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

Cheng, H.

Chapter 1

General introduction and thesis outline

Introduction

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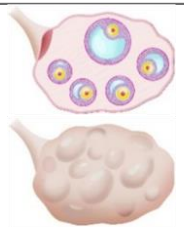
Female infertility is a significant global health concern, affecting millions of women of reproductive age (Hull & Cahill, 1998). It arises from various factors, including age-related decline in ovarian reserve, genetic conditions, hormonal imbalances, anatomical disorders, and medical treatments such as chemotherapy that compromise ovarian function (Lavafian et al., 2023). With advancements in cancer therapies and life-prolonging treatments, an increasing number of women face fertility challenges, necessitating the development of effective fertility preservation strategies (Jeruss & Woodruff, 2009). The female reproductive system, centered around the ovary, undergoes dynamic structural and functional changes throughout life, with follicles—its fundamental units—progressing through distinct developmental stages regulated by intricate molecular pathways (Perheentupa & Huhtaniemi, 2009). Understanding these mechanisms has paved the way for innovations in reproductive medicine, such as oocyte or embryo freezing, ovarian tissue cryopreservation (OTC), and in vitro follicle culture, offering hope to individuals at risk of fertility loss, including cancer patients and those undergoing gender-affirming care.

This research aims to enhance fertility preservation strategies by improving our understanding of ovarian function and optimizing in vitro folliculogenesis. Chapter 1 provides a comprehensive overview of human ovarian development and function, emphasizing its role in reproductive potential and fertility preservation. Chapter 2 investigates the development of multilaminar follicles in child versus adult ovaries, revealing important developmental distinctions. Building on this, Chapter 3 introduces a classification system for ovaries from different donors, offering valuable guidance for clinicians evaluating candidates for ovarian cortex tissue transplantation. Chapter 4 compares the efficacy of two established in vitro culture systems in supporting follicular growth, while Chapter 5 explores a novel component that may enhance the quality and maturation of in vitro–cultured follicles. In contrast, Chapter 6 identifies factors that do not significantly influence follicular development, underscoring the importance of carefully selecting culture conditions to optimize outcomes. By integrating insights from ovarian development, follicular classification, and in vitro culture optimization, this study contributes to the refinement of fertility preservation techniques. Ultimately, these findings hold promise for improving reproductive health outcomes across diverse patient populations.

Ovary Structure and Function Across Different Life Stages after Birth

The ovary is a dynamic organ that undergoes significant structural and functional changes throughout a woman's life, from childhood to adulthood and into postmenopause (Wallace & Kelsey, 2010). As the primary reproductive organ, the ovary plays a crucial role in both gametogenesis (oocyte production) and endocrine function (hormone secretion) (Baerwald et al., 2012). These functions are tightly regulated by the hypothalamic-pituitary-ovarian (HPO) axis and vary according to age and reproductive status (Adashi, 1994; Buffet & Bouchard, 2001).

Table 1. Ovaries from different types of donors.

Features \ Ovaries types		 Type 1	 Type 2	 Type 3	 Type 4	 Type 5	 Type 6	 Type 7	 Type 8
Ovaries surface	Smooth surface	Yes	Yes	Yes	Yes	Yes	Yes/No	Yes/No	Yes
	Visible follicles	Yes	Yes	Yes	Yes	No	No	Yes/No	Yes/No
	With blood	No	No	No	No	No	No	Yes	No
	Softness	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Cross-section	Visible follicles	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
	With blood	No	No	No	No	No	No	Yes	No
	Corpus luteum	No	No	Yes	No	No	No	No	Yes/No
	Ovulate antrum	No	Yes	No	No	No	No	No	No
Ovarian cyst structure		No	No	No	Yes	No	No	No	No

Cryo, cryopreseved; tOVA, tissue from a trans-male; cOVA, tissue from a cis-femle.

During childhood and prepuberty, the ovaries remain in a quiescent state, characterized by the presence of primordial follicles within the ovarian cortex (Gougeon & Chainy, 1987; Hansen et al., 2008). These follicles contain oocytes arrested in prophase I of meiosis, and there is minimal hormonal activity due to the low levels of gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) (Thibaud et al., 1992). The ovarian medulla, rich in blood vessels and connective tissue, remains structurally intact but functionally inactive (Bridges et al., 1993).

In adulthood, the ovary becomes a hub of endocrine and reproductive activity (Fan & Chuva de Sousa Lopes, 2021). The onset of puberty triggers the activation of the HPO axis, leading to cyclic follicular development, ovulation, and steroid hormone production (Monget et al., 2021). The adult ovary is composed of follicles at various stages of development, including primordial, primary, secondary, and antral follicles. Each menstrual cycle, a cohort of follicles is recruited, with one dominant follicle maturing to release a fertilizable oocyte during ovulation (Baerwald & Pierson, 2020; Baerwald et al., 2012). The ovary also produces estrogen and progesterone, which regulate the menstrual cycle, support pregnancy, and maintain secondary sexual characteristics (Fan et al., 2019; Vazakidou et al., 2024). This stage is marked by a delicate balance of follicular growth, atresia, and hormonal feedback mechanisms, ensuring reproductive competence.

During postmenopause, ovarian function declines due to follicular depletion and a cessation of ovulation (Perheentupa & Huhtaniemi, 2009). The ovaries become structurally smaller, with an increase in fibrotic tissue and a loss of active follicles (Faddy & Gosden, 1996). Hormonal production, particularly estrogen and progesterone, significantly decreases, leading to systemic effects such as osteoporosis, cardiovascular changes, and genitourinary atrophy (Sorpreso et al., 2015; Yasui et al., 2012). The lack of ovarian hormone production shifts endocrine regulation to peripheral tissues, such as adipose tissue, which continues to produce small amounts of estrogen (Brailly et al., 1981; Shifren & Schiff, 2000). The structural and functional transformations of the ovary across different life stages highlight its essential role in female reproductive health, with implications for fertility, endocrine balance, and aging-related disorders (Richards & Pangas, 2010; Stringer et al., 2023).

