Advancing fertility preservation: structural and functional insights into the human ovary

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Chapter 2

Multilaminar follicles from cultured human ovarian tissue: a comparative study between child and adult tissue

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Abstract

STUDY QUESTION: Can mature oocyte be obtained from cultured child human ovarian cortical tissue?

SUMMARY ANSWER: Although antral formation was rarely observed in cultured child ovarian cortical tissue, large pre-antral follicles developed and displayed molecular and morphological characteristics more closely resembling in vivo antral follicles than the in vitro–derived antral follicles obtained from adult tissue.

WHAT IS KNOWN ALREADY: Human ovarian cortical tissue contains a large reserve of primordial follicles and represents an important source for fertility preservation. Although early follicular activation has been achieved in vitro, efficient oocyte maturation remain challenging, especially in child tissue. With the increasing number of pediatric cancer patients surviving after gonadotoxic treatments, there is an urgent clinical need to develop reliable in vitro culture systems capable of supporting child ovarian follicle growth and maturation toward functionally competent oocytes.

STUDY DESIGN, SIZE, DURATION: Human ovarian cortical tissue from child (N = 3, aged 7, 8 and 12 years) and adult (N = 3, aged 23, 27 and 32 years) cis female donors were cultured in a defined medium for 8 days, followed by an extended culture supplement with Activin A up to 24 days. Samples were collected at days 0, 8, 16, and 24 for analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ovarian cortical tissue was obtained from child and adult cis female donors undergoing oophorectomy for fertility preservation purposes before chemotherapy. Ovarian cortex was fixed either prior (day 0) or after the culture period. Histological and immunofluorescent analyses were performed to assess follicle survival, growth, and morphology.

MAIN RESULTS AND THE ROLE OF CHANCE: Both child and adult ovarian cortical tissues supported follicular development from unilaminar to multilaminar stage during in vitro culture, with antral follicles appearing exclusively in adult tissues by day 16. Granulosa cells within multilaminar follicles retained proliferative capacity (PCNA+) with low apoptosis and compact morphology. Marker analysis revealed a post-culture reduction in S100B expression in adult antral follicles, whereas its expression increased in pre-antral follicles from child tissues. In contrast, AMH expression remained stable throughout the culture period in both child and adult tissues. KRT19 expression remained low in granulosa cells of multilaminar follicles throughout the culture period. However, STAR was strongly expressed in child cortical tissue theca cells, but weak or absent in adult tissue. Conversely, ACTA2 showed variable baseline expression but was downregulated after culture, suggesting inadequate support for theca cell lineage maintenance.

LIMITATIONS, REASONS FOR CAUTION: The number of donors was limited, and residual cortical regions were used, which may differ from cryopreserved outer cortex.

WIDER IMPLICATIONS OF THE FINDINGS: Child ovarian follicles can progress to the pre-antral stage in vitro and display more physiological characteristics than adult in vitro–derived antral follicles, highlighting their intrinsic developmental potential. Optimizing culture systems to sustain theca cell function and promote complete antral formation will be essential for fertility preservation in pediatric patients.

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Keywords:

Human ovarian tissue, in vitro folliculogenesis, granulosa cells, fertility preservation, child

Introduction

Human ovarian cortical tissue contains a large reservoir of early-stage follicles, offering critical opportunities for fertility preservation and reproductive research. In vitro culture of ovarian cortex has been explored as a strategy to support early follicular growth, particularly in cases where transplantation is not feasible, such as in pediatric cancer patients or individuals at risk of malignant cell reintroduction (Anderson et al., 2017). Although early follicular activation and growth have been achieved in vitro, the generation of morphologically and functionally competent antral follicles remains limited, special in child or adolescent individuals.

Previous studies have demonstrated that the human ovarian cortex is capable of supporting the early developmental progression of primordial follicles toward maturation under appropriate culture conditions (McLaughlin et al., 2018; Xu et al., 2021). Nonetheless, significant variability exists between tissues derived from donors of different ages and hormonal backgrounds (Tsui et al., 2023; Rooda et al., 2024). Notably, child and adult ovarian cortices differ in their follicular composition, stromal organization, and responsiveness to external signals (Anderson et al., 2014; Shen et al., 2023; Masciangelo et al., 2021), which may influence their in vitro developmental potential.

Despite encouraging observations of follicular growth and granulosa cell proliferation in cultured ovarian tissue, the efficiency of antral follicle formation and the functional differentiation of both granulosa and theca cell lineages remain suboptimal (McLaughlin et al., 2018; Xu et al., 2021). In particular, the failure to sustain key markers associated with follicle maturation and steroidogenesis suggests that the current culture systems are insufficient to fully replicate the intricate cellular interactions that govern folliculogenesis (Vazakidou et al., 2024).

