

Morphological rearrangement of the cortical region, in aging ovaries

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Summary. The ovary is a structurally dynamic organ that alters with age. Modifications in the paracrine status influence the capacity of aging oocytes to develop normal embryos. Despite the importance of understanding the cellular and molecular mechanism involved in the process of ovarian aging, histological changes remain poorly understood. Correlating the process of folliculogenesis and somatic cell function during ovarian aging is essential to explain the reproductive decline of aged mammalian species, including humans.

Here, we performed a morphological and immunohistological study on the ovaries of chinchilla rabbits that varied in age from one to 34-months. The spatiotemporal expression of the cholesterol side-chain cleavage cytochrome P450_{scc} (CYP11A) and the smooth muscle actin (SMA) were analyzed.

A significant histological rearrangement of immunodetected cells in theca interna, theca externa and the interstitial tissue around the follicles occurred. The expression of CYP11A1 decreased considerably in antral follicles of aging ovaries. Moreover, we found that the secondary interstitial gland developed extensively, and a remarkable rearrangement of the surface epithelium occurred in aging ovaries. In contrast to ovaries during the reproductive period, the immunohistological changes

demonstrate that the interstitial gland became the most abundant tissue during the aging of ovaries. Thus, the current study provides new data for understanding the alteration of somatic cell function in elderly ovaries and how this affects their declined fertility.

Key words: Aging ovary, Interstitial gland, Theca cells, Cyp11a1, Smooth muscle actin

Introduction

The ovary is an endocrine organ that works as a biological clock in the context of female fertility (Tatone and Amicarelli, 2013). During reproductive life, follicle function depends on the balance of signals between the oocyte, granulosa, and theca interna cells. Functional theca interna cells are the limiting factor in terms of steroidogenesis in granulosa cells and consequently for oocyte maturation (McGee and Hsueh, 2000). Theca cells consist of two layers: the outer layer, known as the theca externa which has cytological features of smooth muscle cells. Likewise, the inner layer is known as the theca interna, consisting of steroidogenic cells that appear epithelioid in shape (Tajima et al., 2007). The theca interna cells persist in the atretic follicles and are incorporated into the interstitium (Erickson et al., 1985). Similarly, the interstitial gland cells originate from the theca interna of atretic follicles (Guraya and Greenwald, 1964).

Estradiol is the final product in the ovary's sex steroid synthesis pathway. However, the efficient

formation of androgens in the ovary requires a preliminary rate-limiting step: the conversion of cholesterol to pregnenolone. The mitochondria of the theca interna cells contain a CYP11A1, the first enzyme in the steroidogenic pathway. Therefore, the initial stage in follicular steroidogenesis takes place in the theca interna cells. However, theca cells remain the ovary's forgotten cells (Young and McNeilly, 2010).

Consequently, the role of the theca cells in aging ovaries of mammals, including humans has received limited attention. Pioneer studies about aging ovaries indicate that 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity decreases considerably in the theca interna cell, in the ovaries of aging rats (Young and McNeilly 2010). Immunolocalization and *in situ* hybridization of steroidogenic enzymes was analyzed in premenopausal human ovaries (Sasano et al., 1989; Tamura et al., 1992; Suzuki et al., 2011) and studies which applied real time-PCR and immunodetection demonstrated that the post-menopausal ovary retains the steroidogenic enzymes to synthesize androgens (Havelock et al., 2006; Laszcynska et al., 2008). A study of immunostained aging ovaries in the macaque showed that detection of androgenic enzymes is diminished in the theca interna layer, in ovaries with a lower number of follicles (Ethun et al., 2012). In the case of the mouse, oxidative damage provoked a significant age-related increase in theca interna cells (Lim and Luderer, 2011).

Reports on the morphological and physiological properties of both kinds of theca cells in the rabbit are still scarce. The rabbit (*Oryctolagus cuniculus*) has been proposed as an alternative model for studying early gonad differentiation (Diaz-Hernandez et al., 2008; Daniel-Carlier et al., 2013) and reproductive pathologies (Banco et al., 2016; Vigueras-Villasenor et al., 2016). The rabbit is the third most common experimental mammal model in the laboratory (Fischer et al., 2012). The phylogenetic distance between rabbit-primate and rodent-primate is the same, however rabbit gene sequences are more similar to those in humans because rodent sequences evolved more rapidly (Graur et al., 1996; Fischer et al., 2012). The rabbit has a life span of approximately seven years and provides a good animal model for studying the process of ovarian aging.

Furthermore, the acceptance of rabbits as pets has increased veterinarian interest in their pathologies (Banco et al., 2016). In the rabbit; meiosis initiates after birth. By the third postnatal week, the ovary forms primordial and primary follicles (Gondos, 1969); then antral follicles appear after the third month (Lee et al., 1996; Hutt et al., 2006). The female reaches fertility at four months of age. To our knowledge, the histological changes underlying the process of ovarian aging in the rabbit remain unknown.

In the current study, we conducted a comprehensive study of ovaries in order to investigate the histological changes of the cells around the follicles and the surface epithelium, during pre-pubertal, pubertal, reproductive and aging ovary. The immunolocalization of the

cholesterol side-chain cleavage cytochrome P450_{scc} (CYP11A1) and smooth muscle actin (SMA) marked the theca interna and theca externa, respectively. The expression of CYP11A1 decreased considerably in antral follicles of aging ovaries. Moreover, we found that the secondary interstitial gland developed extensively and a remarkable rearrangement of the surface epithelium occurred in aging ovaries.

Materials and methods

Animals

We used 23 female rabbits in total (*Oryctolagus cuniculus*), belonging to the European Chinchilla breed. The local Ethics Committee approved this study at the Facultad de Medicina, UNAM. The chinchilla rabbits were sacrificed using an overdose of sodium pentobarbital approved by the National Research Council "Guide for the care and use of Laboratory Animals." Ovaries of different ages were collected and classified into four age groups:

1. Prepubertal: 30 days of age (N=3)
2. Pubertal: 2 months of age (N=3)
3. Reproductive: non-mated estrous, four-months of age (N=5)
4. Mature: 12 to 34 months of age (N=12)

In rabbits, ovulation requires the mating stimulus. Thus, during the reproductive period they are in constant estrous condition. Ovaries in prepubertal and pubertal periods do not manifest gonadotropin dependent cycles (Dal Bosco et al., 2011).

The left ovary was removed and cut in half along a transverse plane and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis MO, USA) overnight and dehydrated with ethanol and embedded in paraffin. Serial tissue sections (7 μ m) from each sample were placed on separate slides for either histology or immunofluorescence.

Histological analysis

The histological study was carried out with Masson's trichrome stain, dehydrated with ethanol, cleared with xylene and mounted with Sub-X Mounting medium (Electron Microscopy Science, PA, USA). Images were collected using Nikon microscope model Eclipse Ni-U (Nikon, Japan). Photo stitching was undertaken manually in power point program, uniting 7 to 12 images.

The microscopic count was performed in five sections, separated by 200 μ m, using a light microscope (Zeiss Axio Scope) combined with digital camera and AxioVision software. We selected one field per section, per ovary, and used magnification x10 to count primordial and primary follicles. The magnification x5 was used to count larger follicles: early and late secondary, atretic and atretic follicle remnants. The images were acquired and imported into FIJI-ImageJ

software, using multi-point function to ensure each follicle was counted only once. The counts from each section were added together to obtain the number of follicles reported per ovary.