Although the ovary generally follows a predictable trajectory of development—from the quiescent state of childhood, through the hormonally active reproductive years, to the functional decline of postmenopause—considerable inter-individual variability in ovarian morphology can be observed at comparable ages. These morphologic differences reflect the dynamic and cyclic nature of ovarian physiology. For instance, in women around the age of thirty, some ovaries exhibit a smooth external surface, soft consistency, and multiple visible surface follicles, indicative of active folliculogenesis. In contrast, others display a more irregular or coarse surface, firmer texture, and an absence of macroscopically detectable follicles. Additional structures such as corpora lutea or ovulatory stigmata may be apparent, signaling recent ovulatory events, while certain follicles may contain blood, corresponding to hemorrhagic follicles or early luteal transformation. Given that these gross morphological characteristics correspond closely with the ovary's functional status and recent cyclic activity, classifying ovaries according to their surface features, consistency, and visible structures provides a useful framework for interpreting ovarian architecture and elucidating the spectrum of normal physiological variation (Table 1).

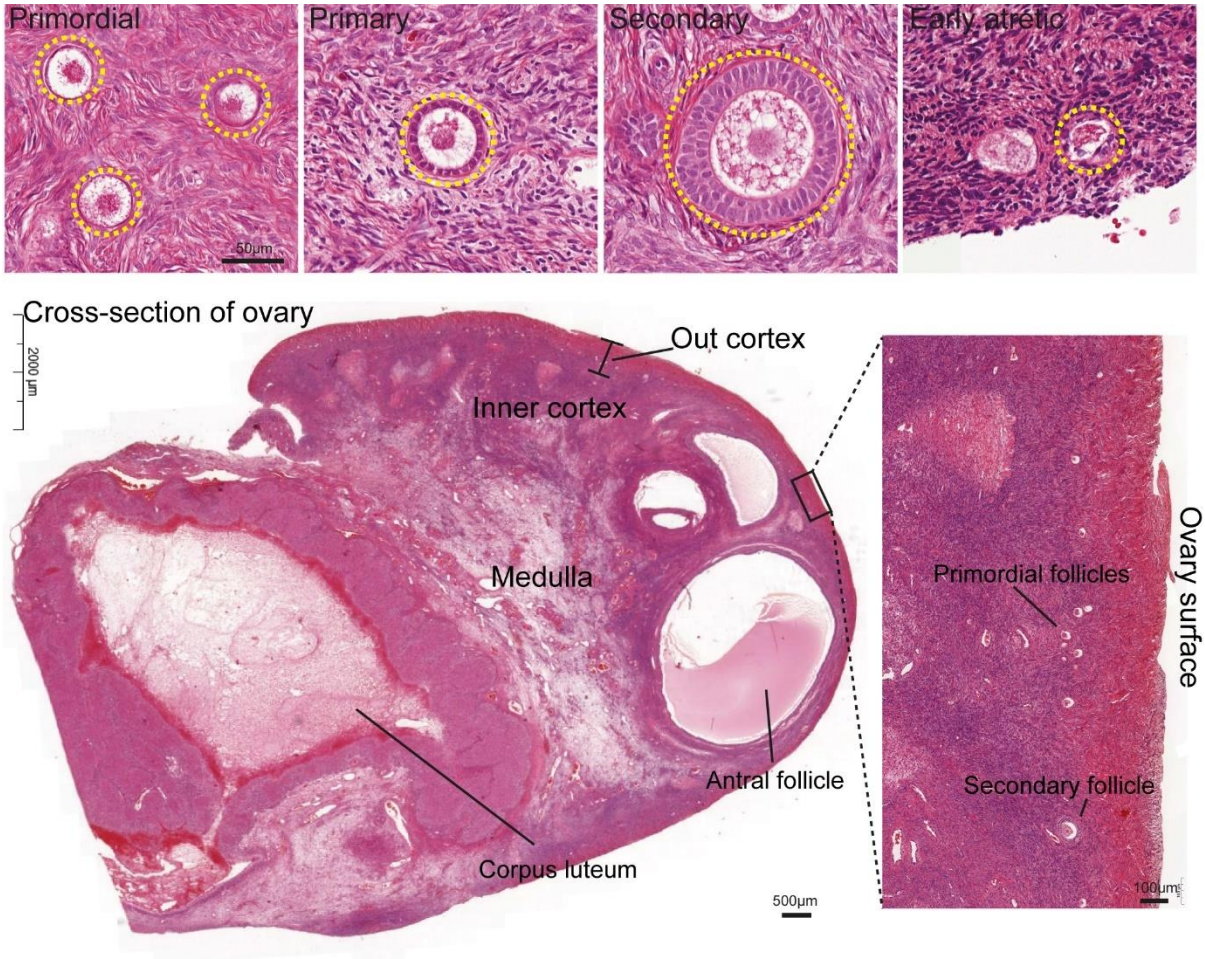


Figure 1. Structure of the human ovary. The figure adapted from Cheng et al., 2024. Hematoxilin-Eosin staining on ovarian histological sections showing different follicular stages, such as primordial, primary, secondary, antral, early atretic follicles and corpus luteum.

