparatus, the precise activity of which is still not understood. The dynamic morphological appearances of the mitochondrial associations suggest a rich employment of energy for the production of a reservoir of new mRNA, proteins and membranes required for subsequent fertilization and early embryonic cleavage (Motta *et al.*, 1988).

During IVF procedures, human oocytes ageing in culture show progressive swelling of vesicular SER culminating in vacuolation and an increased density of the mitochondrial matrix. Mitochondria often cloud together or associate with very large vesicles (Sathananthan, 1997). Similar alterations, accompanied by the appearance of dense oval bodies within the mitochondria, can represent the effects of suboptimal culture conditions (Sathananthan and Trounson, 1989).

Mitochondria during fertilization and development of the early embryo

After fertilization and during the growth of the preimplantation embryo, mitochondria undergo further changes. These alterations involve the relationship mitochondria have with other cellular structures, such as nuclear and SER membranes, as well as their size, shape and internal appearance. The same general changes to the mitochondria have been reported in other mammalian species, and consequently seem to reflect a general stage-specific series of phenomena rather than any species-specific event (Dvorák and Tesarík, 1985). Post-fertilization changes in mitochondria are characterized by a gradual transition of round or oval mitochondria with a dense matrix and few arched cristae to more elongated forms showing a lighter matrix and more numerous cristae oriented transversally to the long axis of the mitochondria (Figure 16) (Dvorák and Tesarík, 1985; Jansen and de Boer, 1998).

The increased uptake and utilization of glucose as a substrate for metabolism appears to coincide with a decrease in density of the mitochondrial matrix and the concomitant increased number of their cristae (Van Blerkom, 1989). However, qualitatively, round-to-oval and more elongated mitochondria can co-exist through the subsequent cleavage stages. The presence of scarcely differentiated, round or oval mitochondria at all stages of preimplantation development could be a reflection of the low level of metabolism exhibited by the early human embryo. Alternatively, such mitochondrial heterogeneity as we have observed could be interpreted as a consequence of differing responsiveness of individual mitochondria to intracellular signals governing mitochondrial differentiation postulated by Jansen and de Boer (1998).

Just after fertilization, voluminous residual MV complexes are still observable in the cortical areas of the pronuclear egg (Smith and Alcivar, 1993), but mitochondria are becoming concentrated in the centre of the oocyte, around the developing pronuclei (Figures 12 and 13), where with pronuclear formation and fusion at syngamy there is presumably a demand for energy (Sathananthan, 1997).

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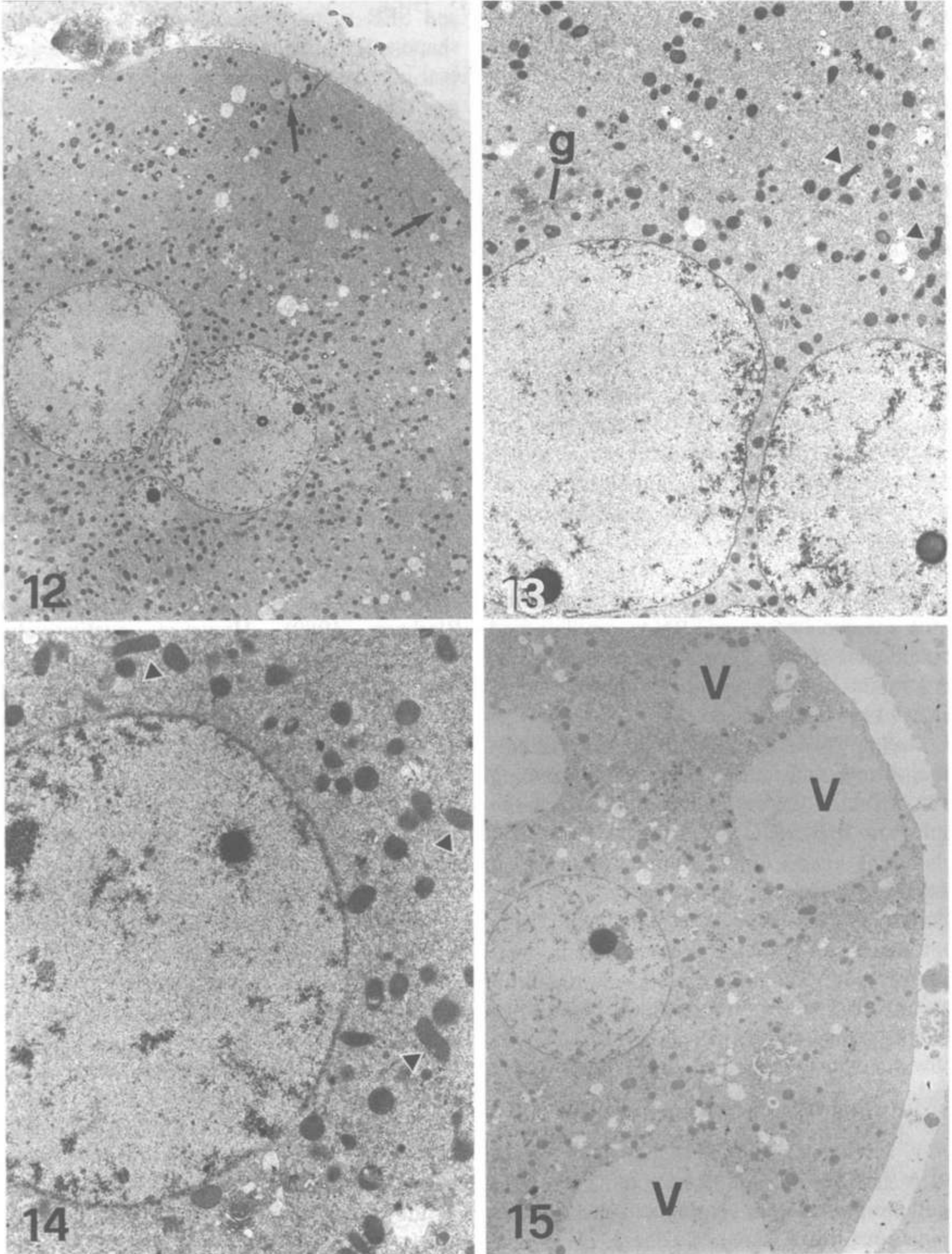

