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Oogenesis in Adult Mammals, Including Humans

A Review

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The origin of oocytes and primary follicles in ovaries of adult mammalian females has been a matter of dispute for over 100 yr. The prevailing belief that all oocytes in adult mammalian females must persist from the fetal period of life seems to be a uniquely retrogressive reproductive mechanism requiring humans to preserve their gametes from the fetal period for several decades. The utilization of modern techniques during last 10 yr clearly demonstrates that mammalian primordial germ cells originate from somatic cell precursors. This indicates that if somatic cells are precursors of germ cells, then somatic mutations can be passed on to progeny. Mitotically active germline stem cells have been described earlier in ovaries of adult prosimian primates and recently have been reported to also be present in the ovaries of adult mice. We have earlier shown that in adult human females, mesenchymal cells in the ovarian tunica albuginea undergo a mesenchymal–epithelial transition into ovarian surface epithelium cells, which differentiate sequentially into primitive granulosa and germ cells. Recently, we have reported that these structures assemble in the deeper ovarian cortex and form new follicles to replace earlier primary follicles undergoing atresia (follicular renewal). Our current observations also indicate that follicular renewal exists in rat ovaries, and human oocytes can differentiate from ovarian surface epithelium in fetal ovaries in vivo and from adult ovaries in vitro. These reports challenge the established dogma regarding the fetal origin of eggs and primary follicles in adult mammalian ovaries. Our data indicate that the pool of primary follicles in adult human ovaries does not represent a static but a dynamic population of differentiating and regressing structures. Yet, the follicular renewal may cease at a certain age, and this may predetermine the onset of the natural menopause or premature ovarian failure. A lack of follicular renewal in aging ovaries may cause an accumulation of spontaneously arising or environmentally induced genetic alterations of oocytes, and that may be why aging females have a much higher chance of having oocytes with more mutations in persisting primary follicles.

Key Words: Oogenesis; ovary; adult; mammals; human.

Introduction

The central question about ovarian reserve and oocytes is whether or not new oocytes can originate in reproductively mature vertebrates. During the late 1800s a discussion was initiated between two groups of reproductive scientists on whether the definitive oocytes in ovaries of adult vertebrates persist from the fetal period of oogenesis *(27)*, or if the fetal primordial oocytes degenerate and definitive oocytes differentiate from the ovarian "germinal" or surface epithelium (SE) before puberty *(46)*. The latter view has been expanded by a suggestion that new oocytes are formed throughout life, in phase with the reproductive cycle, from the SE of the adult mammal at the same time as vast numbers of already formed oocytes become eliminated through atresia *(2,33)*.

However, about 50 yr ago, a dogma evolved that the process of oogenesis follows a uniform pattern, of which there are two main variants. One variant is that the oogenesis appears to continue either uninterruptedly or cyclically throughout the reproductive life, e.g., most teleosts, all amphibians, most reptiles, and, conceivably, few mammals. The other variant is that the oogenesis occurs only in fetal gonads, and oogonia neither persist nor divide mitotically during sexual maturity, e.g., cyclostomes, elasmobranchs, a few teleosts, perhaps some reptiles, all birds, monotremes, and, with a few possible exceptions, all eutherian mammals *(35)*.

Nevertheless, in early 1970s, this belief was felt unwarranted due to a lack of detailed study of adult ovaries. A

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thorough search for oogenesis, using modern techniques at certain stages of the reproductive cycle, has been suggested *(52)*.

Origin of Primordial Germ Cells

The origin of primordial germ cells in vertebrates has been discussed since the proposal by Waldeyer in 1870 that germ cells arise from the proliferation of somatic coelomic (germinal or surface) epithelium of the presumptive gonad *(76)*. A contrary view was Weissmann's theory of the continuity of the germ plasm *(78)*. This theory assumes that during the earliest stages of embryonic development, before embryonic cells become committed along specific pathways, a set of germ cells is set aside, which are destined to give rise to the gametes. It should be noted, however, that Waldeyer later withdrew his earlier conclusions and accepted the evidence against his thesis for a somatic origin of germ cells *(75)*.

Utilization of newer techniques has shown that Weissmann's theory may fit invertebrates (*Caenorhadbitis elegans* and *Drosophila*) and some lower vertebrates (zebrafish and frogs), but not mice, and possibly mammals in general *(1,51)*. Studies of mouse embryos, in which genetically marked cells were introduced at the four- and eight-cell stage blastomere, have shown that such cells can either become germ cells or somatic cells *(45)*. This suggests that no specific germ cell commitment exists prior to implantation. During the postimplantation period, mouse germ cells are not identifiable before approx 7 d after fertilization *(37)* and germ cells differentiate from somatic lineage *(48)*. It has also been shown that cellular differentiation of grafted embryonic cells does not depend on where the grafts were taken, but where they have been placed *(72)*. Additional studies suggest an important role in the development of germ cells for bone morphogenetic protein 4 (BMP4), a member of TGFβ superfamily, as null BMP4 mouse embryos failed to develop primordial germ cells *(47)*.

More recently, oogenesis has been demonstrated in cultured mouse embryonic stem cells. Such oogonia entered meiosis, recruited adjacent cells to form follicle-like structures, and later developed into the blastocysts *(41)*. Cultured mouse embryonic stem cells have also been reported to differentiate into haploid male gametes capable of fertilizing eggs and develop into blastocysts *(36)*. Mitotically active germline stem cells have recently been reported in ovaries of adult mice *(44)*, echoing earlier reports in ovaries of adult prosimian primates *(19,26,43)*.

Altogether, these studies indicate that somatic cells have the potential to develop into germ cells, and some mammalian species possess mitotically active germline stem cells in adult ovaries. Nevertheless, the belief that all primary follicles in adult mammalian females are formed during the fetal period of life is still held by a sizable number of scientists, because of a lack of direct evidence of formation of new primary follicles in adult mammalian ovaries. In this article, we use the term primary for <50 μm diameter follicles (primordial, intermediary, and primary follicle types) and secondary for >50 and <100 μm (growing) follicles.

A number of questions remain: Do mitotically active germline stem cells in adult prosimian and mice females persist from the fetal period of life or differentiate from some type of progenitor cells? Does the number of dividing germ cells determine the number of new primary follicles? What is the source of granulosa cells for newly formed follicles? Is a preservation of germline stem cells in these two species an exception or the rule for other mammals, and particularly humans?

Except in prosimian primates and possibly mice, the persistence of dividing germline stem cells has not been reported. Both our earlier and more recent studies, however, indicate that in adult human ovaries mesenchymal cells in the tunica albuginea (TA) are bipotent progenitors committed to either primitive granulosa or germ cells *(15,17)*.

Tunica Albuginea, Surface Epithelium, and Derived Epithelial Structures in Human Ovarian Cortex

The human TA is a thick fibrous subepithelial layer of loose connective-tissue cells. It does not begin to form until the end of intrauterine life *(53)*. Even then, it is not a true membrane, but merely a collection of loose connective-tissue cells *(68)*. In contrast to the ovarian SE, purportedly the source of a variety of ovarian tumors, the TA has not been well studied.

The ovarian cortex is usually covered by a layer of irregularly shaped special epithelial-like mesothelial cells *(74)*, commonly referred to as the ovarian "germinal" or surface epithelium. This layer is attached to the basal lamina continuous with the subjacent TA by means of collagenous fibrils. Except in the ovaries of newborn animals, mitoses are essentially absent in ovarian SE *(56)*. In functional human ovaries the SE is found in certain areas only, but in women with anovulatory cycles, or patients with polycystic or sclerotic ovaries, the ovarian surface is completely covered with SE *(49)*.

In scanning electron microscopy and submicroscopic studies of ovarian SE during ovulatory cycles, it has been reported that the ovarian surface is frequently evaginated into a series of villous-like projections or papillae, which may vary widely in number, size, and distribution (reviewed in ref. *74*). The SE also invaginates into the ovarian cortex throughout the entire organ. This is true in all mammalian species, including humans. Cortical invaginations appear as subsurface solid cords of epithelial cells and channel-like crypts.

The cord cells are very similar to some of the granulosa cells. In some areas of the ovary, these cords fragment into small "nests" of epithelial cells. Typically, these nests (fragmented cords) consisting of granulosa-like epithelial cells lie in proximity to primary follicles *(56,74)*. The nests are

in contact or are penetrated by nerve terminals, and they appear to contribute epithelial elements to ovarian follicles *(56)*. In contrast, the crypts are hollow, tubular invaginations with lumen, lined by cells possessing the same general features as SE cells. In adult ovaries, the SE retains a relatively embryonic structure *(52)*.

These observations indicate that the SE-derived epithelial nests may represent primitive granulosa cells. On the other hand, the crypt-like subsurface channels are lined by cells which retain the relatively embryonic structure of SE cells.