High-resolution microscopy

Thin transversal sections of ovaries fixed in Karnovsky solution, postfixed with 1% OsO_4 in

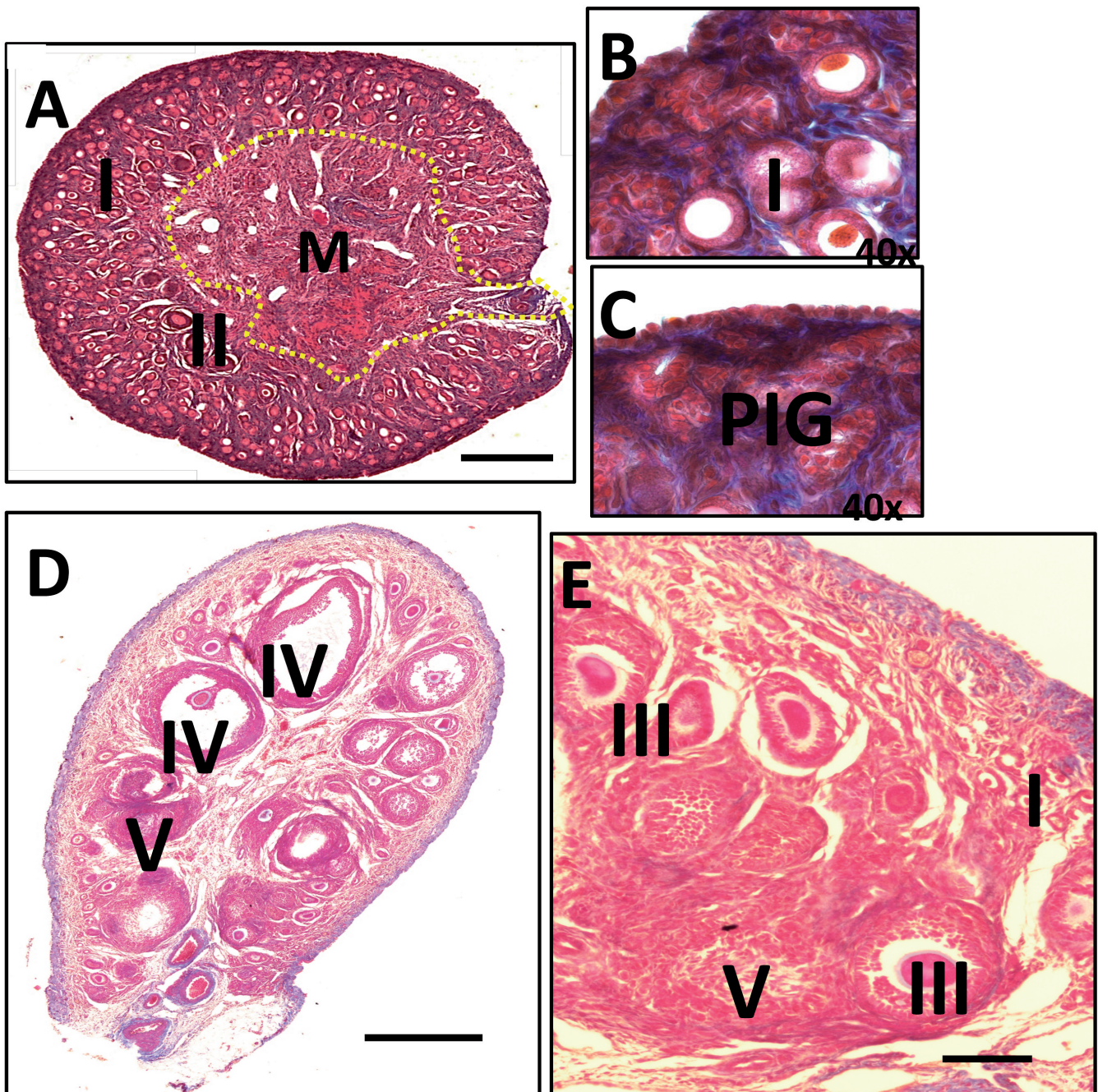


Fig. 1. Histology of the ovary at stages of pre-puberty and puberty, identified using Masson's Trichrome stain. **A.** Photo stitching of one-month-old ovary, showing the follicular reserve integrated by primordial (I) and primary follicles (II). Medullar region (M) delimited by yellow dots. **B.** Greater amplification of primordial follicles. **C.** Amplification of cells that form the primary interstitial gland (PIG). **D.** Photo stitching of 2-month-old rabbit ovary showing the presence of follicles at different stages; antral follicles (IV) and atretic follicles (V). **E.** Greater amplification of the primordial (I), early secondary follicles (III) and atretic follicles (V). Scale bars: A, D, 500 μm ; E, 100 μm .

Zetterqvist's buffer and dehydrated with ascending series of ethanol and embedded in Epon 812. Semithin sections ($1\ \mu\text{m}$ thick) were cut with ultramicrotome (Power Tome Microtomy XL, RMC), using a glass knife and then stained with toluidine blue.

Immunofluorescence

Paraffin was removed with xylol and dehydrated with ethanol. Tissue sections were washed in PBS (Phosphate buffered saline; Gibco by Life Technologies, Grand Island, NY, USA) three times, for 5 min each. Heat induced antigen retrieval was performed by placing the slides in Decloaker chamber (Biocare Medical, Concord, CA, USA) in 1X Diva Decloaker (Biocare Medical, Concord, CA, USA) or Tris buffer pH 10 for 15 min at 110°C . They were then transferred to PBS and

0.5% Triton X-100 in PBS in a sequenza slide rack to immunofluorescence (Ted Pella, Inc, CA, USA). The sections were incubated with 10% horse serum for two hours to block nonspecific binding to proteins. Slices were incubated with primary antibodies to goat anti-Cyp11a1 (Santa Cruz Biotechnology Inc., Dallas, TX, USA), (dilution 1:50) or mouse monoclonal anti-pan-cytokeratin (AE1/AE3+8/18, CM162B Biocare Medical, Concord CA, USA) (dilution 1:100), or mouse anti-smooth muscle actin (Cat. No. sc-58669, Biocare Medical, Concord CA, USA) (1:300) (PD900, Biocare Medical CA, USA), overnight at 4°C . Tissue sections were washed with PBS three times for 5 min. The secondary antibodies, Alexa Fluor 488 donkey anti-goat, Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 555 donkey anti-mouse were incubated (1:350) for 20 minutes at room temperature. Cell nuclei were

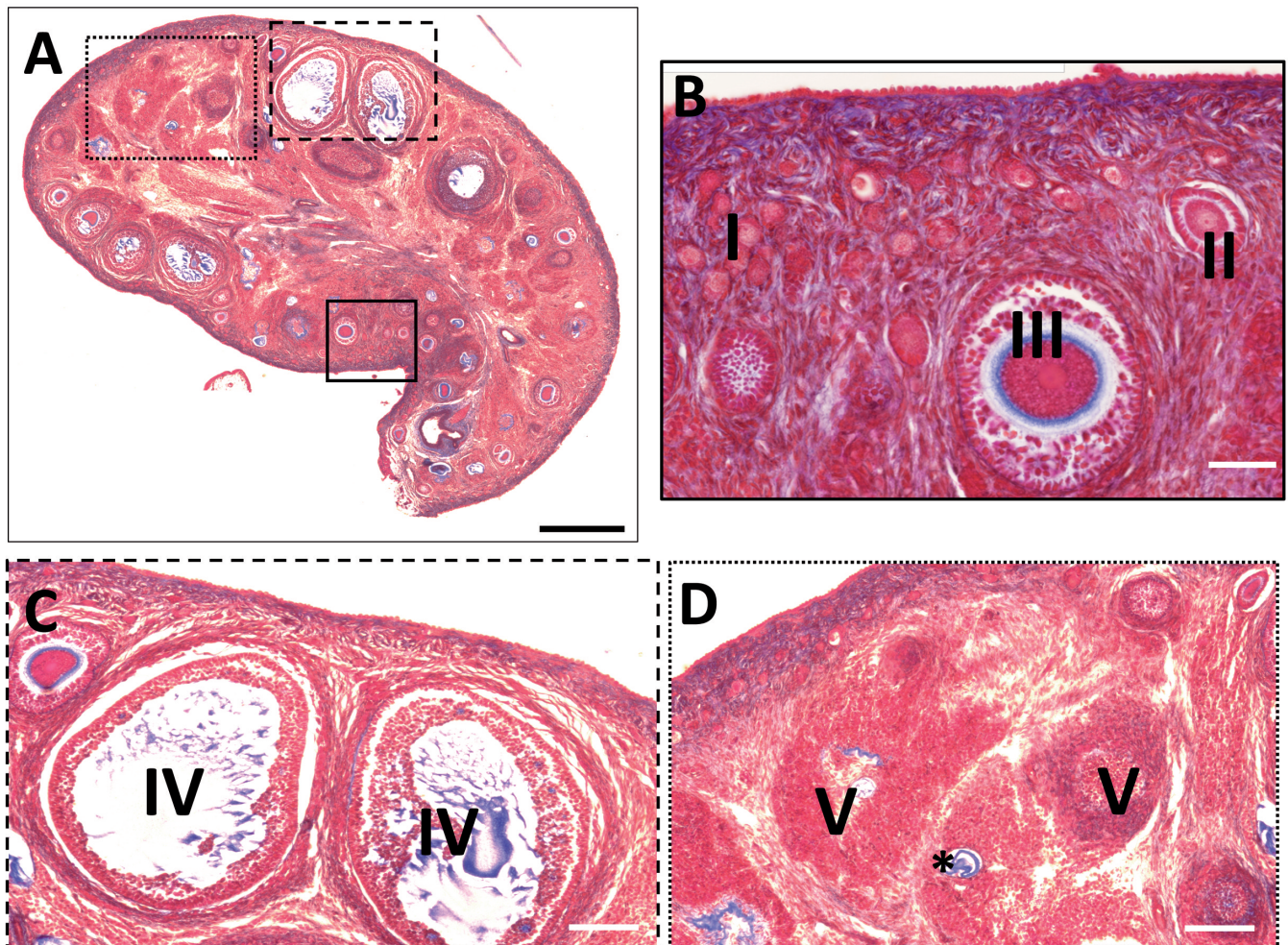


Fig. 2. Histology of ovary at the reproductive stage, identified using Masson's Trichrome stain. **A.** Photo stitching of 4-month-old rabbit ovary exhibits the presence of follicles at different stages of maturity. **B.** Greater amplification of the primordial (I), primary (II), the early secondary follicle (III). **C.** Greater amplification of the dotted area showing antral follicles (IV). **D.** Shows the presence of atretic follicles (V) and atretic follicle remnants (*). Scale bars: A, $500\ \mu\text{m}$; B-D, $100\ \mu\text{m}$.