Structural and Functional Overview of Folliculogenesis

Ovarian follicles are the fundamental structural and functional units of the ovary, responsible for housing and nurturing oocytes while regulating hormone production necessary for female reproduction (McGee & Hsueh, 2000). Folliculogenesis, the process of follicular development, is a dynamic and highly regulated sequence of events that occurs in cyclic phases under the influence of gonadotropins and intraovarian factors (Fiorentino et al., 2023; Hsueh et al., 2015; Rimon-Dahari et al., 2016). Each follicle consists of an oocyte surrounded by layers of granulosa and theca cells, which play critical roles in hormone synthesis and follicular maturation. Follicular development progresses through distinct stages: primordial, primary, secondary, tertiary (antral), and preovulatory (Graafian) follicles, followed by ovulation and the formation of the corpus luteum (Labrune et al., 2022; Telfer et al., 2023)

In the primordial follicle stage, the follicle consists of an oocyte arrested in prophase I of meiosis, surrounded by a single layer of flattened granulosa cells (Telfer & Andersen, 2021). These follicles remain in a dormant state until recruited for further development. Upon activation, primordial follicles transition into primary follicles, where the granulosa cells become cuboidal, and the zona pellucida (a glycoprotein layer essential for sperm binding) begins to form around the oocyte (Ford et al., 2020).

The secondary follicle stage is characterized by further proliferation of granulosa cells and the recruitment of theca cells, which differentiate into the theca interna (involved in androgen synthesis) and theca externa (structural support) (Del Valle et al., 2022; Fan et al., 2019). As the follicle continues to grow, fluid-filled cavities coalesce to form a central antrum, marking the transition to the tertiary (antral) follicle stage (Baerwald et al., 2012; Hennet & Combelles, 2012). This stage is crucial for follicular selection, where only a dominant follicle continues to mature under the influence of follicle-stimulating hormone (FSH), while the remaining follicles undergo atresia (Matsuda et al., 2012; Zhou et al., 2019).

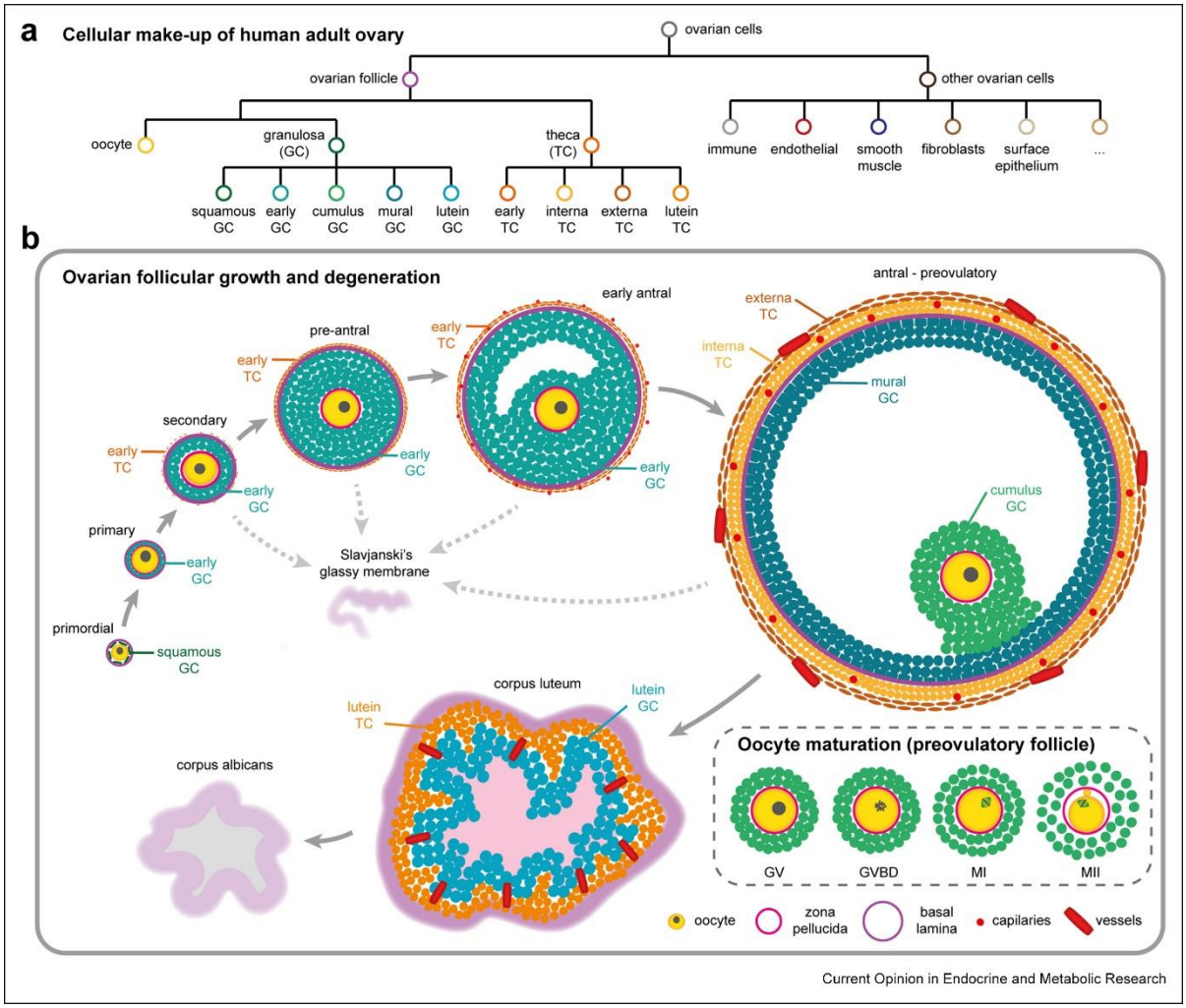


Figure 2. Main cell types in the human adult ovary. The figure is from Xueying et al., 2021. (a) Cartoon overview of the cellular makeup present in the human adult ovary and (b) the development of the oocyte, granulosa cell

(GC), and theca cell (TC) during folliculogenesis. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

The preovulatory (Graafian) follicle represents the final stage of follicular maturation before ovulation. At this stage, granulosa cells differentiate into cumulus and mural granulosa cells, and the follicle is highly responsive to LH (Moorey et al., 2022). The LH surge triggers oocyte meiotic resumption, leading to the release of a mature secondary oocyte during ovulation (Gérard & Robin, 2019). The ruptured follicle transforms into the corpus luteum, a transient endocrine structure that secretes progesterone and estrogen to support potential pregnancy (Devoto et al., 2009). If fertilization does not occur, the corpus luteum degenerates into the corpus albicans, a fibrotic remnant (Kirkendoll & Bacha, 2025).