In this study, we systematically compared the in vitro growth potential of ovarian cortical tissues obtained from child and adult human donors. By culturing cortical cubes over a defined time course, we aimed to evaluate follicular development, granulosa cell morphology and marker expression, and the activation of theca cell lineages. Through histological, quantitative, and immunohistochemical analyses, we assessed the structural and functional integrity of developing follicles in both tissue types. Our findings highlight critical differences in follicular dynamics and cellular marker expression between child and adult ovarian cortices, providing insight into the limitations of current culture systems and informing future strategies for optimizing human folliculogenesis in vitro.

Materials and methods

Ethical permission

The study was conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee of the Leiden University Medical Center, which issued letters of no objection under protocol numbers B16.050, B18.029, and B22.3077. Human ovarian tissue was obtained with signed informed consent from adult or child individuals undergoing oophorectomy for fertility preservation prior to chemotherapy at the Leiden University Medical Center (cis female donors; n = 6).

Ovarian tissue collection and in vitro culture

Fresh ovarian cortical tissue was obtained from child (n = 3) and adult (n = 3) individuals. The main outer cortical layer was removed for clinical cryopreservation, and the remaining inner cortex (from child tissue) or the peripheral cortical boundary (from adult tissue) was dissected for research purposes. Cortical tissue was cut into approximately 1 mm^3 cubes (6–8 cubes/well) and transferred into 24-well plates (92024, TPP, Switzerland) for culture. Each donor was treated as an independent biological replicate.

Tissue cubes were cultured individually in 1mL of McCoy's 5A medium (26600- 023, Invitrogen, UK) supplemented with 1ng/ml recombinant human FSH (rhFSH) (GONAL-F 450U/0.75ML, Merck, USA), 0.1% human serum albumin (HSA) (RVG105901, CSL Behring, UK), 1% Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (100X) (51300044, Thermo Fisher, UK), 3mM glutaMAX (35050-038, Thermo Fisher, UK), 20mM HEPES (15630-049, Thermo Fisher, UK), 0.1% penicillin- streptomycin (15070063, Life Technologies, USA), and 50µg/ml ascorbic acid (A92902, Merck, USA). Cultures were incubated at 37°C in 5% CO_2, with half medium changed every other day.

After 8 days of culture, the medium was replaced with a second-stage formulation to support continued follicular growth. This medium was identical to the first-stage medium with the addition of 100 ng/mL recombinant human Activin A (338-AC-010/CF, R&D Systems, USA). Cultures were continued for up to 24 days, with analyses conducted at day 0, and after 8, 16, and 24 days of in vitro culture.

Histological and immunofluorescent analyses

Histological and Immunofluorescent Analyses similar to that described by Cheng et al., 2024. Briefly, tissue cubes were fixed in 4% paraformaldehyde (PFA) (1.04005.1000, Merck, Germany) overnight, paraffin-embedded, and sectioned at 5 µm thickness. Sections were stained with hematoxylin and eosin (H&E) for morphological assessment of follicular structures. Follicles were classified into five categories based on histological criteria: (1) unilaminar follicles, defined as an oocyte surrounded by a single layer of flattened or cuboidal granulosa cells; (2) multilaminar follicles, with an oocyte enclosed by two or more layers of cuboidal granulosa cells; (3) preantral follicles, characterized by oocyte enlargement and granulosa cell expansion without a visible antral cavity; (4) antral follicles, showing a well-defined antral space; and (5) atretic follicles, identified by signs of degeneration including oocyte shrinkage, nuclear pyknosis, or granulosa disorganization. Only morphologically intact follicles with a visible oocyte nucleus were included in the analysis. To avoid double-counting, follicle enumeration was performed on every eight serial section, and the total number of follicles was calculated in each tissue section (5–8 ovarian cubes per condition).

For immunofluorescence, paraffin-embedded sections were deparaffinized, subjected to antigen retrieval, and blocked using Cheng et al. (2024) same protocols, followed by overnight incubation at 4 °C with primary antibodies. The following markers were assessed: goat anti-FOXL2 (1:250, NB100-1277, Bio-Techne, USA), goat anti-ZP3 (1:100, sc-398359, Santa Cruz, USA), mouse anti-AMH (1:30, MCA2246T, BioRad, USA), mouse anti-STAR (1:100, sc166821, Santa Cruz, USA), mouse anti-PCNA (1:100, sc- 56, Santa Cruz, USA), rabbit anti-S100B (1:200,

AB52642, Abcam, UK), rabbit anti-KRT19 (1:150, ab76539, Abcam, UK), rabbit anti- ACTA2 (1:100, sc-79, Santa Cruz, USA), and rabbit anti-cleaved caspase-3 (1:200, 9661S, Cell Signal technology, USA). After incubation with appropriate fluorophore-conjugated secondary antibodies and DAPI (1:1000, D1306, Life Technologies, USA), and then imaged using a Leica SP8 TC inverted confocal microscope (Leica, Germany) with LAS v3.7.4.23463 software (Leica, Germany).

Statistical analysis

Each experiment was performed in triplicate. Data were analyzed using GraphPad Prism version 9.0.1 software (GraphPad Software Inc, USA). Statistical significance was determined using an unpaired two-tailed Student's t-test (Figure 2G and J) or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Figure 2F and H). Bar graphs show mean ± standard deviation. A p-value of less than 0.05 was considered statistically significant (*P<0.05, **P<0.01, ***P<0.001; ns, not significant).