counterstained with TOTO-3 Iodide (Cat. No. T3604, Life Technologies, NY, USA). Ovaries from 12-34 months manifest a significant amount of

autofluorescence, as reported for other species (Zimon et al., 2006). Thus, sections were incubated with 1X TrueBlack Lipofuscin (Biotium, Inc, CA, USA) to

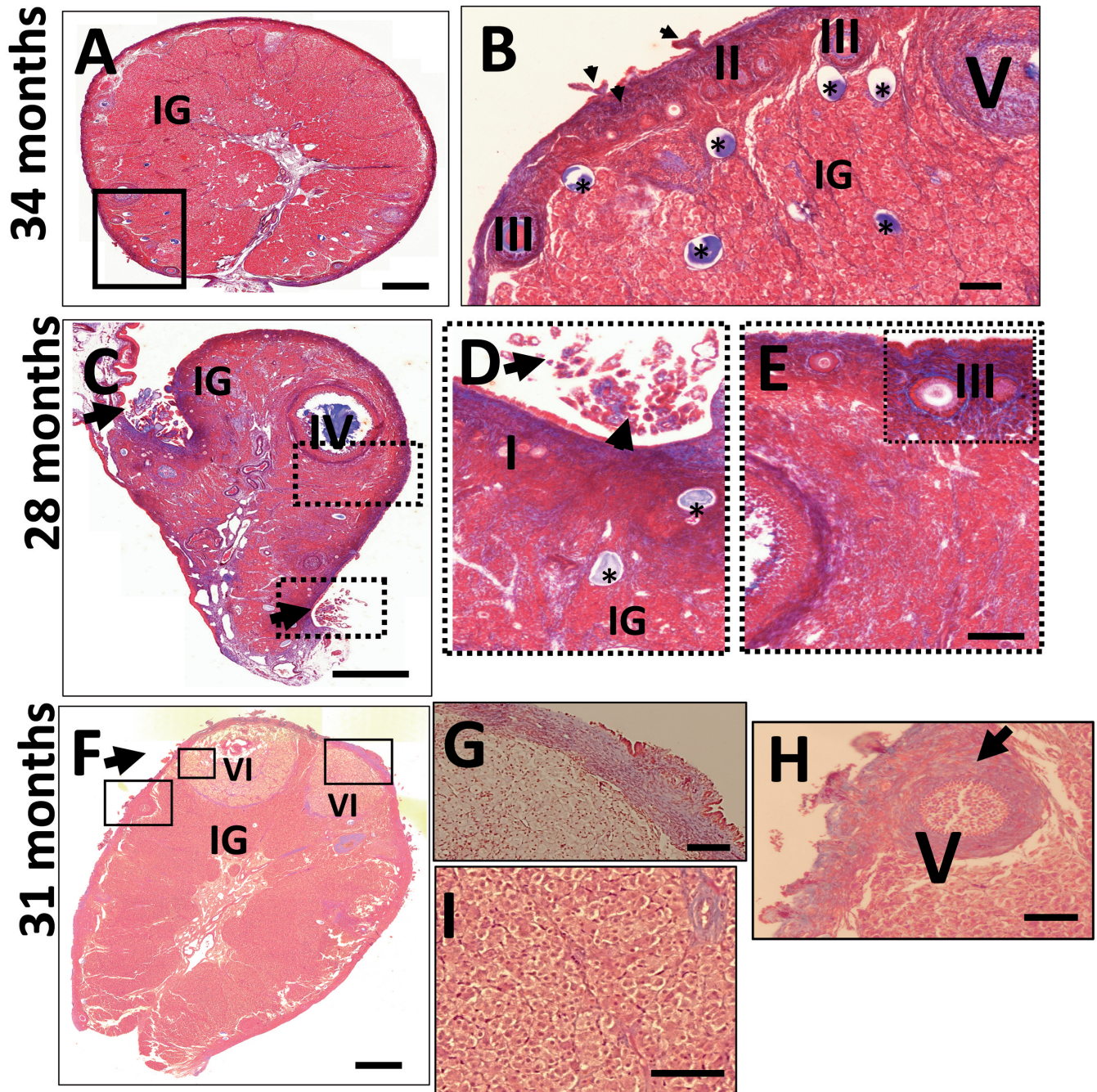


Fig. 3. Histology of aging ovaries identified using Masson's Trichrome stain. Visible increase in the size of the secondary interstitial gland and reduction in the number of follicles. **A, C and F** are representative sections showing the interstitial gland (IG). **B.** shows greater amplification of the marked areas in **A**, and still shows primary (II), early secondary (III) and atretic follicles (V) and abundant atretic follicle remnants (*). **D, E.** Amplification of dotted areas in **C** shows the presence of papillary growths of the ovarian surface epithelium (black arrow) and the presence of follicles (I, III). **G-I.** Amplification of three areas of **F**: corpus albicans (VI). **H.** Amplification of atretic follicle (V), presence of papillary growths (black arrow). **I)** Interstitial gland. Scale bars: A, C, F, 500 μ m; B, D, E, G, H, 100 μ m.

prevent this problem. Finally, tissue sections were mounted with Dako Fluorescence Mounting Medium (Cat. No. S302380, Dako, CA, USA). For immunofluorescence validation, sections of smooth muscle trachea guinea pig and myoid peritubular cells of sections of postnatal rabbit testis were used as positive controls to SMA. Leydig cells of postnatal rabbit testis acted as positive controls to CYP11A1. Negative controls were incubated exclusively with secondary antibodies.

We used a confocal microscope LSM5 Pascal, Zeiss to acquire images and imported these into FIJI-ImageJ software to evaluate immunofluorescence intensity. Representative areas were selected. At least three uniform areas per antral follicle were analyzed for SMA; a total of 11 follicles from 4 different ovaries from 4-month-old and 11 follicles from 12 mature ovaries, and 100 cells selected to show Cyp11a1 immunofluorescence intensity, from 4-month-old (N=4) and mature ovaries (N=12). For display purposes, merged multichannel images were constructed: CYP11A1 (green), SMA (red) cytokeratin (green) and TOTO-3 stained nuclei cells (blue).

Total RNA extraction and RT-PCR

The right ovary was removed and cut in half along a transverse plane, placed in cold RNase-free PBS, then cut into small fragments and stored in RNA later in stabilization solution (Thermo Fisher Scientific, USA), until use. In this section, we only used ovaries from rabbits at the reproductive and mature stage. Total RNA was purified using the RNeasy mini kit (Qiagen, USA) and to prevent DNA contamination, DNase Set digestion (Qiagen) was employed and eluted with RNase-free water. Concentration of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Barrington, IL, USA). Evaluation of RNA purity was by reference to absorbance ratios at 260:230 nm and 260:280 nm. A 1.8 ratio was deemed suitable to proceed with cDNA synthesis. Total RNA was reverse-transcribed to cDNA using transcriptase reverse transcriptase (Roche, Life Science, USA), random primer (Invitrogen, Life Technologies, USA) and 800-1000 ng of RNA. The oligonucleotides were designed according to the sequences reported in the NCBI database. PCR was conducted with the following primers for rabbit: Cyp11a1 Fw: 5'-CTG GCT GCA ATG AGG TCA-3', Cyp11 Rv: 5'-CTG GCT GCA ATG AGG TCA-TGA AGG TGA AGG TCG GTG TGA ACG-3', Hprt1 Fw: 5'-TCT GTG GAT TTG ATC CTG C-3', Hprt Rv: 5'-ATC TGC GTC ATC ATT TCC C-3'. PCR reaction was undertaken using My Taq DNA Polymerase (Bioline, United Kingdom).

