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The Ovarian Stroma as a New Frontier

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Abstract

Historically, research in ovarian biology has focused on folliculogenesis, but recently the ovarian stroma has become an exciting new frontier for research, holding critical keys to understanding complex ovarian dynamics. Ovarian follicles, which are the functional units of the ovary, comprise the ovarian parenchyma, while the ovarian stroma thus refers to the inverse, or the components of the ovary that are *not ovarian follicles*. The ovarian stroma includes more general components such as immune cells, blood vessels, nerves, and lymphatic vessels, as well as ovary-specific components including ovarian surface epithelium, tunica albuginea, intraovarian rete ovarii, hilar cells, stem cells, and a majority of incompletely characterized stromal cells including the fibroblast-like, spindle-shaped, and interstitial cells. The stroma also includes ovarian extracellular matrix components. This review combines foundational and emerging scholarship regarding the structures and roles of the different components of the ovarian stroma in normal physiology. This is followed by a discussion of key areas for further research regarding the ovarian stroma, including elucidating theca cell origins, understanding stromal cell hormone production and responsiveness, investigating pathological conditions such as polycystic ovary syndrome (PCOS), developing artificial ovary technology, and using technological advances to further delineate the multiple stromal cell types.

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AUTHOR CONTRIBUTION STATEMENT

H.M.K., A.S. and V.P. conceived and drafted the review. A.S. provided oversight. H.M.K. and C.E.T. wrote the review. F.L.C. revised the figure. M.B.M., M.X. and F.L.C. provided critical input and edits. All authors read and approved the final version.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Vasantha Padmanabhan is an Associate Editor of *Reproduction*. Vasantha Padmanabhan was not involved in the review or editorial process for this paper, on which she is listed as an author

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WHAT IS THE OVARIAN STROMA AND WHAT DOES IT DO?

Organs are comprised of two components: (1) the parenchyma, or the specialized tissue that performs the function of the organ, and (2) the stroma, which is typically the supporting tissue (Young *et al.*, 2014; Mescher, 2018). Ovarian follicles, which are the functional units of the ovary, comprise the ovarian parenchyma. Conceptualizing the stroma as the inverse of the parenchyma, the ovarian stroma thus refers to the components of the ovary that are *not ovarian follicles*. The ovarian stroma is comprised of general components such as immune cells (Wu *et al.*, 2004), blood vessels (Reeves, 1971), nerves (Neilson *et al.*, 1970), and lymphatic vessels (Brown *et al.*, 2010), as well as ovary-specific components. These ovary-specific components include ovarian surface epithelium (Auersperg *et al.*, 2001), tunica albuginea (Reeves, 1971), intraovarian rete ovarii (Wenzel and Odend'hal, 1985), hilar cells (Neilson *et al.*, 1970), ovarian stem cells (Hummitzsch *et al.*, 2015), a majority of incompletely characterized stromal cells that includes the fibroblast-like, spindle-shaped, and interstitial cells (Reeves, 1971), and possibly other cell types not included in this list. In addition to these cell types, ovarian extracellular matrix (ECM) provides structural and biochemical support to surrounding cells and is a key component of the stroma (Berkholtz *et al.*, 2006) (Figure 1, Table I). Some studies have used the broad terms 'ovarian interstitial stroma' or 'theca interstitial cells' (TICs) to refer to the heterogeneous stromal compartment (e.g. Tingen *et al.*, 2011; Hummitzsch *et al.*, 2019). For the purpose of this review, we will interpret the ovarian stroma as the broadly inclusive non-follicular components of the ovary. We also want to highlight that the term 'stromal cells' does not refer to a single homogenous cell population. Instead, when feasible, we recommend more specific descriptions like 'stromal macrophages' to refer to individual components of the stromal compartment. What is known about the multiple cell types and components of the stroma is detailed below.

General Cell Types of the Ovarian Stroma

Immune Cells—Cells of the immune system appear to play critical roles in supporting ovarian physiologic processes. Immune cells, including macrophages, mast cells, and eosinophils, are present in immature or resting ovaries at low levels throughout the stroma. These levels tend to increase around ovulation, particularly near the theca vasculature, with subsequent migration into developing corpora lutea (Norman and Brannstrom, 1994). Ovarian immune cells serve multiple functions, including phagocytosis and antigen presentation, tissue remodeling via proteolytic enzymes, and secretion of soluble signals including cytokines, chemokines, and growth factors (Norman and Brannstrom, 1994; Wu *et al.*, 2004). Macrophages are a predominant ovarian immune cell type, with other immune cells present including B and T lymphocytes, Natural Killer cells, dendritic cells, neutrophils, eosinophils, and mast cells (Norman and Brannstrom, 1994; Suzuki *et al.*, 1998; Carlock *et al.*, 2013; Kenngott *et al.*, 2016; Fan *et al.*, 2019; Zhang *et al.*, 2020) (Figure 1, Table I). Ovarian macrophages have received ongoing attention with regard to their role in reproductive homeostasis and their regulation by estrogen (reviewed in Wu *et al.*, 2004; Pepe

et al., 2018). Ovaries may contain multiple macrophage subsets, and phenotypes can range from classical inflammatory (M1) to alternative tissue remodeling (M2) during different parts of the ovarian cycle (Carlock *et al.*, 2013; Pepe *et al.*, 2018). Increased proportions of M2 macrophages, monocyte-derived macrophages, and multinucleated macrophages have been seen with murine ovarian aging (Briley *et al.*, 2016; Zhang *et al.*, 2020). Macrophage and other myeloid cell depletion using the CD11b-DTR mouse model has resulted in infertility, with hemorrhagic ovaries, ovarian endothelial cell depletion, impaired corpora lutea formation, and diminished progesterone production (Turner *et al.*, 2011; Care *et al.*, 2013). Although ovarian immune cells, particularly macrophages, have been the subjects of ongoing research, gaps in knowledge remain regarding cyclic, hormonal, and temporal dynamics as well as contributions to ovarian pathologic conditions (Table I).

