Decoding the molecular makeup of the human ovary through single-cell transcriptomics

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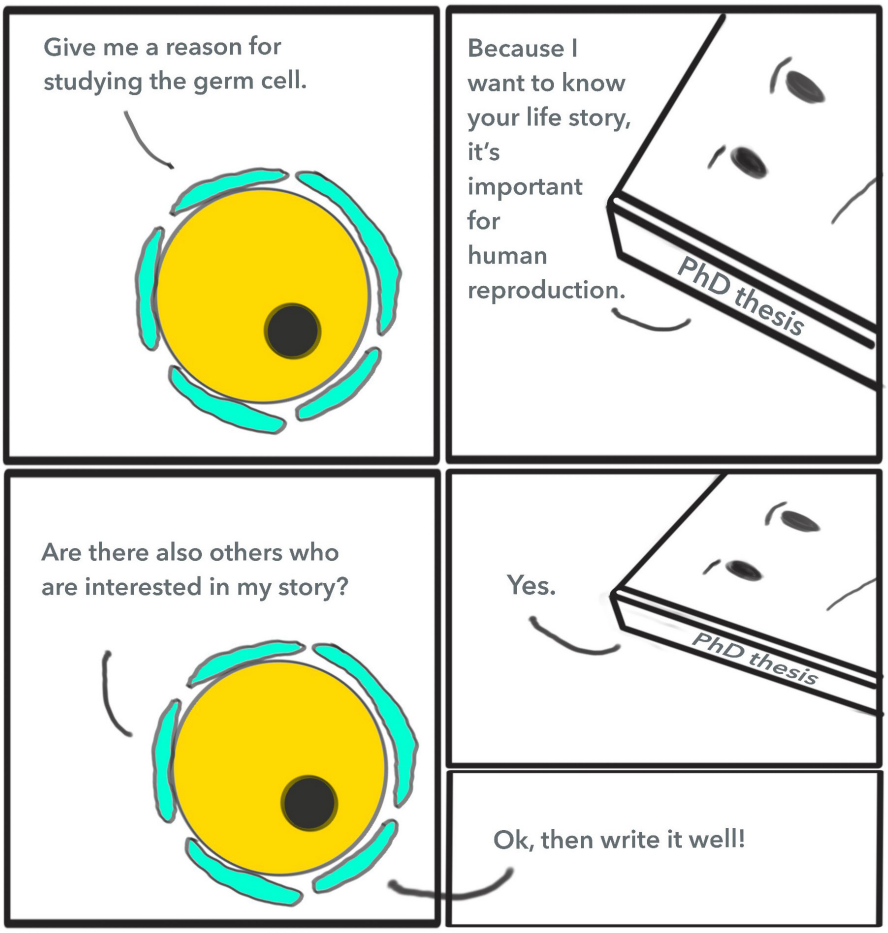
Give me a reason for studying the germ cell.
Because I want to know your life story, it's important for human reproduction.
PhD thesis
Are there also others who are interested in my story?
Yes.
PhD thesis
Ok, then write it well!

Chapter 1

General Introduction and thesis outline

Germ cell and ovarian follicular development: the foundation of human female reproduction

1. Sexual reproduction, germline and the ovary

Sexual reproduction is a common feature of multicellular eukaryotic species, involving the fusion of two haploid gametes to transmit genetic information between generations. In contrast, solely asexual reproduction is rare. From an evolutionary standpoint, sexual reproduction has advantages over asexual reproduction because it introduces novel genetic compositions, thereby allowing for faster rates of adaptation to the environment (Bai, 2015; Ramos and Antunes, 2022; Roze, 2012). The process of sexual reproduction consists of three key events: meiosis, sex differentiation and fertilization (Bai, 2015) (**Figure 1**). Meiosis involves the reduction of the number of chromosomes to half in order to give rise to haploid gametes. Sex differentiation allows haploid gametes to be labeled by sex, which encourages mating between individuals with different traits. Finally, fertilization allows two haploid gametes with different sexes to fuse, resulting in the formation of a new diploid zygote and an update to the genetic information. In multicellular organisms, the germline is responsible for completing this sexual reproduction cycle. Thus, the success of germline development is important for the reproduction of species.

Sexual reproduction in humans relies on specialized reproductive organs, including the ovaries, fallopian tubes, uterus and vagina in females and the testes, epididymis, vas deferens, seminal vesicles, the prostate, bulbourethral glands and the penis in males. Among the reproductive organs of females, the ovary plays a crucial role in germline development, as germ cells reside and mature into competent eggs within the ovary (Oktem and Oktay, 2008). The number of potential eggs that a human female possesses is determined before birth and decreases with age. Therefore, the ovarian reserve of germ cells and the overall health of the ovary are critical factors that affect the female reproductive lifespan.

2. Development of human primordial germ cells

PGC specification and migration

Based on current understanding in humans, the initial germline cell population—primordial germ cells (PGCs) is induced within a developmental window preceding the formation of the three germ layers during gastrulation (Hancock et al., 2021; Ohinata et al., 2005) (**Figure 1**). The timing and mechanisms of human PGC specification in vivo are not yet fully understood. However, the presence of PGCs can be detected around embryonic day (E) 12 by culturing blastocysts in vitro (Chen et al., 2019; Popovic et al., 2019). Around E24, PGCs are present in the dorsal wall of the yolk sac, close to the allantois (Mamsen et al., 2012; Møllgård et al., 2010) then they migrate via the dorsal mesentery of the hindgut to the developing genital ridge around 4-5 weeks post fertilization (WPF) (Fernandes et al., 2018; Hummitzsch et al., 2015; Mamsen et al., 2012; Møllgård et al., 2010). PGCs undergo mitosis during migration and keep proliferating following the arrival in the genital ridge (De Felici et al., 2004). Around 5 WPF (~E36), when PGCs arrive in the genital ridge, coelomic epithelium covering the intermediate mesoderm begins to thicken and develop into

the gonad. A clearly distinguishable gonad structure can be recognized from the neighboring mesonephros by 6 WPF (~E42) (Yang et al., 2019). Both PGCs and the initial gonad are bipotential, and show no sexual differences until 6 WPF (**Figure 1**).

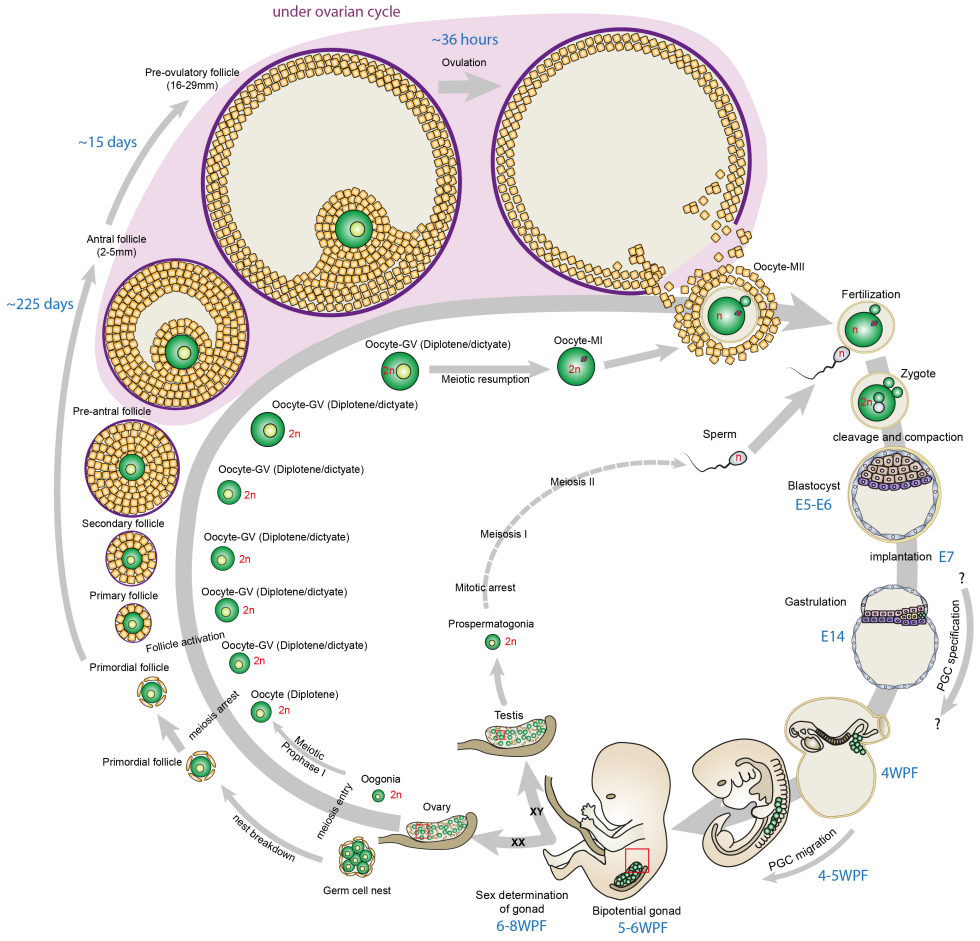

Figure 1. Cycle of human female germline development. The figure is drawn based on Figure 1 from Tang *et al.*, 2016. PGC, primordial germ cell; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; WPF, weeks post fertilization; E, embryonic day; n, haploid; 2n, diploid.