Epitheliomesenchymal Transition In Vitro

In culture, ovarian SE cells undergo an epitheliomesenchymal transition. The resulting mesenchymal-type cells can be stimulated to differentiate back into the epithelial phenotype *(3,29)*. Ovarian SE cells undergoing this epitheliomesenchymal conversion are initially cytokeratin (CK) positive, but lose CK expression with time and passages in cultures *(4)*. Our observations indicate that the diminution of CK expression indicate a resting state for mesenchymal cells in ovarian TA *(15)*.

Expression of Mitogen-Activated Protein Kinases

Normal SE cells express mitogen-activated protein kinases (MAPK) *(22)*, a group of serine/threonine kinases. MAPK is used throughout various species to control cellular responses to external signals such as growth factors, nutrient status, stress, or inductive signals. Transcription factors are substrates for MAPK *(73)*. In adult human ovaries, we have reported prominent cytoplasmic MAPK expression in oocytes of heathy primary follicles, a diminution of oocyte MAPK expression during follicular atresia, and cytoplasmic and nuclear MAPK expression in growing secondary follicles *(10)*. Hence, cytoplasmic MAPK expression appears to be characteristic for resting healthy oocytes. Nuclear translocation of MAPK may signal oocyte growth.

Identification of Germ Cells in Ovarian Surface Epithelium

Scanning and transmission electron microscopy have revealed numerous germ cells (10 μm in diameter) within the ovarian SE of human fetuses from 7 to 24 wk of intrauterine life. Germ cells are easily distinguished from smaller coelomic epithelial cells by their rounded contour, smooth surface, and, in some instances, large ameboid evaginations *(53)*. Using differential interference contrast (DIC) and immunohistochemistry, we previously reported the occurrence of similar putative germ cells within the SE and cortex of adult human ovaries *(17)*. These data indicate that germ cells are present in the SE. They may either invade SE from adjacent structures and are extruded from the ovary *(53)*, originate in SE and invade the ovarian cortex, or both *(17)*.

In addition to their characteristic morphology, germ cells can also be identified by alkaline phosphatase activity *(72)*. However, nonspecific alkaline phosphatase activity has been described in various tissues *(57,63,65,82)*. Zona pellucida (ZP) proteins are more specific markers for oocytes. In postnatal rat ovaries, zona pellucidae first appear in primary follicles adjacent to keratin-positive granulosa cells *(58)*. Some ZP proteins, such as PS1, are also detected in the ovarian SE of rabbit, cat, monkey, baboon, and human, as well as in human ovarian cancers *(28,69–71)*. Hence, expression of ZP proteins in SE cells suggests a relationship between SE and oocytes.

Intravascular Transport of Germ Cells

Germ cells are capable of migration by ameboid movements, but they can also utilize intravascular transport to reach distant destinations *(5,77)*. Why the cell leaves the circulating blood at a certain site is unclear. It has been suggested that there is a trapdoor mechanism that prevents the germ cell from continuing to circulate. Adhesion of primordial germ cells ("cauliflower-like structures") in the aortas of bovine embryos have also been observed *(77)*. Hence, in large mammals, germ cells may migrate to reach adjacent blood vessels, and then utilize vascular transport to reach distant destinations.

Balbiani Body

Oocytes in primary (resting) follicles show a single Balbiani body (named for the 19th century Dutch microscopist) in the cytoplasmic region near the nucleus where the majority of oocyte organelles are concentrated (reviewed in ref. *81*). The Balbiani body contains aggregated mitochondria and can be observed in primary follicles in both fetal and adult ovaries *(21,24,55)*. In a study of turkey hens, no Balbiani body was detected in stage I oocytes, they appeared in stage II oocytes and diminished in the oocytes of growing follicles, coinciding with the dispersion of mitochondria throughout the ooplasm *(21)*. Similar observations were reported in human oocytes *(55)*. Balbiani bodies show immunostaining for CK 8, 18, and 19 *(17,67)*. In primary follicles of fetal and adult human ovaries, follicular (granulosa) cell extensions penetrate deep into the ooplasm, much like a sword in its sheath. There may be as many as three to five "intraooplasmic processes" in one scanning microscopy plane. These intraoocytic invaginations are closely associated with a variety of organelles. They are close to the nuclear zone, and may help activate growth of the oocyte *(54)*.

In mouse fetal ovaries, germ cells are arranged in special clusters (germline cysts) and dividing germ cells remain connected by intercellular bridges called "ring canals." The cysts may allow certain germ cells to specialize as nurse cells *(61)*. One possibility is that such nurse cells in germline cysts help provide oocytes with mitochondria *(60)*. It has been proposed that mitochondria with functional and defective genomes are actively transported into different germ cells, and the quality of each cell's mitochondria might

then determine whether it survived or underwent apoptosis *(61)*. A recent study by Cox and Spradling indicates that during *Drosophila* oogenesis, nurse cells are a source of mitochondria, which enter the oocyte cytoplasm via the "ring canal" to form the Balbiani body, thereby supplying virtually all of the mitochondria of the oocyte *(24)*.

It appears that the Balbiani body contributes to the resting state of the oocyte, because oocyte mitochondria are not dispersed to the ooplasm until the initiation of the follicular growth. In addition, the contribution of granulosa cells to the formation of the Balbiani body in the oocyte cytoplasm indicates the use of these cells in ongoing oocyte assembly.

Numbers of Ovarian Primary Follicles

In adult mammalian ovaries, 60–95% of oocytes are in various stages of degeneration *(31,42)*. Block's quantitative morphological investigations of follicles in women *(7)* showed wide individual variation, but the numbers in the right and left ovaries were similar, with a tendency to decline with age. However, in the 18–38 yr age range, a relationship between age and the number of primary follicles could not be statistically proven *(7)*—see also Fig. 1F. The number of primary follicles in both ovaries varied between 8100 and 290,000. This lack of significant numerical change during the reproductive period also has been reported in cattle *(31)*. Gougeon used the data of Block and his own observations and concluded that the depletion of the primary follicle pool is caused mainly by atresia in younger women and by a decrease in the growing pool in older women, with the cross-over point at 38 ± 2.4 yr of age *(39)*. These observations suggest that in human females new cohorts of primary follicles may replace follicles undergoing atresia until about 38 yr of age, with a lack of follicular replacement after that period causing a significant decrease in the pool of primary follicles.

Origin of Germ Cells and Follicular Renewal in Adult Human Ovaries

Relationship of Tunica Albuginea, Surface Epithelium, and Ovarian Cortex

In most instances, the human ovarian cortex is covered by TA, with or without an SE cover. The TA shows a variable thickness, ranging from almost undetectable to more than 50 μm. Cytokeratin expression of various density is detected in mesenchymal cells of some TA segments, particularly in segments showing an appearance of SE cells. In contrast, no CK expression is detected in mesenchymal cells of the ovarian cortex *(15)*.

The development of SE-derived cortical nests of primitive granulosa cells *(56,74)* coincides with the appearance of SE cells directly connected to the ovarian cortex (without interference of TA), and with appearance of TA flaps extending over the SE (Fig. 1A). Strong CK expression (brown color) in mesenchymal cells exhibiting fibroblast morphology (fb) is apparent. Such cells associate with the inner flap surface covering the SE. At the angle between the flap and SE, the cells show an intermediate morphology between fibroblasts and epithelial cells (fb/se), and appear to contribute to the SE cover (se) of the cortex (arched arrow) *(15,17)*.

These observations collectively indicate that TA fibroblasts, which show CK immunoexpression, differentiate into SE cells by a process of mesenchymal–epithelial transition. However, depending on additional unknown factors, this process may result in either the formation of primitive granulosa cell nests or the differentiation of SE cells covering the TA surface (precursors of germ cells). The nests of primitive granulosa cells descend into the deeper ovarian cortex, where they eventually associate with oocytes to form primary follicles *(15)*.

Epithelial Nests Assemble with Oocytes to Form New Primary Follicles

As noted above, studies of Motta et al. *(56,74)* have shown that in some areas of the ovary epithelial cords fragment and appear as small nests of epithelial cells which lie in proximity to primary follicles *(56)*. In order to study the possible formation of new follicles from epithelial nests and oocytes, we used cytokeratin staining to view the nest (primitive granulosa) cells and DIC to study unstained oocytes. In the lower ovarian cortex, some epithelial nests were found associated with the lumen of ovarian venules. Fig. 1B shows a venule lumen lined by endothelial cells and a CK+ epithelial nest (n) inside the lumen. The nest shows an arm associated with the putative oocyte (o) possibly directing the oocyte outside of the vessel. This seems to be a real stretch.

Ongoing assembly of the epithelial nest with an oocyte is shown in Fig. 1C. The nest exhibits closing "gates." A small portion of the oocyte (dashed line), which still lies outside, is expected to join the complex (arched arrow). Note that the nuclei of stromal cells are visible through the oocyte tail, which may be less thick than the 7 μm thickness of the cryostat section. Cytokeratin+ projections from the nest wall into the oocyte cytoplasm appear to contribute to the formation of the Balbiani body (asterisk) adjacent to the oocyte nucleus (dotted line) *(15)*.

