Application of Single-Cell RNA Sequencing in Ovarian Development

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Contributor: Xiaoqin Gong , Yan Zhang , Jihui Ai , Kezhen Li

The ovary is a female reproductive organ that plays a key role in fertility and the maintenance of endocrine homeostasis, which is of great importance to women's health. It is characterized by a high heterogeneity, with different cellular subpopulations primarily containing oocytes, granulosa cells, stromal cells, endothelial cells, vascular smooth muscle cells, and diverse immune cell types. Each has unique and important functions. From the fetal period to old age, the ovary experiences continuous structural and functional changes, with the gene expression of each cell type undergoing dramatic changes. In addition, ovarian development strongly relies on the communication between germ and somatic cells. Compared to traditional bulk RNA sequencing techniques, the single-cell RNA sequencing (scRNA-seq) approach has substantial advantages in analyzing individual cells within an ever-changing and complicated tissue, classifying them into cell types, characterizing single cells, delineating the cellular developmental trajectory, and studying cell-to-cell interactions.

single-cell RNA sequencing ovary ovarian development intercellular interaction

1. A Single-Cell RNA Perspective of Ovarian Germ Cells

1.1. A Single-Cell Perspective of Fetal Germ Cells

During ovarian development, fetal primordial germ cells (PGCs) originate from the yolk sac and migrate to the gonadal primordium early in fetal life. After undergoing several rounds of mitotic division and proliferation, oogonia are produced [1][2][3]. Then, the majority of oogonia enter meiosis to become oocytes, and dormant oocytes are arrested at the diplotene stage of meiosis I [4][5]. Fetal PGCs and oogonia can be collectively referred to as FGCs. scRNA-seq has been used for research on FGCs. Some studies have focused on the heterogeneity of FGCs and revealed the characteristics of FGCs in the process of the mitotic-to-meiotic transition and the initiation of meiosis.

1.1.1. The Cell Subpopulations and Characteristics of Fetal Germ Cells

The healthy development of FGCs is a key prerequisite for the normal transmission of genetic information from parents to offspring [6][7]. There is great heterogeneity in the gene expression among individual FGCs [8][9], and the subpopulations and developmental trajectories of FGCs are not well-known. To identify clusters of FGCs, Zhao et al. [10] sequenced 19,363 single POU5F1-eGFP^{+} FGCs from E12.5, E14.5, and E16.5 female mouse gonads. *POU5F1* is a pluripotency transcription factor expressed in embryonic stem, early germ, and primordial germ cells [11][12]. After cluster analysis, researchers [10] found that FGCs can be divided into seven clusters according to the

reported markers and differentially expressed genes. The subpopulations of FGCs included PGCs, oogonia, and preleptotene, leptotene, zygotene, early-pachytene, and late-pachytene cells. Each detection time point included several cell clusters, implying that FGCs developed asynchronously, which is consistent with another scRNA-seq study [13]. In addition, using magnetic- and fluorescence-activated cell sorting, c-KIT$^+$ FGCs and larger c-KIT$^-$ cells from 17 female embryos between 4 and 26 weeks after fertilization were isolated [1]. *C-KIT* has been reported to be a cell surface marker for germ cells [9]. Using the t-distributed random neighbor embedding method to analyze scRNA-seq data, Li et al. [1] discovered that FGCs can be divided into four developmental stages: mitotic FGCs, retinoid-acid (RA)-signaling-responsive FGCs, meiotic prophase FGCs, and oogenesis FGCs. Moreover, they found that *NANOG* might act as a specific marker gene for FGCs in the mitotic stage. In addition, RA-signaling-responsive FGCs specifically expressed *STRA8, ZGLP1, ANHX, ASB9*, and *THRA/BTR*. Furthermore, meiotic prophase FGCs are enriched in gametogenesis-specific genes, and *IL13RA2* may serve as a specific marker gene for meiotic prophase FGCs. As for oogenesis FGCs, they highly expressed not only genes related to the cytoskeletal organization, hormone-mediated signaling, microtubule-based movement, and oocyte development, but also programmed cell death and apoptosis genes, implying that some FGCs at that stage were undergoing apoptosis, which was consistent with previous observations that most germ cells became apoptotic as cysts broke down [14]. Oogenesis FGCs specifically expressed *PECAM1*, *ZP3*, and *OOSP2*.