Results

Isolation and in vitro culture of child and adult ovarian tissue

Ovarian samples were initially collected from the IVF laboratory, where the main cortical regions were removed for cryopreservation. The remaining ovarian tissue—specifically, the inner cortical region from child donors and the peripheral (boundary) cortical region from adult donors—was subsequently used for in vitro culture. These residual cortical tissues were cut into 1 mm^3 cubes (6–8 cubes/well; n = 3 donors) and cultured in 24-well plates. After an initial 8-day culture period, the medium was replaced with a second-stage culture medium, and the tissue cubes were further cultured for either 8 or 16 additional days (Figure. 1A and B). Prior to culture, microscopic examination revealed a high density of unilaminar follicles in the inner cortex of child ovaries (Figure. 1C), while hematoxylin and eosin (HE) staining showed the corpus luteum structures (Figure. 1D) and demonstrated significantly higher follicular density in child compared to adult cortical tissue (Figure. 1E). Tissues were derived from donors of varying ages (Figure. 1H), and follicular development was assessed at multiple time points to evaluate the dynamics of in vitro folliculogenesis (Figure. 1F).

Follicular morphology and quantitative analysis in child and adult ovarian tissue during in vitro culture

Next, we evaluated the morphology and developmental progression of follicles following in vitro culture. Histological analysis using HE staining revealed the presence of various types of multilaminar follicles in the cultured ovarian cortical tissue. Granulosa cells (GCs) in these follicles exhibited a more compact arrangement, closely resembling the morphology as observed in vivo. Notably, in adult cortical tissue, antral follicle formation was observed after 16 days of culture. However, in child cortical tissue, no antrum formation was detected even after extending the culture to 24 days. Nevertheless, follicles continued to grow in size over time, and GCs became progressively more tightly packed, similar to in vivo morphology (Figure 2A).

To further characterize follicular development, we first assessed follicle numbers on day 0 (D0) prior to culture. A total of 339 follicles were identified in child ovarian cortical cubes and 198 in adult cortical cubes. In

both groups, the vast majority of follicles were at the unilaminar stage, with an average distribution of 98% unilaminar and 2% multilaminar in child samples, and 99% unilaminar and 1% multilaminar in adult samples (Figure 2B, E). As expected, some cortical cubes did not contain any follicles; however, none of the cubes with follicles contained more than 50% atretic follicles post-culture (Supplementary table 1), and each follicle-containing cube harbored between 3 and 17 follicles (Supplementary table 2).

After 8 days of culture, we quantified 313 follicles in child cortical tissue, of which 61% were unilaminar, 19% multilaminar, 17% unilaminar atretic, and 3% multilaminar atretic. In adult cortical tissue at the same time point, 131 follicles were identified, with 63% unilaminar, 21% multilaminar, 14% unilaminar atretic, and 2% multilaminar atretic (Figure 2C, E). At day 16, child cortex contained 265 follicles (57% unilaminar, 19% multilaminar, 19% unilaminar atretic, 5% multilaminar atretic), while adult cortex had 173 follicles (58% unilaminar, 20% multilaminar, 2% antral, 16% unilaminar atretic, and 4% multilaminar atretic) (Figure 2D, E). By day 24 of culture in child tissue, we observed 225 follicles, consisting of 54% unilaminar, 21% multilaminar, 20% unilaminar atretic, and 5% multilaminar atretic follicles (Figure 2D).

In conclusion, in vitro culture of fresh cOVA ovarian cortical tissue for 8, 16, or 24 days resulted in a significant increase in the proportion of multilaminar follicles compared to D0. Interestingly, the distribution of follicular stages in fresh cOVA tissue was largely similar between child and adult donors (Figure 2F, H). Furthermore, granulosa cells within multilaminar follicles exhibited a more compact morphology after culture, resembling in vivo organization. The diameter of oocytes within non-atretic multilaminar follicles remained comparable to that observed at D0 (Figure 2G, J), suggesting that follicular growth was well supported and that oocyte integrity was maintained throughout the culture period.

Follicular characteristics in the child and adult ovarian cortical tissue

To further assess the quality of multilaminar/antral follicles obtained after in vitro culture of ovarian cortical tissue, we evaluated late-stage apoptosis by detecting cleaved caspase-3, a marker associate with DNA fragmentation. The results indicated absent or minimal apoptosis in stromal cells from both child and adult ovarian tissues. In contrast, granulosa cells (FOXL2+) within multilaminar follicles exhibited positive staining for PCNA, indicating active proliferation following culture (Figure 3A). Additionally, ZP3 was used to delineate oocyte boundaries and assess oocyte size (Figure 3B). These findings suggest that granulosa cells maintained their proliferative capacity in vitro, thereby supporting the developmental competence of cultured multilaminar follicles.