During migration, human PGCs retain pluripotent stem cell potential. Part of them may differentiate into somatic cells or form teratomas (Mamsen et al., 2012; Nicholls and Page, 2021). However, after reaching the genital ridge, PGCs upregulate the expression of germ cell markers such as DAZL and DDX4 while suppress pluripotency factors, such as POU5F1 and NANOG (Overeem et al., 2021; Wen and Tang, 2019). It is worth noting that these processes are asynchronous in humans. In females, PGCs located in the periphery of the ovaries tend to maintain POU5F1

expression until the third trimester, while those in the central region gradually begin to express DDX4 after arrival in the ovary (Anderson et al., 2007; Jrgensen and Rajpert-De Meyts, 2014; Stoop et al., 2005). Although low expression of DDX4 can be observed in POU5F1+ PGCs around 7-8 WPF in the gonad, POU5F1-DDX4+ PGCs are typically detected in the ovary around 9 WPF (Motta et al., 1997; Overeem et al., 2021). The POU5F1-DDX4+ PGCs are committed to gametogenesis, known as "oogonia" in female ovaries and "prospermatogonia" in male testes (Nicholls et al., 2019; Nicholls and Page, 2021; Overeem et al., 2021) **(Figure 1)**.

Gonadal sex determination and sex differentiation of PGCs

The sex differentiation of gonads from the bipotential state is driven by gonadal somatic cells. Prior to sex determination, female somatic cells in the bipotential gonad express WNT4 and RSPO1, thereby priming toward the female fate (Jameson et al., 2012). While in male gonads, the expression of a specific Y chromosome gene—*SRY* between E40-E44 initiates up-regulation of a series of genes, including *SOX9*, *FGF9* and *AMH*. These genes are associated with the differentiation of male Sertoli cells, a testis-specific cell type that supports spermatogenesis (Hanley et al., 2000; Mamsen et al., 2017; Yang et al., 2019). Expression of *SRY* also results in the suppression of the WNT4/RSPO1/CTNNB1 signaling pathway, which prevents the further development of the female reproductive system and controls the organization of testis cords that eventually develop into seminiferous tubules (Eggers and Sinclair, 2012; Mamsen et al., 2017). In XX females, the absence of the inhibitory effect of *SRY* allows CTNNB1 to activate FOXL2 expression in gonadal somatic cells, leading to the differentiation of granulosa cells, which support oogenesis, and thus, promote ovary development (Rotgers et al., 2018).

Interestingly, studies based on mouse models suggest that sex determination of PGCs depends on signals from sexually committed gonadal environments rather than sex chromosomes (Rios-Rojas et al., 2015). In female gonads, oogonia enter meiosis triggered by retinoic acid (RA) and bone morphogenetic proteins (BMPs), such as BMP2, BMP4, BMP5 and BMP7, which are produced by the mesonephros and gonadal somatic cells, respectively (Hamada et al., 2020; Koubova et al., 2006; Miyauchi et al., 2017). Conversely, male Sertoli cells prevent prospermatogonia from entering meiosis by producing the hydroxylase CYP26B1 to degrade RA, and also by suppressing the expression of WNT4 and BMPs (Bowles et al., 2006; Spiller and Bowles, 2022). Instead, prospermatogonia stop proliferating and arrest in the G0/G1 stage of the mitotic cell cycle (Shimada et al., 2021). It has been shown that alteration of the gonadal environment towards the other sex can initiate meiosis in XY germ cells and mitotic arrest in XX germ cells (Adams and McLaren, 2002; Koopman et al., 1991; Zhang et al., 2022).

3. From oogonia to oocytes

Meiosis entry

Around 11 WPF, a subset of oogonia in the ovary begins to express *STRA8*,

which is a key target of RA in regulating meiotic entry (Wang et al., 2022). Studies in mice have demonstrated that RA induced expression of *STRA8* and the cohesin component *REC8*, along with other factors, activates transcription of meiotic and cell cycle genes in oogonia (Ishiguro et al., 2020; Koubova et al., 2014). However, the activation of *STRA8* expression is not solely mediated by RA, as the removal of repressive epigenetic marks from the promoter of *STRA8* allows it to respond to RA (Lowe et al., 2022; Lundgaard Riis and Jørgensen, 2022; Spiller and Bowles, 2022; Vernet et al., 2020). A more comprehensive model regarding sexual dimorphism on meiosis entry remains to be established with future studies.

Meiotic prophase I

Following the last round of mitotic division, oogonia enter G1 phase of the meiotic cell cycle, then replicate the whole genome during the pre-meiotic S phase, and finally enter the meiotic prophase I. Meiotic prophase I is divided into five sub-phases: leptotene, zygotene, pachytene, diplotene and diakinesis (Page and Hawley, 2003). At approximately 12 WPF, some oogonia begin meiosis and progress until they reach the diplotene stage, where they become arrested and eventually develop into primary oocytes (Motta et al., 1997) (**Figure 2**). The resting stage of diplotene before diakinesis is also referred to as dictyate, which can last for years or decades until pre-ovulation (Cohen et al., 2006; TSAFRIRI and KRAICER, 1972). Meiotic prophase I is a crucial stage during which chromosomal crossover (also known as crossing over) occurs. This process involves the exchange of genetic material between maternal and paternal chromosomes, resulting in the creation of new gene combinations. Chromosomal crossover is the primary factor that drives genetic diversity in haploid gametes by enabling the production of a unique set of genes.

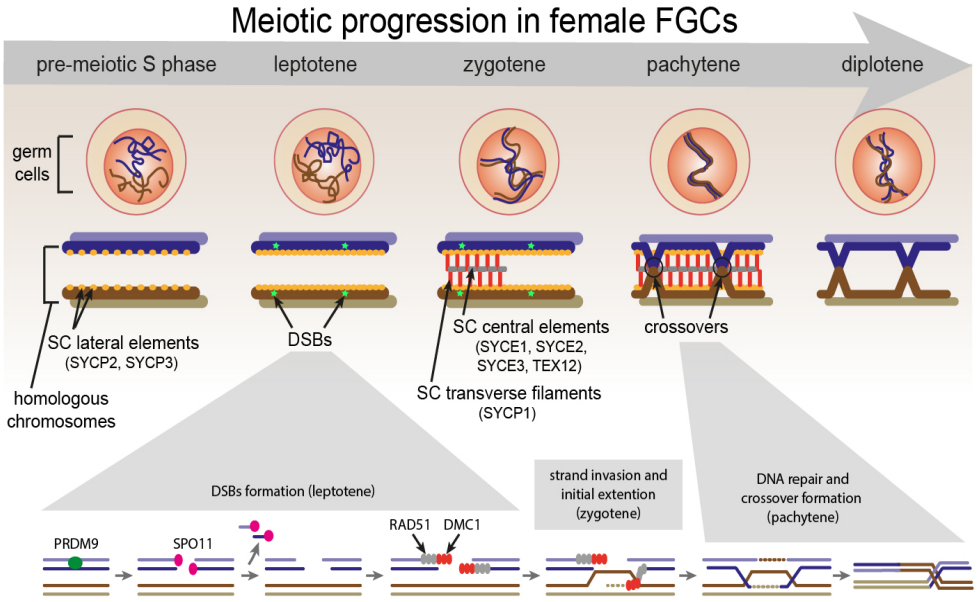

Figure 2. Sub-phases of meiotic prophase I and chromatin dynamics in female fetal germ cells. FGCs, fetal germ cells; SC, synaptonemal complex; DSBs, DNA double strand breaks.