1.1.2. Molecular Mechanisms of the Mitotic-to-Meiotic Transition and the Initiation of Meiosis in Fetal Germ Cells

FGCs migrate to the gonadal ridge during early embryonic development and enter meiosis after mitotic division [15]. The mitotic-to-meiotic transition and initiation of meiosis are critical steps in the successful development of gametes [16], but the molecular mechanisms of these two processes are not well understood. Based on scRNA-seq data, Zhao et al. [10] observed dramatic changes in the gene expression during the transition from mitosis to meiosis in FGCs, with the downregulation of pluripotency marker genes and a marked upregulation of oocyte marker genes. For example, they found that *REC8* may be involved in the transition from mitosis to meiosis and that the transcription factors *MSX1, MSX2, GATA2, CDX2, SOX4*, and *B-MYC* may be related to the initiation of meiosis. In addition, after analyzing scRNA-seq data, Li et al. [1] reported that RA synthesis may have important effects on female entry into meiosis. Furthermore, the scRNA-seq study of Zhao et al. [10] pointed out that the initiation of meiosis first occurs at E12.5. Interestingly, conducting detailed scRNA-seq analysis of more than 52,000 individual cells from the gonadal ridges and ovaries of E11.5–PD5 mouse embryos, Niu et al. [13] also demonstrated that the E12.5 FGCs contained a small number of meiotic cells, which is different from the previous view that female mouse germ cells begin meiosis between E13.5 and E14.5 [17], indicating that scRNA-seq had a unique advantage in finding rare cell populations. In addition, by performing scRNA-seq on 19,387 cells from E11.5–E14.5 mouse gonadal ridges and ovaries, Ge et al. [18] successfully depicted germ cell meiotic initiation using pseudotemporal ordering approaches.

These studies provide detailed information on the transition from mitosis to meiosis and gene expression at the initiation of meiosis in FGCs and offer new avenues for understanding the process of embryonic gonadal development. Defects in meiotic initiation fidelity may lead to some reproductive diseases [19][20]. Understanding the

precise mechanisms of the mitotic-to-meiotic transition and initiation of meiosis of FGCs can help provide a theoretical basis for the future treatment of diseases related to abnormal gametogenesis.

1.2. A Single-Cell Perspective of Oocytes

Oocytes are the main determinants of embryonic developmental competence. Upon recruitment, oocytes undergo growth and maturation. [21][22][23][24]. Oocyte maturation can be affected by some ovarian-related diseases (for example, polycystic ovarian syndrome (PCOS)) [25]. In addition, some oocytes age with aging [26]. The oocyte undergoes dramatic changes in its transcriptional profile during these processes. In addition, with the increase in work and social pressures, the proportion of advanced pregnancies and infertility has been increasing over the past few decades [27][28]. The progress of assisted reproductive technology has brought hope of procreation to these populations [29][30]. However, the effects of in vitro culture techniques and age on the development and quality of oocytes should not be ignored [31][32]. Therefore, a new technological approach is needed to reveal potential mechanisms, thereby improving therapeutic effects. In addition, as the largest cell in the human body, the oocyte is rich in RNA, which gives it a unique advantage in scRNA-seq studies [33][34].

1.2.1. Oocyte Characteristics from Different Follicular Stages

As one of the most pivotal functional cell types in ovaries, oocytes play a decisive role in embryonic development, and their quality is extremely important for female fertility [35][36]. As follicles develop to a higher stage, oocytes constantly change [37]. The emergence of scRNA-seq makes it possible to study the gene expression of oocytes at the single-cell level, which enriches the understanding of oocyte development. Some scRNA-seq studies demonstrated that human oocytes expressed *GDF9, ZP3, DDX4, SYCP3, DAZL, FIGLA, OOSP2, ZP2, SOX30*, and *ZAR1* [38][39][40]. Furthermore, cynomolgus monkey oocytes specifically expressed *GDF9, ZP3, DDX4, SYCP3, LMOD3, RBM46*, and *NET01* [41], while mouse oocytes highly expressed *DDX4* and *DAZL* [42][43][44]. In addition, by integrating single-cell sequencing data from cells in the adult ovarian cortex with diameters less than 35 μm and scRNA-seq data from the fetal ovary, Wagner et al. [38] found that compared with fetal PGCs, the pluripotency marker genes *NANOG* and *POU5F1* were less expressed in most developmental stages of adult oocytes, which may be associated with the stemness of PGCs [8][39].