Negative controls were prepared without template or RNA total. The amplification conditions were 5 min at 95°C, followed by 1 min at 95°C, 1 min at 56°C and 1 min at 72°C (29 cycles), with a final extension of 10 min. The reactions were carried out in duplicate. PCR

products were electrophoresed in 1% agarose gel with GelRed (Biotium, USA). The PCR products corresponded to the estimated length. Gels were photographed with Mini HD6 (Uvitec, Cambridge), and amplicon identity was validated by sequencing. Optical density was calculated using ImageJ Software.

Data analysis

We determined statistical differences between groups by applying an unpaired Student's t-test when only two groups were compared. When more than two groups were compared, we carried out a one-way analysis of variance, followed by Tukey-Kramer Multiple Comparison test, using the GraphPad InStat Software Vers 3.05. Data were expressed as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$ bimarginally.

Results

Histological changes in aged ovaries

This histological study initiated at the prepubertal stage. Ovary from one-month-old rabbit consists of cortex and medulla. The cortex contained: [I] primordial follicles (oocytes surrounded by a single layer of flattened granulosa cells) and [II] primary follicles (oocytes surrounded by cuboidal granulosa cells), located near the medullary region (Fig. 1A,B). Primary

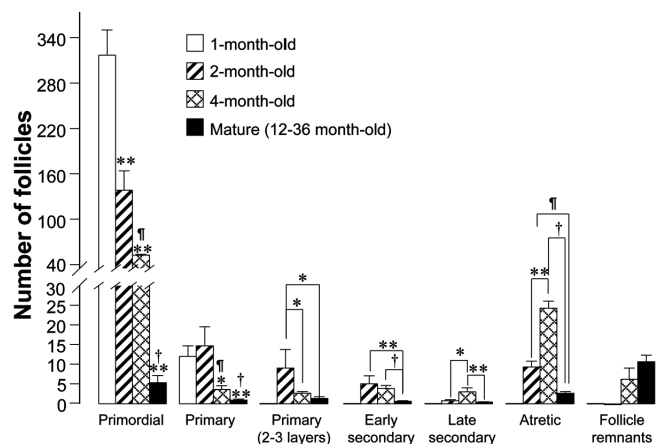


Fig. 4. Histogram of the number of primordial, primary, early and late secondary, atretic and atretic remnants at different ages: 1 month (n=3), 2 months (n=3), 4 months (n=5) and mature (n=12). Each box represents the mean of value in each group. Data were expressed as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$ bimarginally. In the primordial and primary groups, * $p < 0.05$, ** $p < 0.01$ when compared vs 1-month-old group; † $p < 0.01$ when compared 4-month-old group vs 2-month-old group. In the primordial group, ‡ $p < 0.01$ when compared mature group vs 2-4-month-old groups and in the primary group only with the 2-month-old group. In the remaining groups, * $p < 0.05$, ** $p < 0.01$, † $p < 0.01$, ‡ $p < 0.01$.

interstitial gland was located below the surface epithelium in the cortex (Fig. 1C). At this age, atretic follicles were still absent. Subsequently, prepubertal ovaries in two and four-month-old rabbits (reproductive age) developed: [III] early secondary follicles (oocyte surrounded by more than one layer of cuboidal granulosa cells, without theca interna cells) and, [IV] antral follicles (presence of antrum and the theca cell layer). Moreover, [V] atretic follicles, and their remnants were visible (Fig. 1D-E, 2A-D).

The mature females used in this study were multiparous and initially showed a decrease in fertility, after which they rejected copula. The histological analysis of ovaries from 12 to 32-month-old chinchilla rabbits revealed dramatic changes in their tissue rearrangement. To compile a general view of entire mature ovaries, we made photo stitchings of representative ovaries. Among the most salient features was the presence of a hypertrophic interstitial gland (Fig. 3A,C,F). Even though there was an evident gross

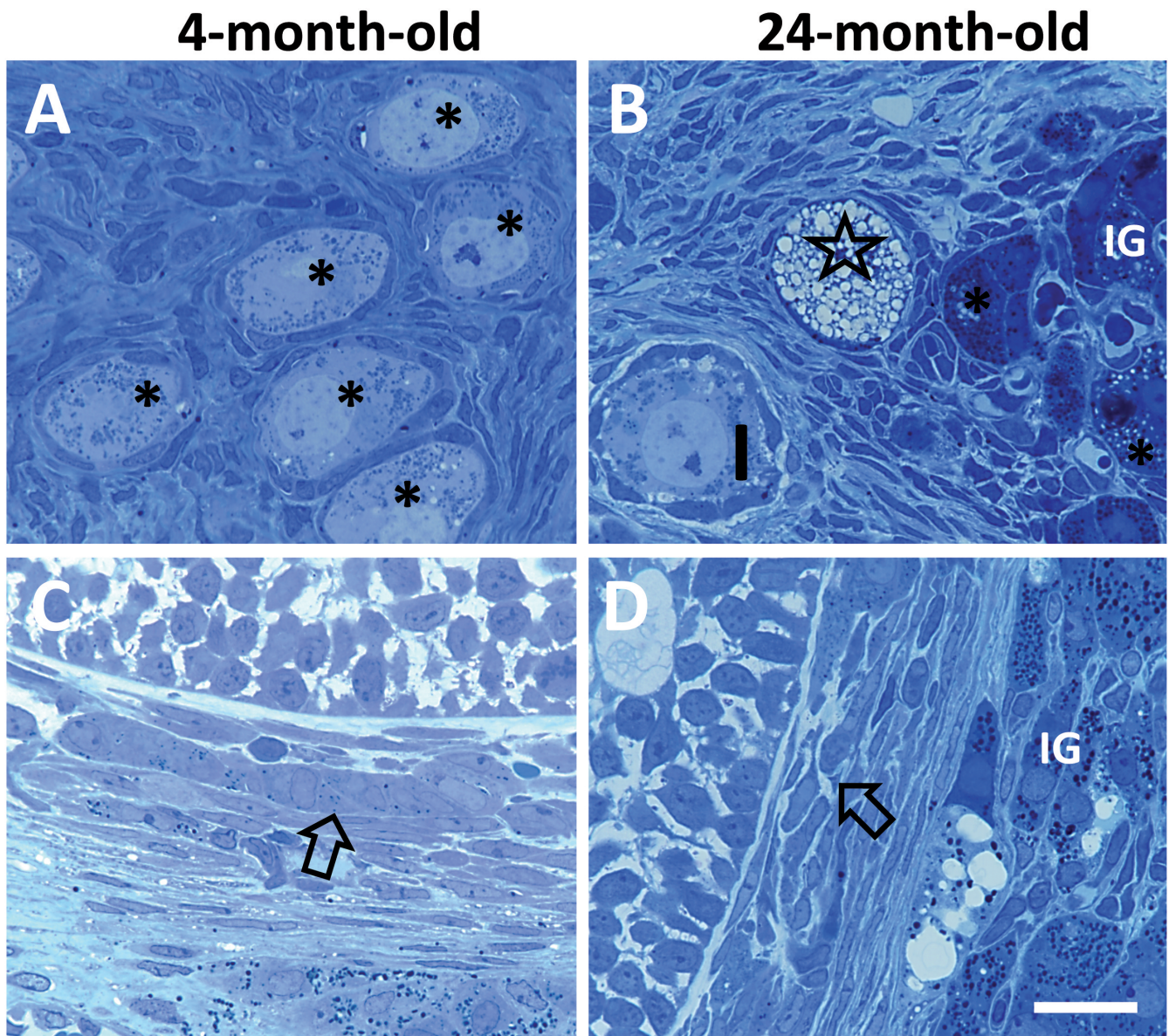


Fig. 5. Representative semi-thin sections of ovaries at reproductive and mature stage. **A.** Shows a cluster of primordial follicles (*) of four-month-old rabbits. **B.** The mature ovaries show isolated primordial follicles (I) some of them have abundant vacuoles as oocyte remnants (STAR). Part of the interstitial gland (IG). **C, D.** Show theca interna cells (empty arrow) of antral follicles of reproductive and mature ovaries respectively. IG: interstitial gland. Scale bar: A-D, 20 μm.

reduction in the number of mature follicles; all mature rabbit ovaries still contained primordial and primary follicles (Fig. 3B,D,E), as well as scarce antral follicles (Fig. 3C). Numerous atretic follicle remnants and some corpus albicans were evident in analyzed ovaries (Fig.

3B,D,G,H).