Chromosomal crossover requires the pairing of homologous (maternal and paternal) chromosomes, which is termed "synapsis" (Cohen et al., 2006). During synapsis, two homologous chromosomes are physically tethered together and aligned with each other by a zipper-like structure—synaptonemal complex (SC) (**Figure 2**). The SC consists of three elements: lateral elements that coat the chromosome axes, transverse filaments that create the bridge to link two lateral elements, and central elements that locate in the center of the SC (Gao and Colaiácovo, 2018; Heyting, 1996) (**Figure 2**). To form the bivalent (paired homologous chromosomes that are tethered together for crossovers), lateral elements are loaded along each chromatid, followed by the assembly of transverse filaments and central elements. In mammals, at least eight proteins have been identified as components of SC structure, including lateral element proteins SYCP2 and SYCP3, transverse filament protein SYCP1, and central element proteins SYCE1, SYCE2, SYCE3, TEX12 and C14orf39 (Dunne and Davies, 2019; Gao and Colaiácovo, 2018). In addition to synapsis, the generation and repair of DNA double strand breaks (DSBs) are also necessary steps to form crossovers (**Figure 2**). DSBs tend to occur in specific genomic regions, and these so called "hot spots" are positioned mainly by PRDM9, a zinc finger protein with histone methyltransferase activity (Diagouraga et al., 2018; Powers et al., 2016). To initiate DSBs, PRDM9 binds to DNA at hot spots and promotes the recruitment of the topoisomerase enzyme SPO11 to cleave DNA and produce resected 3′single-stranded DNA (ssDNA) tails (Paiano et al., 2020). DNA recombinases RAD51 and DMC1 subsequently bind to ssDNA tails and guide strand invasion to the homologous double strand DNA. Finally, by DNA synthesis using invaded strands as templates, the homologous chromatins form a crossover intermediate that is eventually resolved into crossovers through a DNA mismatch repair mechanism (Baudat et al., 2013; Gerton and Hawley, 2005; Manhart and Alani, 2016; Singh et al., 2021) (**Figure 2**).

Sub-phases of meiotic prophase I can be identified based on the progression of meiotic events, including the SC assembly, formation of DSBs, chromosome pairing, repair of DSBs, synapsis, and disassembly of SC. The lateral element proteins of the SC, including SYCP3, begin to accumulate as early as pre-meiotic oogonia. During leptotene, these proteins are loaded onto the stretched chromosome axes, while homologous chromosomes achieve parallel alignment by attaching and clustering telomeres to the nuclear envelope (Roig et al., 2004; Waters and Ruiz-Herrera, 2020; Tsai and McKee, 2011). Meanwhile, during leptotene, the genome-wide generation of DSBs (marked by γ-H2AX) and the chromosomal localization of DSB repair proteins (e.g., RAD51, DMC1) also start to take place (Baudat and De Massy, 2007; Hunter et al., 2001; Mahadevaiah et al., 2001; Roig et al., 2004). Homologous chromosomes initiate synapsis in zygotene, which is mediated by assembly of transverse filaments and central elements (e.g., SYCP1, SYCE3) (Fraune et al.,

2012; Schramm et al., 2011). Pachytene starts when SC assembly is complete and crossovers are formed between fully synapsed chromosomes (Roig et al., 2004). Subsequently, the homologous chromosomes segregate at non-recombination sites, and the SC dissolves in diplotene (**Figure 2**). The molecular study of these meiotic events and the formation of meiotic crossover was challenging due to limited techniques and models. Further studies with the application of new approaches, such as super resolution microscopy and high-throughput single-cell sequencing techniques, will extend our understanding of this field.

Primordial follicle formation and the ovarian reserve

In the fetal ovary, oogonia develop in clusters called germ cell nest and are surrounded by somatic cells, forming the ovarian cords. Oogonia within the same nest tend to be connected by intercellular bridges (Grive and Freiman, 2015; Motta et al., 1997). At around 16 WPF, some of the oocytes in the diplotene stage are encapsulated by a layer of granulosa cells, forming a structure known as the primordial follicle (Mizuta et al., 2022) (**Figure 1**). Studies in mice have proposed that granulosa cells and the oocyte establish adhesive contacts (e.g., E-cadherin), and communicate through signaling pathways (e.g., JNK, NOTCH, KIT) to facilitate the primordial follicle assembly (Grive and Freiman, 2015; Niu et al., 2016; Tingen et al., 2009; Wang et al., 2017; Xu and Gridley, 2013).

Once all oocytes have formed primordial follicles, the ovarian reserve constituted by these primordial follicles is non-renewable. During the period of establishing the ovarian reserve, germ cell loss is also observed. According to an estimation through histological quantification, the number of germ cells in the fetal ovary peaks at around 5 months (20 WPF) and then decreases. From the fifth month, studies have observed a degeneration of oogonia and oocytes displaying cellular abnormalities, such as: a swollen nuclei in oogonia and vacuolization of cytoplasm in oocytes. It is likely that the degeneration of germ cells is not only via apoptosis, as only a small number of apoptotic oocytes were detected by TUNEL assay in fetal ovaries between 17 and 31 WPF (Abir et al., 2002). Additionally, extrusion of germ cells onto the ovarian surface have been detected during fetal ovary development, which may also contribute to the germ cell loss (BAKER, 1963; Motta et al., 1997).

4. Oocyte growth and maturation

Parallel growth and degeneration of the oocyte and the follicle

Before resuming meiosis, primary oocytes undergo a process of growth and enlargement, increasing in diameter from 35 μm in primordial follicles to about 120 μm in pre-ovulatory follicles (Griffin et al., 2006) (**Figure 1**). The growth of the oocyte and the follicle in the ovary are interdependent, as the follicle is the fundamental unit that undergoes growth and degeneration as a whole.

The number of oocytes ovulated during each ovarian cycle varies among species. Unlike poly-ovulatory species, such as mice, rats and pigs, humans are mono-ovulatory and typically release only a single oocyte per cycle (Dalbies-

Tran et al., 2020). Although many growing follicles are able to be stimulated by gonadotropins at the same time, only one will become dominant for ovulation, and the rest undergo a process known as follicular atresia, which involves the shrinking and eventual disappearance of the follicle from the stroma of the ovary. Based on estimates, around 700,000 primordial follicles are present at birth, and this number decreases during childhood, leaving approximately 300,000 follicles by puberty (Kerr et al., 2013; Monniaux et al., 2014; te Velde, 2002; Wallace and Kelsey, 2010). Throughout a woman's reproductive lifespan, only approximately 500 oocytes will ovulate, and the remaining oocytes will be lost from the ovary through follicular atresia (Motta et al., 1997).

Meiotic maturation of oocytes

Oocytes can be categorized into three stages based on their meiotic progression states: germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) (**Figure 1**). This classification is commonly used in oocyte research and clinics to evaluate oocyte maturation. GV oocytes are recognized by a large vesicular structure of the nuclear envelope, which encloses a prominent nucleolus and chromosomes that are still arrested in diplotene of the prophase I (De La Fuente, 2006). Meiotic resumption starts with GV breakdown (GVBD), during which the nuclear envelope dissolves, followed by the assembly of microtubules within the nuclear region and their attachment to chromosome kinetochores. As the microtubules develop into barrel-shaped bipolar spindles, the chromosomes become individualized and align along the spindle equator, resulting in the MI stage. To ensure asymmetric division, the spindle migrates close to the oocyte cortex, pulls homologous chromosomes toward opposite poles, and leads to the extrusion of the first polar body (Holubcová et al., 2015; Swain and Smith, 2021). After completing the first meiotic division, the spindle disassembles and reassembles near the first division site to form the bipolar structure again with chromosomes organized on the equator, which represents the MII stage (Roeles and Tsiavaliaris, 2019) (**Figure 1**). Instead of proceeding to the second meiotic division, the oocyte is arrested in the MII stage and released from the ovary. The transition of oocytes from GV to MII in the ovary is triggered by a surge of luteinizing hormone (LH), and it takes roughly 36 hours from the onset of LH surge to ovulation (Williams and Erickson, 2000).

The MII oocyte (egg) enters the fallopian tube after ovulation. Only if fertilization occurs within a time window of up to 24 hours, the egg resumes meiosis and completes the second meiotic division by extruding the second polar body (Wilcox et al., 1998). Fertilization with sperm induces a rise in intracellular Ca^{2+} levels in the egg, which in turn triggers a series of events, including completion of meiosis, formation of the female pronucleus, decompaction and remodeling of paternal genome, and transition to the cleavage division (Clift and Schuh, 2013). Ultimately, fertilization completes the germ cell development cycle by allowing the egg to finish meiosis and develop into a totipotent embryo (**Figure 1**).

Oocyte competence

Not all MII oocytes are capable of being fertilized and developing successfully into the blastocyst stage. In addition to meiotic maturation, other factors, such as genomic, epigenetic and cytoplasmic maturation, are also crucial in determining the oocyte quality for fertility (He et al., 2021).

During follicular growth, GV oocytes undergo a genomic remodeling process that results in the change of chromatin configurations from a non-surrounded nucleolus (NSN) to a more condensed ring-shaped surrounded nucleolus (SN) state (De La Fuente, 2006). This transition has been linked to the suppression of oocyte transcriptional activity and the ability to resume meiosis in humans (Combelles et al., 2002; Miyara et al., 2003; Tan et al., 2009). Epigenetic modifications, such as histone methylation and deacetylation, are involved in the compaction of chromatins in GV-SN oocytes (Huang et al., 2012; Liang et al., 2012; Zhang et al., 2012). In a later section, we will introduce the epigenetic reprogramming during germ cell development in more detail.