Follicular atresia is a vital physiological process that eliminates most ovarian follicles before maturation, ensuring ovarian homeostasis and the selection of high-quality oocytes for reproduction (Hillier & Tetsuka, 1997; Wei et al., 2023). Occurring throughout a female's reproductive life, atresia is primarily driven by granulosa cell apoptosis, leading to follicular degeneration (Vaskivuo & Tapanainen, 2003). This process involves basement membrane disruption, immune cell infiltration, and eventual shrinkage and absorption of the follicle by the ovarian stroma (Irving-Rodgers et al., 2001). As a natural selection mechanism, atresia ensures that only the most viable follicles progress to ovulation, optimizing reproductive potential.

Signaling Pathways in Folliculogenesis: Focus on Steroidogenesis

Follicular growth is a highly complex and tightly regulated process essential for female reproduction (Fan & Chuva de Sousa Lopes, 2021). It involves the recruitment, development, and selection of ovarian follicles, leading to the maturation of a dominant follicle capable of ovulation (Quirk et al., 2004). This process is orchestrated by multiple intracellular signaling pathways, hormonal interactions, and local ovarian factors, ensuring proper folliculogenesis and oocyte development (Xie et al., 2023). The major pathways involved in follicular growth include the gonadotropin signaling pathway, PI3K/Akt pathway, mTOR pathway, Hippo signaling pathway, and TGF-β superfamily signaling (Clark et al., 2022; Devillers et al., 2023; Liu et al., 2022; Liu et al., 2006; Zhou et al., 2022). Among these, the steroidogenesis signaling pathway plays a crucial role in ensuring proper follicular development by regulating the synthesis of estrogens, androgens, and progestogens in response to hormonal cues (Zheng et al., 2023).

Steroidogenesis pathway also plays a fundamental role in ovarian folliculogenesis, supporting oocyte maturation, granulosa cell differentiation, and overall follicle viability (Chakraborty et al., 2021). Within the ovarian follicle, steroid hormones are primarily synthesized by two key cell types: granulosa cells (GCs) and theca cells (TCs). GCs and TCs are two essential somatic cell types within the ovarian follicle that work together to support folliculogenesis and oocyte maturation. GCs surround the oocyte and respond to FSH, promoting cell proliferation and converting androgens into estrogens via the enzyme aromatase. TCs, located outside the granulosa layer, respond to LH and produce androgens from cholesterol, which are then used by granulosa cells

for estrogen synthesis (Kotsuji & Tominaga, 1994). This cooperative interaction, known as the two-cell, two-gonadotropin model, is critical for proper hormonal signaling, follicular growth, and the development of a healthy oocyte capable of ovulation and fertilization (Ben-Chetrit et al., 1996).