Additionally, Zhang et al. [39] analyzed the scRNA-seq data of human oocytes and found that the marker genes of oocytes from various follicular stages exhibited different expression patterns. For instance, *DDX4, ZP2, ZP3*, and *ZP4* showed stable expression in oocytes from follicles at different stages of development, but *ZP1, GDF9*, and *H1FOO* were gradually upregulated with oocyte maturation. Data from single-cell transcriptome sequencing of monkey oocytes also showed that the expression of *ZP1* and *GDF9* was gradually upregulated during follicular development [41]. Moreover, with the maturation of oocytes, the activity of some ten-eleven translocation family genes was low in adult oocytes at all follicular stages, and *DNMT1, DNMT3A*, and *DNMT3B* expression increased gradually [39]. The expression of *DNMT1* and *DNMT3A* in monkeys gradually increased with the development of oocytes [41]. Considering that ten-eleven translocation family proteins are associated with active DNA

demethylation, and the DNA methyltransferase family is related to the maintenance of DNA methylation [45][46], the findings indicated that the oocyte maturation was accompanied by an increased DNA methylation.

More importantly, by analyzing the regulatory network of transcription factors in human oocytes at each follicular stage, Zhang et al. [39] found that *SOX30* may play an important role in the transition from primordial to primary follicles, and *SOX13* and *SOX15* may be necessary for antral follicle formation. Wang et al. [41] elaborated on the detailed stage-specific regulatory networks of transcription factors that regulate cell-type-specific markers of oocytes from various follicular stages. *ELF4* and *FOS* may be essential for oocytes from primordial follicles. In addition, *RPS4X* and *FIGLA* may play vital roles in oocytes from primary follicles. Moreover, by comparing the single-cell transcriptome profiles of oocytes to the previously reported RNA data of mice, researchers found that humans not only shared some genes with mice, but also had some special regulatory mechanisms in oogenesis, which may provide a better understanding of oocyte development between humans and mice [39].

1.2.2. The Characteristics of In Vitro Matured Oocytes

Oocyte maturation is a determinant step for embryo development, providing sufficient energy and nutritional materials for early-stage embryonic growth, and is influenced by many factors, among which the environment is of great importance [47][48]. In vitro matured (IVM) oocytes have poor developmental competence, and the unsynchronized cytoplasmic maturation and nuclear saturation may be the reason. However, the underlying molecular mechanisms remain ambiguous [49]. Using scRNA-seq, Zhao et al. [50] studied three IVM oocytes and three in vivo (IVO) matured oocytes. They showed that exposure to the in vitro environment can lead to a decline in the activity of CoA-related enzymes, such as *ACAT1* and *HADHA*, ultimately contributing to a decrease in energy metabolism. The energy metabolism capacity is known to play a significant role in regulating the gene expression, protein translation, and protein modification, and a decrease in the oocyte energy metabolism capacity may impair the developmental potential [48][51]. These findings imply that targeting genes involved in energy metabolism may help improve the adverse outcomes of IVM oocytes. Furthermore, the increased level of NADP+ resulting from the high expression of nicotinamide nucleotide transhydrogenase enhances the ability of humans to repair DNA double-strand breaks, thus maintaining euploidy. This may be a key reason why, although the developmental potential of some fertilized eggs is low, they can complete the normal maturation process in vitro. The results will likely yield further insights into the mechanisms of human oocyte maturation, thus providing benefits to some patients with infertility.

1.2.3. The Characteristics of Oocytes from Aged Females

It is well-known that the quality of oocytes decreases with age, thus impairing the embryo's developmental potential [52]; however, the molecular mechanisms have not been explained clearly.