Next, we examined the expression of three complementary granulosa cell markers: keratin 19 (KRT19), an intermediate filament protein associated with ovarian surface epithelium and transiently expressed in early-stage granulosa cells during the primordial-to-primary follicle transition, reflecting epithelial lineage characteristics (Wamaitha et al., 2023); anti-Müllerian hormone (AMH), a secreted factor marking granulosa cells of preantral and small antral follicles, widely used as an indicator of early follicular development and ovarian reserve (Karaviti et al., 2025; Visser et al., 2006); and S100 calcium-binding protein B (S100B), a cytoplasmic

marker of differentiated granulosa cells in growing follicles, with enriched expression during the secondary-to-antral transition (Fan et al., 2021).

Consistent with their expression at day 0 (D0), granulosa cells in unilaminar follicles after culture maintained high KRT19 expression. In contrast, KRT19 expression was markedly lower in multilaminar follicles and absent in pre-antral/antral follicles in both child and adult ovarian tissues. AMH expression remained comparable to D0 levels across all follicle stages in both tissue types. S100B, which was highly expressed in antral follicles and showed low or no expression in multilaminar follicles at D0, exhibited a differential pattern after culture—its expression decreased in adult antral follicles but increased in child pre-antral follicles (Figure 3B and C).

Finally, we investigated the expression of two complementary markers of theca cells. Steroidogenic acute regulatory protein (STAR), a key marker of steroidogenic activation, is expressed in both theca and granulosa cells during the transition to the antral stage, indicating increased mitochondrial cholesterol transport and initiation of steroid hormone biosynthesis (Yahya et al., 2024). Smooth muscle actin (ACTA2) marks theca cell progenitors located at the periphery of the follicular basement membrane during the primary-to-secondary follicle transition (Guahmich et al., 2023).

At D0, STAR expression was observed surrounding granulosa cells in antral follicles. Interestingly, strong STAR expression was detected around granulosa cells in child ovarian tissue, while no expression was observed in adult tissue (Figure 3B). Additionally, ACTA2 was strongly expressed in theca cells surrounding follicles before culture; however, its expression was diminished or absent in both child and adult tissues after culture (Figure 3D), potentially indicating the arrest or loss of theca cell lineage commitment under current culture conditions.

In summary, the in vitro culture of human ovarian cortical tissue preserved the structural integrity and proliferative capacity of granulosa cells within multilaminar follicles, as evidenced by low apoptosis and sustained PCNA expression. The dynamic expression patterns of granulosa cell markers—KRT19, AMH, and S100B—reflected partial progression of follicular maturation, though key features of late-stage differentiation appeared attenuated. Furthermore, differential expression of theca cell markers STAR and ACTA2 suggested limited activation or maintenance of the theca cell lineage under current culture conditions. These findings highlight both the potential and limitations of current in vitro culture systems in supporting human folliculogenesis and underscore the need for further optimization to achieve full functional maturation of follicular compartments.

Discussion

Our study demonstrates that human ovarian cortical tissue from both child and adult donors can sustain follicular survival and early development in vitro, though with distinct limitations in later-stage maturation. These findings advance the field of ovarian tissue culture but also highlight critical gaps that must be addressed to achieve clinically relevant outcomes for fertility preservation.

The observed increase in multilaminar follicles in cultured tissue confirms the potential of this system to support early folliculogenesis. However, the failure of child follicles to form antral structures—despite prolonged

culture—suggests intrinsic or microenvironmental barriers difference between child and adult tissue. This aligns with prior reports of developmental disparities between juvenile and adult ovaries (Rooda et al., 2024; Vazakidou et al., 2024), possibly reflecting differences in paracrine signaling or steroid sensitivity. The preservation of oocyte diameter and granulosa cell (GC) proliferation (PCNA+) underscores the system's ability to maintain follicular health, yet the persistence of unilaminar follicles alongside growing cohorts indicates asynchronous development, a challenge also noted in other in vitro models (Yang et al., 2020).

The divergent expression of S100B following in vitro culture—marked by decreased levels in adult antral follicles and increased expression in child pre-antral follicles—may reflect differences in developmental stage and age-related sensitivity to the in vitro environment. Adult antral follicles are more dependent on endocrine support (Eppig et al., 2001; McGee et al., 2000), which is often suboptimal in culture systems. This insufficiency may induce granulosa cell (GC) stress or dysfunction, resulting in the downregulation of S100B. In contrast, child follicles, which are typically quiescent in vivo, may become activated under in vitro conditions (Ghezelayagh et al., 2022). The upregulation of S100B in these follicles may reflect an early stress response or a survival mechanism triggered by this activation. These observations suggest that current culture conditions may be more supportive of early follicular activation than of maintaining the function and integrity of more mature follicle stages. This highlights the need for stage-specific culture optimization tailored to the metabolic and hormonal requirements of different follicular stages. Encouragingly, the retention of granulosa cell proliferative capacity (PCNA+) and low levels of apoptosis (cleaved caspase-3–) indicate a degree of cellular health that holds promise for clinical applications. However, the functional competence of these granulosa cells—particularly their ability to support oocyte maturation—remains to be fully determined.