Blood Vessels—The vasculature of the ovary supports critical ovarian functions, and includes blood vessel endothelial cells, pericytes, and smooth muscle cells (Figure 1, Table I). Ovarian blood vessels travel through connective tissue to provide tissue oxygenation, hormone trafficking, and nutrients, in addition to supporting waste removal. The medulla of the human ovary typically contains the larger blood vessels and at the cortico-medullary junction, small medullary arteries branch to cortical arterioles (Reeves, 1971). These cortical arterioles form vascular arcades of interconnected short straight vessels of fixed length running along the connective tissue fascicles. With pressure, the cortical arterioles could be compressed to form avascular regions as part of the formation of stigma for ovulation (Reeves, 1971). Medullary vessels include spiraling arteries and arterioles, which may allow expansion with growth (Reeves, 1971). The microvasculature of the ovary contributes to folliculogenesis and corpora lutea formation. Follicles contain a basal lamina between their granulosa and theca cell compartments, allowing for a blood-follicle barrier (Siu and Cheng, 2012). With the formation of the theca cell layer, follicles develop microvasculature between the theca cells that supports the increased growth and development of the follicle, yet never passes beyond the basal lamina before ovulation. The formation of the corpus luteum, a highly vascular structure, occurs after theca microvasculature invades into the granulosa layer following ovulation (Rolaki *et al.*, 2005). Gaps in knowledge remain around the role of oxygen tension, as regulated by ovarian vasculature. Oxygen tension may have regulatory effects in the ovary, with in vitro studies demonstrating that oxygen levels can impact bovine granulosa cell luteinization and rat corpora lutea progesterone production (Gafvels *et al.*, 1987; Baddela *et al.*, 2018). Dysfunction of ovarian vasculature has been implicated in the pathophysiology of PCOS (Di Pietro *et al.*, 2018), and additional studies are needed to address the pathologic role and therapeutic management of altered ovarian angiogenesis (Table I).

Nerves—Neilson *et al.*'s (1970) review of ovarian innervation describes widespread innervation present in the ovarian stromal compartment, noting that some nerves follow blood vessels in the medulla while others branch among the cells in the stroma (Figure 1, Table I). In mouse gonadal development, neural crest neurons colonize the ovary, differentiate into neurons and glia, and form dense neural networks in the medulla that extend towards cortical regions (McKey *et al.*, 2019). Functionally, both sympathetic and parasympathetic innervation of the ovary has been demonstrated, and regulation by the

sympathetic nervous system has been shown to inhibit estradiol secretion and cause vasoconstriction (reviewed in Uchida, 2015). In a PCOS model, estradiol-treated rats demonstrated increased ovarian sympathetic activity and cystic anovulatory ovaries, with improvement noted in cyclicity and corpora lutea formation following superior ovarian nerve transection (Barria *et al.*, 1993). Further study is warranted regarding the neuronal regulation of different cells types in the stroma, physiologic consequences of denervation, and neuronal contributions to pathology (Table I).

Lymphatic Vessels—Lymphatic vasculature includes small capillaries comprised of endothelial cells without a basement membrane that have large gaps between cells to allow fluid, cellular, and macromolecular transport. These capillaries feed into larger collecting vessels with basement membranes, valves, and smooth muscle (Figure 1, Table I). The ovary has a rich lymphatic network, closely associating with blood vasculature, extending from the medulla into the cortex adjacent to developing follicles, with some species variability in regard to presence in the corpus luteum (Brown and Russell, 2014). The lymphatic system typically helps to maintain fluid homeostasis by returning extravascular fluid and proteins back to the bloodstream and participating in immune cell trafficking. In the developing mouse ovary, lymphatic vessels only appeared postnatally, potentially arising from the extraovarian rete ovarii, as seen in a *Prox1*-EGFP mouse model, where *Prox1* expression marks the commitment of endothelial cells to the lymphatic lineage (Svingen *et al.*, 2012). Lymphatic vasculature has been shown to remodel in response to hormonal regulation in mouse ovaries (Brown *et al.*, 2010). Although lymphatic vasculature plays essential physiologic roles in the ovary, the dynamic regulation and pathologic relevance of the ovarian lymphatics remains to be fully elucidated (Table I).

Ovary-Specific Cell Types of the Ovarian Stroma

Ovarian Surface Epithelium—The surface epithelium of the ovary is a heterogenous flat to cuboidal epithelial layer derived from the mesoderm, also called the “germinal epithelium” because of the false past belief that it contributed to germ cell formation (Auersperg *et al.*, 2001) (Figure 1, Table I). The keratin-rich ovarian surface epithelial cell layer helps to facilitate repair after ovulation and dynamically expands and contracts with cyclic ovarian changes (Xu *et al.*, 2018; Hartanti *et al.*, 2020). Scanning electron microscopy and immunofluorescence of the surface epithelium of developing fetal bovine ovaries demonstrated expansion from the hilar region to surround the entire ovary, with changes corresponding to underlying stromal rearrangement (Hartanti *et al.*, 2020). Although fetal ovarian surface epithelial cells had been previously thought to be a developmental source for granulosa cells, more recent studies suggest that ovarian surface epithelial cells instead share a common progenitor with granulosa cells, known as the Gonadal Ridge Epithelial-Like (GREL) cell (Auersperg *et al.*, 2001; Hummitzsch *et al.*, 2013). Although definitive markers have not been identified, surface epithelial cells have increased expression of the cytokeratins 7, 8, 18, and 19 as well as plakophillin-2 and desmoglein-2 (Hummitzsch *et al.*, 2013; Hartanti *et al.*, 2020) (Table I). Further work remains regarding identifying definitive markers, understanding heterogeneity, and clarifying the pathologic contributions of the ovarian surface epithelium.

Tunica Albuginea—The ovarian tunica albuginea, positioned beneath the surface epithelium, is a thin and hypocellular connective tissue sheath, which serves as a protective layer for the ovary (Reeves, 1971). The tunica albuginea is collagen-rich and undergoes remodeling prior to ovulation. Using electron microscopy, Okamura *et al*, (1980) observed a decrease in presence of collagen bundles at the human follicular apex as follicles reached the preovulatory stage. This degradation was paralleled by an increase in apical fibroblasts with developed cytoplasm and lysosome-like granules, which were suspected to contain collagenases for degradation of the tunica albuginea (Okamura *et al.*, 1980). There has been limited study of the ovarian tunica albuginea and further work can help to clarify physiologic and pathologic roles and regulation (Figure 1, Table I).

Intraovarian Rete Ovarii—The rete ovarii are remnants of the mesonephric (Wolffian) ducts that typically form part of the male reproductive tract and regress in the female reproductive tract. They are often found as groups of tubules lined by cuboidal or columnar epithelium in the hilus of the ovary or extending through the medulla, as well as in the extraovarian space (reviewed in Wenzel and Odend'hal, 1985) (Figure 1, Table I). There has been limited investigation into the function of the rete ovarii, particularly after development where they may play relevant roles. In a study of murine theca cell lineages, one of the two identified progenitor populations of theca cells migrated from the adjacent mesonephros and was potentially related to the rete ovarii (Liu *et al.*, 2015; Rotgers *et al.*, 2018). Ovarian lymphatic vasculature origins have also been connected to the rete ovarii (Svingen *et al.*, 2012). Although not necessarily specific markers, increased levels of cytokeratin 19 and vimentin have been noted in human rete ovarii (Russo *et al.*, 2000). Further study is needed to elucidate the physiologic role of the rete ovarii in adults as well as the pathologic relevance (Table I).