Double-Color Immunohistochemistry (Fig. 1D)

In order to better visualize assembly of oocytes with epithelial nests, we used double-color immunohistochemistry for CK and ZP proteins. These sections were not counterstained with hematoxylin, but enhanced DIC has been used. Figure 1D shows an association of the oocyte (blue ZP immunoexpression) with epithelial nest (brown CK immunoexpression) resembling an occupied bird's nest. This phase of assembly is similar to that shown in Fig. 1C.

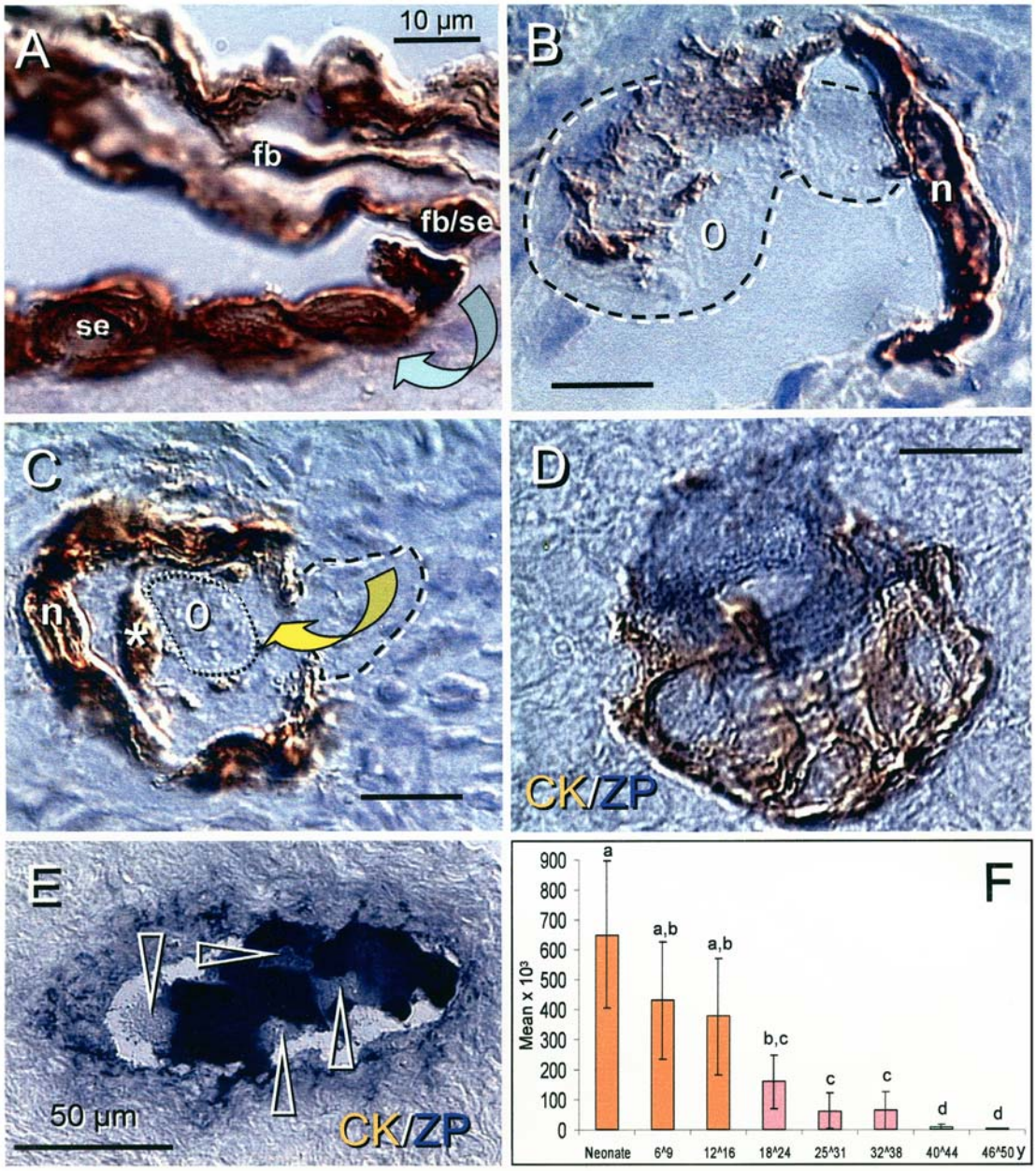


Fig. 1. Follicular renewal in adult human ovaries. CK18 (**A–C**) and CK/zona pellucida (ZP) staining (**D** and **E**). (**A**) Transition of TA fibroblasts (fb) into SE cells (se). (**B**) The intravascular oocyte (o) is "caught" by epithelial nest (n). (**C**) Formation of Balbiani body (asterisk) during oocyte/nest assembly. (**D**) Oocyte (blue)/nest (brown) assembly. (**E**) Degenerating ZP+ oocytes in a medullary vessel (unstained nuclei indicated by arrowheads). (**F**) Number of primary follicles in human ovaries. Bars in A–D = 10 µm. For details see text and http://www.rbej.com/content/2/1/20.

Origin of Germ Cells

Most of the ovarian SE cells showed CK immunostaining, but immunoexpression of ZP proteins was restricted to certain SE segments. While most ZP proteins were also detected in the zona pellucida of oocytes during and after assembly within epithelial nests (including primary, secondary, preantral, and antral follicles), the PS1 meiotically expressed porcine oocyte carbohydrate antigen *(70)* was not detected in zona pellucida of oocytes in human ovarian follicles (contrary to porcine ovaries). The PS1 is also found in some human ovarian cancers *(71)*.

The immunoexpression of PS1 in human SE cells was cytoplasmic. Cells descending from the SE into TA also showed nuclear PS1 staining. The dividing SE cells showed an asymmetric distribution of meiotically expressed nuclear PS1, suggesting meiotic activity. Double-color immunostaining for PS1 and CK revealed an asymmetric distribution of PS1 in putative germ cells descending from the SE, resulting in two distinct (CK+ or PS1+) daughter cells. Larger putative germ cells with nuclear PS1 staining were detected in TA. Such cells divided symmetrically (both daughter cells

expressed PS1) and entered the adjacent ovarian cortex. In the cortex, the putative germ cells showed a translocation of nuclear PS1 immunoexpression to cytoplasmic staining, and an association with cortical vasculature showing minute amounts of PS1 immunoexpression in adjacent endothelial cells. In addition, PS1 immunoexpression was detected in putative germ cells migrating from CK+ epithelial crypts into the adjacent cortex—an alternative pathway for germ cell origin *(15)*.

Staining for MAPK revealed symmetrically dividing putative germ cells in TA with prominent nuclear MAPK immunoexpression, similar to those showing nuclear staining for PS1. However, nuclear MAPK immunoexpression persisted in putative germ cells in the upper ovarian cortex. Intravascular putative germ cells showed an increase in size (20 μm) accompanied by an appearance of MAPK cytoplasmic clusters in addition to persisting nuclear MAPK immunoexpression. During association of oocytes with CK+ nests in the lower ovarian cortex, strong MAPK immunoexpression was apparent in the oocyte nucleus and cytoplasm. Fresh follicles with an established ring of granulosa cells in the adjacent ovarian cortex showed strong nuclear and cytoplasmic staining but poor MAPK immunoexpression in the paranuclear (Balbiani) body *(15)*.

These observations indicate that in adult human females there are no persisting oogonia or germline stem cells, but germ cells nonetheless originate from asymmetrically dividing SE cells. Such germ cells symmetrically divide in TA (crossing over) and eventually reach oocyte size assembling with nests of primitive granulosa cells to form new primary follicles. Such follicular renewal may replace earlier primary follicles undergoing atresia.

Follicular Atresia

Degeneration may affect groups of primary and secondary follicles. Immunohistochemically, the follicles undergoing atresia release ZP proteins into the neighboring stroma. This is associated with an altered oocyte morphology and disorganization of the follicular CK+ granulosa layer. In addition, there is a considerable influx of large macrophages into the area from accompanying vessels *(15)*. Some investigators have claimed that characteristic morphological features of primary follicle atresia is often difficult to determine (reviewed in ref. *5*), while others are more confident *(39)*. In our immunohistochemical study, the assembly of oocytes with epithelial nests was also associated with release of ZP proteins. Formation of new follicles was characterized by a well-defined oocyte nucleus, intraooplasmic CK+ extensions from the nest cell wall, and formation of the Balbiani body, i.e., structures and processes not apparent during follicular regression. Resting normal primary follicles and growing secondary/preantral follicles show a regular morphology, with no leakage of ZP proteins, and only occasional small tissue macrophages associated with the developing theca *(15)*.