It is well acknowledged that fertilization and early embryogenesis almost entirely rely on maternal cytoplasmic materials, such as mRNA, proteins and organelles, until the embryonic genome becomes functional (Bentsen et al., 2020; Lu et al., 2017). Studies using transmission electron microscopy indicate that during oocyte maturation from GV to MII stage, cytoplasmic changes involve the redistribution of organelles throughout the ooplasm, disintegration of the Golgi apparatus, deposition of cortical granules in the subcortical area, and the organization of mitochondria-endoplasmic reticulum complexes (Trebichalská et al., 2021). Additionally, the quantity and quality of oocyte mitochondria are linked to energy metabolism and oocyte competence, as the number of mitochondria, mitochondrial DNA (mtDNA) copy number, and mitochondrial ATP content increase with oocyte growth and maturation to MII stage (Jansen and De Boer, 1998; Zhang et al., 2017).

5. Ovarian follicles and follicular endocrine cells

Structure of the ovary and the ovarian follicle

The human ovary is a highly organized organ, consisting of two distinct regions arranged from the peripheral to the central part: the cortex and medulla (**Figure 3**). The cortex contains a layer of ovarian surface epithelium (OSE), a zone of connective tissue with lower cell density known as the tunica albuginea, and an inner connective tissue containing ovarian follicles at various stages and abundant stromal cells. On the other hand, the medulla region contains loose connective tissue, prominent blood vessels, lymphatic vessels and nerves (Kitajima et al., 2018). Primordial follicles are stored in the outer cortex region, which lies beneath the tunica albuginea. As follicles grow, they tend to locate in the inner cortex region, adjacent to the medulla. Following ovulation, the ovary develops the corpus luteum, which is a yellowish structure containing follicular cells that have undergone luteinization (**Figure 3**).

Ovarian follicles provide the microenvironment for the developing oocyte. The two main ovarian specific somatic cell types that are involved in construction of

follicles are granulosa cells (GCs) and stroma-derived theca cells (TCs). GCs are cells that surround the oocyte and are present since the primordial follicle stage. Outside of GCs there is a layer of basal lamina composed by extracellular matrix. TCs are eventually localized on the exterior of the basal lamina (Heeren et al., 2015) (**Figure 4**). These cells play indispensable roles in oocyte growth mainly in two ways: (1) They set up intercellular communications to promote the growth of the follicle and oocyte. For example, GCs secrete factors that maintain oocyte meiotic arrest and activate oocyte growth (Grosbois and Demeestere, 2018; He et al., 2021; Li and Albertini, 2013). (2) They are able to secrete and respond to hormones from the hypothalamic-pituitary-ovarian (HPO) axis.

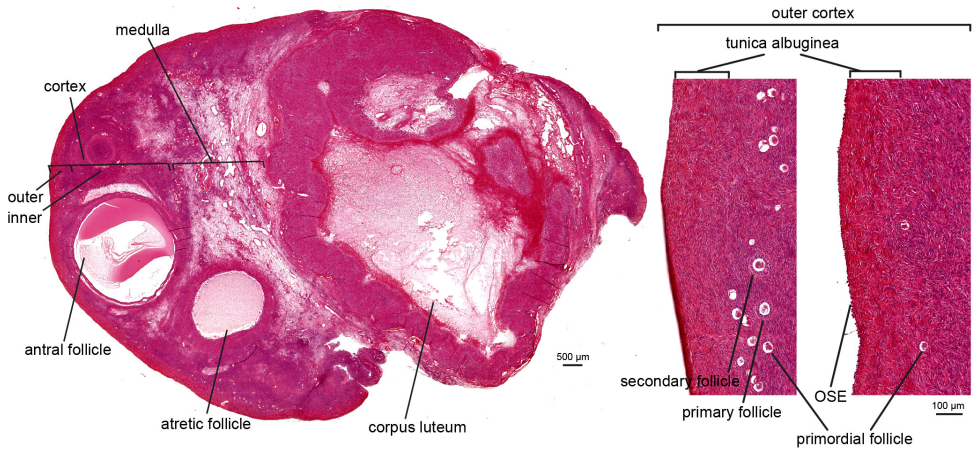

Figure 3. Structure of the human ovary. The figure is adjusted based on Figure 1 from Valle *et al.*, 2022. OSE, ovarian surface epithelium.

In addition to GCs and TCs, follicular growth also requires vasculature to provide nutrients, oxygen and hormone supplies. The vascular network is organized within TC layers, and the number and size of vessels increase as follicles expand (Brown and Russell, 2014). Leukocytes, namely macrophages have also been found to be present in theca layers of growing follicles, and they have been proposed to play multiple functions involved in follicular growth and atresia (Best et al., 1996; Wu et al., 2004) (**Figure 4**). In vivo, follicles grow as a whole with primary cells (oocyte, GCs and TCs) and supportive cells (e.g., vascular and immune cells). Although oocytes are the cells responsible for human reproduction, their survival is highly dependent on other components of the follicular microenvironment.

Granulosa cells

GCs are initially squamous epithelial-like cells when they enclose oocytes in primordial follicles. The OSE is believed to be the origin of squamous GCs during fetal stage, but it is still controversial whether the OSE is the exclusive

source of GCs, or if cells originating from the mesonephros can also give rise to GCs (Auersperg et al., 2001). Once dormant primordial follicles are activated, GCs change their morphology from squamous into cuboidal and undergo proliferation. As GCs continue to develop, two subtypes can be recognized by their spatial location and endocrine functions within antral follicles: mural and cumulus GCs (**Figure 4**). Mural GCs, which are located adjacent to the basal lamina, are responsible for estrogen synthesis and will eventually develop into hypertrophied granulosa lutein cells (lutein GCs) to produce progesterone after ovulation. Cumulus GCs are several layers of cells that are in contact with oocytes through gap junctions, jointly, they form a cumulus-oocyte complex (COC) that collaboratively regulates oocyte meiotic arrest and resumption (**Figure 4**) (Baena and Terasaki, 2019; Buccione et al., 1990). The establishment of distinct cumulus and mural GCs is morphologically based on the formation of the follicular antrum, which is a cavity full of fluid within the follicle. As the antrum enlarges, further molecular and functional distinctions between these two subtypes of GCs are thought to be driven by oocyte-derived paracrine factors (e.g., GDF9, BMP15) and gonadotropins (e.g., FSH) (Diaz et al., 2007; Eppig, 2001). The specific factors and mechanisms that are responsible for the differentiation of cumulus and mural GCs are yet to be fully resolved.

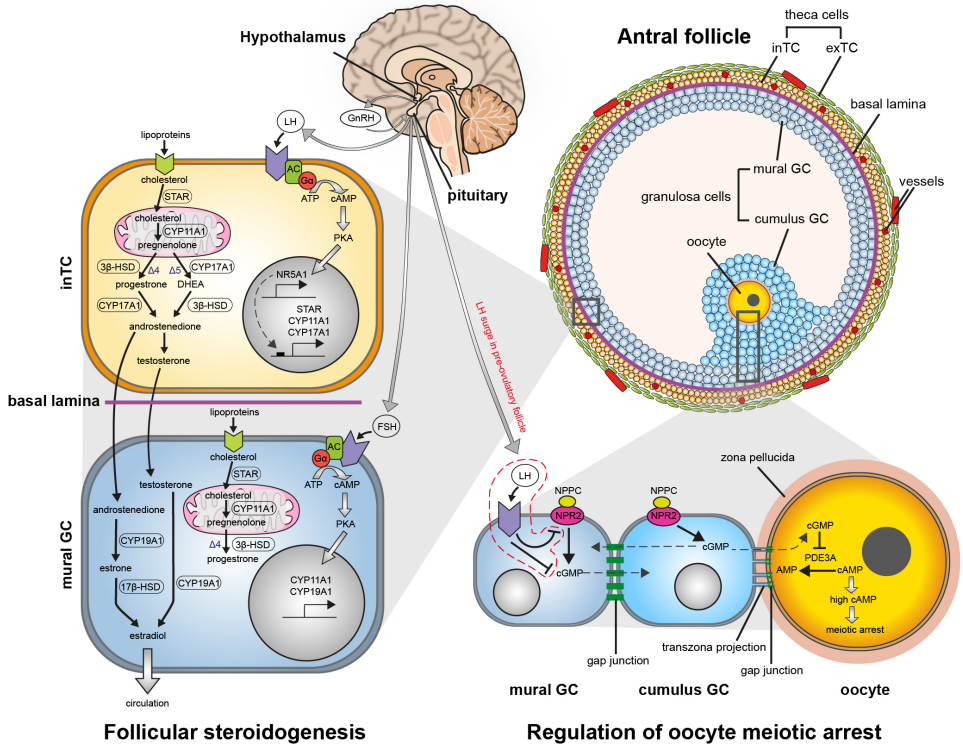

Figure 4. Roles of GCs and TCs in follicular steroidogenesis and oocyte meiotic resumption. inTCs are responsible for the synthesis of androstenedione and testosterone,

which can diffuse into GCs and be further converted into estradiol. The maintenance of meiotic arrest in oocytes depends on the level of cAMP, which is stabilized by diffusion of cGMP from GCs via gap junctions. An LH surge triggers meiotic resumption by disrupting the cGMP flow from GCs to the oocyte. GC, granulosa cell. inTC, theca interna cell; exTC, theca externa cell.