Hilar Cells—There are reports of distinct cells located in the ovarian hilus with Reinke crystals, which are commonly found in testicular Leydig cells (Neilson *et al.*, 1970) (Figure 1, Table I). These cells are frequently located in clusters associated with a nerve trunk (Neilson *et al.*, 1970), and synthesize and secrete androgens in response to LH stimulation, although their physiologic role has not been well-established (Erickson *et al.*, 1985). Hyperplasia of these hilar cells has been implicated in virilization in postmenopausal women (Delibasi *et al.*, 2007). Cellular markers have not been established and the physiologic role and pathologic relevance of these cells remains generally uncharacterized.

Ovarian Stem Cells—The ovary may contain stem cells for a variety of different cell types, including somatic (e.g., granulosa, surface epithelial, thecal, stromal) and germline stem cells (reviewed in Hummitzsch *et al.*, 2015) (Figure 1, Table I). The presence and importance of ovarian germline (oogonial) stem cells has been a controversial topic, although the ovarian follicular reserve is generally lost with age without substantive renewal. Putative ovarian oogonial stem cells were first isolated through DEAD [Asp-Glu-Ala-Asp] box polypeptide 4 (DDX4, also known as VASA) tagging and cell sorting and have been shown to develop into oocytes, although isolation of DDX4 positive cells has been questioned, particularly related to assumptions about cytoplasmic versus surface expression and antibody cross-reactivity (Johnson *et al.*, 2004; Zarate-Garcia *et al.*, 2016). Others have

disputed the presence of oogonial stem cells, noting that oogonial stem cells were not detectable using sensitive single-cell lineage-tracing in adult female mice (Lei and Spradling, 2013). Additionally, postnatal DDX4-expressing cells generated using a *Rosa26*$^{rbw/+}$*; Ddx4-Cre* fluorescent reporter mouse were not seen to be mitotically active nor participating in follicular renewal (Zhang *et al.*, 2012). A recent single-cell sequencing study isolated human Abcam DDX4-positive cells and concluded these cells were perivascular cells rather than oogonial stem cells (Wagner *et al.*, 2020). In contrast, cell line establishment of female germline stem cells has been described using cells from human ovarian cortical tissue fragments present in follicular aspirates, which differentiated into oocyte-like cells (Ding *et al.*, 2016). Isolated, purified, and cultured female germline stem cells from an EGFP-transgenic mouse were shown to differentiate into oocytes, capable of restoring function and generating offspring in a mouse model of premature ovarian failure (Wu *et al.*, 2017). The presence of ovarian germline stem cells continues to be a highly contested topic, generally eclipsing the discussion of somatic stem cells. The addition of human mesenchymal stem cells originating from amniotic fluid has also been used to help restore ovarian function in mouse models of premature ovarian failure, suggesting a role for somatic stem cells in improving altered paracrine signaling and the stroma microenvironment (Liu *et al.*, 2019).

Incompletely Characterized Stromal Cells—The majority of the ovarian stroma is comprised of a mixed population of incompletely characterized cells commonly referred to as stromal cells (Reeves, 1971). This includes the populations of cells also described as fibroblast-like, spindle-shaped cells, or interstitial cells (Figure 1, Table I). In general, fibroblasts secrete ECM proteins, such as collagen, for cellular support, scaffolding, and repair. A retrospective study of histologic sections from non-pathologic human ovaries from 167 women ages 17–79 carried out with the goal of describing the morphology of various types of stromal cells identified five types of fibroblast-like/interstitial stromal cells (Reeves, 1971). While recent human single-cell RNA-sequencing studies (e.g., Fan *et al.*, 2019) confirm the presence of multiple stromal cell clusters, a comprehensive and complete characterization of stromal cell types throughout the ovary is lacking. The distribution and subtypes of stromal cells will likely differ with their location in the ovary (e.g. cortex vs. medulla). The stromal cell distribution is also likely to be affected by cyclic structural changes, as follicles grow and ovulate, and corpora lutea develop. Changes are also evident over the reproductive lifespan, including increases in fibrotic collagen as demonstrated in aging murine and primate ovaries (Briley *et al.*, 2016; Wang *et al.*, 2020). Some possible cellular markers that have been identified include COUP-TFII and/or ARX (Hummitzsch *et al.*, 2013; Rotgers *et al.*, 2018). Other studies have used *DCN* and *LUM* to identify populations of human theca/stroma cells (Fan *et al.*, 2019). Higher expression in some of the human cells considered to be stroma was demonstrated for markers *PDGFRA*, *DCN*, *COL1A1*, *COL6A1*, *STAR*, and/or *CYP17A1* (Wagner *et al.*, 2020). A different study delineated nonhuman primate ovarian stroma by expression of *TCF21*, *COL1A2*, and/or *STAR* (Wang *et al.*, 2020). For these incompletely characterized stromal cell types, careful ontology, further marker identification, and attention to nuances of regionality and steroid production are critical next steps (Table I).

Extracellular Matrix (ECM) Components

Structure & Definition—The ECM is composed of fibril- and network-forming proteins, proteoglycans, and glycosaminoglycans, the composition of which is unique to each tissue (Figure 1, Table I). Cells secrete soluble ECM components to the extracellular compartments where cell-secreted enzymes such as lysyl oxidase (LOX) crosslink the ECM precursors into large networks (Theocharis *et al.*, 2016). These matrices regulate cellular functions including adhesion, migration, and proliferation through cell receptor interactions, mechanotransduction, and cell interaction with ECM-sequestered growth factors (Taipale and Keski-Oja, 1997).

Several reviews have covered the extensive list of ECM components that exist broadly in tissues and specifically in the ovary; most notably, collagen types I, III, IV, and VI, fibronectin, and laminin (Berkholtz *et al.*, 2006; Irving-Rodgers and Rodgers, 2006). Collagens I and III have been shown to be distributed in concentric layers connected by bundles in human cortical stroma (Lind *et al.*, 2006). A recent proteomic study examining the ECM of the human ovarian cortex revealed that collagens comprise nearly half of the ECM proteins and associated factors, the most dominant of which was collagen VI, a basement membrane-anchoring ECM protein (Ouni *et al.*, 2019). Another recent proteomic study examined ECM compositional differences between porcine cortex and medulla, showing increased expression of collagen I, agrin, elastin microfibril interfacer 1, and fibronectin in the cortex compared to the medulla (Henning *et al.*, 2019). These proteomic studies both identified over 80 ECM and ECM-associated proteins, in categories of collagens, glycoproteins, proteoglycans, ECM-affiliated proteins, ECM regulators, and secreted factors (Henning *et al.*, 2019; Ouni *et al.*, 2019).