Figures 12–15.

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Some mitochondria are attached to the nuclear envelope, and to membranes of the endoplasmic reticulum and annulate lamellae. This relation, typical for both the male and the female pronucleus, is retained even after the process of pronuclear formation is completed (Dvorák *et al.*, 1984). Interestingly, mitochondrial polarization near the nuclear envelope reiterates several times during oogenesis and fertilization and seems to acknowledge a particular demand of the oocyte, with a need for the special support of mitochondrial activity whenever a remodelling of nuclear material is taking place. It might also be relevant that mtDNA replication in somatic cells is preferentially located close to the nucleus (Davis and Clayton, 1996).

Mitochondria of the pronuclear oocyte are similar in morphology to those found in prefertilization stages. Their size varies from about 0.4–0.6 μm. They are round or oval with a few cristae parallel to the outer mitochondrial membrane. The mitochondrial matrix is electron-dense (Figures 12, 13 and 16) (Zamboni *et al.*, 1966; Soupart and Strong, 1974; Dvorák and Tesarík, 1985). However, elongated forms with a central constriction ('dumb-bell' shaped mitochondria), commonly interpreted as dividing mitochondria (but plausibly fusing), are also observable, especially during early pronuclear stages, when synthesis of pronuclear membranes is active (Figures 13 and 16) (Soupart and Strong, 1974; Dvorák and Tesarík, 1985). It has been suggested that mitochondrial duplication might be necessary in order to maintain a constant number of organelles in daughter cells (Soupart and Strong, 1974).

The outer leaflet of the nuclear membrane in pronuclear oocytes and early cleaving embryos can be dilated by evaginations occupied by membrane-bound vesicles that contain granular material of low electron density. This is a normal feature of early stages of development, both in the human (Sundstrom *et al.*, 1981; Dvorák *et al.*, 1982, 1984; Trounson and Sathananthan, 1984; Pereda and Coppo, 1987; Motta *et al.*, 1988; Pereda *et al.*, 1989) and in other mammals (Szollosi, 1965). Similar structures in the pronuclei of the rat have been curiously interpreted as tertiary nucleoli (Szollosi, 1965). It has also been suggested that these structures could be primordial mitochondria (Baker and Franchi, 1969). For now the nature and functional significance of these prominent membrane-bound vesicles is unknown.

In later stages of pronuclear oocyte development, pronuclei become closely associated with each other in the centre of the cell; then the nuclear envelope between them breaks down and the maternal and paternal chromosomes finally come together, thus establishing the genome of the new individual. This event,

Figure 12. Transmission electron micrograph of pronuclear-stage zygote (three pronuclei). Mitochondria, similar in size, shape and form to those of the preovulatory oocyte, are concentrated around the pronuclei in the centre of the cell. MV complexes (arrows) can still be observed at the periphery of the oocyte cytoplasm. Original magnification ×1750.

Figure 13. Transmission electron micrograph of pronuclear-stage zygote (three pronuclei), higher power. Mitochondria are seen around the pronuclei, even in the interpronuclear space, including 'dumb-bell'-shaped forms (arrowheads), possibly indicative of division or fusion. Note the dense, compact nucleolus in each pronucleus. g = Golgi membranes. Original magnification ×2500.