Follicular atresia and luteal regression are essential mechanisms required for the elimination of unnecessary structures and normal ovarian function. Elimination of antral follicles undergoing atresia and degenerating corpora lutea during reproductive years in human females is a fast process, associated with infiltration by activated macrophages *(12,13)*, and there is no reason to expect that similar processes accompanying regression of primary and secondary follicles *(15)* will last longer than several days. If at least 60% of oocytes are in various stages of degeneration, one may conclude that without follicular renewal the ovarian function will cease within a few months. However, in aging ovaries, the elimination of degenerating ovarian structures appears to be affected, possibly due to the age-induced alterations of the immune system function *(14)*. Hence, atresia may not affect primary follicles in aging ovaries *(39)*, and such follicles may persist in spite of an accumulation of spontaneously arising or environmentally induced genetic alterations of oocytes.

Epithelial Crypts— An Alternative for Germ Cell Origin

Enhanced follicular atresia was accompanied by the appearance of epithelial nests (fragmented epithelial cords) in adjacent segments of the ovarian cortex. We have shown that these nests are small CK+ spheroidal cell clusters of 20–30 μm in diameter. There were also epithelial crypts, likely originating from deep SE invaginations. These do not communicate with the ovarian surface, as evidenced from serial sections. The movements of epithelial nests and crypts appear to be caused by a rearrangement of stromal bundles. Their migration is probably guided by HLA-DR+ (activated) tissue macrophages. We have also described an alternative germ cell origin, from epithelial crypts in the lower ovarian cortex, accompanied by an accumulation of primary follicles in the neighborhood *(15)*.

These observations indicate that adult human ovaries exhibiting atresia of primary and secondary follicles initiate formation of new epithelial nests with granulosa cell features *(56)*. This is one of the prerequisites for formation of new primary follicles. Cortical crypts, consisting of epithelial cells retaining a relatively embryonic structure of SE cells *(52,56)*, appear to be an alternate source of germ cells. Germ cells entering the vasculature may reach epithelial nests at distant destinations, although vascular proximity is not a requirement for follicular development from nests approaching the cortical crypts producing germ cells.

Oocyte Remnants in Medullary Vessels (Fig. 1E)

Is the number of newly formed follicles in human ovaries determined by the number of available epithelial nests or the number of generated germ cells? In the first instance, the isolated nests will either persist or degenerate. In the second, degenerating oocytes not utilized in new follicle formation might be detected. To answer this question we uti-

lized double-color immunohistochemistry to search for ZP+ oocytes not assembled with CK+ structures. Figure 1E shows an accumulation of degenerating ZP+ oocytes in a medullary vessel. An accumulation of oocytes in occasional ovarian medullary vessels was observed in 4 of 12 cases studied (33%). This suggests that the differentiation of oocytes during the reproductive period is a relatively frequent event. These ovarian samples showed either preparation for or the ongoing formation of new primary follicles. The accumulation of oocytes in some medullary vessels was present in the samples from both ovaries. Yet, there were eight cases showing no such activity. These ovaries rarely showed primary follicles in the ovarian cortex. This agrees with the observations of Block *(7)*, who observed a similarity between oocyte numbers in the right and left ovaries but wide variation between cases during the reproductive period.

Our observations support the idea that the formation of new primary follicles in adult human ovaries is a cyclical process, which may occur during a certain period of the ovarian cycle. This idea was first proposed by Evans and Swezy *(33)*. Although we did not study a large number of patients, our observations indicate that new primary follicles are likely to be formed during the late luteal phase, as evidenced from the patient's history, ovarian (CL) immunohistochemistry, and endometrium morphology *(15)*. However, the preparation for follicular renewal consists of two sequential steps: formation of primitive granulosa nests, followed later by differentiation of new germ cells. This may be initiated earlier during the ovarian cycle. For example, follicular atresia is most prominent during the late follicular phase (follicular selection), when the formation of epithelial nests may be initiated, followed by an appearance of germ cells and the development of oocytes later during the cycle.

Interactions of Morphoregulatory Molecules and Tissue Macrophages with Ovarian TA and Developing Follicles

We have observed that the formation of primary follicles in adult human ovaries is a complex process initiated in certain segments of the TA. It is important to know what are the signals involved in the initiation of both the mesenchymal–epithelial transition and the transition of some SE cells into germ cells. Such an event may require a specific milieu of factors involved in developmental processes, such as immune system-related morphoregulatory molecules and cells.

Molecules belonging to the immunoglobulin gene superfamily (IgSF) and involved in morphoregulatory processes include the Thy-1 differentiation protein (Thy-1) and the neural cell adhesion molecule (N-CAM). It has been suggested that the involvement of Ig-related molecules in tissue interactions is more primitive than their involvement in the immune system, and the immune functions evolved from the sets of molecules mediating tissue interactions *(23)*. The Ig-related molecules have a diversity of functions, but in most cases the common denominator is a recognition at the cell surface *(79)*.

The Thy-1 consists of a single variable Ig domain and belongs to a group of glycosylphosphatidylinositol-anchored molecules involved in primitive recognition at the cell surface, with the consequences of recognition being due to the differentiated state of the cells. It requires that the correct ligand and receptor are expressed on the appropriate cells at the right time *(23,79)*. Thy-1 is one of the smallest and oldest IgSF molecules from which other members of IgSF have probably evolved during phylogeny. Thy-1 expression within the tissues of different animal species varies, but it is expressed by vascular pericytes and neural tissues in all species studied *(11,79)*.

Thy-1 and "Targeted Delivery" of Paracrine Substances

We have shown that the pericytes release intercellular Thy-1+ vesicles among neighboring tissue (parenchymal or epithelial) cells. These intercellular vesicles have been shown by immunoelectron microscopy to exhibit Thy-1 surface expression and contain a substance lacking Thy-1 staining *(10)*. They may represent a unique paracrine mechanism, so-called "targeted delivery," by which a potent growth factor lacking tissue specificity (vesicle content) is specifically delivered via chemotaxis to certain type of target cells expressing a putative receptor for Thy-1 ligand.

We have reported that Thy-1+ vesicles migrate through the epithelial basement membranes, and after reaching target cells they release content and collapse into Thy-1 "spikes." This is associated with a maturation of parenchymal and epithelial cells (reviewed in ref. *11*). The "targeted delivery" of certain growth factors by intercellular Thy-1 vesicles to the particular cell types is a novel aspect in the regulation of cellular differentiation, and it can be enabled by tissue specificity of Thy-1 glycoprotein carbohydrate moieties described earlier *(6)*. For example, the pericytes in follicular theca interna may produce Thy-1 vesicles with affinity to the neighboring thecal cells, and pericytes associated with follicular basement membrane vesicles with Thy-1 affinity to the granulosa cells *(12)*.

Neural Cell Adhesion Molecule

Neural cell adhesion molecule is a single chain consisting of five constant Ig domains. N-CAM plays a central role in development, repair, and regeneration of tissues *(30)*. N-CAM appears on early embryonic cells and is important in the formation of cell collectives and their boundaries at the sites of morphogenesis. Later in development it is found in various differentiated tissues and is a major cell adhesion molecule, mediating adhesion among neurons and between neurons and other tissues. The N-CAM molecule has been found to be identical to the CD56 determinant of natural killer cells (reviewed in ref. *17*).

Immune System–Related Cells

Immune system–related cells (tissue macrophages and intraepithelial T cells) are involved not only in immunity but also in the regulation of differentiation of both epithelial and parenchymal cells (reviewed in ref. *11*). We have reported that the differentiation of SE cells from TA progenitors is accompanied by activated (HLA-DR) macrophages and Thy-1 differentiation protein. Isolated segments of the SE also showed the presence of N-CAM and lacked MHC class I expression. In such segments clear spherical putative germ cells differentiated from SE precursors in the presence of activated tissue macrophages and T cells. Activated tissue macrophages also accompanied migration of tadpole-like germ cells and their association with cortical vessels expressing Thy-1 *(17)*.

These observations suggest that the differentiation of SE and germ cells from TA precursors require the involvement of immune system–related cells and morphoregulatory molecules. We speculate that some factors required for the differentiation of germ cells from SE precursors may disappear with age-dependent functional alterations of the immune system. Immune system dysfunction may cause a cessation of follicular renewal in aging ovaries. Indeed, the onset of immune system alteration occurs between 35 and 40 yr of age *(50)*, coinciding with the onset of a significant diminution of primary follicles in human ovaries.

An "Ovary-Within-the Ovary" Pattern of Thy-1 Differentiation Protein Distribution

Why do human primary follicles form in the lower ovarian cortex, not just near the origin of their components, the primitive granulosa and germ cells? We reported previously that groups of follicles lie in isolated areas of the cortex, exhibiting an oval arrangement of stromal cells *(17)*. In a subsequent study, staining for Thy-1 revealed that groups of primary follicles reside in rounded stromal areas, extending approx 400–1200 μm from the ovarian surface. They exhibit a diminution of Thy-1 immunoexpression, and show an "ovary-within-the ovary" pattern. In addition, the growth of some follicles in a given cohort is associated with Thy-1+ secretion from vascular pericytes *(15)*. Hence, a lack of Thy-1 differentiation protein may maintain primary follicles in the resting state, and Thy-1 secretion by vascular pericytes may stimulate follicular growth.