Many studies of ECM have focused on matrix within follicles during development. Follicles have a unique pericellular matrix called the basal lamina, composed primarily of laminin and type IV collagen stabilized by nidogen and perlecan which separates the granulosa and theca cell compartments (Irving-Rodgers and Rodgers, 2006). As follicles grow, they continuously remodel the basal lamina to allow for expansion of the follicle as granulosa cells proliferate. Granulosa cells have been shown to produce the major components of the basal lamina, although theca and other cells in the ovarian stroma may contribute to basal lamina deposition in later stages (Rodgers *et al.*, 1999). The basal lamina also plays a role in mediating granulosa cell growth and antrum formation through growth factor sequestration and signaling. Perlecan in the basal lamina is able to bind growth factors and is charge- and size-selective, serving as a barrier to diffusion of growth factors between the granulosa and theca cell compartments, allowing the follicular fluid and basal lamina to become reservoirs of factors to promote healthy folliculogenesis (McArthur *et al.*, 2000).

Mechanics—The ovary has two major compartments which differ in their ECM composition and structure – a stiff cortex where primordial follicles reside in dormancy, and a less dense medulla where antral follicles vigorously remodel the ECM through proteolytic degradation as they reach preovulatory stages. Decellularized human and bovine ovarian tissue reveals radially-aligned collagen fibers in the cortex, lending to its increased stiffness, whereas the medulla is composed of a network of pores with anisotropic collagen fibers,

suggesting differences between cortical and medullary ECM-producing stromal cells (Laronda *et al.*, 2015; Chiti *et al.*, 2018). The prominence of ovarian cortical and medullary regionalization can differ across species, and is notably reduced in rodent ovaries when compared to human ovaries (Jiménez, 2009). The mechanical properties of these regions have important roles in mechanotransduction for the follicles as they activate and develop. Primordial follicle dormancy has been shown to be regulated by the Hippo signaling pathway, where rigidity of the ovarian cortex inactivates yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) to inhibit growth (Kawamura *et al.*, 2013). Follicle activation can be initiated with disruption of the Hippo signaling pathway, for example when follicles are isolated from the cortex, further illustrating the importance of ECM mechanical properties in maintenance of the follicular reserve (Kawamura *et al.*, 2013). After activation, early stage follicle growth and survival is still dependent on a stiff matrix, as has been shown in vitro (Hornick *et al.*, 2012). As follicles grow, they require a softer matrix for expansion as provided by the medullar region of the ovary, and in vitro studies have shown improved growth, survival, and steroidogenesis of later stage follicles in permissive matrices (West *et al.*, 2007; West-Farrell *et al.*, 2009).

Function: Signaling and Remodeling—ECM components play a large role in regulating cell functions through both direct and indirect signaling. Fibronectin and laminin contain integrin-binding sequences (most notably Arg-Gly-Asp, or RGD) which allow cells to directly interact with the ECM and initiate signaling cascades for proliferation and differentiation as follicles develop (Monniaux *et al.*, 2006). ECM also has an indirect role in signaling as it acts as a reservoir of growth factors and cytokines and mediates their presentation to cells both when they are bound and when they are released upon ECM degradation. ECM is a dynamic structure in tissues, continuously being remodeled by the cells which reside in it through matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), and plasminogen activators (McIntush and Smith, 1998). Follicles and other ovarian stromal cells secrete these enzymes to soften the surrounding ECM and allow for follicular expansion, and in this process cytokines and growth factors bound to the ECM are released. Several growth factors known to be key regulatory molecules in folliculogenesis including fibroblast growth factor, transforming growth factor beta, platelet derived growth factor, hepatocyte growth factor, and insulin-like growth factor have ECM-binding motifs or can be sequestered within the ECM through binding factors such as follistatin (Logan and Hill, 1992). In this way, ECM remodeling is a mechanism by which growth factor bioavailability can be mediated or disrupted in some pathological conditions (McIntush and Smith, 1998). If dysregulated, ECM degradation may also trigger pathogen-free inflammation. For example, hyaluronan is a glycosaminoglycan that forms low molecular weight fragments during turnover, which have been shown in cultured murine stromal cells to increase the secretion of type 2 inflammatory cytokines and activate genes involved in eosinophil recruitment, while also leading to adverse effects on cultured follicles (Rowley *et al.*, 2020).

At the final stages of follicular maturation the ECM again plays an important role in ovulation. Follicles are stimulated by the LH surge to produce large amounts of MMPs and plasminogen activator to degrade the ECM at the apical region of the follicle (Curry and

Smith, 2006). This process is further amplified by the release of tumor necrosis factor-alpha (TNF-a) from the degraded ECM to promote collagenase production and apoptosis of ovarian epithelial cells (Curry and Smith, 2006). The weakened cellular and ECM components at the apical region, along with pressure from the follicular fluid and increased vascular pressure, facilitate follicular rupture and expulsion of the oocyte into the periovarian space (Matousek *et al.*, 2001).

KEY AREAS FOR FURTHER RESEARCH AND FUTURE PERSPECTIVES

Understanding the origins of the theca cells

The theca cell layer is divided into the theca interna, with cytoplasmic lipid droplets characteristic of its role in steroid production, and the theca externa, which is a mix of fibroblasts and smooth muscle cells that are more contiguous with the broader ovarian stroma (reviewed in Young and McNeilly, 2010; Richards *et al.*, 2018). The relationship between the supporting cells of the ovarian stroma and the theca cells has not been definitively established, although it is generally agreed that the theca cells originate at least in part from stromal cells (Young and McNeilly, 2010; Rotgers *et al.*, 2018).

Murine theca cells have been shown to arise from two types of progenitors: *Wt1*-positive cells in the fetal ovary and *Gli1*-positive cells migrating from the mesonephros adjacent to the ovary (Liu *et al.*, 2015). Near birth, desert hedgehog and Indian hedgehog paracrine signals from granulosa cells appear to prompt expression in undifferentiated stromal progenitor cells of the theca lineage marker *Gli1*. Microarray analysis suggested differences based on theca progenitor population, with increased steroidogenesis in the mesonephros-derived *Gli1*-positive cells (Liu *et al.*, 2015). The steroidogenic androgen-producing theca cells may arise from the mesonephros derived progenitors, while the theca fibroblasts, perivascular smooth muscle cells, and possibly the interstitial ovarian cells may arise from the ovarian WT1+ progenitors (Richards *et al.*, 2018).