Comparisons of follicle counts are presented in figure 4. The primordial follicles decreased significantly with age: one-month-old vs two-months, one-month-old vs 4-month-old and one-month vs mature (** $p < 0.01$);

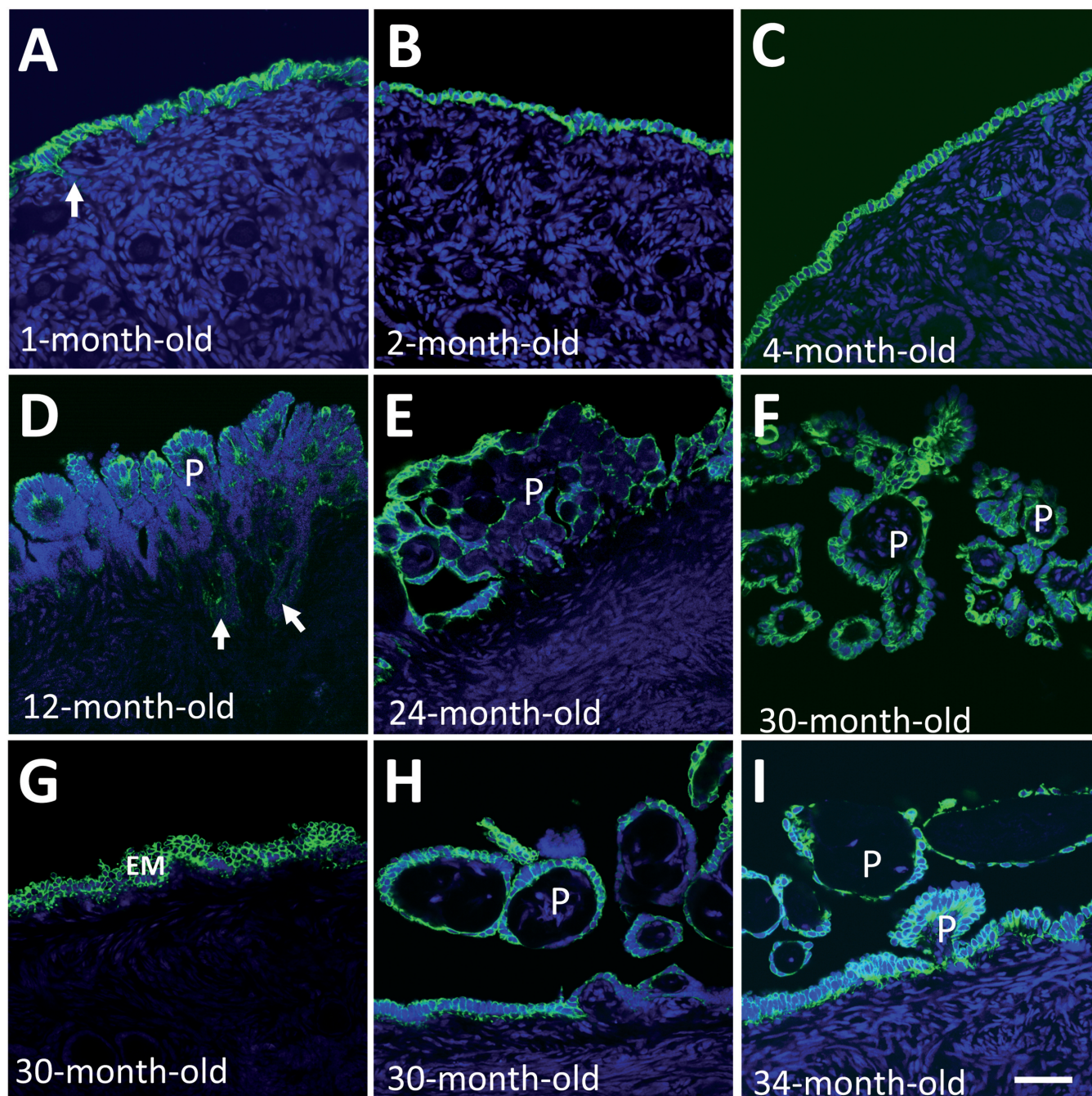


Fig. 6. Immunodetection of cytokeratin (green) in the ovarian surface epithelium (OSE) of rabbit at different ages. **A-C.** Prepubertal (1 month, **A**) pubertal (2 months, **B**) and reproductive age (4 months, **C**) respectively, showing normal morphology with a single layer of ovarian surface epithelium. **D-I.** Hyperplastic OSE as observed in aging ovaries. Papillary (P) growths on the surface epithelium, deep surface invaginations (arrow) and a multilayer epithelium (EM) are visible. Cell nuclei stained with TOTO-3 iodide (blue). Scale bar: 50 μm .

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two-month vs four-month and two-month vs mature ($\dagger$ $p < 0.01$); four-month vs mature ($\P$ $p < 0.01$).

The youngest group (one-month-old), have

significantly more primary follicles than the ovaries from four-month-old ($*p < 0.05$) and from the mature ovaries group ($**p < 0.01$). Similarly, two-month-olds

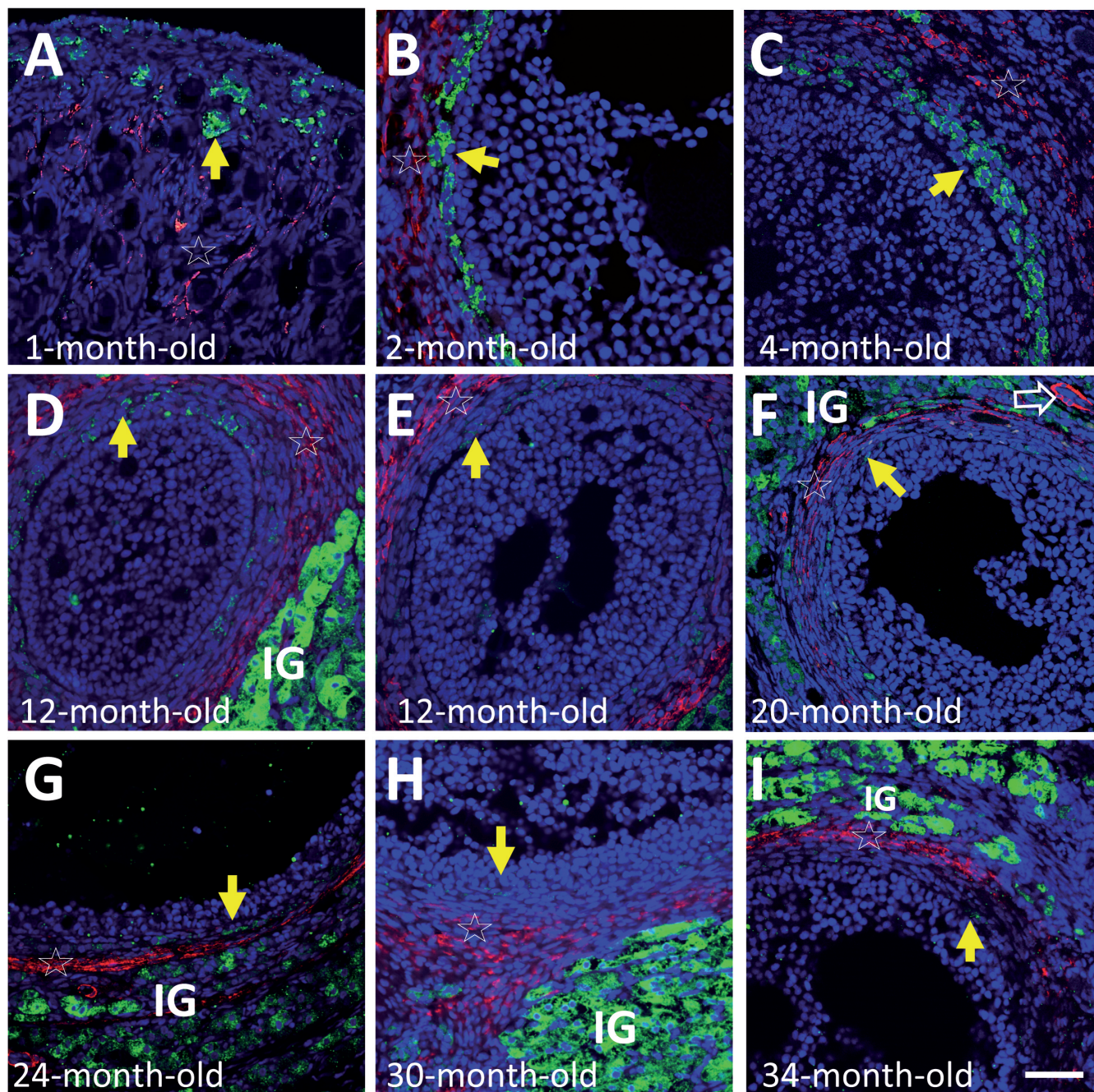


Fig. 7. Immunodetection of Cyp11a1 (green) and smooth muscle actin SMA (red) in chinchilla rabbit ovaries, at different ages. **A.** Prepubertal ovary (one month) showing Cyp11a1 in scattered steroidogenic cells (yellow arrow) in the ovarian cortex. There are cells positive to SMA around the primary follicles (STAR). **B, C.** Represent ovaries at pubertal (2 months) and reproductive age (4 months), respectively. Antral follicles show Cyp11a1 (green) and SMA (red) positive cells in the theca interna and theca externa, respectively. **D-I.** Show ovaries from 12 to 34-month-old chinchilla rabbits. In the theca interna, the number of cells expressing Cyp11a1 and their intensity decreases (yellow arrow), whereas these two features increase in the interstitial gland (IG). The cell nuclei stained with TOTO-3 iodide (blue). Scale bar: 50 μ m.

have significantly more primary follicles than four-month-old and mature groups ($\S$ and $\dagger$, respectively $p < 0.01$).