A Role for Thy-1 Differentiation Protein in Follicular Growth and Selection

The growth of preantral and antral follicles is accompanied by activated macrophages and the abundant secretion of Thy-1 from thecal vascular pericytes *(12,17)*. However, most women preserve and ovulate a single selected follicle, while other growing follicles undergo subsequent atresia during the typical menstrual cycle. The mechanism by which the selected follicle is protected from atresia remains unclear. We have shown that during follicular selection the selected (dominant) follicle exhibits a marked retardation of thecal development compared to regressing (nondominant) follicles, which show an accelerated differentiation of the thecal layer. However, once the dominant follicle reaches maturity (preovulatory follicle) with expression of the LH receptor by granulosa cells *(16)*, the Thy-1 pericytes under the follicular basement membrane are highly activated and the theca interna also shows advanced differentiation *(12)*.

These data support an idea that temporary retardation of thecal development, and low androgen secretion until granulosa cells mature and express aromatase, may be a decisive mechanism in follicular selection. One possibility is that the activity of thecal Thy-1+ pericytes is temporarily inhibited in the selected follicle by autonomic innervation accompanying follicular vessels (see ref. *12* for details). This may ensure that only a single follicle from antral follicles growing in both ovaries is selected to ovulate during each cycle. Atresia of other antral follicles may be caused by high levels of thecal androgens affecting immature granulosa cells lacking aromatase.

Origin of Germ Cells in Ovaries of Midpregnancy Human Fetuses

An important question is whether germ cells could also originate from somatic SE cells in human fetal ovaries. Ovarian differentiation begins before follicles form, and it is characterized by the development of oocytes, organization of the rete ovarii, and the evolution of the SE from peritoneal mesothelial cells. In human embryos, primordial germ cells arise outside the urogenital ridge, in the dorsal endoderm of the yolk sac at 24 d of age. They migrate by ameboid movements to indifferent gonadal primordia at 28–35 d. At the age of 9 wk, female gonads show a marked development of rete cords with lumen formation. The rete reaches the center of the ovary at 12 wk. Meiosis of oocytes begins at 3 mo. The nuclei of the germ cells lie close together in clusters without clearly defined cell membranes.

Degeneration of oocytes in fetal gonads is a frequent event, and one possibility is that these syncytia (nuclei without defined cell surfaces) may represent degenerating oocytes originating from primitive germ cells and/or oocytes not utilized for the formation of primary follicles. Another possibility is that these syncytia are analogous to the mouse ovarian germ cell cysts of interconnected cells that form by incomplete cytokinesis, i.e., endoreplication of the nuclei without cell division. In the mouse, the ovarian germ cell cysts undergo programmed breakdown by the end of the fetal intrauterine life, during which approx 33% of the oocytes survive to form primordial follicles *(61)*.

The syncytia in human fetal ovaries are surrounded by slender stromal (mesenchymal) cells. The first primary follicles are formed after the fourth fetal month. Follicle formation always begins in the innermost part of the cortex, close to the rete ovarii. This structure is essential for folli-

cular development, as if it is removed before folliculogenesis has started, follicles will not form *(20)*. Formation of the follicle requires the attachment of granulosa cells to the oocyte surface and closure of the basement membrane around this unit. At 5 mo of fetal age, the ovary contains its peak population of germ cells. At 7 mo of intrauterine life the last oogonia enter meiosis (reviewed in ref. *62*). Possible reasons for cessation of fetal oogenesis and its resumption at menarche are discussed bellow.

The surface of the ovary is covered by a serous membrane made of SE that is continuous with the peritoneal mesothelium. During the early stages of ovarian development there is a rapid proliferation of SE cells, resulting in cellular stratification, nuclear pleomorphism, and nuclear irregularities. Toward the end of intrauterine life the SE is reduced to a single layer of cells, exhibited in the adult ovary in a cuboidal, columnar, or squamous pattern. Many cells from the SE enter the cortex in fetal ovaries and become associated with oocytes *(62)*.

The SE has been implicated in the formation of oocytes *(2,17,33,34)*, and it also has been suggested that the SE is a source of granulosa cells in adult ovaries *(9,56)*.

It is possible that ovarian development is influenced by mesenchymal-epithelial interactions, which accompany differentiation of epithelial cells in adult tissues *(11)*. This may depend on the ability of specialized mesenchymal cells (SMC) (tissue macrophages and T cells) first to recognize the character of primary germ cells, which populate the gonadal anlage from the extragonadal source during embryonal development, and then to induce replication of this process within the fetal ovary. Ovarian surface epithelium, owing to its rapid proliferation and pleomorphism, as well as its capacity to differentiate into various cell types (ovarian cancers), might be a target of SMC in this process. To our knowledge, a relationship of SMC to ovarian structures in developing human gonads has not been reported. It has, however, been shown that SE of human fetal ovaries from 7 to 24 wk of intrauterine life contain numerous germ cells (10 μm in diameter) *(53)*.

Studies of Fetal Ovaries

The morphological and immunohistochemical observations described from human fetal ovaries represent previously unpublished material. The tissue samples were received during the 1980s from the Department of Pathology of the Institute for the Care of Mother and Child, Prague, Czech Republic, and processed in the same place for immunohistochemistry as indicated in *(17)*, without staining for ZP proteins.

Figure 2A shows Papanicolaou's polychromatic staining (PAP) in the ovary of a 5-mo-old human fetus. The germ cells within the SE (asterisks) are smaller when compared to those positioned under the SE (white arrowhead). In deeper ovarian cortex, germ cells with well-defined cytoplasm show a further increase in size (black arrowhead). They lie among smaller cells with round or elongated nuclei. Beneath the layer of well-defined germ cells lies a nuclear cluster (nc), or syncytium, of germ cells, and the entire area is surrounded by mesenchymal cell cords (mcc), i.e., extensions of centrally located rete cords into the cortex *(62)*.

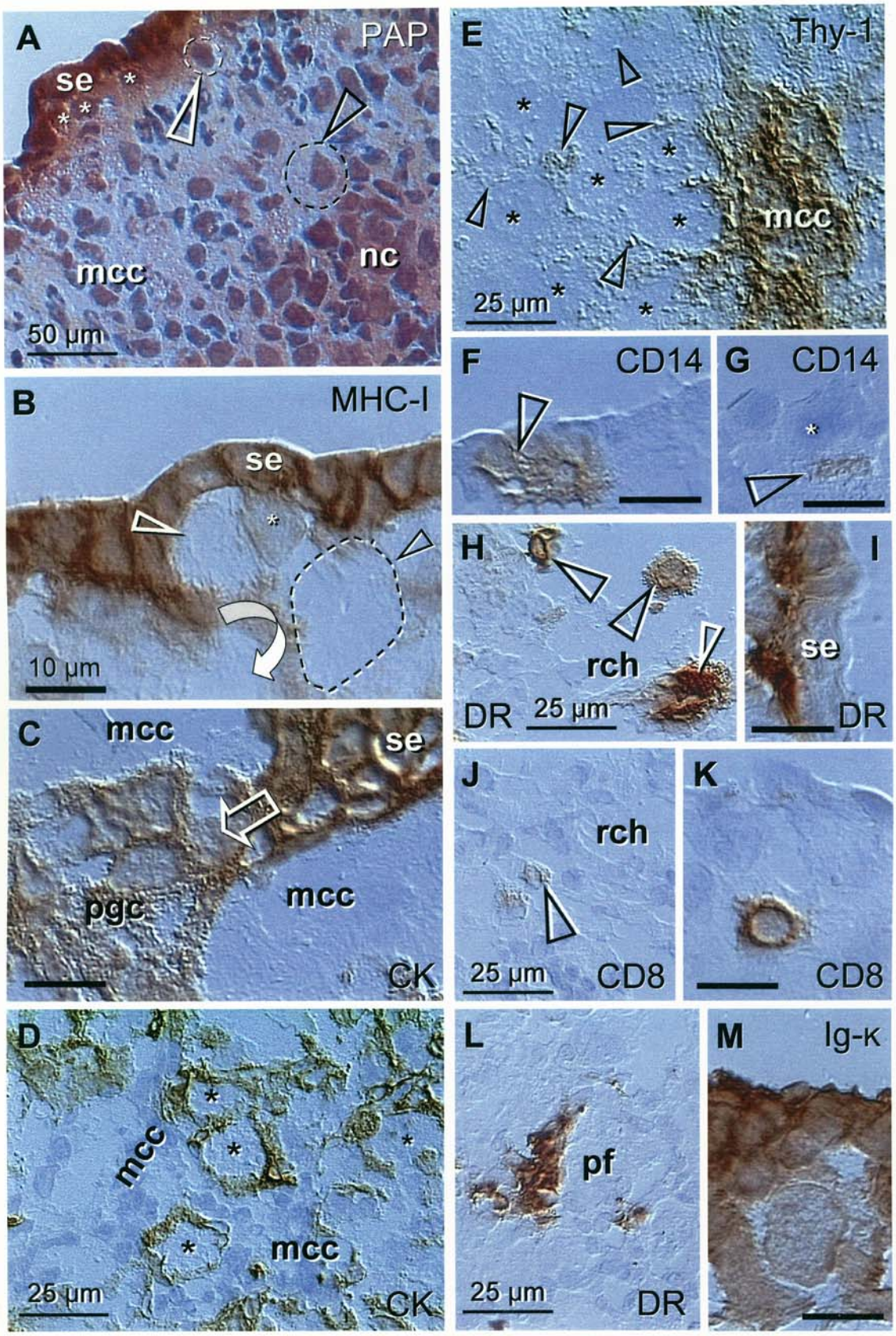
Further immunohistochemical investigation of human fetal ovaries may help to trace the origin of germ cells in midtrimester fetuses, the origin of primitive granulosa cells, and an association of mesenchymal cells with these processes.