Additional undifferentiated stromal cell progenitors (possibly positive for *Lhx9, Mafb, Coup-tfII, and Arx)* may yield a nonsteroidogenic stromal cell population, possibly expressing *Coup-tfII* and *Arx*. Overlapping expression of COUP-TFII and ARX in the same population of cells has not been established (Rotgers *et al.*, 2018). Sonic hedgehog signaling has been shown to regulate expression of COUP-TFII, which was identified in murine theca interna cells and in mesenchymal cells around the corpus luteum (Krishnan *et al.*, 1997; Takamoto *et al.*, 2005). COUP-TFII is likely expressed in steroidogenic cells, as haploinsufficient female mice demonstrated altered reproduction function, including reduced expression of steroidogenic enzymes needed for progesterone synthesis and reduced vascularization (Takamoto *et al.*, 2005). Three populations of somatic cell precursors have been demonstrated in murine fetal ovaries, marked by mutually exclusive expression of COUP-TFII and the granulosa cell markers FOXL2 and LGR5 (Rastetter *et al.*, 2014). Mutually exclusive FOXL2 and COUP-TFII expression was also seen in early fetal human ovaries, with COUP-TFII expression in the stromal cell population. Several 46,XX *SRY*-negative children with mutations in the gene encoding COUP-TFII were virilized, with testicular tissue confirmed in one child, suggesting a “pro-ovary” and “anti-testis” role for COUP-TFII in developing human female gonads (Bashamboo *et al.*, 2018).

A transgenic mouse study suggests the presence of at least two steroidogenic cell types for ovarian theca and interstitial gland cells. In postnatal mouse ovaries, only a portion of the steroidogenic theca and interstitial gland cells expressed enhanced green fluorescent protein (EGFP) as a reporter of the fetal Leydig enhancer (FLE) of the *Nr5a1* gene (SF-1). SF-1 regulates expression of steroidogenic *CYP* genes. In testes, the FLE differentiates fetal from adult Leydig cells. In these ovaries only approximately 16% of the SF-1 postive cells were positive for EGFP, suggesting at least two cell populations (Miyabayashi *et al.*, 2015).

A transcriptome analysis of the bovine ovarian stroma found that populations isolated by laser microdissection were similar between general interstitial stroma and what they labeled as pre-theca cells (stroma adjacent to preantral follicles). They combined them for the purpose of analysis, and the subsequent stroma was found to be different from both the tunica albuginea and the theca interna (Hummitzsch *et al.*, 2019). The theca interna of small antral follicles had an upregulation of genes associated with steroid hormone and cholesterol synthesis as compared to the stroma (Hummitzsch *et al.*, 2019).

Of note, the concept of theca interstitial cells (TICs) has been used as a catch-all for the residual ovarian tissue husk once follicles had been punctured (Tingen *et al.*, 2011; Tian *et al.*, 2015). When cultured, theca interstitial cells from mouse ovaries take on a fibroblast-like appearance that is distinct from granulosa cells (Tian *et al.*, 2015). The heterogeneity of the TICs has been noted, with a reported shift in populations over a 12-day co-culture with follicles. At the beginning of the culture, the population contained predominantly lipid droplet-containing cells resembling theca cells as well as fibroblast-like cells, whereas the cells were mainly macrophages by day 12 (Tingen *et al.*, 2011). This transition in cell phenotype may be due to differential survival in culture of the different starting cell populations, emphasizing that TICs are not a homogenous grouping.

Further understanding the stromal compartment may aid in better identification of theca progenitors (Figure 2). Additionally, studies using mixed populations of TICs may benefit from greater categorization of these non-follicular populations to aid in interpretation and reproducibility of findings.

Stromal cell hormone production and responsiveness

Some of the ovarian stromal cells are capable of steroid hormone production and contain hormone receptors. For instance, estrogen receptor alpha and beta have been identified in the cytoplasm and nucleus of bovine interstitial cells, which were described as oval cells with lipid droplets and vacuoles that were distinguishable from fibroblasts (Kenngott *et al.*, 2016). Progesterone receptor alpha has been identified in stromal cells and interstitial cells of pregnant and post-partum rabbit ovaries (Abd-Elkareem, 2017). Interstitial cells with features of steroid production have been documented in early gestation in the human fetal ovary (Konishi *et al.*, 1986). Postmenopausal ovarian stromal cells have been postulated to produce androgens, although a study of in vitro isolated postmenopausal human stromal cells found that the predominant population had negligible expression of a key steroidogenic enzyme in the androgen biosynthesis pathway, *CYP17A1*, and did not appear to have significant steroidogenic potential (Jabara *et al.*, 2003). Additionally, they found that transcripts for certain steroidogenic enzymes (*STAR*, *CYP11A1*, and *HSD3B*) were much

less abundant in the in vitro isolated stromal cells than in theca cells, with the exception of *STAR* which had more transcript abundance in stromal cells than in fibroblasts (Jabara *et al.*, 2003). In contrast, localization of CYP17A1 shifted from exclusively the theca interna in control mice to patches in the interstitial stroma in DHT-treated mice, supporting a potential role for the stroma in androgen production following certain perturbations (Candelaria *et al.*, 2019). Single-cell RNA sequencing studies have also demonstrated subpopulations of stromal cells expressing *CYP17A1* and *STAR* (Wagner *et al.*, 2020; Wang *et al.*, 2020). Although stromal cells have demonstrated varied hormone production and responsiveness, definitive characterization of these dynamics and their functional significance remains to be established (Figure 2).

Pathological ovarian stromal changes: polycystic ovary syndrome as an example

Polycystic ovary syndrome (PCOS) has been defined by the Rotterdam Criteria (2004) as two of the three characteristics – hyperandrogenism, oligo or amenorrhea, and follicular cysts as noted on ultrasound (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group., 2004). Polycystic ovarian morphology includes the following features – thickening of the tunica albuginea, ovarian stromal hyperplasia, stromal cell luteinization, and large cystic antral follicles (Hughesdon, 1982). The thickness of the cortical stroma is increased by one third and the subcortical stroma by five-fold (Hughesdon, 1982). In detailed ultrasound assessment, women with PCOS were found to have significantly increased ovarian volume, stromal volume, and stromal peak blood flow velocity as compared to controls (Buckett *et al.*, 1999). In contrast, no difference was found in ovarian stromal blood flow between women with PCOS and a control group explicitly excluding patients with low ovarian reserve (Younis *et al.*, 2011). The ratio of ovarian stromal area to total ovarian area (S/A) by ultrasound was a good predictor of hyperandrogenism in lean Italian women with PCOS, with increased ovarian vascularization and blood flow noted in PCOS patients as compared to controls (Battaglia *et al.*, 2012), and S/A ratio has been proposed as a method to refine the Rotterdam PCOS classification (Belosi *et al.*, 2006). In contrast, the S/A ratio was found to have limited predictive value as a PCOS diagnostic in reproductive-aged Thai women with PCOS (Leerasiri *et al.*, 2015). Another study found increased ovarian stromal area with PCOS, but was unable to demonstrate a relationship between stromal area and PCOS hormonal characteristics (Kaleli *et al.*, 1998). Ovarian angiogenesis dysfunction including increased ovarian stromal vascularization, lower impedance to flow (Alcázar and Kudla, 2012), and alterations in angiogenic factors levels in PCOS, have been further reviewed elsewhere (Di Pietro *et al.*, 2018), with possible implications that restoration of appropriate vessel formation could improve folliculogenesis and ovulation. Inflammation-related gene expression was downregulated in the ovarian stroma and upregulated in granulosa cells for PCOS women as compared to controls, although the downregulation in the stroma may have been affected by a reduced abundance of leukocytes in the PCOS stroma as measured by *CD45* mRNA levels (Schmidt *et al.*, 2014). A reduction in theca-associated activated/memory T lymphocytes has also been seen in PCOS ovaries as compared to controls, without notable differences in macrophage or neutrophil levels across multiple ovarian compartments (Wu *et al.*, 2007). Broadly, PCOS may impact stromal volume, tunica albuginea thickness, stromal luteinization,

vascularization, blood flow, inflammation and immune cell distribution, although the causes and functional impacts of these stromal changes have not been fully elucidated.