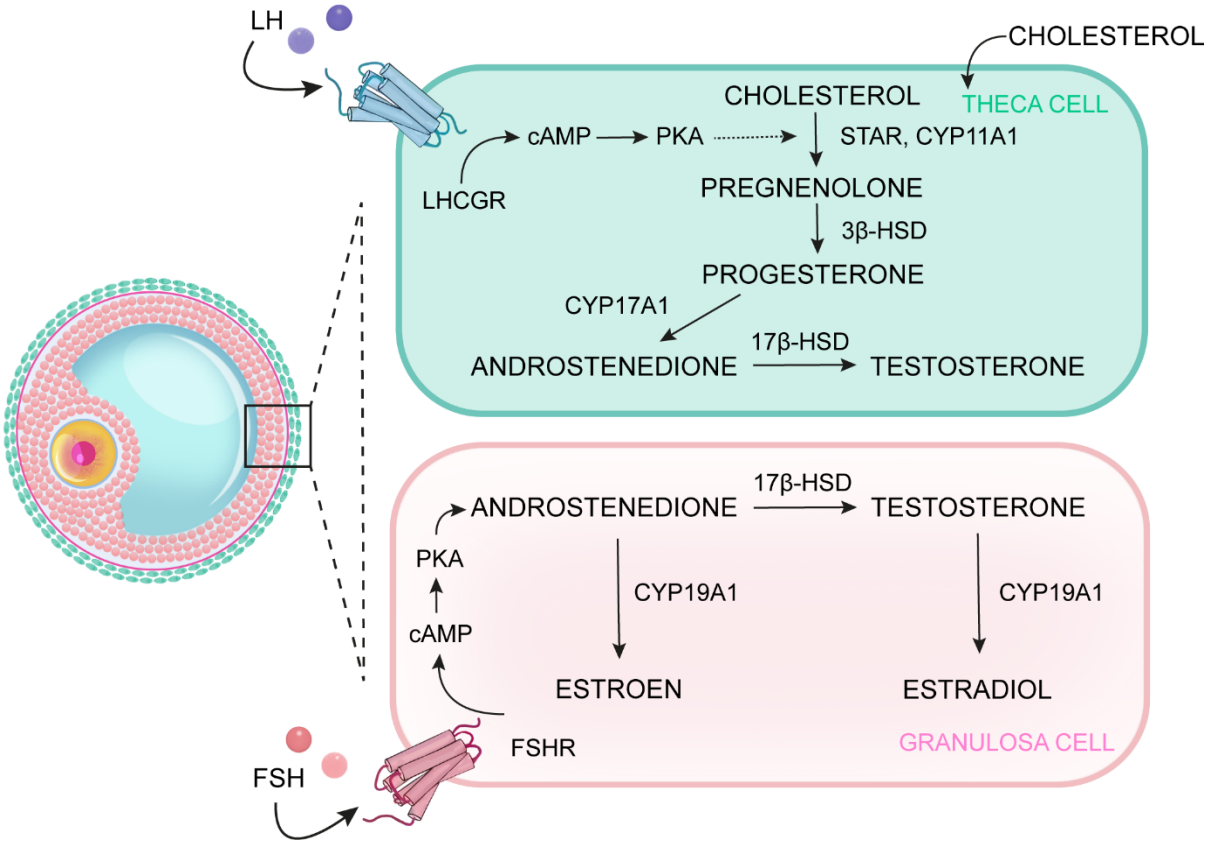


Figure 3. Ovarian steroidogenesis: two cell, two-gonadotropin theory. The figure is adapted from Jozkowiak et al., 2022. Ovarian steroidogenesis begins with cholesterol, which enters the theca cells from the circulation and is transported into mitochondria under the control of STAR. Binding of LH to its receptor (LHCGR) enhances the expression of enzymes required for androgen biosynthesis. Cholesterol is first converted to pregnenolone by CYP11A1, and then, within the smooth endoplasmic reticulum, pregnenolone is transformed into progesterone through the action of 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone is subsequently metabolized by CYP17A1 to androstenedione, which can either be reduced to testosterone by 17β-HSD or transferred into granulosa cells. In granulosa cells, aromatase (CYP19A1) catalyzes the conversion of androstenedione to estrone and testosterone to estradiol. Estrone may also serve as a substrate for 17β-HSD to produce estradiol.

This coordinated process, begins when LH stimulates theca cells to convert cholesterol into progestogens, and convert progestogens into androgens (such as androstenedione and testosterone) through a series of enzymatic steps involving STAR (Steroidogenic Acute Regulatory Protein), CYP11A1 (cholesterol side-chain cleavage enzyme), and CYP17A1 (17α-hydroxylase/17,20-lyase) (Chakraborty et al., 2021). These androgens then diffuse into adjacent granulosa cells, where FSH upregulates the expression of aromatase (CYP19A1), allowing the conversion of androgens into estrogens—mainly estradiol (E2). Estradiol, in turn, supports granulosa cell

proliferation, oocyte growth, and follicular antrum formation (Kobayashi et al., 2006). The expression of STAR in granulosa cells, which is typically absent in early-stage follicles, becomes upregulated as follicles mature or under certain in vitro conditions. However, premature or ectopic expression of STAR—especially in culture—may indicate abnormal or dysregulated steroidogenic activity, potentially disrupting normal follicular development and oocyte competence (Cheng et al., 2024).

Thus, proper regulation of the steroidogenesis pathway is essential for maintaining a physiological hormonal milieu during folliculogenesis. Any alterations in this pathway, such as early STAR activation or imbalance in hormone synthesis, may compromise follicular function and oocyte quality, particularly in in vitro systems where external signals may not fully replicate the ovarian environment.

Female Fertility Preservation

Female fertility preservation is an emerging field in reproductive medicine aimed at safeguarding reproductive potential for individuals who may face infertility due to aging, medical treatments, or other factors (Jadoul et al., 2010). Fertility preservation strategies are particularly crucial for women undergoing gonadotoxic treatments such as chemotherapy and radiation therapy, as well as those with conditions like premature ovarian insufficiency (POI), endometriosis, or autoimmune diseases (Anderson et al., 2020; Antunes et al., 2023). Additionally, social and elective fertility preservation is increasingly sought after by individuals who wish to delay childbearing due to personal, professional, or medical reasons (Cheng et al., 2024).

The biological basis of female fertility preservation is rooted in the finite ovarian reserve, as women are born with a limited number of oocytes that progressively decline in both quantity and quality with age (Mulder et al., 2021). Unlike men, who continuously produce sperm throughout life, female fertility is constrained by follicular depletion and age-related chromosomal abnormalities, leading to reduced reproductive potential after the mid-30s (Colmorn et al., 2021).

Several techniques have been developed to preserve fertility, including oocyte cryopreservation (egg freezing), embryo cryopreservation, OTC, and ovarian suppression through gonadotropin-releasing hormone (GnRH) analogs (Taylan & Oktay, 2019). Among these, oocyte vitrification is the most widely used and clinically established method, allowing mature eggs to be frozen and later used in assisted reproductive technologies (ART) such as in vitro fertilization (IVF) (Da Luz et al., 2022). Embryo cryopreservation, which involves fertilizing an egg before freezing, is another effective option, though it requires sperm and is often chosen by couples (Prades et al., 2011). Ovarian tissue cryopreservation, an experimental but promising technique, involves freezing and reimplanting ovarian tissue to restore endocrine and reproductive function (Bahroudi et al., 2022).