The loss of theca cell markers (STAR, ACTA2) in cultured tissue is a critical finding, as theca-derived signals are essential for antral formation and steroidogenesis (Young et al., 2010). The residual STAR expression in child tissue hints at retained steroidogenic potential, but its absence in adult cultures may explain the limited follicular progression. This aligns with studies showing that theca-stromal interactions are difficult to maintain in vitro (Orisaka et al., 2006). Future work should test co-culture systems or hormonal supplementation to restore theca cell function (Liu et al., 2015).

The higher follicular density in child cortex aligns with established ovarian biology, and while the similar distribution of follicular stages (unilaminar vs. multilaminar) between age groups at D0 suggests comparable developmental potential, the subsequent divergence during culture likely reflects microenvironmental or hormonal differences (Wallace et al., 2010). This distinction is particularly relevant for fertility preservation, as most existing in vitro growth (IVG) protocols have been developed using adult ovarian tissue-leaving their suitability for child tissue inadequately explored (Roness et al., 2015). Addressing this critical gap through optimization of culture conditions, particularly to sustain theca cell function and promote antral formation in child tissue, could significantly enhance the clinical utility of in vitro folliculogenesis for young patients undergoing gonadotoxic treatments (Ghezelayagh et al., 2022; Gellert et al., 2018).

Conclusions

This study advances the understanding of human ovarian cortical culture, demonstrating its ability to sustain follicular growth but revealing limitations in late-stage maturation. The differential responses between child and adult tissue highlight the need for further refinement of IVG protocols. By addressing theca cell support and refining granulosa cell differentiation to promote proper follicular development. Such refinements will be essential to achieve complete and functionally competent follicular development, especially in the context of fertility preservation for young patients.

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Author's roles

HC and SMCDSL conceived the study. HC, FW, GSKP, LAJVDW, SMCDSL contributed to material collection. LAJVDW, SMCDSL arranged the ethical permits. HC, FW generated data. All authors contributed to data analysis. All authors contributed to manuscript writing. All authors approved the submitted version. All authors provided the final approval of the version to be published.

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Conflict of interest

The authors have no conflicts of interest to declare.

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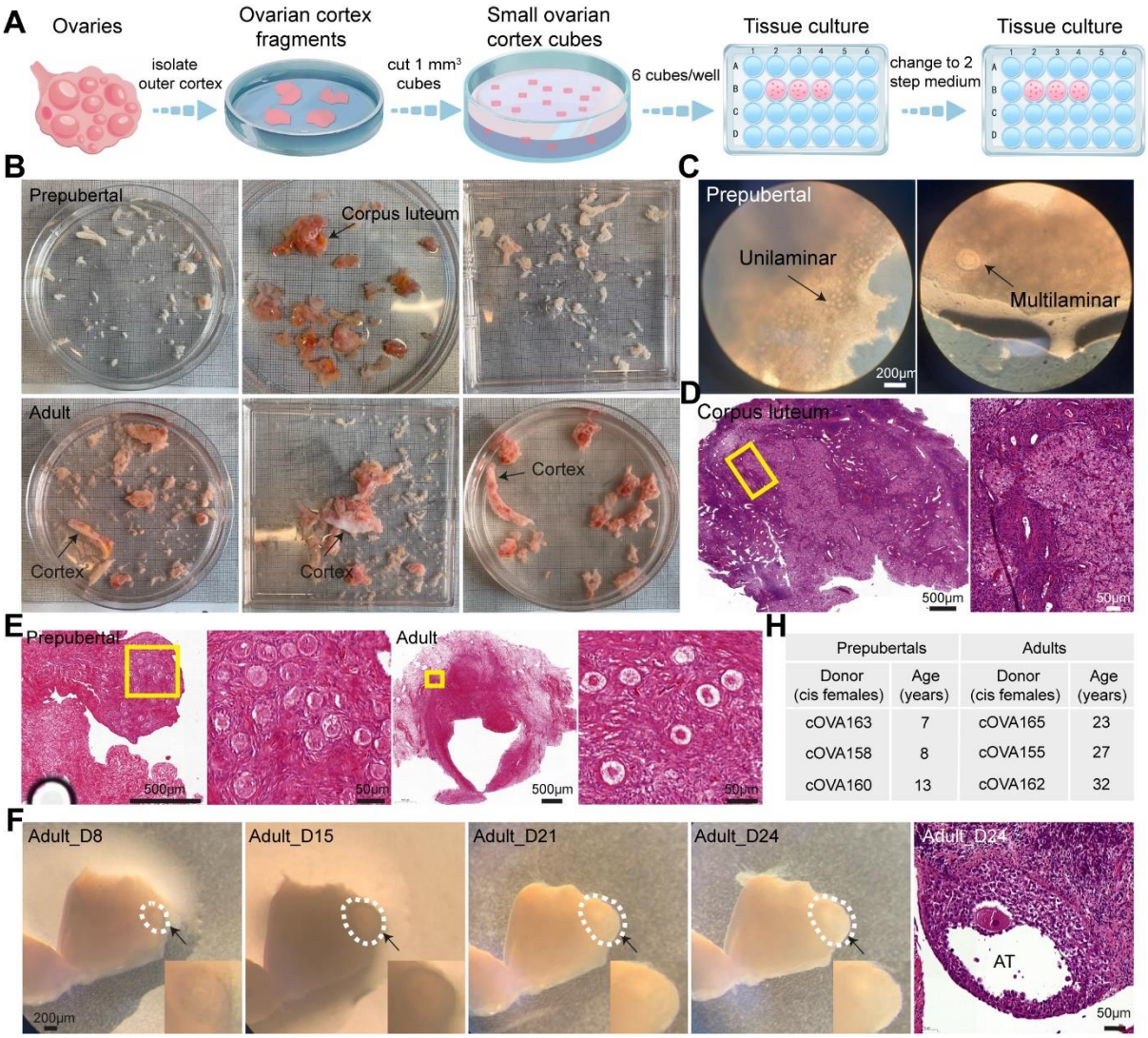
FIGURES AND SUPPLEMENTARY MATERIALS