Hyperandrogenism, one of the common aspects of PCOS, has been shown to drive certain stromal alterations. For instance, in transgender men given exogenous testosterone therapy, increases were noted in tunica albuginea collagenization, stromal hyperplasia, and stromal luteinization with clusters of luteinized stromal cells (Spinder *et al.*, 1989; Ikeda *et al.*, 2013), as well as increased stromal androgen receptor staining (Chadha *et al.*, 1994). Multiple cell types in polycystic ovaries may produce androgens, as immunohistochemistry revealed the presence of steroidogenic enzymes for androgen synthesis in follicular theca cells, luteinized stromal cells, hilar cells, and sporadic non-luteinized stromal cells (Kaaijk *et al.*, 2000). Mice treated with dihydrotestosterone (DHT) also demonstrated stromal changes, including less dense, hyperplastic, and lipid-filled stroma when compared to age-matched controls. These mice also had an overexpression of multiple genes in the mechanically-separated stroma between controls and DHT-treated mice (Candelaria *et al.*, 2019). This included increased *Vcam1* expression (which may impact vascular and immune responses) in thecal and stromal cells, while theca-specific androgen receptor knockout mice (ThARKO, *Cyp17a1*-iCre, $AR^{f/f}$ mice) demonstrated a lack of DHT-induced *Vcam1* elevation (Richards *et al.*, 2018; Candelaria *et al.*, 2019). ThARKO mice were also shown to retain much of their reproductive function, including cyclicity and fertility as compared to controls when treated with DHT (Ma *et al.*, 2017). For mice with DHT-induced stromal changes, superovulation rescued at least some of the abnormal stromal morphology (Candelaria *et al.*, 2019).

Several changes occur in the ovarian ECM in polycystic ovary syndrome. The cortex and basal laminas of follicles thicken and become more collagenous with reduced glycosaminoglycan content (Salvetti *et al.*, 2003). A comparison of human PCOS to control ovaries in both the follicular and luteal phases revealed significantly lower pro-collagen IV expression compared to control ovaries, and this decrease in collagen IV was postulated to contribute to premature luteinization (Oksjoki *et al.*, 2004). PCOS patients tend to have increased MMP-9 secretion as well, which may be related to the inability of follicles to undergo normal atresia (Dambala *et al.*, 2019).

Stromal contribution to artificial ovary technology

The term ‘artificial ovary’ typically references an ovary constructed using a combination of ovarian follicles (or hormone-producing cell types) within a supportive scaffold (Figure 2). The creation of an artificial ovary as a means of fertility preservation and endocrine support has been a persistent challenge from biological and engineering perspectives as follicle development requires a complex symphony of soluble signals and mechanical cues, some of which may derive from the ovarian stroma.

Co-culture of follicles with stromal feeder cells has shown promise for providing the key soluble factors to promote growth of early stage murine follicles in vitro (Tingen *et al.*, 2011). With regard to directly sourcing ovarian stromal cells, ideal collection strategies may differ between stromal cells and follicles. Human stromal cells have been shown to be better preserved after vitrification than slow freezing, with slow freezing increasing necrosis and

collagen bundle disruption in the stromal cells, while follicles were similarly preserved in both vitrification and slow freezing (Keros *et al.*, 2009). Isolating human stromal cells from fresh medullary tissue was shown to be superior to isolation from ovarian cortex in slow frozen and fresh samples, and led to increased cell yield, better viability, and improved vascularization when encapsulated in fibrin and implanted in the peritoneal pockets of nude mice (Soares *et al.*, 2015). For xenograft models, the importance of transplanting stromal endothelial cells has been demonstrated (Dath *et al.*, 2011). Isolated human ovarian cortical stromal cell suspensions containing stromal endothelial cells yielded well-vascularized and organized grafts after one-week implantations in mice, in contrast to grafts depleted of stromal endothelial cells, which were smaller, necrotic, and poorly vascularized (Dath *et al.*, 2011).

It is also challenging to develop a supportive scaffold that fully recapitulates the ovarian ECM. Multiple three-dimensional hydrogel culture systems such as alginate, fibrin, and poly(ethylene glycol) (PEG) aim to recapitulate the mechanical properties of the ovarian environment to maintain the spherical structure of follicles and allow for their expansion; however, these systems are lacking the biological functionality of ECM and the ability to sequester growth factors (Luyckx *et al.*, 2014; Smith *et al.*, 2014; Kniazeva *et al.*, 2015; Kim *et al.*, 2016; Chiti *et al.*, 2018; Rios *et al.*, 2018). Several groups have attempted to restore the biological function of ECM in these artificial ovaries by encapsulating follicles in ECM matrices such as Matrigel or decellularized tissues (Scott *et al.*, 2004; Laronda *et al.*, 2015). Unfortunately, these matrices do not include all of the components present in native ovarian ECM and also face challenges in translation in regard to availability of tissue and batch-to-batch variability.

While each of these systems incorporates key components necessary for follicle growth, there is yet to be a system that truly mimics the ovarian microenvironment in both complexity of cell populations and extracellular matrix composition which can be translated for clinical use. Part of this limitation relates to scarcity of knowledge as it pertains to the cell types and functions of the ovarian stroma.

Identification of ovarian stromal cells

The multiple populations of cells referred to as stromal cells are incompletely characterized and categorized, leading to confusion across studies that report findings about stromal cells without further identification (Figure 2). Regional differences (e.g. cortex vs. medulla) likely influence the distribution and subtypes of stromal cells. Immunofluorescent imaging using known markers for follicular or stromal cells has advanced our understanding of the ovarian stroma, including the delineation of at least two distinct populations of steroidogenic theca and interstitial gland cells in postnatal murine ovaries, as well as the identification of at least three different somatic cell lineages in murine fetal ovaries (Rastetter *et al.*, 2014; Miyabayashi *et al.*, 2015). With developments in single-cell sequencing technologies to complement these detailed imaging studies, we may soon have the ability to better characterize the cells commonly called stromal cells and refer to them with more precise names as we understand their individual roles in physiologic and pathologic processes.