Germ cells within the SE show a diminution (asterisk, panel B) or lack of MHC class I expression (white arrowhead), and their size substantially increases under the SE layer, where they exhibit a tadpole shape (dashed line). Note a lack of tunica albuginea. When leaving SE, the germ cell tail appears to be oriented toward the cortex, resembling our observations on the back out of emerging germ cells from the epithelial crypts in adult human ovaries *(15)*.

Primitive granulosa cells (pgc, panel C) show a decrease of cytokeratin 18 expression when compared to the SE cells. They originate from sprouts of SE cells extending into the ovary between mesenchymal cell cords (mcc), and associate with oocytes in the deeper ovarian cortex to form primary follicles (asterisks, panel D). Note the lack of CK18 Balbiani bodies in oocytes of primary follicles in midpregnancy fetal ovaries. The mesenchymal cell cords are rich in expression of Thy-1 (panel E), which is secreted (arrowheads) among primary follicles (asterisks).

Primitive macrophages expressing CD14 show extensions among SE cells (arrowhead, panel F) and were found to be associated with appearance of intraepithelial germ cells (asterisk, panel G). Large activated tissue macrophages (HLA-DR+) reside in rete cords (white arrowhead, panel H), and monocyte- (right black arrowhead) and lymphocyte-type cells (left black arrowhead) in rete channels (rch). The rete cords also show prominent Thy-1 expression (data not shown). The accumulation of activated tissue macrophages is apparent under the SE (panel I). Rete channels also contain CD8 T cells (panel J). Such cells were found in the vicinity of the SE (panel K). Activated tissue macrophages are also associated with larger primary follicles (pf, panel L). In contrast to SE cells, the intraepithelial germ cells do not show a binding of immunoglobulins (Ig-κ, panel M).

Taken together, these observations indicate that small germ cells (10 μm) differentiate from SE cells in the ovaries of midpregnancy human fetuses. After leaving SE, the germ cells increase in size and mature into oocytes contributing to the development of primary follicles in a milieu rich in Thy-1 differentiation protein. The SE is also a source of primitive granulosa cells. Hence, as in adult ovaries, the midpregnancy human SE cells are bipotent progenitors with a commitment for both germ and granulosa cells. It is possible that tissue macrophages residing in rete cords carry a memory on the germ cells populating the ovary during embryonal period of life. Such memory could be transferred to monocytes and T cells migrating through the rete chan-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nels during midpregnancy. The migrating cells reaching the SE may stimulate a transformation of SE cells into germ cells. In addition, the mesenchymal cell cords rich in Thy-1 may participate in the transformation of SE cells into the primitive granulosa cells. In this way, different potentials of SE cells may be realized, depending on the local influence derived from migrating mesenchymal cells. Pluripotency of progenitor cells is not unusual. It persists in bone marrow throughout life. This "one cell, two fates" phenomenon has been described for vascular progenitor cells *(80)*.

Possible Role of LH/hCG in the Stimulation of Oogenesis

Why the oogenesis ceases at a certain period of fetal development, while other tissues continue to growth, remains unclear. The source of germ cells in midpregnancy fetal ovaries appears to be some isolated cells in the SE, and this transition of SE cells into germ cells occurs in the presence of SMC resembling the oogenesis in ovaries of adult females *(17)*. The SE cells represent the bipotent progenitor cells with a capacity to differentiate into primitive granulosa and germ cells. These transitions into one or another cell type may reflect a plasticity of progenitor cells in a particular microenvironment. They may occur under the influence of the extracellular matrix, cytokines (some of which are secreted by SMC), adhesion molecules, membrane receptors, intercellular junctions, signaling pathways, or transcription factors, commonly produced in the embryo and less frequently in adult organisms. Such transitions are examples of manifestations of cell plasticity and subsequent dramatic changes resulting in lineage commitments into certain cell types (reviewed in ref. *15* and *64*). We speculate that the particular microenvironment required for the transformation of SE cells into germ cells (association of SMC with SE cells) also requires certain hormonal condition. The SE expresses LH/hCG receptor *(16)*, and high levels of hCG in early pregnancy decline with pregnancy advancement *(40)*. In addition, a development of the placental hCG barrier causes virtually zero hCG values in the blood of human term fetuses *(59)*. If the association of SMC with the SE requires LH/hCG binding to its receptor, it may resume during ovulatory LH peak, which does not occur until menarche. In other words, we propose that there is a lack of SMC-induced oogenesis between the seventh month of intrauterine life and menarche, owing to the lack of saturation of LH receptors in ovarian SE. The preparation of oogenesis may also require high levels of estrogens, corresponding to the preovulatory peak of 17β estradiol, as we experienced in our recent studies of human oogenesis in vitro *(18)*. A working model on age-associated changes of SMC and hormonal signals (LH/hCG & E2) required for the initiation and resumption of oogenesis in human ovaries is given in Table 1.

Human Oogenesis In Vitro

After these observations in adult and fetal human ovaries, we asked ourselves if a similar potential for SE cells could be demonstrated in vitro. Our current observations show that oocytes and granulosa type cells can develop along with fibroblasts in pure SE and mixed SE/cortical (alternative pathway) human ovarian cultures *(18)*.

Figure 3A shows 3-d mixed culture in phase contrast. It consists of undifferentiated rounded stem cells of variable size. The large cells (50 μm in diameter) show more prominent clusters of organelles (arrow) as compared to the 15 μm small cells (arrowhead). No oocytes were detected on d 3, but fibroblasts were found to differentiate in some cultures. In 5-d cultures two oocyte-type cells are presented in panel B (arrows). They are 60 and 80 μm in diameters and centrally located germinal vesicle, and are accompanied by elongated fibroblasts adjacent to their surface (arrowheads). A larger oocyte (100 μm) with well-defined surface (arrow) and attached fibroblasts (arrowheads) is shown in panel C. Panel D shows a follicle-like structure, which consists of a large oocyte (180 μm) accompanied by small (15 μm) rounded cells (arrowheads), resembling granulosa cells.

Staining for ZP protein [sperm receptor *(66)*] on d 5 (panel E) shows nuclear but not cell surface (white arrow) ZP expression in a middle sized (120 μm) oocyte. Note thick zona pellucida, a well-defined nuclear envelope (black arrowhead), and attached fibroblast (white arrowhead). Panel F shows cytoplasmic CK5 expression in differentiated fibroblasts and a weak endogenous peroxidase staining of the nuclei in other cells. The large oocyte in panel G (200 μm) shows ZP expression at the majority of the cell surface (black arrow). The structure indicated by the white arrowhead resembles a polar body. Note a lack of ZP staining in the adjacent cell surface segment (white arrow). The cell shows no attached fibroblasts, a narrow zona pellucida and

Fig. 2. *(Opposite page)* Origin of oocytes and granulosa cells in midpregnancy human fetal ovaries. (**A**) Papanicolaou's staining (PAP) shows surface epithelium (se) containing small germ cells (asterisks), showing increase in size in the adjacent (white arrowhead) and distant cortex (black arrowhead). nc, nuclear cluster, mcc, mesenchymal cell cord. (**B**) MHC class I expression diminishes in small (asterisk) and disappear in larger germ-like cells (arrowheads) leaving the SE (arched arrow). (**C**) CK18 staining of a cluster of primitive granulosa cells (pgc) descending from the SE (arrow) between mesenchymal cell cords. (**D**) Primary follicles (asterisks) in the lower cortex, without CK18+ Balbiani bodies in oocytes. (**E**) Thy-1 is secreted (arrowheads) from the mesenchymal cell cord among primary follicles (asterisks). (**F**) CD14 primitive macrophages associate with SE and developing germ-like cells (asterisk, **G**). (**H**) Large HLA-DR+ cells reside in the rete (white arrowhead), and smaller monocyte- (right black arrowhead) and lymphocyte-type cells (left) migrate through the rete channels (rch). (**I**) Association of activated tissue macrophages with the SE. (**J**) CD8 T cells in rete channels (arrowhead) and beneath the SE (**K**). (**L**) HLA-DR cells also associate with some primary follicles (pf). (**M**) Immunoglobulins (Ig-κ) have a low affinity to germ like cells but heavily bind to SE cells. Bar in **B** for **C**, **F**, **G**, **I**, **K**, and **M**.