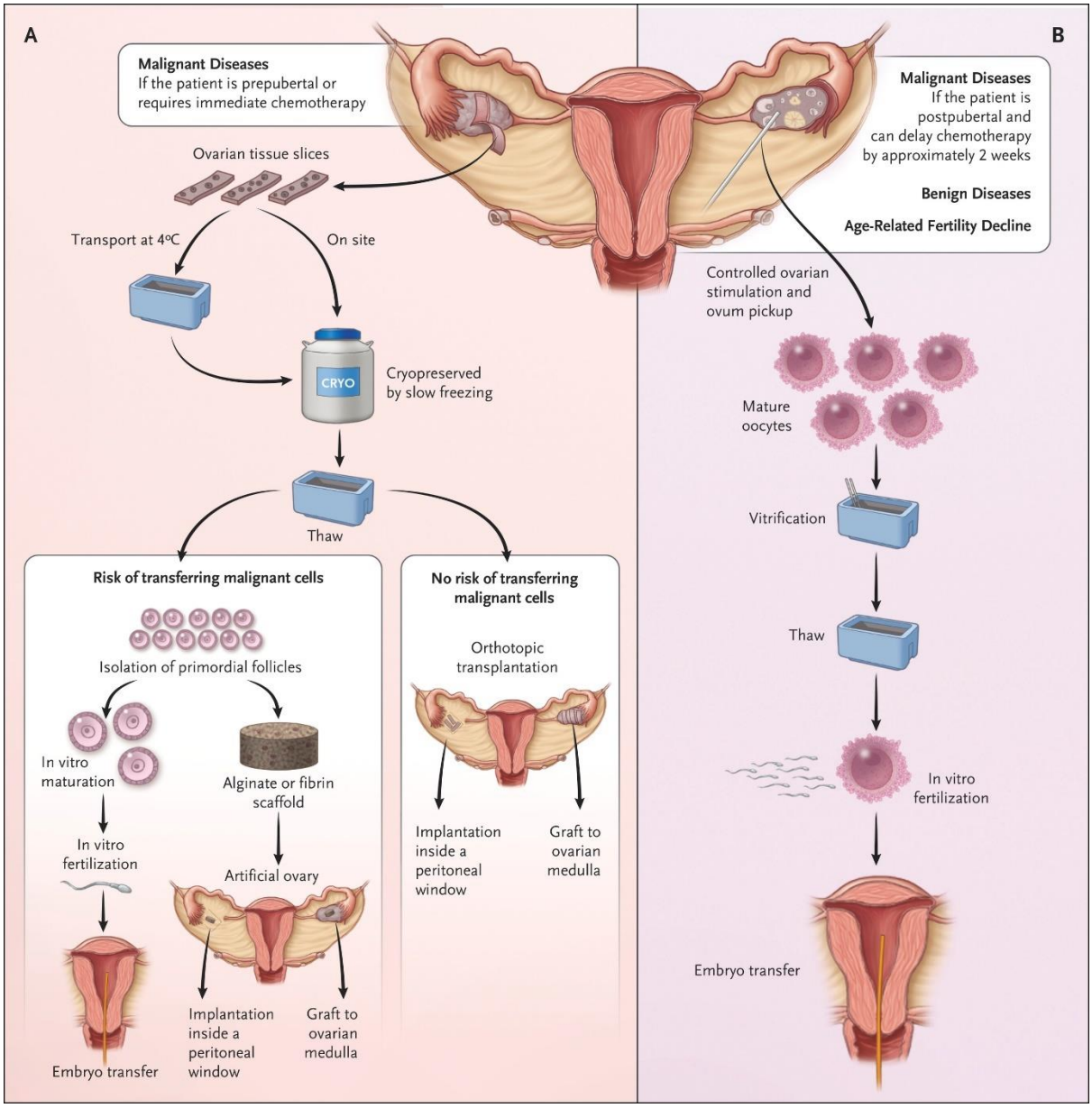


Figure 4. Options for female fertility preservation. The figure is from Donnez J et al., 2017. For prepubertal patients or those requiring urgent chemotherapy (Panel A), ovarian tissue can be collected either through multiple biopsies or removal of an entire ovary, which is then dissected into cortical strips. The tissue is cryopreserved on site using slow-freezing protocols or transported at 4 °C to a specialized facility for processing. Following thawing, if malignant cell transmission is not a concern, the tissue may be transplanted back—either into the ovarian medulla (when an ovary remains) or into a surgically created peritoneal pocket. In cases where malignant contamination is possible, individual ovarian follicles may be isolated and cultured in vitro until mature oocytes are obtained, which can subsequently be fertilized and transferred to the uterus. Alternatively, isolated follicles can be encapsulated within biomaterial scaffolds (alginate or fibrin) to generate an “artificial ovary” suitable for grafting to the ovarian medulla or peritoneal site. For postpubertal patients in whom chemotherapy can be postponed for about two weeks (Panel B), controlled ovarian stimulation can be performed to retrieve

mature oocytes, which are vitrified immediately. Upon thawing, these oocytes may be fertilized and transferred as embryos. This strategy is also applicable to women with nonmalignant conditions or age-related fertility decline. In certain cases, the two approaches (Panels A and B) may be combined, with ovarian-tissue cryopreservation followed by stimulation and vitrification of mature oocytes.

Ovarian Tissue Cryopreservation: Clinical Applications and Limitations

OTC is an advanced fertility preservation technique that involves the surgical removal, freezing, and potential reimplantation of ovarian cortical tissue (Bahroudi et al., 2022). Unlike oocyte or embryo cryopreservation, which requires ovarian stimulation and mature egg retrieval, OTC can be performed without hormonal stimulation, making it a viable option for individuals who require urgent fertility preservation (Hončová, 2023). This technique is particularly beneficial for prepubertal cancer patients who are not yet producing mature oocytes and transmasculine individuals who may wish to preserve their reproductive potential before undergoing gender-affirming medical or surgical treatments (Cheng et al., 2024).

For cancer patients, gonadotoxic treatments such as chemotherapy and radiation therapy pose a significant risk of POI and infertility. OTC offers a fertility preservation strategy that can be performed before cancer treatment, allowing for the later restoration of ovarian endocrine function and reproductive potential. Once the patient is ready to conceive, the cryopreserved tissue can be transplanted back into the body, where it may resume follicular activity and allow natural conception or ART (Fraison et al., 2023).

In transmasculine individuals, gender-affirming hormone therapy can suppress ovarian function, and gender-affirming surgeries, such as hysterectomy and oophorectomy, may result in irreversible loss of fertility. OTC provides an opportunity for fertility preservation before the initiation of medical or surgical transition.

Despite its promising applications, ovarian tissue cryopreservation remains an evolving technique with ongoing research focused on optimizing tissue viability, follicular activation, and artificial maturation strategies (Kolibianaki et al., 2020). Ethical considerations, including autonomy and informed consent, are particularly relevant for young cancer patients and transmasculine individuals, necessitating a patient-centered approach in reproductive counseling (Anderson et al., 2020). As advancements continue, OTC is emerging as a critical fertility preservation method that expands reproductive choices for individuals facing medical and gender-related fertility challenges.