Prepubertals		Adults	
Donor (cis females)	Age (years)	Donor (cis females)	Age (years)
cOVA163	7	cOVA165	23
cOVA158	8	cOVA155	27
cOVA160	13	cOVA162	32

Figure 1. Follicular morphology in child and adult ovarian cortex before culture.

(A) Schematic overview of the two-stage in vitro culture workflow for freshly isolated child and adult cis-female ovarian cortical tissue (cOVA). **(B)** Gross morphology of child and adult ovarian tissue after dissection. Black arrowheads indicate the cortex or corpus luteum structures. **(C)** Representative image of unilaminar and multilaminar follicles (black arrowheads) under the microscope in child ovarian cortical tissue at day 0 (D0), prior to culture. Scale bar: 200 µm. **(D)** Hematoxylin and eosin (H&E) staining of freshly isolated child cOVA tissue at D0, showing corpus luteum structure. Right panel shows a magnified view of the corpus luteum. Scale bar: 50 µm. **(E)** H&E staining of freshly isolated child and adult cOVA cortical tissue at D0, highlighting unilaminar follicles. Scale bar: 50 µm. **(F)** In vitro development of unilaminar follicles in adult cOVA cortical tissue across time points, with representative H&E staining of antral follicles observed at day 24 (D24). Scale bars: 200 µm and 50 µm, respectively. **(H)** Data represent comparisons among different age groups between pediatric and adult individuals.