Table 1
Working Model on Age-Associated Changes
of Specific Mesenchymal (SMC) and Hormonal Signals (LH/hCG and E2)
Required for the Initiation and Resumption of Oogenesis in Human Ovaries

Period of life	SMC[c]	LH/hCG[d]	E_2[e]	Oogenesis
First trimester–midpregnancy	yes	yes	yes	yes[f]
Last trimester–newborn	yes	**no**	yes	**no**[f]
Postnatal–menarche	yes	**no**	**no**	**no**[g]
Reproductive period[a]	yes	yes	yes	yes[f]
Premenopause[b]	**no**	yes	yes	**no**[g]
Postmenopause	**no**	yes	**no**	**no**[f]

[a]From menarche until 38 ± 2 yr of age.
[b]From 38 ± 2 untill menopause.
[c]Specialized mesenchymal cells (tissue macrophages and T cells) with commitment for stimulation of SE to germ cell transformation.
[d]Levels corresponding to the mid cycle LH peak, or more [hCG levels should be 10X more, because it has a 10% affinity to the LH receptor compared to that of LH *(8)*.
[e]Levels corresponding to the preovulatory E2 peak, or more.
[f]Confirmed.
[g]Predicted.

poorly defined nuclear envelope (black arrowhead), compared to cell in panel E. A lack of nuclear "membrane" is a characteristic feature for secondary oocytes undergoing the first meiotic division *(62)*.

These observations indicate that the oocyte- and granulose-type cells can differentiate in SE cultures. The association of granulosa (nurse) cells appears to contribute to advanced oocyte growth (panel D) while attached fibroblasts may induce the development of zona pellucida layer (panels C and E). Isolated large eggs show the characteristics of secondary oocytes after the first meiotic division, with polar body extrusion and surface ZP protein expression.

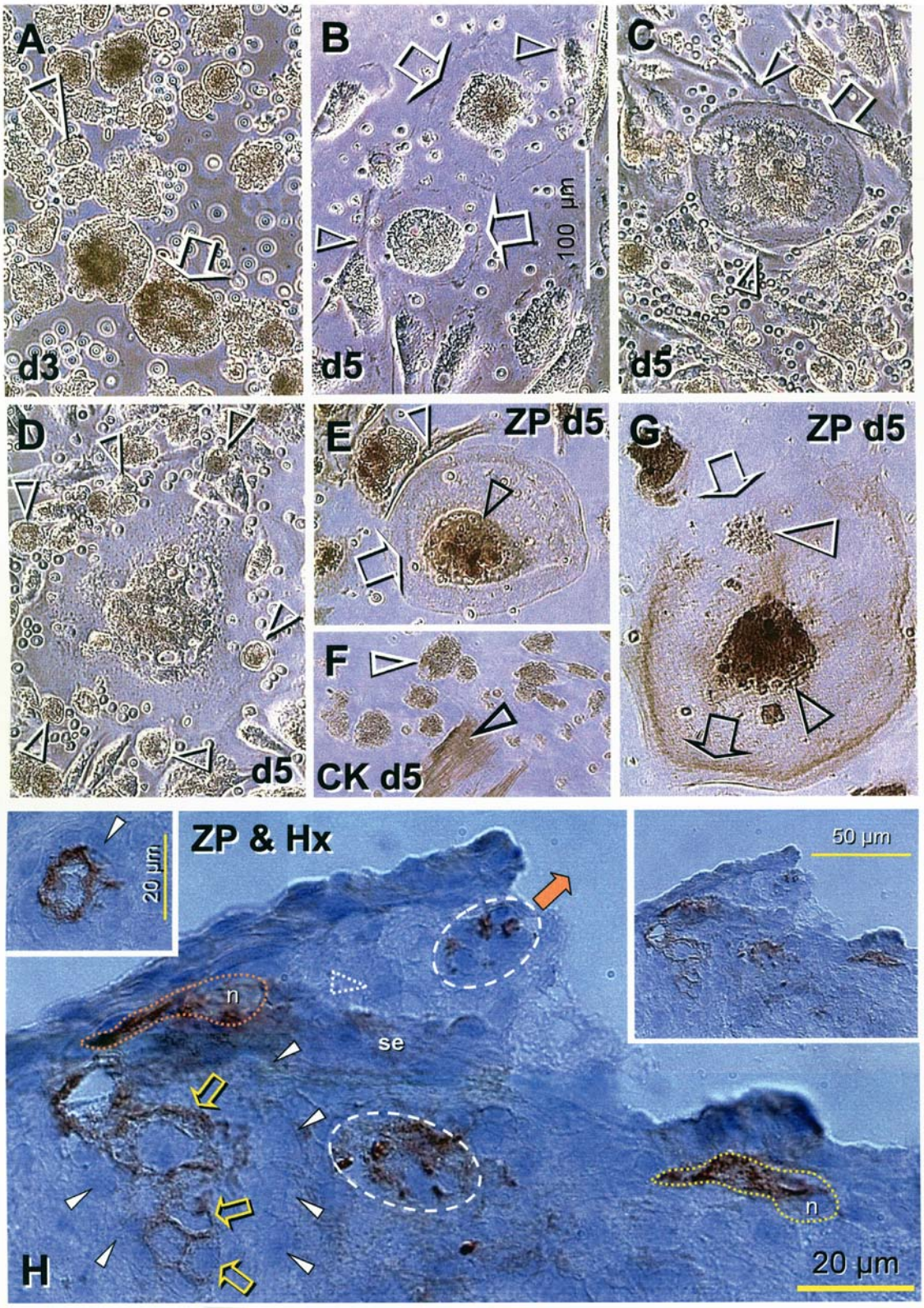
Rat Ovaries

Another interesting question is whether or not the oogenesis we observed in the ovaries of adult women can be seen in other mammalian species. Recent studies of follicular renewal in mammalian ovaries indicated that in postnatal mice the oocytes originate from large (approx 40 μm) ovoid cells, resembling germ cells of fetal mouse ovaries and expressing germ cell markers, in the surface epithelial layer covering the ovary. Histomorphometric studies at 30 d postpartum revealed the presence of 63 ± 8 such cells per ovary (n = 4 mice), a number close to that expected for a small pool of asymmetrically dividing germ cells *(44)*.

We have studied serial sections of prepubertal (d 35) and postpubertal rat ovaries, and were unable to detect such cells in the ovarian SE. There were, however, occasional degenerating oocytes leaving the ovary and described in the past in mouse ovaries (reviewed in ref. *5*), often accompanied by granulosa cells (solid arrow, Fig. 4H). This view also was recently expressed by others *(38)*.

It is important to note that rat ovaries show some distinct general features compared to human ovaries. In contrast to humans, the adult rat ovaries are permanently covered by SE cells. A distinct TA layer of loose connective tissue and the deep cortex of compact stromal cells are not apparent. The ovaries are significantly smaller (5–7 vs 35–40 mm), and follicular turnover is much frequent—selection of approx 10 follicles in both ovaries and accompanying follicular

Fig. 3. *(Opposite page)* Mixed culture of human surface epithelium and stromal cells (**A–F**), and follicular renewal in rat ovaries (**G**). (**A**) Day 3 culture consists of small (arrowhead) and large stem cells (arrow). In 5-d cultures (**B–F**) oocyte-type cells are present. The cells reach 60–80 μm size (arrows, panel **B**), and 100 μm (arrow, **C**), and are accompanied by fibroblast-type cells (arrowheads). Large oocytes develop in the presence of small (15 μm) rounded granulosa-type cells (arrowheads, panel **D**) forming follicle-like structures. Immunostaining for ZP proteins (brown color) shows no expression in a thick zona pellucida surface layer of the moderate size oocyte (arrow, panel **E**) exhibiting well-defined nuclear envelope (black arrowhead) and attached fibroblast cell (white arrowhead).Panel **F** shows CK 5 expression in differentiated fibroblasts (black arrowhead) and background staining (endogenous peroxidase) in the nuclei of other cells (white arrowhead). Large cell resembles secondary oocyte with ZP staining at the surface (black arrow, panel **G**), extrusion of polar body (white arrowhead) and poorly defined nuclear envelope (black arrowhead). Note a lack of ZP expression in surface segment (white arrowhead) adjacent to the putative polar body showing only non-specific expression of endogenous peroxidase (compare with strong ZP staining of the nucleus). (**H**) The rat ovary stained for ZP (brown) and hematoxylin (blue) shows two tadpole-like cells with ZP+ tails (dotted lines) and ZP unstained nuclei (n), two degenerating primary follicles (dashed lines) with possible expulsion (solid arrow), a cluster of putative germ cells (open arrows), and adjacent granulosa cells (arrowheads), possibly derived from the surface epithelium (se). The cells under the SE flap exhibit weaker staining of the nuclei (dotted arrowhead) compared to the SE cells. The right inset shows the same area without symbols, the left inset shows ZP expression in the normal primary follicle. Details in text.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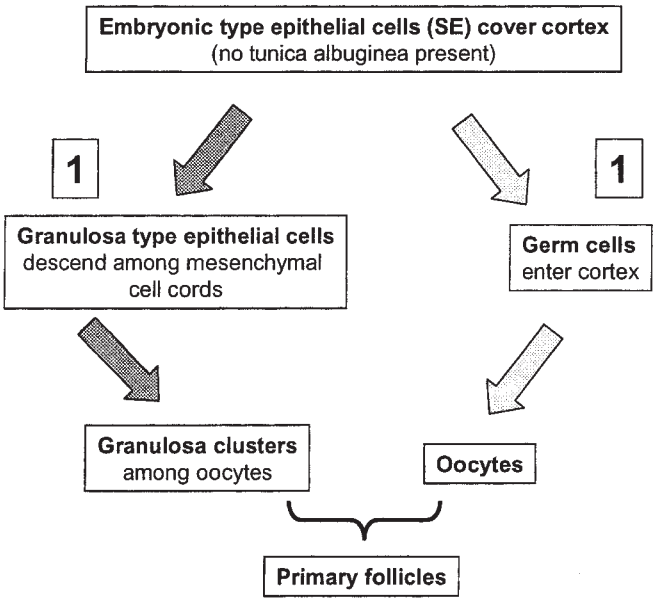