In Vitro Culture of Female Ovarian Cortical Tissue

In vitro culture (IVC) of female ovarian cortical tissue is an innovative approach in reproductive medicine aimed at supporting the growth and maturation of ovarian follicles outside the body (Cheng et al., 2024). This technique is particularly significant for fertility preservation in individuals who are unable to undergo traditional ART, such as prepubertal cancer patients and those with ovarian insufficiency (Del Valle et al., 2022). By providing an artificial environment that mimics the physiological conditions of the ovary, IVC enables the activation, growth,

and maturation of follicles from cryopreserved or fresh ovarian tissue, potentially leading to the generation of viable oocytes for fertilization (McLaughlin et al., 2018; Xu et al., 2021).

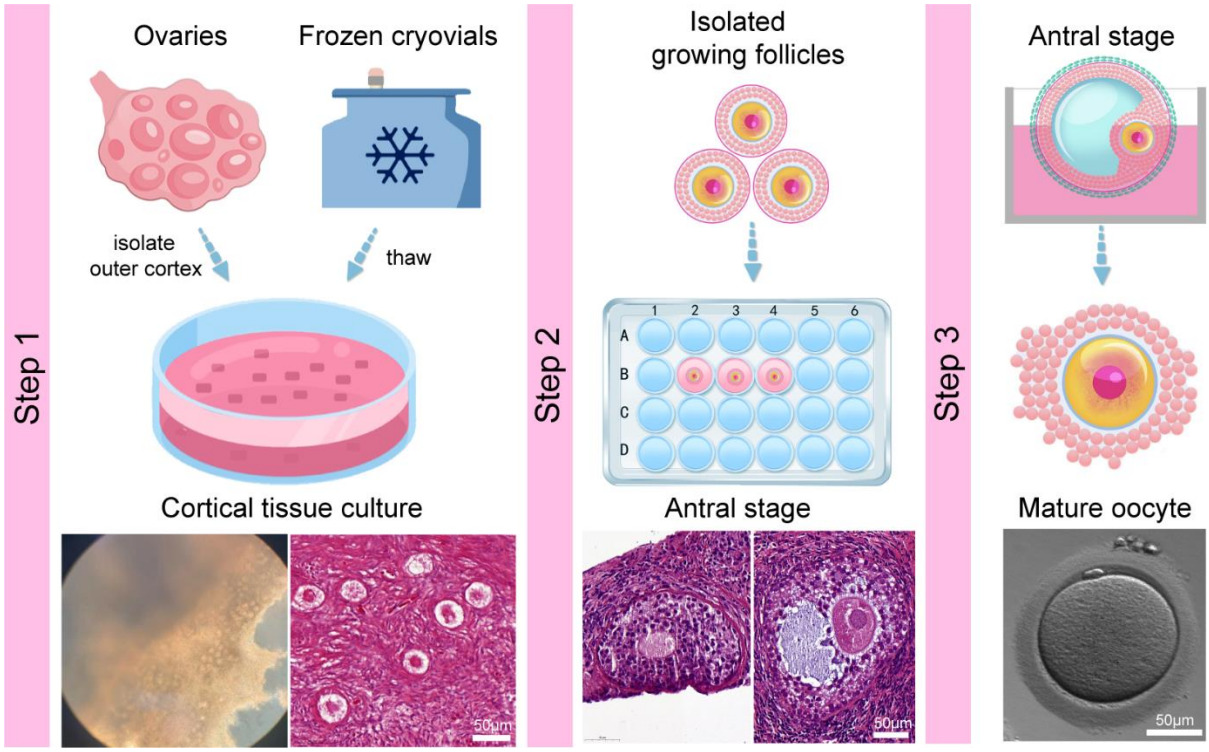


Figure 5. Multi-step in vitro culture system supporting the growth (IVG) and maturation of human primordial follicles into oocytes. Step 1: Freshly isolated or cryopreserved–thawed ovarian tissue is cultured in a free-floating medium. The lower panels show histological analysis of ovarian tissue at Day 0. Once follicles reach the multilaminar stage, they are mechanically isolated using fine needles. Step 2: The isolated follicles are cultured individually for 8 days, supporting their progression from the preantral to the antral stage. Representative HE staining illustrates the secondary and antral follicles obtained at this step. Step 3: To achieve the final stages of oocyte growth and maturation, the oocyte–cumulus complex is retrieved from the antral follicle and cultured together with its surrounding granulosa cells. These complexes are then placed in maturation medium for in vitro maturation (IVM). The resulting oocytes are subsequently assessed for meiotic competence, as indicated by the presence of a metaphase II spindle and a polar body.

The ovarian cortex, which houses the primordial follicle pool, serves as a primary target for in vitro culture (Gougeon, 2010). However, successful follicular development in vitro remains a challenge due to the complex interplay of endocrine, paracrine, and autocrine signals required for folliculogenesis (Telfer, 2019a). Early-stage follicles must progress through key developmental phases, including primordial follicle activation, granulosa and theca cell proliferation, antrum formation, and meiotic maturation of the oocyte (Telfer, 2019b). To support this process, in vitro culture systems utilize specialized media enriched with growth factors, gonadotropins, cytokines, and extracellular matrix components, which aim to replicate the ovarian microenvironment.

The in vitro development of oocytes from unilaminar follicles to the metaphase II (MII) stage remains a significant challenge, with only two studies (McLaughlin et al., 2018; Xu et al., 2021) having successfully reported this using a multi-step culture system. However, the developmental competence of these MII oocytes—specifically, their ability to be fertilized and progress to pre-implantation embryos—has yet to be assessed. Moreover, these protocols have only been applied to freshly obtained ovarian cortex tissue from adult cisgender female donors. In contrast, ovarian tissue preserved for fertility purposes, whether from cisgender females or transmasculine individuals, is typically cryopreserved. Therefore, optimizing in vitro culture systems for use with cryopreserved ovarian cortex tissue is critical for advancing future clinical applications.