Figure 14. Transmission electron micrograph of blastomere, 6-cell embryo. More elongated mitochondria (arrowheads) are seen together with rounded elements. In this embryo, the mitochondrial matrix remains dark and the nucleolus compact. Original magnification ×5000.

Figure 15. Transmission electron micrograph of blastomere, degenerating 6-cell embryo. Several voluminous vacuoles (V) are only partially surrounded by mitochondria. Original magnification ×1750.

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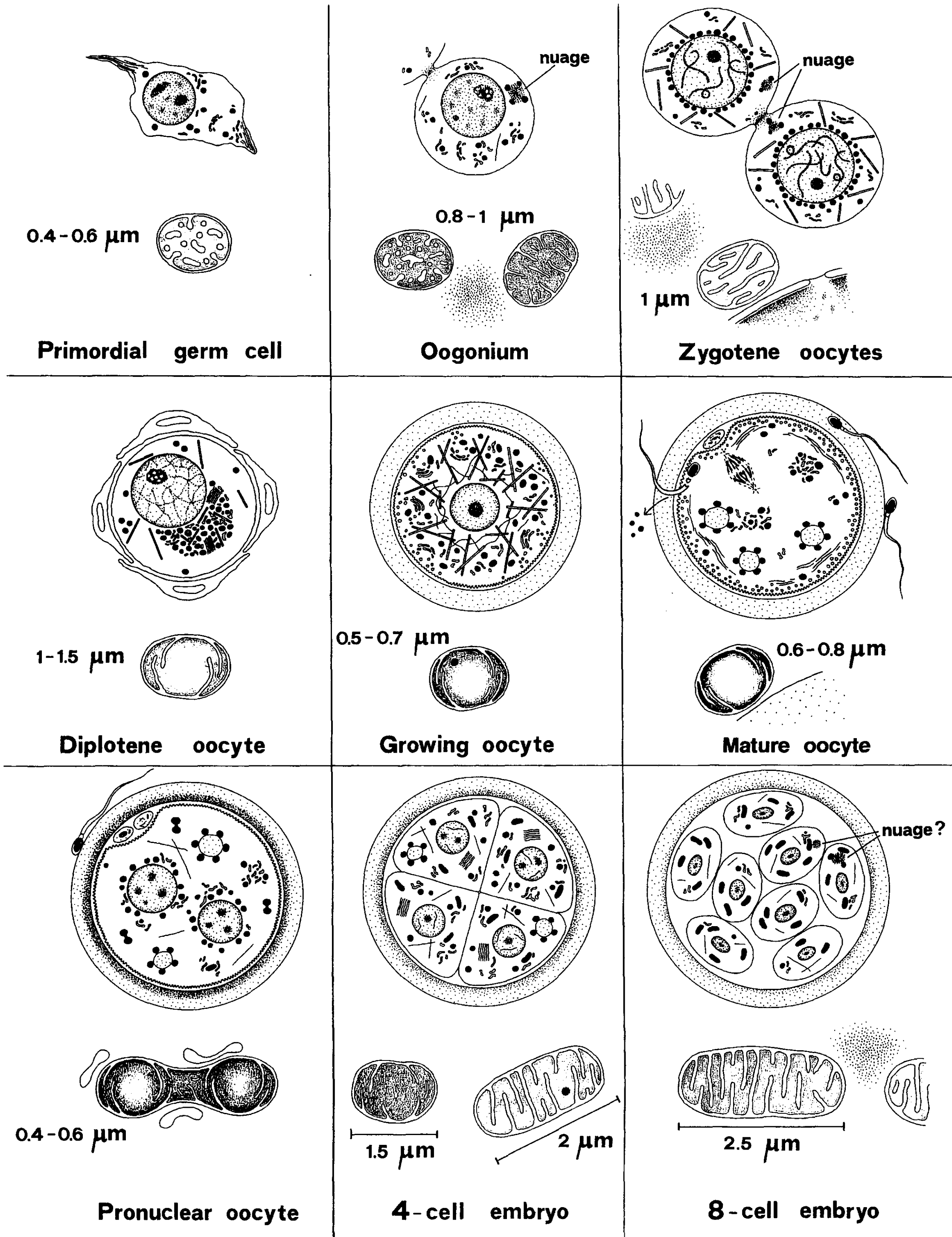

Figure 16. Diagram of mitochondrial morphodynamics in human oocytes and embryos, showing changes in microtopography, size, shape, configuration of cristae and matrix density of the mitochondria.

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called syngamy, is considered to represent the end of the fertilization process (Dvorák *et al.*, 1984; Sathananthan *et al.*, 1993). Then the first embryonic cleavage occurs.