The one-month-old ovaries only present primordial and primary follicles, thus, this group was removed from the comparative statistical analysis of primary follicles consisting of 2-3 layers, secondary follicles, atretic follicles and atretic remains. A significantly reduced number of early secondary follicles comparing 2-month-old vs mature ($**p < 0.01$); and four-month-old vs. mature ($\dagger p < 0.01$). A significantly smaller number of primary follicles of 2-3 layers was observed when a comparison was made between 2-month-old vs. 4-month-old and vs. mature ovaries ($*p < 0.05$). While a significantly increased number of late secondary follicles was observed when a comparison was made between 2-month-old vs 4-month-old ($*p < 0.05$) and a reduction when a comparison was made between 4-month-old vs

mature ($**p < 0.01$).

A significantly higher number of atretic follicles at 4-month-old was observed when compared vs 2-month-old ($**p < 0.01$) and mature ($\dagger$, $p < 0.01$). We also found statistical differences when a comparison was made between 2-month-old vs. mature ($\S p < 0.01$). A comparison was only made for the count of atretic follicle remnants between 4-month-old vs. mature, but there were no apparent significant differences.

Examination of semi-thin sections from four-month-old rabbits stained with toluidine blue showed clusters of primordial follicles and an oocyte with abundant organelles throughout the cytoplasm, surrounded by a layer of squamous granulosa cells. The mature ovaries displayed isolated primordial follicles with a large number of vacuoles between flattened granulosa cells and the oocyte and the atretic follicle remnants also presented abundant vacuoles (compare Fig. 5A vs 5B).

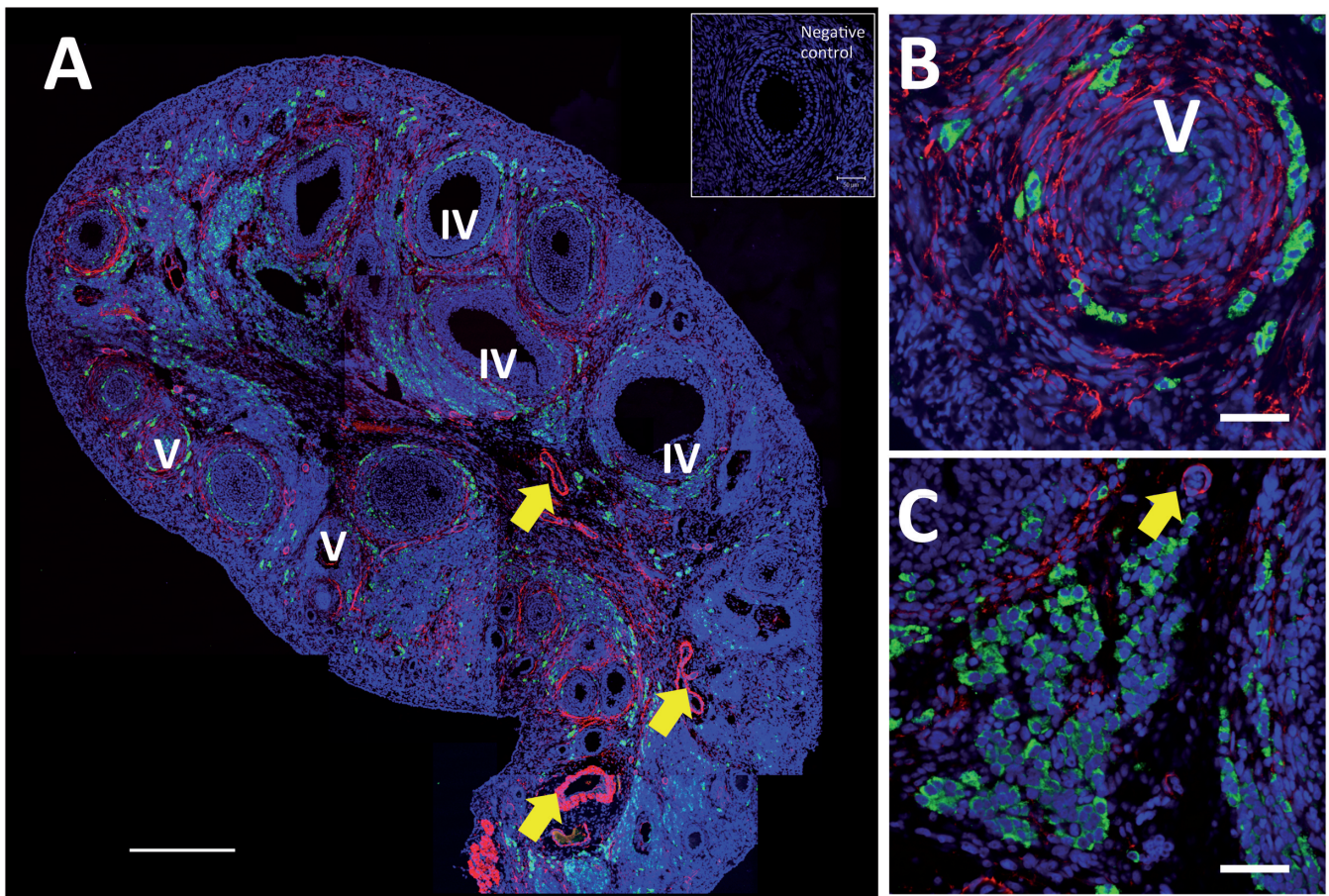


Fig. 8. Immunolocalization of Cyp11a1 and SMA in a four-month-old ovary, during the reproductive stage. **A.** Photo stitching of an entire ovary section, taken at low magnification. The distribution of Cyp11a1 (green) and SMA (red), around follicles at different stages, are shown. Antral follicles (IV) and atretic follicles (V), sectioned along diverse planes are visible. The SMA reveals the tunica media of blood vessels in the medullary area (yellow arrow). The inset in panel A corresponds to a representative negative control without primary antibodies. **B.** This is a magnification of an atretic follicle (V) with cells positive to Cyp11a1 (green) and SMA (red). **C.** The higher resolution of cells of the interstitial gland show Cyp11a1 staining in the cytoplasm. SMA is seen in the tunica media of blood vessels, located between the secondary interstitial gland and the follicles (yellow arrow). Scale bar: 50 μ m.

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The antral follicle has a well preserved membrana granulosa and theca interna cells show a typical epithelioid shape. In contrast, theca interna cells of antral follicles in mature ovaries exhibit a scattered rearrangement more like fibroblast cells. Moreover, the interstitial gland presents a large number of vacuoles (compare Fig. 5C vs 5D).

Evidence of ovarian superficial epithelium (OSE) hyperplasia in mature ovaries

The formation of exuberant epithelial evaginations on OSE was a propensity among all mature chinchilla rabbits (Fig 3). We used immunodetection of the intermedia filament, known as cytokeratin to corroborate the epithelial identity of these structures. In ovaries between one and 4-month-old, OSE was formed by monostratified cuboid epithelial cells with few invaginations (Fig 6A-C). However, in the ovaries of mature chinchilla rabbits of 12-33-month-old, the OSE

manifested papillary growths formed from a simple epithelium, deep surface invaginations and epithelial multilayering (Fig 6D-I).