Recently, using the scRNA-seq technique, Zhang et al. [53] discovered that compared to in oocytes from younger women, genes related to oxidative stress, transcriptional activation, and immune function in those from older women were upregulated. Additionally, a single-cell study sequenced a total of six matured oocytes from younger (≤30 years) and older (≥40 years) patients and found that some genes associated with oxidative stress were

dramatically upregulated in oocytes from older women compared to in those from younger women, while the gene *TOP2B*, devoted to promoting double-strand break repair after oxidative stress, was downregulated in oocytes from older women [53]. In addition, Wang et al. [41] analyzed high-quality transcriptomes of 418 oocytes collected from four young and four aged cynomolgus monkeys at a single-cell resolution. They demonstrated that antioxidant genes, such as *GPX1* and *GSR*, were downregulated in the oocytes of aged cynomolgus monkeys, which could be responsible for the increased oxidative damage during ovarian aging.

Considering that oxidative stress may damage the oocyte proteome with negative consequences for meiosis, fertilization, and embryonic development [54][55], these results provide a new way to evaluate the quality of oocytes in older women and elucidate the causes of ovarian aging at the molecular level, thus contributing to the discovery of new biomarkers to improve the oocyte quality. As more women worldwide are delaying the age of reproduction, resolving the mechanism of decreased oocyte quality with increasing age will be helpful for family health and social stability.

1.2.4. The Characteristics of Oocytes from Polycystic Ovarian Syndrome Patients

The oocytes in PCOS patients are often of poor quality, leading to lower fertilization, cleavage, and implantation rates [56]. It is essential to elucidate the mechanism behind this in detail. By analyzing the scRNA-seq data from 14 oocytes from seven healthy fertile women and 20 oocytes from nine patients with PCOS at the germinal vesicle (GV) stage, metaphase I stage, and metaphase II (MII) stage, Qi et al. discovered that some genes associated with mitochondrial function (for example, oxidative phosphorylation), such as *COX6B1*, *COX8A*, *COX4I1*, and *NDUFB9* were prematurely activated at the GV stage of PCOS oocytes, whereas it occurs at the MII stage in healthy oocytes [57]. Mitochondria plays an essential role in the oocyte maturation, meiotic spindle assembly, fertilization, and subsequent preimplantation embryogenesis [58]. Abnormal function of the mitochondria may account for the low-quality oocytes in PCOS patients, which provides a new idea for improving reproductive outcome of PCOS patients.

1.3. A Single-Cell Perspective of Oogonial Stem Cells

A heated debate about whether there are OSCs in the female ovary that can produce new oocytes has been consistently ongoing. Some studies have suggested that mammalian oocytes are formed in the early stages of fetal life, and their finite number represents female fertility, which decreases with increasing female age [59][60]. Nevertheless, some researchers have recently indicated that OSCs found in the ovarian cortex of female mammals have the ability to self-renew, clone, expand, and differentiate into oocytes [61][62]. When these OSC-derived oocytes combine with sperm, offspring can be produced, which might provide a basis for the clinical application of OSCs [63][64][65]. However, little is known regarding the biological characteristics of OSCs. Some studies have reported that DEAD box polypeptide 4 (DDX4) can be used to sort OSCs [66][67]. Recently, using immunomagnetic bead sorting with DDX4-based antibodies to enrich OSCs, Wu et al. [65] identified the developmental characteristics of transplanted OSCs by single-follicle RNA sequencing of follicles transplanted with GFP-positive OSCs in recipient and wild-type (WT) mice. The results showed that OSCs expressed *DDX4, DPPA3, IFITM3, POU5F1,*

DAZL, and *PRDM1*. Single-follicle RNA data indicated that the expression profiles of preantral follicles of WT mice were similar to those of OSC-derived preantral follicles. In addition, small antral follicles of WT mice and OSC-derived antral follicles showed similar gene expressions, indicating that follicles from WT mice and OSC-derived follicles have analogous developmental mechanisms. For instance, they found that the PI3K-AKT signaling pathway plays an essential role in the development of OSC-derived preantral follicles to small antral follicles, which is consistent with previous studies on the follicular development [68][69].