Intercellular communication between the oocyte and granulosa cells

GCs interact with the oocytes through gap junctions and juxtracrine/paracrine signaling factors (Eppig, 2018). Gap junctions between the oocyte and GCs are mainly composed of GJA4 (connexin-37) proteins, while GJA1 (connexin-43) proteins are detected between GCs. Gap junctional communication starts in the primordial follicles, and is maintained in growing follicles through the formation of transzonal projections (TZPs), which are specialized filopodia of GCs that can penetrate zona pellucida (El-Hayek et al., 2018) (**Figure 4**). Juxtracrine signaling transduction, which involves direct communication through cell surface molecules, is also involved in the regulation of follicular growth. For instance, the recognition of receptor Tyrosine Kinase KIT and its ligand KITLG between the oocyte and GCs is proposed to activate oocyte growth upon follicle awakening (Hutt et al., 2006; Vo and Kawamura, 2021). Regarding paracrine signaling transduction, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are the best defined oocyte factors that regulate GC activities (Knight and Glister, 2006; Chang et al., 2016). *In vitro* studies have shown that BMP15 and GDF9 can inhibit luteinization of GCs by downregulating StAR expression and progesterone production (Chang et al., 2013; Yamamoto et al., 2002). Additionally, heterodimers of BMP15 and GDF9 have been found to synergistically stimulate the proliferation of GCs in humans (Mottershead et al., 2015; Peng et al., 2013; Sanfins et al., 2018). Exploring cellular interactions within the follicle is challenging due to the complex and dynamic nature of the follicular microenvironment. However, utilizing high-throughput molecular profiling techniques on the follicle could provide valuable insights.

Theca cells

Compared to studies on GCs, TCs have received much less attention. TCs are not initially present in primordial follicles, but are recruited from the ovarian stroma when the follicle has developed two to three layers of GCs (Erickson et al., 1985). The origin of TCs in the ovary remains unclear. It has been hypothesized that a population of unspecialized mesenchymal precursor cells may respond to signals from growing follicles, leading to their differentiation into TCs. Based on studies in mouse models, several factors such as GDF9 from oocytes and DHH and IHH from GCs, may be involved in the recruitment and development of TCs (Magoffin, 2005; Richards et al., 2018; Young and McNeilly, 2010). The newly recruited TCs are fibroblast-like cells. As follicles grow, two distinctive layers of TCs can be observed: the theca interna layer adjacent to the basal lamina, and the outer theca externa layer

organized between the theca interna and the ovarian stroma. The theca interna and externa layers are mainly composed of endocrine theca interna cells (inTCs) and theca externa cells (exTCs), respectively, along with vascular tissue, immune cells and extra cellular matrix components (**Figure 4**). inTCs exhibit a typical polygonal shape when they become active for steroidogenesis, and following ovulation, they undergo luteinization and develop into theca lutein cells (lutein TCs) (Erickson et al., 1985). Studies have shown that exTCs exhibit smooth-muscle cell characteristics, as evidenced by an enrichment of smooth muscle actin and myosin. It is hypothesized that exTCs not only provide structural support for the follicle, but also to assist the ovulation process and compaction into a corpus luteum through their contractile capability (O'shea, 1981; Walles et al., 1990).

Steroidogenesis in granulosa and theca cells during the ovarian cycle

Estradiol and progesterone are the two primary circulating hormones secreted by ovarian antral follicles and the corpus luteum (Mikhail, 1970). The occurrence of ovulation triggers changes in the levels of these two hormones, which in turn determines the follicular phase (before ovulation) and the luteal phase (after ovulation). Together, the follicular phase, ovulation and luteal phase constitute the ovarian cycle. During the follicular phase, follicles enlarge in response to gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and produce more estradiol than progesterone. In contrast, during the luteal phase, the corpus luteum produces more progesterone than estradiol (Baerwald and Pierson, 2020).

Estradiol is produced by GCs and lutein GCs. However, inTCs are responsible for converting cholesterol into androstenedione and testosterone, which are substrates for estradiol synthesis in GCs (**Figure 4**) (Andersen and Ezcurra, 2014). This process is promoted by FSH and LH, and is known as the "two-cell-two-gonadotropin" theory. The theory is based on the fact that the FSH receptors FSHR and aromatase (CYP19) for estradiol synthesis are only detected in GCs, while 17α-Hydroxylase (CYP17) for androgen synthesis is present exclusively in inTCs (Andersen and Ezcurra, 2014; Millier et al., 1994; Patel et al., 2010) (**Figure 4**). By binding to their receptors respectively on GCs and inTCs, FSH and LH activate the expression of key factors for steroidogenesis, such as: CYP17 in inTCs, CYP19 in GCs, and steroidogenic acute regulatory protein (StAR) in both cell types (**Figure 4**). According to androgen levels in the follicular fluid, androstenedione is the main androgen produced by follicles (Brailly et al., 1981). Follicular steroidogenesis is regulated not only by gonadotropins but also by intrafollicular factors and follicle-produced estradiol. For example, AMH expression in GCs is high in small antral follicles (< 4 mm), and this inhibits FSH-induced CYP19 activity (Weenen et al., 2004). Additionally, the expression of insulin-like growth factors IGF-II (and also IGF-I) in GCs is involved in up-regulating CYP19 activity in response to FSH (Baerwald et al., 2012a). Furthermore, GCs from follicles larger than 10 mm release inhibin A and inhibin B to increase estradiol production. Inhibin B, rather than

inhibin A, forms a negative feedback loop together with estradiol to reduce FSH secretion from the pituitary gland (Andersen, 2017).

After ovulation, lutein GCs and TCs are responsible for progesterone synthesis. Nevertheless, a small amount of progesterone can also be synthesized by pre-ovulatory follicles, which is assumed to be associated with triggering the LH surge and ovulation (Dozortsev and Diamond, 2020; Mikhail, 1970). Both mural GCs and inTCs are capable of progesterone synthesis, but it is unclear which cell type is primarily involved in its production before ovulation (Andersen and Ezcurra, 2014; Jamnongjit and Hammes, 2006) (**Figure 4**).

6. Follicular growth and degeneration

Follicular growth from primordial to antral stage

In humans, it takes around 240 days for a recruited primordial follicle to mature until ovulation (Stringer and Western, 2019) (**Figure 1**). The first wave of primordial follicle activation commences from 23-26 WPF during fetal development. Upon follicle activation, the primordial follicles with squamous GCs first develop into primary follicles when the layer of squamous GCs all becomes cuboidal. In the meantime, the oocyte enlarges and acquires a glycoprotein layer known as the zona pellucida. The cuboidal GCs proliferate and form a multilayer of GCs, leading to the formation of the secondary follicle (<0.15 mm). At this stage, the fibroblast-like TCs are recruited to surround the secondary follicle, and the follicular vasculature begins to organize. As the GCs continue to proliferate, the follicle reaches the preantral stage (0.15–0.2 mm), acquiring multiple (>5) layers of GCs, as well as theca interna and externa layers. The follicle progresses to the antral stage, where an antrum cavity develops inside the GC layer. The formation of the antrum results from the accumulation of follicular fluid, which is assumed to be derived from the bloodstream along with components secreted by GCs. Preantral and even small antral follicles (< 2mm) are found in the ovary of children, indicating that their growth relies on factors from the local microenvironment rather than gonadotropins (Peters et al., 1978). Further growth of antral follicles is a gonadotropin-dependent process, as their enlargement occurs only after puberty (Hennet and Combelles, 2012; McGlacken-Byrne and Conway, 2022).

From the selectable antral follicle to ovulation

Antral follicles that reach the selectable stage (2-5 mm) are subjected to stimulation of FSH and LH. FSH promotes GC proliferation, steroidal hormone synthesis, as well as the expression of the LH receptor LHCGR in mural GCs and inTCs, while LH promote steroidal hormone synthesis and is involved in triggering ovulation (**Figure 4**) (Casarini et al., 2022; Yung et al., 2014). From a cohort of selectable antral follicles, only one “dominant” follicle is able to grow until preovulatory stage (16-29 mm) during each ovarian cycle, while all other “subordinate” follicles normally undergo atresia before they grow beyond 10 mm (Baerwald et al., 2012b). According to current hypotheses on follicle selection, the

ability to attain the highest FSH or LH sensitivity (e.g., the highest expression level of FSHR or LHCGR) is crucial for antral follicles to become "dominant" (Baerwald et al., 2012b; Macklon and Fauser, 2001).