Fig. 4. Working model of possible pathways for formation of primary follicles in fetal human ovaries.

atresia occurs during a short (4–5 d) cycle. Unfortunately, the granulosa cells in adult rat ovaries do not express cytokeratins *(25)*.

Expression of ZP proteins in the ovary of postpubertal rat in the first estrus is shown in Fig. 3H. Dotted lines indicate two tadpole like cells with ZP+ tails and unstained nuclei (n). They resemble the tadpole-like germ cells found in adult human ovaries *(15)*. The left side tadpole cell appears to migrate toward the SE flap opening into the extraovarian space, and the right side tadpole cell migrates under the SE. The SE flap resembles a similar structure observed in human ovaries (see Fig. 1A), but in the rat ovary it covers an area containing cells with poorly stained nuclei (dotted arrowhead, Fig. 3H). A possibility is that these cells are derived from the SE and represent bipotent stem cells with a potential to differentiate either into the granulosa or germ cells.

The dashed lines indicate two degenerating small primary follicles with dispersed ZP fragments of oocytes, either leaving the ovary (solid arrow) or regressing under the ovarian surface. Note a normal primary follicle in the left inset with a single layer of granulosa cells (arrowhead) and surface ZP staining of the oocyte. Open arrows indicate a cluster of putative germ cells with surface ZP expression, arrowheads indicate adjacent cells, possibly descending from the SE like primitive granulosa cells in human fetal ovaries (see Fig. 2C). The right inset shows the same area without symbols.

These observations indicate that tadpole-like germ cells may develop in the adult rat ovaries. These cells may, but do not necessarily, originate from the SE. An alternative site is the ovarian hilar region, which contains sex cords expressing an intrinsic BMP system replete with bone morphogenetic protein (BMP) ligands and receptors *(32)*. Indeed, alternative pathways for germ cell origin were also found in adult human ovaries *(15)*. The tadpole putative germ cells expressing ZP migrate under the ovarian surface, and they may settle and give rise to the clusters of dividing germ cells. The rat SE cells may give rise to clusters of primitive granulosa cells available for the formation of primary follicles like in human fetuses.

Another possibility to be considered, and possibly more suitable for adult rat ovaries, is that the cells descending from a segment of the SE are initially uncommitted stem cells, some of which give rise to the proliferating germ cells (approx 10 μm in diameter, bottom open arrows, Fig. 3G), and other become primitive granulosa cells available for *in situ* follicular renewal (arrowheads). The proliferating germ cells either differentiate into oocytes (approx 20 μm in diameter, upper open arrow), or migrate under the SE to the neighboring regions of the ovary.

In adult rat ovaries, the primary follicles characteristically lie under the SE. Because the granulosa cells do not have a capacity to migrate, the migrating germ cells should reach a source of granulosa cells in order to form primary follicles. The occurrence of rat follicular renewal may also vary during the ovarian cycle. This may be associated with an elimination of older primary follicles by atresia, as suggested by others *(2,33)*. In contrast to adult human ovaries, the number of new primary rat follicles may be determined by the number of new germ cells and not epithelial cell nests, which do not develop in the rat ovaries. On the other hand, however, the oogenesis and formation of primary follicles in the adult rat ovary resembles a situation in human fetal ovaries. In both instances, the granulosa cell clusters are adjacent to the proliferating and differentiating germ cells, and the number of primary follicles is determined by a number of available oocytes.

Conclusions

Additional studies of postnatal oogenesis and follicular renewal are required in distinct species, as available data indicate that interspecies differences exist in the origin of germ cells, availability of granulosa cells, and formation of new primary follicles. In adult prosimian primates and possibly mice, the female germline stem cells persist and divide, and may contribute to the follicular renewal by association with granulosa cells derived from a yet unknown source. The adult rat ovaries show migrating tadpole-like germ cells of unknown origin under the SE. They may settle and proliferate, and recruit adjacent granulosa cells derived from the SE to form primary follicles.

The character and occurrence of follicular renewal during the fetal, postnatal, reproductive, and postreproductive periods may also vary among species. The transition of SE cells into germ cells in certain SE segments alternates with

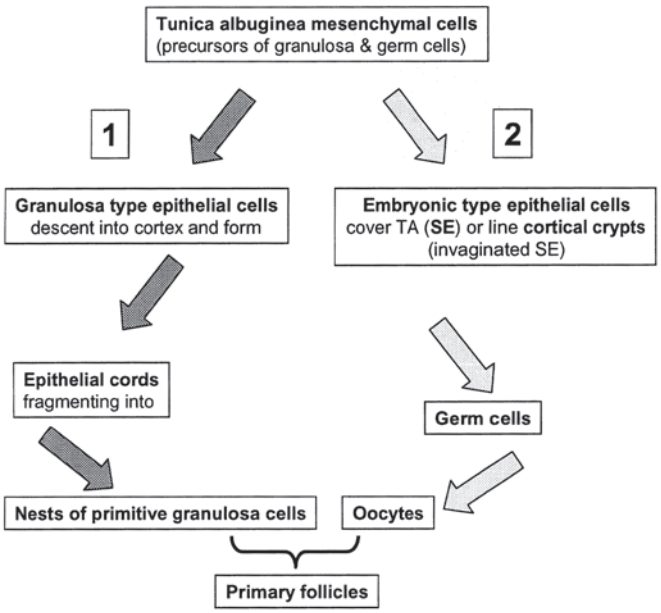

Fig. 5. Working model of possible pathways for formation of primary follicles in adult human ovaries.

the formation of primitive granulosa cell clusters in the same midpregnancy human fetal ovary (Fig. 4). The germ cells migrate to the adjacent cortex, develop into oocytes, which associate with available granulosa cells and form fetal primary follicles. Similar events were observed in adult rat ovaries. There is, however, a marked delay between the fetal and reproductive period in human females, associated with a significant diminution of follicle numbers. It is possible that follicular renewal may not be required until the menarche, or at least onset of puberty, are attained. The TA is formed during the perinatal period associated with a lack of development of new oocytes and follicles. We speculate that mesenchymal cells in TA originate from SE cells (epithelial–mesenchymal transition; see ref. *15* for details).

During the optimum reproductive period in human females, follicular renewal has been detected, but the process is more complex (Fig. 5) as compared to the formation of primary follicles in midpregnancy fetal ovaries. In ovulating ovaries, the SE differentiates in certain ovarian segments only. The differentiation of SE encompasses a mesenchymal–epithelial transition of TA cells back into the SE, formation of epithelial cords and fragmentation into nests, translocation of epithelial nests to deeper cortex, and their association with the vascular lumen. This is followed by formation of germ cells and their local and vascular transport, oocyte–nest assembly, and formation of the Balbiani body.

Finally, follicular renewal may diminish in aging ovaries (between ages 35 and 40), and preservation of ovarian function until menopause may depend on the number of persisting follicles. We suggested that the lack of follicular renewal in aging human ovaries may be accompanied by an accumulation of spontaneous and environmental aberrations of oocytes in these persisting primary follicles. Thus, a lack of follicular renewal may be a cause of increased incidence of fetal chromosomal aberrations, with age (reviewed in ref. *15*).

Altogether, the differences in follicular renewal between mammalian species may exist due to the differences in phylogeny (prosimians vs human females), size and structure of ovaries, length of the ovarian cycle and number of ovulations, the gap between the fetal and reproductive period of life, the reproductive life span, and a need for preservation of ovarian function long (approx 10 yr) after the termination of natural follicular renewal (human vs rat). The differentiation of primitive granulosa and germ cells from the bipotent mesenchymal cell precursors of TA in adult human ovaries appears to be a more sophisticated variation on a theme seen throughout most vertebrates.

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