Currently, scRNA-seq has emerged as a valuable tool for identifying rare cell populations [70]. To explore whether OSCs are in the ovary, Wagner et al. utilized scRNA-seq to investigate the transcriptome of more than 24,000 cells in the adult ovarian cortex at a single-cell resolution. Using unbiased cluster analysis, they discovered that most cells collected by the DDX4 antibody belonged to perivascular cells that did not express DDX4 transcripts, rather than to OSCs. Furthermore, OSCs were not found in the ovarian cortex [38]. Given that single-cell transcriptome sequencing technology has significant advantages in discovering rare cell types, the process of obtaining single cells may have harmful effects on cellular activity and may even lead to the death of rare cells [71]. In addition, Bhartiya et al. [72] thought that the size of OSCs was so small that they needed to be collected with high-speed centrifugation, such as at 1000 g, and a low speed does not allow OSCs to settle. Hence, it is possible this is the reason why current studies did not capture OSCs. In the future, improving the process of collecting single cells and expanding the sample size may be an effective way to confirm the presence of OSCs in the ovary.

2. A Single-Cell RNA Perspective of Ovarian Somatic Cells

2.1. A Single-Cell Perspective of Pregranulosa Cells

Pregranulosa cells (pre-GCs) originate from ovarian surface epithelium cells and are precursors of granulosa cells [73][74]. It is believed that pre-GCs are essential for the development of primordial follicles and the establishment of ovarian reserves [75][76]. By sequencing 563 XX *NR5A1-GFP*$^{+}$ somatic cells from E10.5, E11.5, E12.5, E13.5, E16.5, and PD6 mouse ovaries at the cellular level, Ste'vant et al. [77] found that pre-GCs were first observed as early progenitors on E11.5, using transcriptome analysis. Pre-GCs remained in an early differentiation stage, expressing stem-cell-related genes until E16.5, and then continued to differentiate through folliculogenesis. Furthermore, they revealed that pre-GCs expressed genes associated with lipid metabolic processes and hormone secretion, implying that pre-GCs had the potential to provide hormones at the fetal stage, which was in accordance with the results of a previous study [78].

In addition, two types of pre-GCs have been reported in mammalian embryonic ovaries [79][80]. To explore the cellular origin, division timing, and gene expression profiles of pre-GCs, Niu et al. [13] obtained single-cell transcriptomic data of over 52,500 single cells from between E11.5 and postembryonic day 5 from 10–18 mouse gonads/ovaries. They demonstrated that pre-GCs specifically express *WNT4, WNT6, KITL*, and *FOXL2*. They also revealed that bipotential pre-GCs (BPG) originated from bipotential precursors, differentiated into wave 1 follicles that began to develop immediately after birth in the medullar region and were related to the onset of fertility, while epithelial pre-GCs (EPG) derived from the ovarian epithelial progenitor cells became the granulosa cells of the

primordial follicles in the ovarian cortex, which represented the ovarian reserve. Bipotential precursors were derived from the ovarian epithelial progenitor cells. The two cell types had similar, but distinct, gene expression profiles. For instance, bipotential precursors had a nonmitotic signature, with low MKI67 and *HIST1H1AP* but high *CDKN1B* expressions, and the ovarian surface epithelium had mitotic characteristics, with high *MKI67* expression. Furthermore, BPG cells expressed *FOXL2* early, while EPG cells expressed *LGR5* and abundant *FOXL2* until after birth, which was consistent with the results of the single-cell transcriptome sequencing in the study by Wang et al. [42]. Moreover, using lineage tracing, they found that with the development of the embryo, cortical BPG cells were replaced by EPG cells, and EPG cells never significantly resided in the ovarian medulla.

2.2. A Single-Cell Perspective of Granulosa Cells

GCs play a critical role in maintaining the oocyte quality and endocrine ovarian function [81]. During oocyte development and folliculogenesis, GCs undergo proliferation and differentiation [82]. Importantly, GCs also participate in ovulation [83][84]. Moreover, many GCs become atretic during follicular development [85]. The quality of GCs declines with aging [86]. Recently, scRNA-seq was conducted to characterize GCs at different cellular stages.