Expression of CYP11A1 and SMA

Immunodetection of CYP11A1 revealed the primary interstitial gland in the cortex region of the one-month-old ovaries (Fig. 7A). Similarly, in two and four-month-old ovaries, there was evident CYP11A1 staining in antral and atretic follicles and formation of the secondary interstitial gland (Figs. 7B,C, 8A-C). Contrastingly, in the ovaries of 12-33-month-old chinchilla rabbits, there was an evident decrease in intensity in antral follicles (Fig. 7D-I) and abundant immunostaining of Cyp11a1 was evident in the interstitial gland (Fig. 9A-F). We analyzed semiquantitative immunofluorescence laser confocal microscopy. Results showed that Cyp11a1 detection significantly decreased in theca interna cells of antral

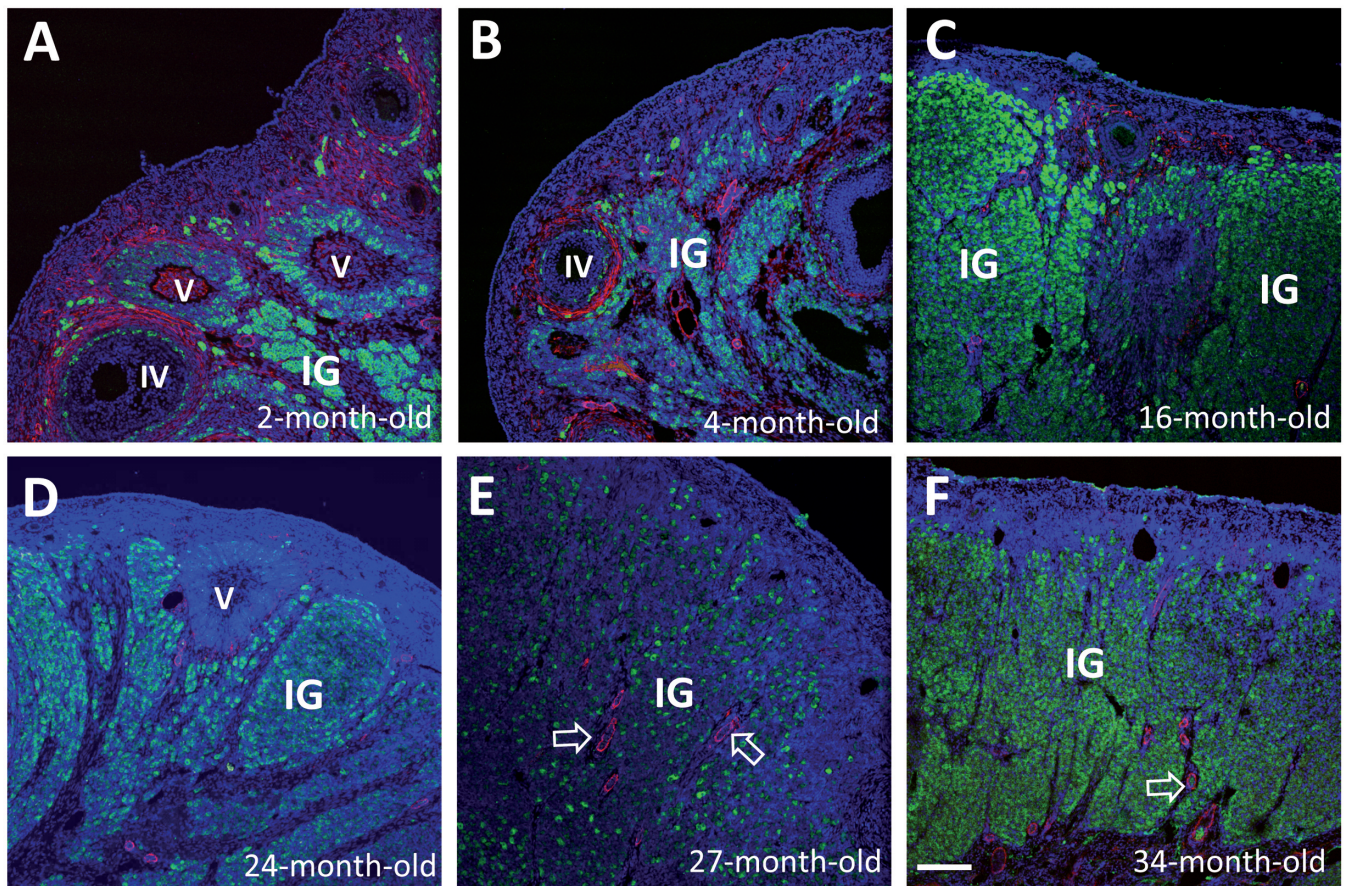


Fig. 9. Images of representative ovaries show the increased area occupied by the interstitial gland (IG) with advancing age among chinchilla rabbits. **A-B.** Pubertal (2-month-old) and reproductive age (4-month-old) ovaries express Cyp11a1 (green) in antral (IV) and atretic follicles (V) and the forming secondary interstitial gland (IG). **C-F.** The IG covers much of the ovarian cortex, indicated by the display of abundant immunostaining of Cyp11a1. SMA (red) expression is evident in large blood vessels (arrows) between the IG and around any remaining atretic follicles. Scale bar: 50 μ m.

follicle from mature ovaries (** $p < 0.01$), whereas detection significantly increased in cells of the interstitial gland from mature ovary (Fig. 10A) (* $p < 0.05$). The levels of *Cyp11a1* mRNA determined by RT-PCR showed that the expression increased in mature ovaries of 24-month-old chinchilla rabbits (Fig 11).

Immunostaining caused by SMA was evident around the primary follicles of one-month-old ovaries (Fig. 7A). From 2 to 4-month-old, SMA immunostaining was evident in cells located in the theca externa of primary, early secondary follicles and antral follicles, indicating that these cells acquired characteristics of smooth muscle cells when follicular maturation initiated (Fig. 7B,C). Likewise, SMA was immunodetected in the tunica media of blood vessels (Fig. 8A). Atretic follicles and the tunica media of blood vessels located in the interstitial gland were also SMA positive (Fig. 8A-C). SMA positive cells were immunodetected in the tunica media of blood vessels (Fig. 9C-F). We analyzed semiquantitative immunofluorescence particularly from the theca externa layer of antral follicles from 4-month-old and mature ovaries, finding no significant differences (Fig. 10B).

Discussion

In the current study, we show that both primordial and secondary follicles declined significantly with age, similar to that reported for other models such as

macaque and vervet monkey ovary (Nichols et al., 2005, Atkins et al., 2014), whereas atretic follicles were abundant during the reproductive stage.

Steroidogenic tissue was identified by the presence of CYP11A1, an enzyme necessary for the initiation of steroidogenesis. Results indicate that the interstitial gland occupies much of the ovarian cortex in 12-34-month-old chinchilla rabbits. In contrast, the cells of the theca interna of antral follicles decreased the expression of CYP11A1 in mature ovaries, which correlates with the loss of the epithelioid shape of the theca interna cells from ovaries of mature rabbits.

The postmenopausal ovary is hormonally active, retains capacity to express steroidogenic enzymes (Havelock et al., 2006; Laszcynska et al., 2008) and produce steroid like testosterone, androstenedione, and estradiol (Fogle et al., 2007; Brodowski et al., 2012). Similarly, in the aging atrophic ovary of women, although some estrogen-producing follicles remain, the secondary interstitial gland produces higher levels of androgens (Erickson et al., 1985; Adashi, 1994). Thus, the interstitial gland is the most important steroidogenic tissue in both humans and rabbits (Mossman et al., 1964). The interstitial gland derives from the theca interna of atretic follicles (Guraya and Greenwald, 1964). The interstitial gland, which is abundant in mated females, reveals itself in early ovaries of young rabbits.