It takes about 15 days for selectable follicles to go through selection and allow the dominant one to grow into a preovulatory follicle (**Figure 1**). Subsequently, the LH surge causes a reduction in gap junctions between mural GCs and later cumulus GCs, leading to a decrease of cGMP levels in cumulus GCs and the oocyte, which in turn allows meiosis to resume (Jaffe and Egbert, 2017) (**Figure 4**). Various morphological, mechanical and molecular changes occur within the preovulatory follicle that may be associated with follicle rupture and oocyte release, such as: the disruption of basal lamina, the invasion of vessels into GCs, the infiltration of leukocytes and secretion of cytokines (Duffy et al., 2019). To facilitate the healing of the post-ovulation ovarian surface wound, it is suggested that OSE plays an important role, perhaps through its ability to proliferate and transdifferentiate (Auersperg et al., 2001).

Development of the corpus luteum after ovulation

After ovulation, the dominant follicle collapses and undergoes reorganization into the corpus luteum, which is composed of lutein GCs and lutein TCs, as well as fibroblasts, endothelial cells, perivascular cells, and immune cells. The corpus luteum produces large amounts of progesterone and moderate levels of estrogen (Abedel-Majed et al., 2019; Bagnjuk and Mayerhofer, 2019). If the pregnancy does not occur, the corpus luteum reaches its peak size 6 days after ovulation and then undergoes luteolysis (Baerwald et al., 2005). Luteolysis triggers apoptosis and necroptosis of lutein cells, leading to the breakdown of the corpus luteum structure. Around 14-16 days after ovulation, progesterone levels return to baseline. Eventually, the corpus luteum regresses into the corpus albicans, which is a white and fibrous scar within the ovarian stroma, and disappears in the ovary (Stocco et al., 2007).

7. Follicular atresia

Compared to rare dominant follicles, becoming subordinate follicles and undergo degeneration via atresia is more common in the ovary. Although atresia can occur at any stage of follicular growth, studies on primates have shown that the highest incidence of follicular atresia occurs during selectable stage (Gougeon, 2004). Moreover, follicular atresia is postulated to be highly associated with the ovarian cycle, as the majority of antral follicles observed during the luteal phase are atretic (Baerwald et al., 2012b).

Follicular atresia is typically assessed by morphological changes, although abnormal changes can vary among atretic follicles. Histological studies have shown that oocyte degeneration are signs of early atresia in follicles before antral stage (**Figure 5**). However, in antral follicles, early atresia is characterized by nuclear pyknosis in GCs caused by apoptosis, and hypertrophy of inTCs. Further atresia usually involves detachment of the COC, disappearance of mural GCs, presence of

apoptotic GCs in the follicle fluid, macrophage infiltration, fibroblasts surrounding the antrum, thickening of the basal lamina and shrinkage of the follicle (Gougeon and Testart, 1986; Gougeon, 2004) (**Figure 5**). The death of GCs is not only mediated by apoptosis, alternative pathways such as autophagy and necrosis are also involved in their depletion (Regan et al., 2018; J. Zhou et al., 2019). In addition to morphological changes, atretic follicles exhibit low aromatase activity, resulting in a greater androgen/estrogen ratio (Baerwald et al., 2012b; Brailly et al., 1981). It is likely that follicle atresia at different stages is caused by failures in the development of different compartments. For instance, follicular angiogenesis and neovascularization are proposed to be related to atresia of selectable antral follicles (Robinson et al., 2009). Before developing a high dependence on the vascular network, the decrease in gap junctions and adherens junctions between oocytes and GCs could be one of the main reasons for inducing atresia in secondary and preantral follicles (Yan et al., 2019). Due to the lack of molecular features of follicular cells during atresia, how follicles initiate atresia and coordinate a series of degeneration events remains a mystery.

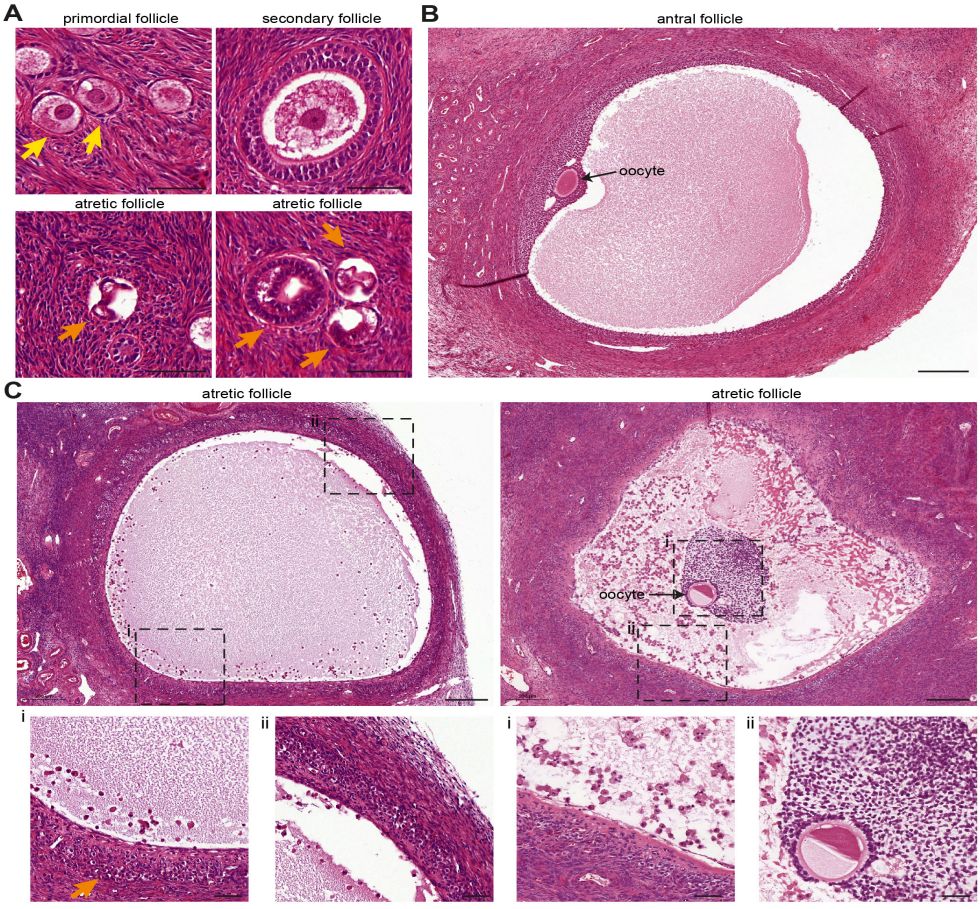

Figure 5. Morphology of ovarian atretic follicles. (**A**) Morphology of atretic follicles between primordial and secondary stage. Yellow arrows indicate normal primordial follicles and orange arrows indicate atretic follicles. Scale bars are 50 μm. (**B**) Morphology of normal antral follicles. Scale bar is 200 μm. (**C**) Morphology of atretic antral follicles. Magnified images in dashed squares are shown in the bottom panels. The orange arrow indicate hypertrophied theca interna cells. Scale bars are 200 μm in overview images and 50 μm in magnified images.

8. Epigenetic reprogramming and dosage compensation in germ cells

Sex chromosomes and dosage compensation

In humans, X and Y chromosomes are responsible for the sex determination, and an X-X combination represents a female genotype, while an X-Y combination represents a male genotype. According to current understanding, sex chromosomes are evolved from a pair of autosomes, with the acquisition of sex-determining loci leading to the suppression of recombination between the two chromosomes. As a result, the X and Y chromosomes have independently accumulated mutations over time, causing them to become increasingly different from each other (Ellegren, 2011; Sangrithi and Turner, 2018). The human X chromosome contains over 800 protein-coding genes and 600 non-coding genes, whereas the Y chromosome harbors 568 genes, of which only 71 are protein-coding (Maan et al., 2017; Schurz et al., 2019). Due to the substantial gene loss of the Y chromosome during evolution, the expression of the lost genes would only come from the homologous X chromosome, which caused an imbalance of dosage between male autosomal and sex chromosomal genes (Bellott et al., 2014; Sangrithi and Turner, 2018). In 1967, Susumu Ohno proposed a theory of dosage compensation that described a twofold hyperexpression of the X chromosome in males to balance the dosage of autosomal and sex chromosomal genes. Furthermore, to achieve a similar expression level of X-linked genes between sexes, females have one active X chromosome that is upregulated and one inactive X chromosome that is suppressed (Ohno, 1967) (**Figure 6**). Subsequent studies have confirmed the phenomenon of X chromosome inactivation (XCI) in mammals, whereas the idea of X chromosome upregulation (XCU) was controversial for a long time and was found to be associated with specific tissues and dosage sensitive genes (Galupa and Heard, 2018; Pessia et al., 2014; Sangrithi and Turner, 2018).