2.2.1. The Characteristics of Granulosa Cells from Different Stages of Follicles

When it comes to the possible marker genes of GCs, some scRNA-seq studies suggested that human GCs expressed *AMH, WT1, FOXL2, STAR, SERPINE2, GSTA1, CYP11A1, INHBA, CDH2, GJA1*, and *TNNI3* [1][38][39][40][87], nonhuman primate GCs expressed *AMH, WT1*, and *INH4* [41], and mouse GCs expressed *AMH, AMHR2, KITL, FSHR*, and *CYP19A1* [42][44][88]. Moreover, Zhang et al. [39] observed that genes related to steroid production are dynamically expressed in GCs during folliculogenesis. For example, the expression levels of *HSD17B1, HSD3B2*, and *NR5A1* in GCs gradually increase with follicle growth, indicating that steroid hormones are important for follicular development. Furthermore, their analysis revealed that transcription factor regulatory networks in GCs may be involved in the regulation of folliculogenesis. For instance, *GREB1, NFKB1, MEF2A, PIASI, FOSL2, KLF13*, and *PRDM4* may potentially participate in regulating primordial activation, which will provide new clues for further studies on folliculogenesis.

As for GC subpopulations, Zhang et al. [39] performed scRNA-seq on adult GCs from primordial to preovulatory follicles and then analyzed the data using the principal component unsupervised method. They showed that GCs could be divided into five clusters according to the stages of follicular development (i.e., primordial, primary, secondary, antral, and preovulatory follicular GCs) and presented a subset of stage-specific genes as candidate cell-type-specific markers for GCs during folliculogenesis, including *MGP, PTK6, KIF20A*, and *VTN*, that might separately serve as signature genes for primary, secondary, antral, and preovulatory follicular GCs. However, this was demonstrated using sequencing data based on the gene expression from a small number of GCs from each follicular stage, and the reliability requires validation through further experiments. They also showed that the aggregation of GCs at different stages of follicular development showed some overlap, with more overlap between primary and secondary follicular GCs, indicating that the transcriptional patterns of the two stages were similar. GCs of primordial follicles are distinct from those of antral follicles. Similarly, Wagner et al. [38] also discovered

there was no overlap between primordial and antral follicular GCs from the inner ovarian cortex, implying that the more separated the GC developmental stages, the more different the gene expression profiles.

In addition, scRNA-seq studies demonstrated that mural and cumulus GCs from small antral follicles gathered together showed high expression levels of *WT1* and *EGR4* but low expression levels of *VCAN* and *FST.* However, when mural and cumulus GCs from selective follicles were divided into two groups, human cumulus GCs expressed high levels of *IGFBP2, INHBB, IHH, VCAN, FST*, and *HTRA1*, while human mural GCs showed high expression levels of *CYP19A1, KRT18, AKIRIN1, LIHP*, and *CITED2* [40][87]. In mice, cumulus GCs from selective follicles can be identified by *HAS2* and *NPR2*, whereas mural GCs from selective follicles can be identified by *FSHR, BMPR2*, and *NPPC* [88]. It has been reported that early GCs undergo metabolic reprogramming as they become cumulus GCs [88], suggesting that mural GCs and cumulus cells from early antral follicles undergo differentiation.

2.2.2. The Characteristics of Granulosa Cells during Ovulation

The ovary experiences repeated ovulation during the reproductive period, and ovulation is driven by the surge of luteinizing hormone (LH) [89]. An LH surge can result in an altered cell function and gene expression in mural and cumulus GCs. When stimulated by ovulatory signals, luteinizing GCs undergo a transition from proliferation to differentiation and expansion [90], and GCs in preovulatory follicles will express ovulation-related genes, such as *PGR* and *PTGS2*. Additionally, previous studies have shown that GCs play an important regulatory role in ovulation [83][91], the role of individual GCs or certain GC subsets in ovulation has not been revealed, and the related mechanisms need to be further discussed. The advent of scRNA-seq has made it possible to study individual cells or choose a subpopulation for analysis based on the gene expression.