The so-called "interstitial gland" was first described in the human ovary in 1902 (Limon, 1902). However,

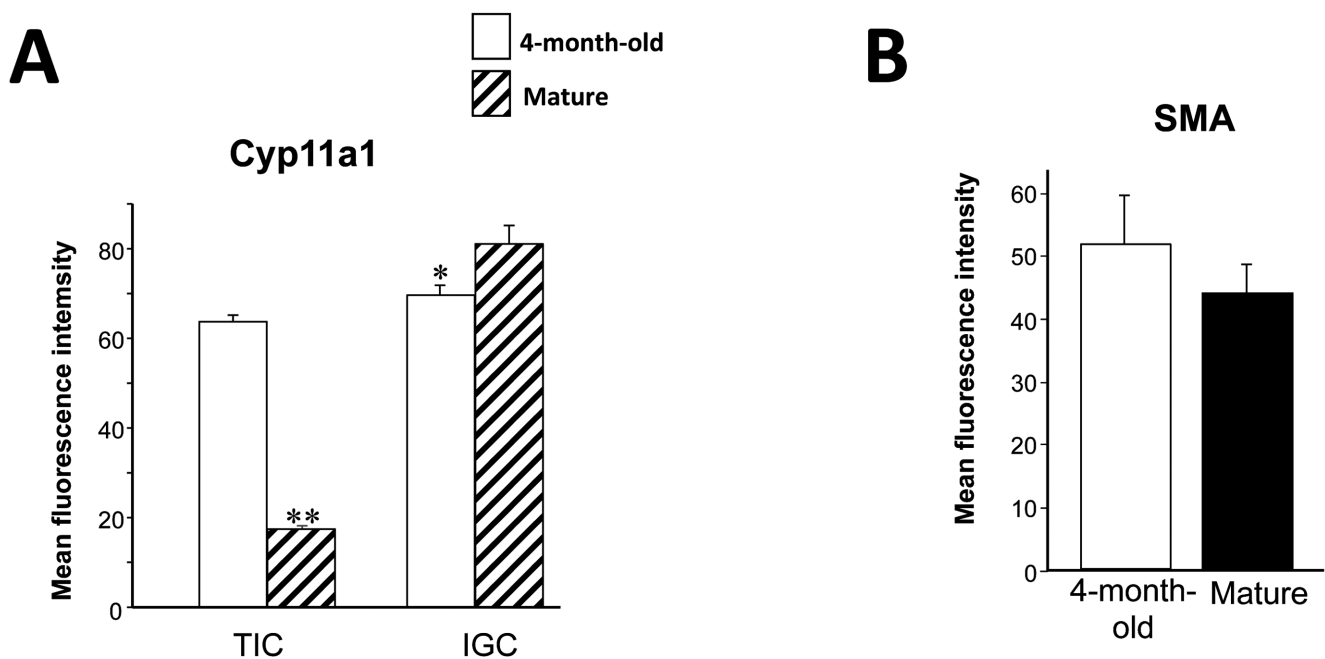


Fig. 10. Immunofluorescence quantification of Cyp11a1 and SMA intensity of antral follicles in ovaries of reproductive and mature chinchilla rabbits. Cyp11a1 intensity was quantified in cells of the theca interna (TIC) and in the interstitial gland cells (IGC). SMA detection was quantified in three different areas of 11 antral follicles. Fluorescence intensity data was shown as mean $\pm$ ES. (* $p < 0.05$, ** $p < 0.01$).

there are only limited studies describing the role played by the tissue that forms the interstitial gland in aging ovaries. The reason may be due to the ambiguous identification of the interstitial gland, using conventional stains in classic mammalian models: the rat and the mouse (Mossman et al., 1964). The hypertrophic interstitial gland is linked to the aging ovary. As a highly steroidogenic tissue, it becomes a source of reactive oxygen species, possibly affecting follicular viability (Ramalho-Santos and Amaral, 2013).

To identify molecular changes during ovarian aging, some authors made genome-wide gene expression analysis, using microarrays (Wood and Rajkovic, 2013; Wei et al., 2015). However, although they reported that the expression of Cyp11a1 is upregulated in aged ovaries of rhesus monkey, immunodetection of the tissue expressing the enzyme was not undertaken (Wei et al., 2015). In the human post-menopausal ovary, mRNA and protein of Cyp11a and other steroidogenic enzymes were detected (Jabara et al., 2003; Havelock et al., 2006). In the present work, we performed both RT-PCR and immunofluorescent of Cyp11a1 in the aging ovaries of rabbit. This result is consistent with some studies in human postmenopausal ovaries (Havelock et al., 2006). And as in the rhesus monkey, the mRNA of the enzyme is upregulated in mature rabbit ovaries. Complementarily, the current results show that the immunofluorescence has modifications concerning both the intensity and distribution of Cyp11a1 among follicles and the developing interstitial gland. The stroma tissue conformed by the secondary interstitial cells retains steroidogenic capacity.

The ovarian surface epithelium (OSE) of the chinchilla rabbit showed important architectural changes, dependent on age. Aging rabbit OSE underwent exuberant villous epithelial processes, similar to those observed in human and monkey ovaries (Wei et al., 2015). In humans, the origin of ovarian cancer seems to be heterogeneous. Recent results suggested that the most common type of ovarian cancer derives from the fallopian tube (Kroeger and Drapkin, 2017). However, it is possible that surface epithelium may contribute to other types of ovarian cancer, complying with the “incessant ovulation” hypothesis (Fathalla, 2013). The evident formation of conspicuous papillae in aging ovaries identified in the current study corroborates the potential for OSE hyperplasia.

In postmenopausal ovary, the localization and expression of androgen receptor, progesterone receptor, and estrogen receptor- α were shown in surface epithelium (Brodowska et al., 2010). Steroid hormones and their receptor play a role in carcinogenesis from ovarian surface epithelium (Lau et al., 1999), it would be interesting to analyze immunolocalization of steroid hormone receptor in mature rabbit ovaries.

The smooth muscle functions as a structural framework that provides mechanical support to the mammalian ovary (Madekurozwa, 2007; Madekurozwa et al., 2010). In the present study, SMA appeared in

primary follicles prior to the differentiation of steroidogenic cells in the theca interna. As shown here, the expression of SMA in the theca externa is evident in reptiles, birds and other mammals (Van Nassauw and Callebaut, 1991; Van Nassauw et al., 1991; Maretová and Maretta, 2002; Madekurozwa, 2007; Madekurozwa et al., 2010). The SMA positive cells of the theca interna form a dynamic tissue and adjust their arrangement for several ovarian processes: recruiting follicles, follicular atresia, formation of corpora lutea and ovulation (Motta and Familiari, 1981; Maretová and Maretta, 2002).

In the present study, we found a close similarity between aging rabbit ovaries to the histological changes described in aging human ovaries. Even though older ovaries retain conspicuous steroidogenic tissue, its function gradually transfers from follicles to the secondary interstitial gland. Although the theca interna has been considered a “forgotten cell type” (Young and McNeilly, 2010), the interstitial gland has also been overlooked. Morphological and immunohistological similarity with mature human ovaries makes the rabbit a better animal model than most rodents, for improving our understanding of the aging process.

In the current work, we used mature multipary rabbit to reveal changes in the cortical region of ovaries during the entire estrous cycle, including ovulation, and pregnancy. However, in women, nulliparity has been associated with an increase in reproductive cancer, including ovarian cancer (Britt and Short, 2012; Gleicher 2013). Further investigation is necessary to provide better understanding of the aging process in the context of nulliparity to guarantee prevention strategies for

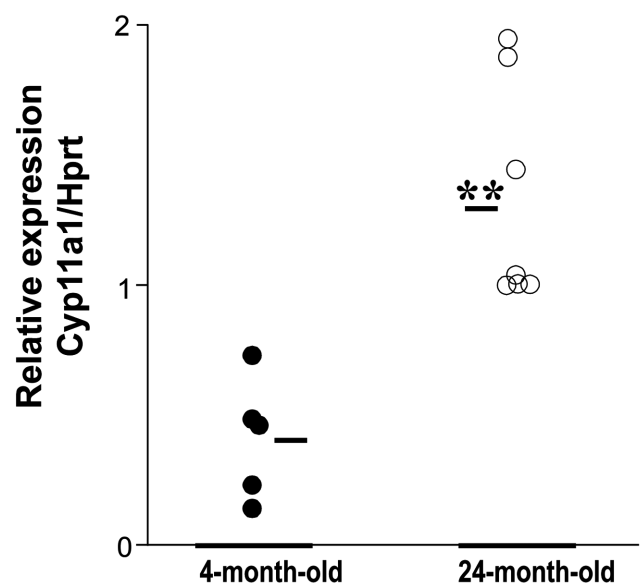


Fig. 11. Levels of mRNA for Cyp11a1 in ovaries of reproductive and mature chinchilla rabbits by RT-PCR. Densitometry results, showing significant differences (** $p < 0.01$).

reproductive cancers. Recent reports show that in mice, virgin-nulliparous ovaries increase ovarian cancer risk and accumulate higher concentrations of lipofuscin ("age pigment") (Urzua et al., 2018).

Among rabbits ovulation is induced by coitus or mechanical stimulation and therefore mature follicles grow and regress continuously (Dal Bosco et al., 2011). The aging of the ovary of nulliparous rabbit may be an interesting model to discriminate between dependent ovulation alterations of OSE and a decrease in follicular reserve and hypertrophy of the interstitial gland in the absence of ovulation.

Conclusions

In contrast to ovaries during the reproductive period, immunohistological changes demonstrate that the interstitial gland becomes the most abundant tissue during the aging of ovaries. Thus, the current study provides new data for understanding the alteration of somatic cell function in elderly ovaries, which underlies their declined fertility. Further studies are needed to understand the role of the interstitial gland in follicular development and oocyte quality.

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