The establishment of XCI is mediated by the long non-coding RNA *XIST* (X-inactive specific transcript), which silences X-linked gene expression by spreading the transcripts in cis across the X chromosome and recruiting chromatin modifying factors, such as histone deacetylases, histone methyltransferases, and DNA methyltransferase, among other factors. This results in a stable heterochromatin structure known as the "Barr body" (Barr and Bertram, 1949; Galupa and Heard, 2018; Loda and Heard, 2019; Minajigi et al., 2015; Wutz, 2011). The XCI state is normally recognized by a dispersed cloud of *XIST* RNA over the inactive X chromosome with co-accumulation of repressive histone marks, such as H3K27me3 (trimethylation of Histone H3 lysine 27), and monoallelic expression of most X-linked genes

from the active X chromosome (Fang et al., 2019). Although XCI leads to a highly repressed state of the inactive X chromosome, a subset of genes remains expressed (Fang et al., 2019). In humans, approximately 15% of X-linked genes constitutively escape XCI, and are potentially involved in the development of sexual dimorphism (Carrel and Willard, 2005; Galupa and Heard, 2018). These escapee genes tend to be located on the outer surface of the compacted three-dimensional (3D) structure of the inactive X chromosome and lack transcriptional repressive histone marks on their chromatin (Fang et al., 2019). Despite extensive studies on XCI, mechanisms of XCU remain poorly understood. Nevertheless, a recent study has shown that XCU is achieved globally through increased burst frequencies of RNA transcription on the X chromosome (Larsson et al., 2019).

Epigenetic reprogramming and X-chromosome reactivation

Epigenetics refers to mitotically stable modifications that control gene expression without any change in the DNA sequence. The main epigenetic processes include DNA (cytosine) methylation, modification of histone proteins, remodeling of chromatin, and RNA-based regulation (Gibney and Nolan, 2010). Germ cells undergo dramatic epigenetic changes during their development, which are crucial for germ cell development and also impact offspring due to the inheritability of parental epigenetic marks. In contrast to somatic cells, female germ cells undergo reprogramming of DNA methylation and epigenetic imprints, as well as the X-chromosome reactivation (XCR) to develop into gametes (**Figure 6**).

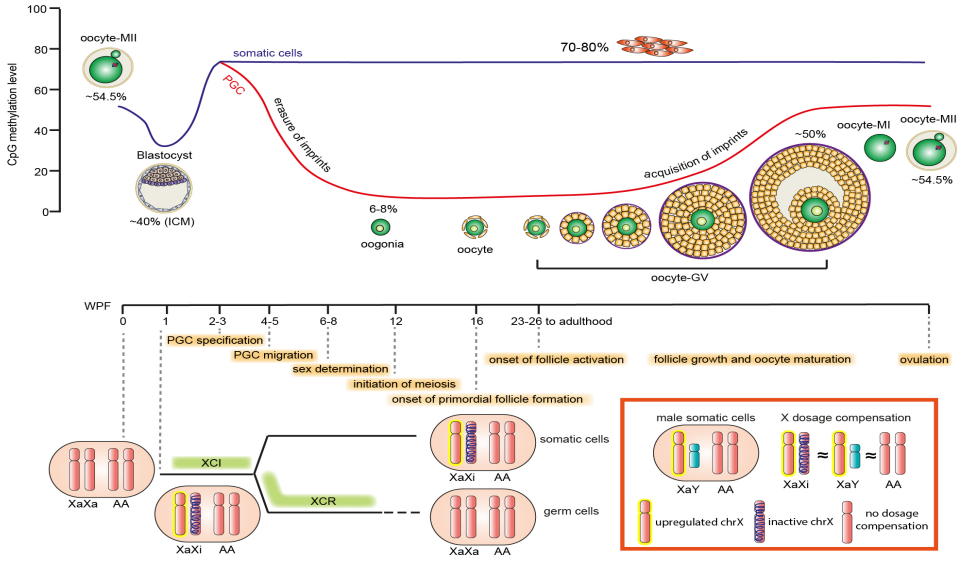

Figure 6. Epigenetic reprogramming and dosage compensation in human female germline. PGC, primordial germ cell; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; ICM, inner cell mass; XCI, X-chromosome inactivation; XCR, X-chromosome reactivation; WPF, weeks post fertilization; chrX, X chromosome; Xa, active X chromosome; Xi, inactive X chromosome; A, autosome.

In humans, a first wave of global DNA demethylation occurs soon after fertilization and reaches its bottom in the blastocyst stage. Subsequently, the DNA methylation level gradually increases in the three cell lineages of blastocyst: trophectoderm (TE), epiblast (EPI) and primitive endoderm (PE) (Gkountela et al., 2015; F. Zhou et al., 2019). However, PGCs undergo a second wave of more rigorous global DNA demethylation after specification, and the methylation level at cytosine-phosphate-guanine (CpG) sites reaches its lowest point around 6-8% at 10-11 WPF (Wen and Tang, 2019). Afterwards, de novo DNA methylation gradually builds up in oocytes during follicular growth. The methylation level rises to more than 50% in GV oocytes from antral follicles and remains relatively stable until the MII stage (54.5%) (Qian and Guo, 2022; Yu et al., 2017; Zhu et al., 2018) (**Figure6**).

Successful embryogenesis requires not only two sets of homologous parental genomes but also parental genomes with distinct epigenetic marks. Imprinted genes exhibit monoallelic expression of either maternal or paternal allele, and are clustered on chromosomes controlled by imprinting control regions (ICRs). These imprinted genes and ICRs contain differentially methylated regions (DMRs), which allow parental alleles to show different gene expression patterns (Abramowitz and Bartolomei, 2012). In humans, parental imprints (epigenetic marks that ensure monoallelic expression from a specific allele) are erased during the second wave of DNA demethylation in PGC development, and then the oocyte re-establishes its genome imprints during follicular growth (**Figure 6**). The acquisition of DNA methylation marks in different imprinted genes is asynchronous among DMRs, but they are largely methylated in GV oocytes from antral follicles (Arnaud, 2010; Monk et al., 2019; Sato et al., 2007). Aberrant DNA methylation levels at ICRs are associated with multiple human syndromes. For example, 35-65% of patients with Silver-Russell syndrome show hypomethylation at the H19-ICR on the paternal allele, and hypomethylation at the KvDMR1-ICR on the maternal allele has been found in around half of the patients with Beckwith-Wiedemann syndrome (Ishida and Moore, 2013).

The XCR is normally recognized by an incomplete XCI state which is characterized by a diminished *XIST* cloud, loss of the repressive histone mark H3K27me3, and biallelic expression of X-linked genes (Fang et al., 2019). It has been proposed that XCI is established between E6 and the fourth week of pregnancy in humans, after which somatic cells maintain the XCI pattern, while the specified female PGCs gradually reactivate the silenced X chromosome until meiosis entry (Chitiashvili et al., 2020; Patrat et al., 2020). XCR in human PGCs has already occurred by 4WPF, which coincides with the second wave of global DNA demethylation (**Figure 6**) (Chitiashvili et al., 2020; Patrat et al., 2020). Studies on the development of female PGCs suggest that the progression of global DNA demethylation mainly depends on fetal age, regardless of the heterogeneity of PGCs within the ovary. In contrast, XCR appears to be more dependent on the developmental phase of PGCs, rather than on fetal age (Chitiashvili et al., 2020; Li et al., 2021; Vértesy et al., 2018). The regulation of XCR and dosage balancing between two X chromosomes during

PGC development remains unclear, new techniques that enable tracking the behavior of each X chromosome will be valuable for understanding this process.

9. Studying molecular signatures by single-cell RNA sequencing

From morphology to single-cell transcriptomics

In the past, studies have mostly relied on characterization of morphological changes to understand the developmental dynamics in cells and tissues. However, without knowing molecular signature changes, it is difficult to fully explore the biological mechanisms involved. The emergence of transcriptomics, which studies the cellular RNA transcripts produced by the genome, enables researchers to investigate cellular activities by gene expression patterns. Later, the breakthroughs achieved in single-cell resolution and high-throughput DNA sequencing have finally opened the door for studying unbiased transcriptome-wide information of the mRNA at the single cell level in heterogeneous samples (Ambrosi et al., 2012; Svensson et al., 2018; Tang et al., 2009).