To investigate the characteristics of GCs during ovulation, Dong et al. [36] performed scRNA-seq analysis of hundreds of cumulus cells from two women with normal ovarian function and found a CD24(+) cumulus GC subpopulation, which played a crucial role in triggering ovulation. The CD24(+) cumulus GC subpopulation can activate the EGFR-ERK1/2 pathway and induce the expression of prostaglandin synthases and transporters, such as *PTGS2, PLA2G4A, SLCO2A1*, and *ABCC4*. PGR has been reported to have an important role in ovulation [92] [93]. Additionally, repeated ovulation is accompanied by inflammation, and the ovary can maintain a dynamic balance for several years [94][95]. To explore the mechanism by which the ovary protects itself from repeated ovulatory inflammation, Park et al. [96] applied scRNA-seq to the ovaries of *ESR2-PGR* knockout (KO) and WT mice. Their results demonstrated that *PGR* can indirectly inhibit the synthesis of *PTGS2* and *PGE2* in GCs by inhibiting *NF-κB*, thereby reducing the ovarian damage caused by oxidative stress and the DNA damage induced by ovulatory inflammation.

2.2.3. The Characteristics of Granulosa Cells in Atretic Follicles

In female mammals, more than 99% of follicles undergo atresia at various stages [97]. GCs play a crucial role in regulating follicular atresia [98]. Using scRNA-seq, FAN et al. [40] found that GCs from atretic follicles highly express genes related to myeloid leukocyte activation, such as *FCER1G, CD53, AIF1*, and *CX3CL1*. Macrophages have

been observed in GC layers from atretic follicles, and excessive T-helper 1 in the ovary leads to follicular atresia, implying that immune cells play a role in follicular atresia [99][100][101][102]. In addition, another scRNA-seq study by FAN et al. [87] discovered that GCs in the early stages of atresia had lower levels of *GJA1* and *CDH2*. *GJA1* and *CDH2* are associated with cell connections [103][104][105], indicating that the cell–cell communication in atretic follicles decreased. Follicular atresia is important for maintaining homeostasis of the ovarian internal environment, and a better exploration of the characteristics of atretic GCs will facilitate the understanding of the molecular mechanisms of follicular atresia.

2.2.4. The Characteristics of Granulosa Cells from Aged Females

The characteristics of GCs change with the progression of ovarian aging. Some studies have shown that oxidative stress causes damage to GCs during ovarian aging, thus affecting the normal development of oocytes and influencing the fertility of women [86][106]. The analysis of scRNA-seq results of juvenile and old monkey GCs showed that the expression of apoptosis-related genes in GCs from aged female ovaries was upregulated, while the expression of genes related to oxidoreductase activity was downregulated. In addition, the transcriptional regulatory network demonstrated that key genes, including *ELF4* and *FOSB*, which are crucial in regulating reductase-activity-related genes such as *IDH1* and *NDUFB10*, were downregulated [41]. A decrease in antioxidant enzyme activity leads to a damaged antioxidant response, resulting in the enhanced production of reactive oxygen species in GCs [107][108]. Therefore, the antioxidant capacity of GCs is impaired with increasing age, contributing to a decline in the GC quality. The scRNA-seq study more specifically explains the damage mechanism of oxidative stress in senescent GCs, which will help put forward anti-aging strategies in the future.

2.3. A Single-Cell Perspective of Ovarian Stromal Cells

Approximately 83% of ovarian cortex cells are classified as stroma [38], and the majority of ovarian stroma are stromal cells [109]. Furthermore, “ovarian stromal cells” comprise multiple cell populations [110]. The emergence of scRNA-seq makes it possible to better characterize stromal cells. Using this method, researchers found that some genes, such as *DCN, COLLA1, LUM, APOE*, and *COL3A1*, could serve as cellular markers for human stromal and theca cells [40][87]. Another scRNA-seq study discovered that human stromal cells expressed mesodermal lineage markers (*DCN* and *PDGFRA*) and extracellular matrix proteins (*COLLA1* and *COL6A1*) [38]. In nonhuman primate ovarian stromal cells, cellular markers have been reported, such as *TCF21* and *COLLA2*, and some cells of this population show high expression levels of the theca cell marker steroidogenic acute regulatory protein [41]. Because theca cells are generated from stromal cells [111], they may have the same cell markers. In addition, mouse stromal cells specifically express *TCF21* and *NR2F2* [42]. These studies demonstrated the characteristics of stromal cells under normal physiological conditions.