In the two-cell embryo, organelles generally appear uniformly dispersed in the blastomeres of embryos developing *in vitro*, with a slight tendency to assume a perinuclear arrangement (Dvorák *et al.*, 1982; Trounson and Sathananthan, 1984). At this stage, both *in vitro* and *in vivo*, a large number of mitochondria observed by TEM still show a rounded shape with a dense matrix and few small peripheral cristae as observed both by TEM (Dvorák *et al.*, 1982; Trounson and Sathananthan, 1984; Pereda and Coppo, 1987) and by high-resolution SEM of the freeze-fractured surface ODO-treated blastomeres (Makabe *et al.*, 1997). Mitochondria are rarely found in associations with the envelope of the blastomeres' nuclei or annulate lamellae (Dvorák and Tesarík, 1985). Residual small MV complexes are still observed. *In vivo,* the cytoplasm of blastomeres at these stages of development clearly appears partitioned into three concentric areas. The most peripheral area shows a loose, finely granular appearance and is known to contain few MV complexes. Numerous mitochondria, intermingling with the most part of the remaining organelles, occupy the middle largest zone, whereas a narrow rim of cytoplasm almost devoid of organelles surrounds the nuclei (Pereda and Coppo, 1987).

In the 4-cell embryo developed *in vitro*, elongated mitochondria (the major axis of which measures 1.5 –2.5 μm) with numerous transverse cristae are more abundant than in the previous stage, suggesting an increase in mitochondrial activity. The matrix is lighter, with pale areas, and in some cases intra-mitochondrial granules are present (Figure 16) (Sundstrom *et al.*, 1981; Trounson and Sathananthan, 1984). MV complexes are even more reduced in number and size, and vesicles are sometimes replaced by small tubular aggregates (Dvorák and Tesarík, 1985). Clumping of mitochondria can be seen in the blastomeres of embryos affected by slow or arrested development during IVF procedures (Trounson and Sathananthan, 1984). Observations of the mitochondrial population of the blastomeres of 4-cell embryos *in vivo* is less differentiated than that of 4-cell embryos developing *in vitro* (Pereda *et al.*, 1989), being more like that of the 2-cell embryo *in vitro*. This difference has been attributed to supposed differences between mitochondrial metabolic activity of embryos at the same developmental stage grown in different microenvironments (Smith and Ord, 1983).

Mitochondrial morphology and distribution in 6- to 8-cell embryos and in morulas developed *in vitro* is characterized by the occurrence of an increased number of elongated forms, which reach up 2.5 μm in length (Figure 14) (Sundstrom *et al.*, 1981; Dvorák and Tesarík, 1985; Sathananthan *et al.*, 1993). At this stage, the presence of numerous typical MV complexes (Dvorák and Tesarík, 1985), as well as the occurrence of several large vacuoles only partially surrounded by mitochondria (Figure 15) probably both reflect a response of blastomeres to a cellular damage, presumably due to prolonged culture under suboptimal conditions. On the other hand, ultrastructural analysis of a 7-cell embryo that had developed *in vivo* and which consisted of both pale and dark blastomeres revealed small, round or oval, scarcely developed mitochondria in the pale, electron-lucent blastomeres, whereas elongated forms with numerous transverse cristae were most commonly found in the denser blastomeres (Pereda and Croxatto, 1978); among these elongated forms small distended SER cisternae were sometimes contiguous with the mitochondrial membrane. The functional basis for the morphological differences between human embryos develop-

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ing *in vitro* and *in vivo* is not known and deserves to be investigated.

Mitochondrial differentiation inexorably proceeds during further preimplantation stages as blastomeres come to loose their totipotency. However, as they transform into the cells of the inner and outer mass during formation of the blastocyt, no differences in appearance of mitochondria have been noted between embryoblast and trophoblast cells (Dvorák and Tesarík, 1985). Finally, after implantation, gastrulation is followed by formation of the first pre-migratory PGCs in the posterior wall of the yolk sac. A new mitochondrial cycle has begun and mitochondria have been put aside for the new generation (Jansen and de Boer, 1998).

Conclusion and future perspectives

This paper summarizes, from an ultrastructural point of view, the most significant traces of the passage of mitochondria through the human female germ line from the maternal PGCs to the fertilized oocyte and blastomeres of the newly-formed embryo (Figure 16). We have found that oocyte mitochondria change in morphology, populate different domains within the cell, and establish complex relations with other cell organelles, presumably according to the different energetic-metabolic requirements of the germ cell during its differentiation, maturation, fertilization, and ultimate endowment to the blastomeres of the forming embryo. We have pointed out that the final steps of this process might not proceed identically in embryonic development *in vitro* and *in vivo*.

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