Single-cell RNA sequencing techniques

The single-cell RNA sequencing (scRNA-seq) workflow includes single cell isolation, RNA extraction, reverse transcription (conversion of RNA into cDNA), library preparation and sequencing (Haque et al., 2017) (**Figure 7**). Depending on the preparation method, libraries can be generated by selectively capturing the 3' end or the entire length of mRNA transcripts. Several scRNA-seq platforms are available for both 3' library and full-length sequencing, and the most representative platforms for these two types of sequencing are 10X Genomics Chromium (10X) and Smart-seq2/Smart-seq3 methods (Jovic et al., 2022) (**Figure 7**). The 10X method, published in 2017, is a 3' RNA sequencing platform that uses a high-throughput microfluidics encapsulation-based method to capture single cells (Zheng et al., 2017). During encapsulation, a gel bead with barcoded oligonucleotides, lysis and reverse transcription reagents is loaded in the same vesicle droplet with the cell. This approach allows RNA extraction, reverse transcription and cDNA amplification from tens of thousands of cells to be processed in a single run (**Figure 7**). 10X also uses unique molecular identifiers (UMIs) to barcode each individual mRNA during the reverse transcription step. This enables recognition of amplified copies from the same transcript and quantification of the absolute counts of mRNA detected from a gene (Kivioja et al., 2012). By using high-throughput barcoding and sacrificing full-length coverage, the 10X method allows pooling of cDNAs from large numbers of cells for a given number of sequencing reads. Thus, 10X has been frequently used for profiling cell types in heterogeneous tissues (Wang et al., 2021). Smart-seq2 and Smart-seq3 are plate-based full-length sequencing methods. Smart-seq2 was published in 2013, and an improved version incorporating UMIs was released as Smart-seq3 in 2020 (Hagemann-Jensen et al., 2020; Picelli et al., 2013). Full-length sequencing methods, such as Smart-seq2, perform better at detecting mRNAs with different spliced variants or low abundance. However, because Smart-seq methods

uses 96 or 384 well plates, only hundreds of cells can be processed per batch (Baran-Gale et al., 2018) (**Figure 7**).

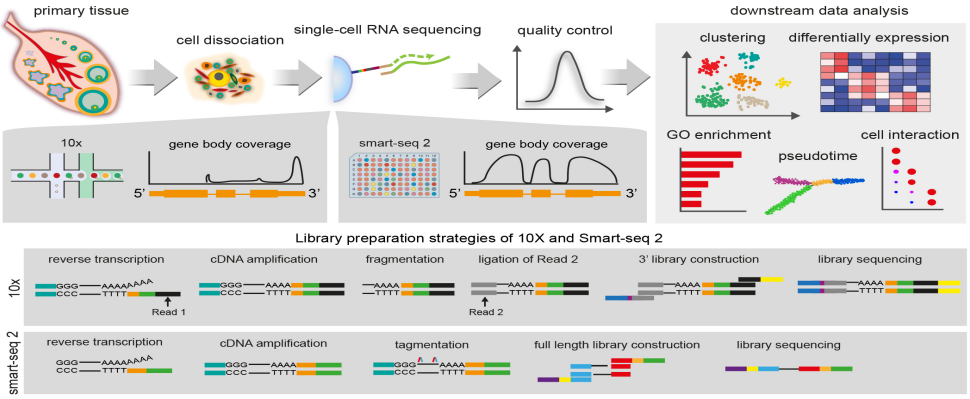

Figure 7. The workflow of single-cell RNA sequencing experiment and analysis. 10X Genomics Chromium (10X) and Smart-seq2 methods are showed here as representatives for 3' library and full-length sequencing. During library construction, primers that only recognize fragmented molecules containing the Read1 sequence and Read2 sequence are used in 10X protocol. Smart-seq2 use a tagmentation approach to acquire fragments that are assumed to cover the entire length of mRNA transcripts.

Studying human organs by single-cell transcriptomic analysis

Although all human cells share the same genetic material, only a subset of genes is expressed at any given time, which defines the specific morphology and functions of each cell type. scRNA-seq analysis can reveal similarities in gene expression profiles among cells, allowing them to be grouped into clusters using mathematical algorithms. This approach enables the identification of different cell types, sub-types, transitional states, and rare cell populations. Furthermore, downstream analyses such as differential expression, gene ontology (GO) enrichment, pseudotime and ligand-receptor analysis can provide valuable information about cellular activity, cell function, cell lineage development and cell-cell interactions (Jovic et al., 2022) (**Figure 7**). Together, scRNA-seq technologies have opened up an exciting era in which human tissues and organs can be studied by their cellular composition and features. By studying various organs, it is possible to map all cells across the human body and build a comprehensive human cell atlas (Elmentaite et al., 2022).

10. Scope and outline of the thesis

Approximately 15% of individuals are estimated to suffer from infertility, with about 70% of cases being caused by either female factors alone or a combination of female and male factors (Cox et al., 2022). Among causes of the female infertility, ovarian factors such as anovulation, polycystic ovarian syndrome, and decreased oocyte quality due to advanced maternal age, are the leading reasons for seeking

clinical assistance (Mustafa et al., 2019; Sahana Devi and Yendapalli, 2023). However, the development of effective treatments for ovary-related infertility is hindered by a lack of knowledge about the responsible cell types and underlying mechanisms. To gain a more comprehensive molecular understanding of these ovarian causes of female infertility, it is essential to establish a map of ovarian cells and their transcriptomes throughout human oogenesis (from PGC to egg) and folliculogenesis (from primordial follicle to ovulation).

Previous researches on human oogenesis and folliculogenesis were mainly based on morphological changes due to limited access to human tissue and a lack of high-throughput and high-resolution molecular analyzing techniques. However, to extend our understanding of these processes, it is necessary to utilize scRNA-seq technique to uncover cellular signatures during germ cell development and follicular growth and degeneration. Although several pioneering studies have employed scRNA-seq to analyze the development of germ cells during the fetal stage, single-cell transcriptomics of the different sub-phases of meiotic prophase I and ovarian folliculogenesis has not yet been explored. **To address these gaps in knowledge, this thesis aims to characterize fetal female germ cells during meiotic prophase I and adult ovarian cells that are related to folliculogenesis in human through single-cell transcriptomics analysis.** By identifying different cell types, subtypes or developmental states and profiling gene expression of these populations, we aim to provide a better understanding of the cellular composition of the ovary and the development of follicular cells during oogenesis and folliculogenesis. With this knowledge, we hope to pave the way for the development of targeted interventions that will ultimately improve female reproductive health.

Chapter 2 focuses on the single-cell transcriptomics analysis of fetal female premeiotic and meiotic germ cells. To study the meiotic progression of human female fetal germ cells, we characterize the transcriptional profiles of female meiotic prophase I sub-phases. To further investigate the sexual dimorphism during meiotic prophase I, we compare molecular features of each sub-phase in females to those in males. Our analysis aims to reveal potential key players that are involved in the process of meiosis for both sexes, and genes that are associated with the sex-specific development of germ cells.

In **chapter 3**, we perform scRNA-seq using human adult ovaries and analyzed gene expression profiles of human adult ovarian somatic cells, as well as characterize molecular signatures of growing and regressing (atretic) follicular populations, including granulosa and theca cells. The work provides a transcriptomic landscape of human adult ovarian somatic cells.

With the advances of single-cell transcriptomics, several studies, including our work in **chapter 3**, have analyzed features of human adult ovarian cells. The collective efforts of these studies have expanded our knowledge of cell populations and their

molecular signatures in the human adult ovary. Therefore, in **chapter 4**, we aim to review the current knowledge and provide a comprehensive summary of the main cell types in the human adult ovary, along with specific markers that can be used to identify these ovarian cells, particularly oocyte, granulosa and theca cells at different sub-stages of folliculogenesis.

In **chapter 5**, to study the oocyte-granulosa crosstalk in small antral follicles, we perform scRNA-seq of oocytes and their surrounding granulosa cells. The analysis focuses on the characterization of antral follicles by their oocyte and granulosa cell signatures, and the investigation of ligand-receptor interactions between oocyte and granulosa cells in these antral follicles.

Together, the works presented in this thesis provide valuable transcriptomic datasets of human ovarian cells, contributing to the establishment of the molecular landscape of human oogenesis and folliculogenesis. Alongside our contributions, research efforts that focus on reconstructing human oogenesis and folliculogenesis at the molecular level through scRNA-seq are driving the field forward. **Chapter 6** discusses the advancements and gaps in this field, as well as the potential of using other high-throughput techniques and in vitro models to expand our understanding of oogenesis and folliculogenesis. Furthermore, **chapter 6** includes insights provided by this thesis for future studies involving ovarian follicular cells, sex differences in germ cell development, and scRNA-seq analysis. Finally, the importance of integrating in vivo knowledge with in vitro approaches to advance the development of innovative treatments for female fertility is also discussed in **chapter 6**.

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