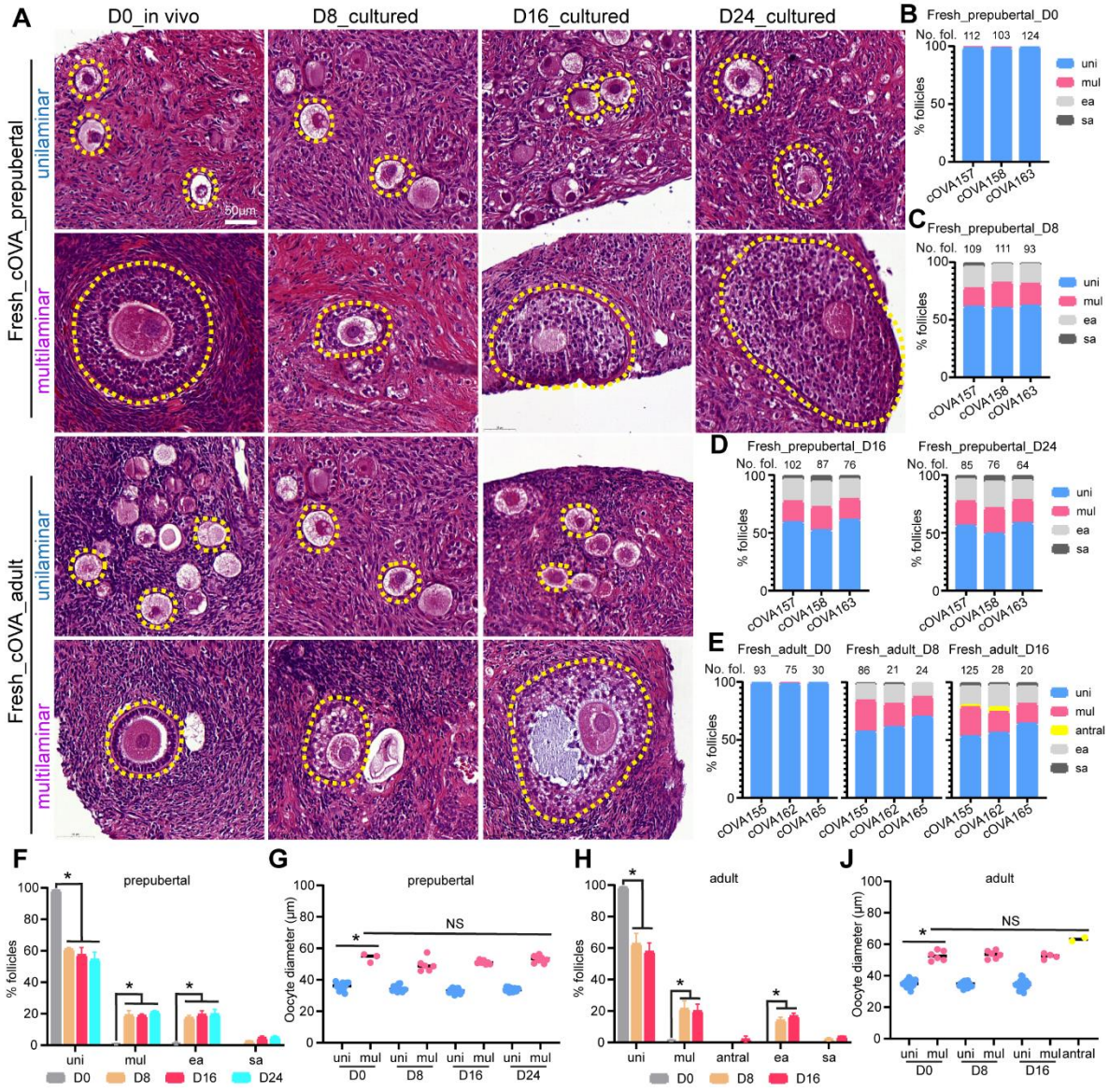


Figure 2. Follicular distribution in child and adult ovarian cortex before and after culture.

(A) Hematoxylin and eosin (HE) staining of freshly isolated child and adult cOVA cortical tissue before (D0) and after culture (D8, D16 and D24), showing different types of follicles (dashed line). Scale bars are 50 µm. S1, first step of culture; S2, second step of culture. **(B, C, D)** Percentage of follicular distribution in freshly isolated child cOVA cortical tissue per donor before (D0) and after culture (D8, D16, D24). In the top of the graph are the total number of follicles counted per donor. **(E)** Percentage of follicular distribution in freshly isolated adult cOVA cortical tissue per donor before (D0) after culture (D8, D16). In the top of the graph are the total number of follicles counted per donor. **(F)** Percentage of follicular distribution in freshly isolated child and adult cOVA **(H)** cortical tissue per follicular stage before and after culture. The results shown are mean ± standard deviation from three independent experiments; *P<0.05, **P<0.01, ***P<0.001. **(G)** Oocyte diameter was measured in uni/mul follicles and antral follicles in freshly isolated child and adult cOVA **(J)** cortical tissue before and after culture. *P<0.05, compared to mul follicles at D0. uni, unilaminar; mul, multilaminar; ua, unilaminar atretic; ma, multilaminar atretic.