Single-cell RNA-sequencing experiments have already made progress in identifying major ovarian cell types, transition stages, and markers for cell identification. These studies have significantly contributed to mapping the signatures of human and murine oocytes and granulosa cells from multiple follicular stages (Zhang *et al.*, 2018). Yet, data about the ovarian stroma remain elusive and comparatively scarce. An investigation of somatic cells only in the inner cortex was performed in women undergoing fertility preservation procedures, detecting five clusters of granulosa cells, five clusters of theca and stromal cells, two clusters of smooth muscle cells, three clusters of endothelial cells, and four clusters of immune cells (Fan *et al.*, 2019). They confirmed the presence of adaptive immune cells including T lymphocytes, Natural Killer cells, and B lymphocytes, as well as innate immune cells including monocytes and macrophages. This study also identified upregulation of the complement system (including C1R, C1S, and C7) by theca and stromal cells as a potential contributor to ovarian tissue remodeling (Fan *et al.*, 2019). A subsequent single-cell analysis of the human ovarian cortex reported six clusters, including oocytes, granulosa cells, immune cells, endothelial cells, perivascular cells, and stromal cells. They classified a majority of cells (83%) as stroma, noting shared expression of mesodermal lineage markers (*PDGFRA, DCN*), ECM proteins (*COL1A1, COL6A1*), as well as expression of *STAR* and *CYP17A1* by some cells in the stromal cluster. Although they isolated many stromal cells, their study mainly focused on discerning whether cells isolated using the Abcam DDX4 antibody were oogonial stem cells (Wagner *et al.*, 2020). A single-cell transcriptomic study of ovarian aging in nonhuman primate ovaries identified seven ovarian cell types, including oocytes, granulosa cells, stromal cells, smooth muscle cells, endothelial cells, Natural Killer T cells, and macrophages (Wang *et al.*, 2020). The stromal cell cluster specifically expressed *TCF21* and *COL1A2*, with some cells in the stromal cluster expressing high levels of *STAR* (Wang *et al.*, 2020). A time series single-cell RNA sequencing study was performed for cells labeled with the gonadal somatic cell marker *Nr5a1* (steroidogenic factor 1, SF-1) in the developing mouse ovary from E10.5 to postnatal day 6. Four distinct populations, including early progenitors, stromal progenitors, pre-granulosa cells, and postnatal granulosa cells were identified from their sequencing. Using their time series, they analyzed cell conversion from early progenitors to both the stromal progenitor lineage (E13.5) and the granulosa cell lineages (E11.5-E12.5) (Stévant *et al.*, 2019). These studies are supported by precise immunofluorescent characterization of at least three somatic cell populations in fetal mouse ovaries, including COUP-TFII-positive possible pre-theca progenitors, LGR5-positive cortical granulosa cell progenitors, and FOXL2-positive medullary granulosa cell progenitors (Rastetter *et al.*, 2014). Although single-cell sequencing studies allow for greater granularity in understanding the nuance of different ovarian cellular populations, including the stroma, it remains important to continually reflect on the possible limitations of any starting cellular populations (e.g. inner cortex only), with the overall goal of broadening our understanding of the entire ovarian microenvironment.

Future perspectives

As the majority of ovarian research studies focus on the ovarian follicles, a thorough understanding of the components and functions of the ovarian stroma is an active area of current research. The support provided by the ovarian stroma is essential for three-dimensional follicular maintenance and the integration of signals to support folliculogenesis.

The stromal compartment is heterogeneous and analyses using bulk methods or gross dissection may lose the granularity that could be observed between low density specialized cellular populations. In addition to precise immunohistochemical and immunofluorescent studies for specific stromal cell population identification and lineage tracing, single-cell sequencing studies will continue to allow for more in-depth analysis of physiologic and pathologic changes occurring to specific cell types that might otherwise be grouped together. These sequencing studies must be conducted with critical reflection on the specifics of the origin of the sequenced cells. Greater understanding and careful ontology of the different populations of stromal cells would reduce ambiguity between studies. Further study integrating phenotypic changes in specific stromal cellular populations with functional changes would also help determine how changes in the ovarian stroma occur over time and may interact with folliculogenesis, position, and hormone production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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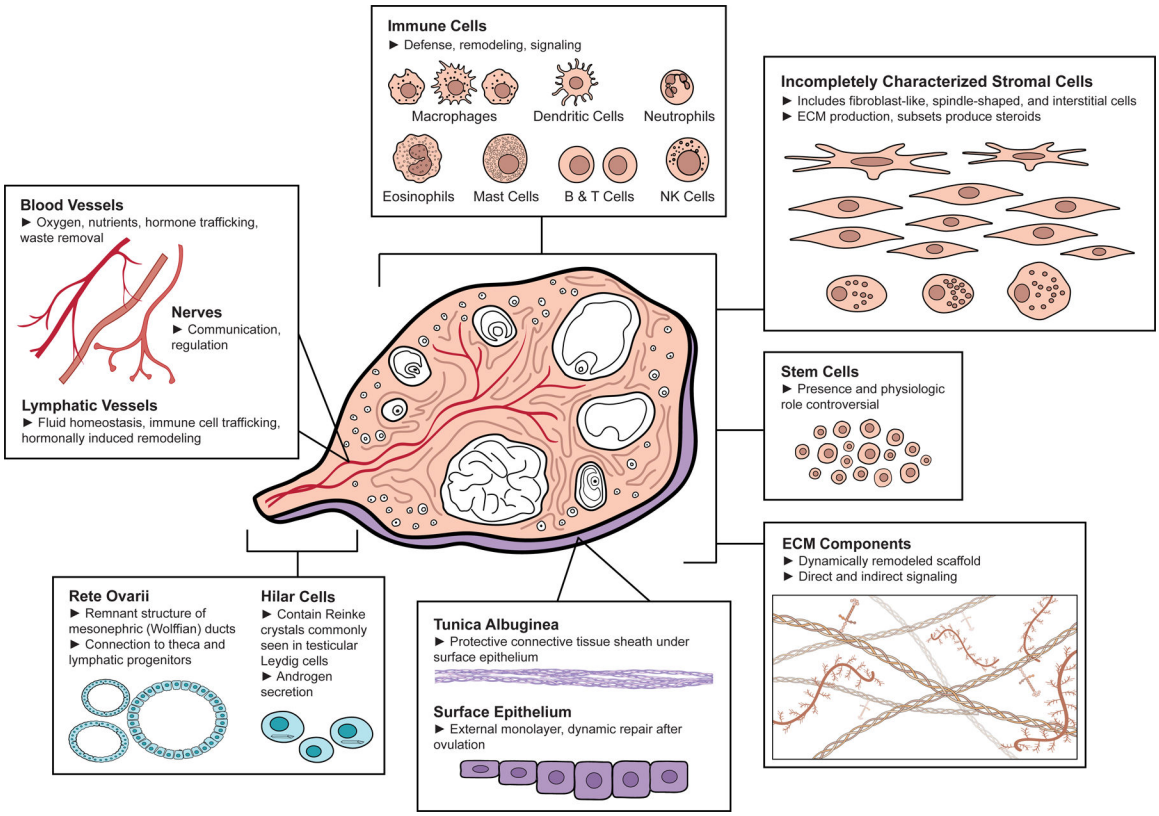

Figure 1.