Recent innovations, including three-dimensional (3D) culture systems, biomimetic scaffolds, and organ-on-a-chip technologies, have shown promise in improving follicular survival and maturation rates. These advancements bring in vitro follicle culture closer to clinical translation (Cheng et al., 2024; Del Valle et al., 2022; McLaughlin et al., 2018; Xu et al., 2021). Successfully maturing oocytes in vitro from ovarian cortical tissue could open new fertility preservation pathways for cancer survivors, individuals with POI, and transgender individuals seeking non-invasive reproductive options.

Despite its potential, several challenges remain, including optimizing culture conditions, ensuring oocyte quality, and reducing risks of chromosomal abnormalities (McLaughlin et al., 2018; Xu et al., 2021). Further research is needed to refine culture protocols and enhance the efficiency of in vitro folliculogenesis. As advancements continue, the in vitro culture of ovarian cortical tissue holds promise for expanding reproductive options and improving fertility preservation strategies in diverse patient populations.

Scope and Outline of the Thesis

Fertility preservation has become a vital field in reproductive medicine, providing individuals at risk of premature ovarian failure, infertility, or reproductive aging with the opportunity to retain their reproductive potential (Oktay et al., 2018). Among fertility preservation strategies, ovarian tissue cryopreservation (OTC) has emerged as a promising approach. OTC enables the restoration of both endocrine function and fertility by transplanting cryopreserved ovarian tissue back into the patient (Anderson et al., 2020; "Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion," 2019). To date, this technique has resulted in over 200 live births worldwide, demonstrating its viability for prepubertal girls, cancer patients, and individuals undergoing gonadotoxic treatments (Fraison et al., 2023). However, despite its success, several challenges remain, including low follicular survival rates, limited long-term ovarian function post-transplantation, and the potential risk of reintroducing malignant cells in cancer patients (Bastings et al., 2013; Rosendahl et al., 2013). Additionally, autologous transplantation is not a viable option for trans masculine individuals. From both a social and medical perspective, developing clinical protocols that support efficient follicular growth and maturation in vitro—resulting in functional oocytes for use in assisted reproduction—would be highly beneficial, as it could eliminate the need for ovarian tissue transplantation.

Significant progress has been made in the in vitro culture of human ovarian cortical tissue, with studies demonstrating successful early follicular activation. However, most current systems fail to sustain growth beyond the secondary follicle stage, preventing the development of fully mature oocytes (McLaughlin et al., 2018; Xu et al., 2021). Even when follicles develop, the resulting oocytes often exhibit poor developmental competence, limiting their potential for in vitro fertilization (IVF). Furthermore, most research has been conducted using fresh ovarian tissue from cisgender women, making it challenging to translate these findings into clinical applications for diverse patient populations. To address these gaps, **this thesis aims to characterize ovarian and follicle structure across different life stages, providing a comprehensive understanding of their morphological, cellular, and functional characteristics. Additionally, this work seeks to advance culture conditions, follicular activation strategies, and tissue engineering approaches to improve follicular survival and oocyte quality.** However, long-term follicular survival, oocyte maturation, and clinical translation remain significant hurdles. Continued research and technological innovations will be essential for developing safe and effective protocols, ultimately expanding reproductive options for individuals facing fertility challenges.

In **Chapter 2**, we investigated the development of multilaminar follicles from cultured human ovarian cortical tissue, comparing samples from child and adult donors. Our findings demonstrated that the in vitro culture system preserves early follicular architecture and supports granulosa cell proliferation in both age groups, allowing partial follicular maturation. However, limited theca cell support and reduced expression of late differentiation markers indicate that the system remains insufficient to achieve full functional maturation. These results provide an important foundation for refining culture conditions and advancing both fertility preservation strategies and in vitro folliculogenesis models.

In **Chapter 3**, we classified ovaries from different donors based on various characteristics. It is well known that individual ovaries vary in size, shape, and morphology, and each of these factors can influence follicle quality. Based on these variations, we classified the ovaries into four types and discovered that while size and shape did not significantly impact follicle quality, the presence of blood and the number of antral follicles did. This classification provides clinicians with valuable insights when considering ovarian cortex tissue transplantation for patients.

In **Chapter 4**, we evaluated different culture conditions to allow follicular growth. Two different publications have reported follicle growth to maturation starting from fresh-collected human adult ovaries (McLaughlin et al., 2018; Xu et al., 2021). We have compared the efficiency of the two published culture-media to culture fresh/cryopreserved ovaries of transgender and cryopreserved ovaries of cisgender donors. The main conclusion in this chapter is Telfer's medium was superior to Xu's medium, as indicated by both HE staining and IF staining in the three type donors.

Base on **Chapter 4**, we selected the best culture condition used in the **Chapter 5** and tested different components to enhance the quality of the secondary follicles further. We identified one component (CYP19A1 inhibitor) that improved quality and increased the percentage of secondary follicles. We used markers for granulosa cells (KRT19, STAR, CYP17A1, CYP19A1) and oocytes (ZP3) to assess the quality. Additionally, we

perform Stereo-seq analysis to compare the gene expression of antral follicle and corpus luteum. The main conclusion of this chapter is the CYP19A1 inhibitor had no impact on cisgender donors, but they were effective in increasing the quantity of follicles in transgender donors.

In **Chapter 6**, we collected the data from Chapter 4 with the components that did not influence follicular growth, we use the HE and IF staining to evaluate the quality of the follicles, highlighting the need for caution when people selecting such components in research or application in clinic.

In **Chapter 7**, we discuss the main findings of this thesis, and potential novel targets for further research aimed at enhancing our understanding of the folliculogenesis in the ovary. We expect that these studies will create new opportunities for advancing clinical application of fertility preservation and approaches that promote ovarian cortical tissue culture in vitro.

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