Additionally, stromal cells from atretic follicles show high levels of *XBP1* and *SELK*, which participate in endoplasmic-stress-induced apoptosis [87]. Apoptosis is the underlying mechanism of follicular atresia [112], implying that the apoptosis of stromal cells may play a role in follicular atresia. More importantly, stromal cells from atretic follicles express genes related to the complement system, such as *C1R, C1S*, and *C7*. As the complement system

plays an important role in immune and inflammatory responses [113], activation of the complement system may contribute to ovarian remodeling and the maintenance of ovarian homeostasis. Given that many follicles undergo atresia before reaching ovulation in every ovarian cycle, an understanding of the features of stromal cells from atretic follicles can provide new insights into the process of ovarian and follicular remodeling.

2.4. A Single-Cell Perspective of Ovarian Smooth Muscle Cells

Smooth muscle cells (SMCs) were discovered in the ovary around the follicles, corpora lutea, atretic follicles, and between groups of interstitial cells [114]. They play an essential role in ovulation, regulating the growth and development of the corpus luteum and the collapse of the ruptured follicle [115][116][117]. scRNA-seq technology has contributed to identifying markers. Two studies on SMCs from the inner cortex of adult human ovaries found that they can be recognized by *TAGLN* and *RGS5* [40][87]. Another scRNA-seq study of the human ovarian cortex identified SMCs expressing *TAGLN, RGS5, MYH11, MCAM*, and *RERGL* [38]. Single-cell transcriptomic analysis of nonhuman primate ovaries revealed that SMCs specifically expressed *DES* and *ACTA2* [41]. These studies have contributed significantly to the mapping of ovarian SMC signatures.

2.5. A Single-Cell Perspective of Ovarian Endothelial Cells

Ovarian endothelial cells (OECs) play an essential role in neovascularization, follicular development, and the formation and function of the corpus luteum [118][119][120]. scRNA-seq has enabled significant progress in the understanding of OECs. Several scRNA-seq studies have demonstrated that human OECs can be identified based on the expression of *CDH5 (CD144), vWF, CLDN5, PECAM1 (CD31)*, and *CD34* [1][38][40][87]. An investigation of nonhuman primate ovaries also confirmed that OECs can be identified by the high expression of *CDH5* and *vWF* [41]. In addition, three single-cell transcriptomic studies of mouse E16.5 to PD3 ovaries revealed that OECs can express *APLNR* and *EGFL7* [42][43][44]. Understanding the heterogeneity and clarifying the pathological contributions of OECs remain important tasks.

2.6. A Single-Cell Perspective of Ovarian Immune Cells

Ovarian immune cells comprise macrophages, monocytes, B lymphocytes, T lymphocytes, and natural killer cells [110]. Immune cells play a key role in supporting optimal ovarian function and contribute to folliculogenesis, ovulation, and corpus luteum formation and regression [121][122]. Recently, scRNA-seq has yielded significant achievements in the characterization of ovarian immune cells. Two scRNA-seq studies of humans showed that *CD53, CXCR4, CD69*, and *ITGB2* may be marker genes of ovarian immune cells; human innate immune cells highly express *CD68* and *IFI30*; human ovarian T lymphocytes specifically express *CD2, CD3G*, and *CD8A*; and human antigen-presenting cells specifically express *CD14, HLA-DRA, B2M*, and *HLA-DQB1* [38][87]. A study of nonhuman primates suggested that natural killer cells expressed *CD3D* and *KLRB1*, whereas macrophages expressed *CD68* and *CD14* [41]. In mice, ovarian immune cells specifically express *ELANE, MPO*, and *TYROBP* [42][43][44]. A scRNA-seq investigation of mouse follicles indicated that T lymphocytes can be marked by *AW112010*

and *CD3G*, B lymphocytes can be marked by *IGHM* and *CD37*, and monocytes or monocyte-derived cells can be marked by *CD14* [88]. These studies provided an experimental basis for exploring ovarian immune cells in vitro.

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