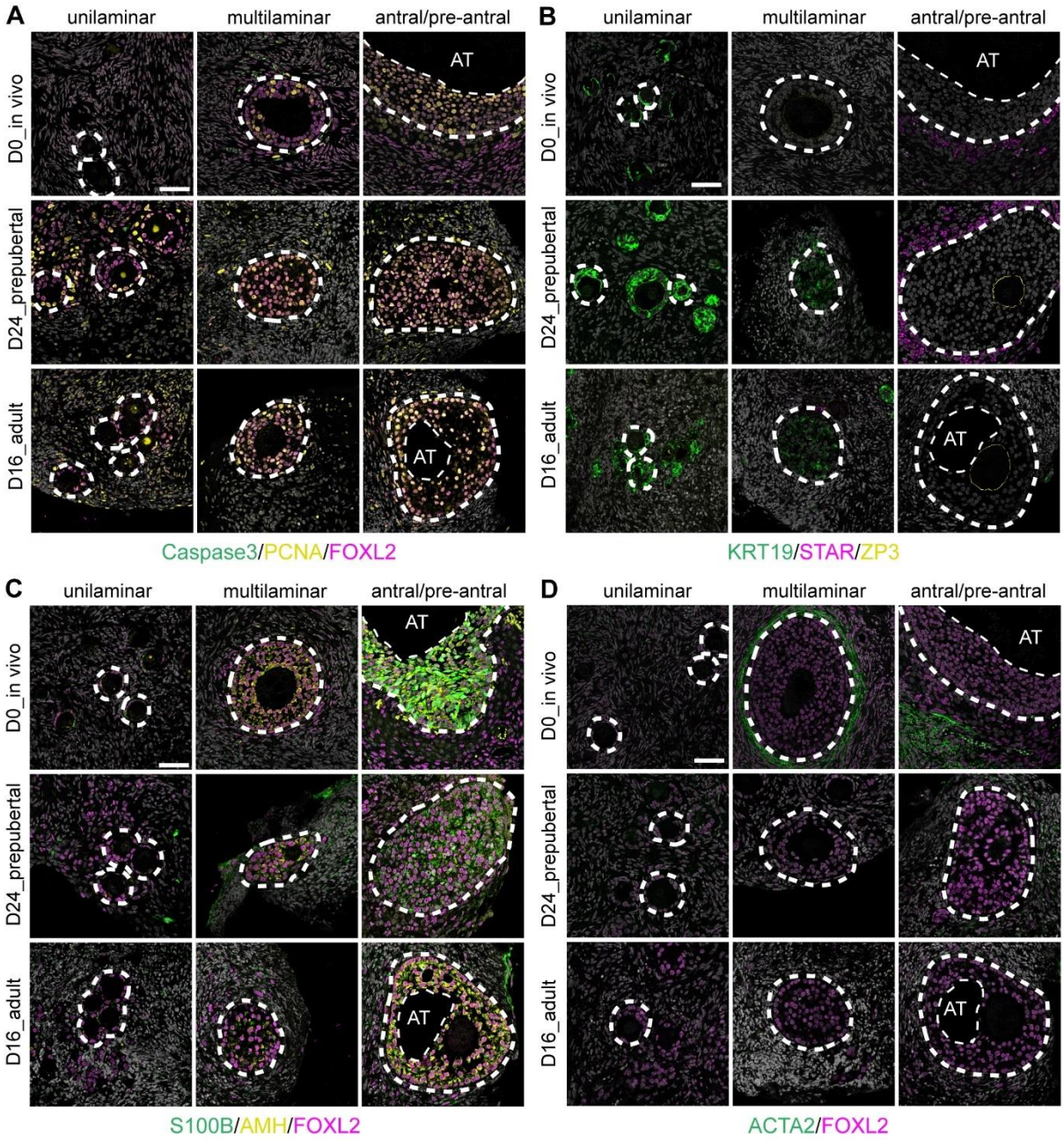


Figure 3. Characterization of different cell types associated with multilaminar follicles.

Immunofluorescence staining was performed to detect Cleaved-Caspase 3 (green), PCNA (yellow), KRT19 (green), STAR (magenta), S100B (green), AMH (yellow), ACTA2 (green), ZP3 (yellow), and FOXL2 (magenta) in unilaminar, multilaminar, and antral/pre-antral follicles. These follicles were present in freshly isolated adult cOVA cortical tissue prior to culture (D0), as well as in freshly isolated child and adult cOVA cortical tissue following in vitro culture. Dashed lines outline individual follicles. Scale bar = 50 µm.

Table 2. Characteristics of follicles in cOVA tissue cubes.

Culture days	Donor[a]	Cubes without follicles/total cubes (%)	Cubes with only atretic follicles/cubes with follicles (%)	Cubes with > 50% atretic follicles/cubes with follicles (%)	Cubes with secondary follicles/cubes with follicles (%)
D8_child	cOVA157	1/8(13)	0/7(0)	0/7(0)	5/7(71)
	cOVA158	2/8(25)	0/6(0)	0/6(0)	4/6(67)
	cOVA163	3/7(43)	0/4(0)	0/4(0)	3/4(75)
D16_child	cOVA157	2/7(29)	0/5(0)	0/5(0)	4/5(80)
	cOVA158	2/6(33)	0/4(0)	0/4(0)	2/4(50)
	cOVA163	3/8(38)	0/5(0)	0/5(0)	3/5(60)
D24_child	cOVA157	2/8(25)	0/6(0)	0/6(0)	4/6(67)
	cOVA158	3/7(43)	0/4(0)	0/4(0)	3/4(75)
	cOVA163	3/8(38)	0/5(0)	0/5(0)	4/5(80)
D8_adult	cOVA155	2/7(29)	0/5(0)	0/5(0)	4/5(80)
	cOVA162	3/6(50)	0/3(0)	0/3(0)	2/3(67)
	cOVA165	4/7(57)	0/3(0)	0/3(0)	2/3(67)
D16_adult	cOVA155	2/8(25)	0/6(0)	0/6(0)	5/6(83)
	cOVA162	5/8(62)	0/3(0)	0/3(0)	2/3(67)
	cOVA165	4/7(57)	0/3(0)	0/3(0)	2/3(67)

[a]c, cis female

Table 3. Total number of follicles used in each conditions													
Child	conditions	D0			D8			D16			D24		
	patients	cOVA157	cOVA158	cOVA163	cOVA157	cOVA158	cOVA163	cOVA157	cOVA158	cOVA163	cOVA157	cOVA158	cOVA163
	total follicle	112	103	124	109	111	93	102	87	76	85	76	64
	total cubes(with follicles)	8	7	9	7	8	7	6	6	7	7	6	7
	follicles/cube	14	15	14	16	14	13	17	15	11	12	13	9
Adult	conditions	D0			D8			D16			–		
	patients	cOVA155	cOVA162	cOVA165	cOVA155	cOVA162	cOVA165	cOVA155	cOVA162	cOVA165	–	–	–
	total follicle	93	75	30	86	21	24	125	28	20	–	–	–
	total cubes(with follicles)	7	11	9	8	8	8	9	7	7	–	–	–
	follicles/cube	13	7	3	11	3	3	14	4	3	–	–	–

cOVA, tissue from a cis-femle.