Components of the Ovarian Stroma. Central diagram of a human ovary (adapted from Gray, 1918) surrounded by boxes highlighting different ovarian stromal components including (clockwise from top center): immune cells including macrophages, dendritic cells, neutrophils, eosinophils, mast cells, B & T cells, and Natural Killer (NK) cells; incompletely characterized stromal cells (including fibroblast-like, spindle-shaped, and interstitial cells); stem cells; extracellular matrix (ECM) components; surface epithelium and tunica albuginea; rete ovarii and hilar cells; and blood vessels, lymphatic vessels, and nerves. Made using ©BioRender - biorender.com.

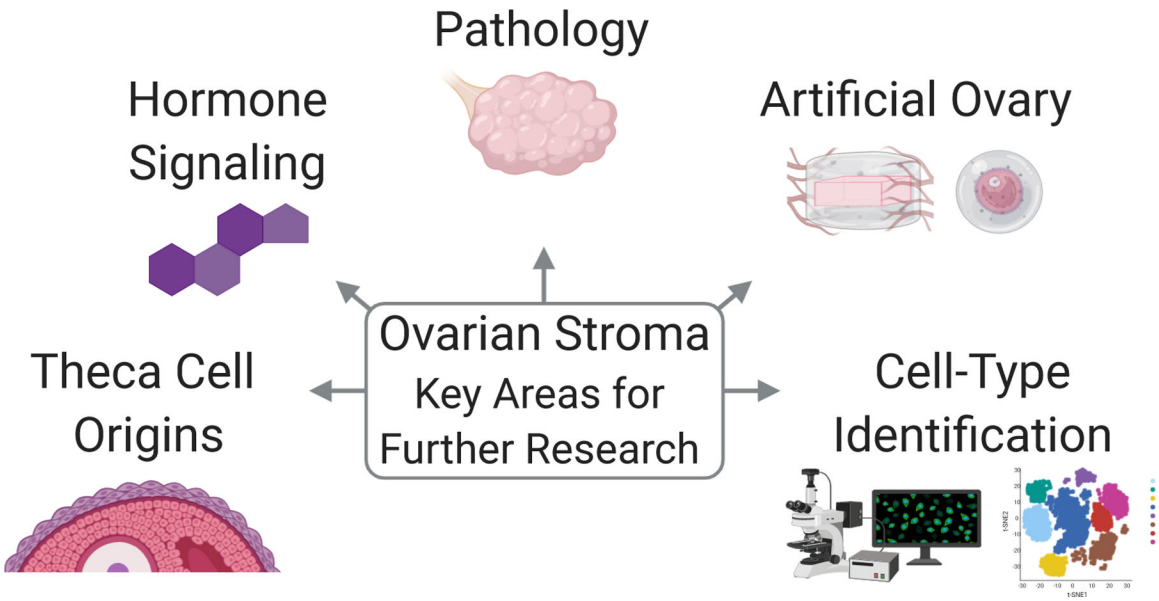

Figure 2.

Ovarian Stroma Key Areas for Further Research. Includes (clockwise from left): theca cell origins, hormone signaling, pathology, artificial ovary, and cell-type identification. Made using ©BioRender - biorender.com.

Table I:

General and Ovary-Specific Components of the Ovarian Stroma

	Subsets	Regionality	Function	Example Cellular Markers†	Further Research Required
General Components					
Immune Cells	Macrophages, dendritic cells, neutrophils, eosinophils, mast cells, B Lymphocytes, T Lymphocytes, Natural Killer cells	Throughout stroma, around theca vasculature	Defense, remodeling, signaling	Leukocyte: CD45 Myeloid: CD11b Macrophages: CD68 Dendritic Cells: CD11c Neutrophils: CD16 Eosinophils: CD193 Mast Cells: CD117 Lymphoid: B Lymphocytes: CD19 T Lymphocytes: CD3 Natural Killer: CD56[1]	Cyclic, hormonal, temporal dynamics, pathologic relevance
Blood Vessels	Endothelial cells, pericytes, smooth muscle cells	Branching medullary spirals to cortical arcades	Oxygen, nutrients, hormone trafficking	Endothelial cells: VE-Cadherin Pericytes: PDGFRB Smooth muscle cells: α-SMA[2]	Dynamic role of oxygen tension, pathologic role and management in PCOS
Nerves	Neurons, glial cells	Branching medulla to cortex	Communication, can regulate hormone secretion and vasoconstriction[3]	Neurons: MAP2 Glia: SOX10[4]	Neuronal regulation of stromal cell types, pathologic relevance
Lymphatic Vessels	Endothelial cells, smooth muscle cells	Branching medulla to cortex, vasculature association	Fluid homeostasis, immune cell trafficking, hormonally induced remodeling[5]	Endothelial cells: LYVE1[6] Smooth muscle cells: α-SMA	Dynamic regulation, pathologic relevance
Ovary-Specific Components					
Surface Epithelium		External monolayer of ovary	Supports repair after ovulation, dynamic	CK7, CK8, CK18, CK19, Plakophilin-2, Desmoglein-2[7]	Heterogeneity, pathologic contributions
Tunica Albuginea		Outer layer under surface epithelium	Protection	Minimal cellularity	Physiologic and pathologic contributions
Intraovarian Rete Ovarii		Hilar region, medulla	Connection to progenitor populations, including theca and lymphatics[8]	CK19, Vimentin[9]	Physiologic role in adults, pathologic relevance

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	Subsets	Regionality	Function	Example Cellular Markers†	Further Research Required
Hilar Cells		Hilar region, nerve trunk association	Contain Reinke crystals commonly seen in testicular Leydig cells, androgen secretion[10]	Not established	Physiologic role, pathologic relevance
Stem Cells			Physiologic role in adults not established	Oogonial: DDX4 or IFITM3 (controversial)[11]	Presence and potential physiologic role in adults
Incompletely characterized stromal cells	Up to 5 different types of fibroblast-like/spindle-shaped/interstitial cells identified[12]	Subsets mainly in cortex, subsets mainly in medulla	Subsets associated with production of collagens I or III, subsets are steroid producing with cytoplasmic lipids and vacuoles[12]	COUP-TFII[13]; COUP-TFII and/or ARX[14]; DCN, LUM for theca/stroma[15]; some express PDGFRA, DCN, COL1A1, COL6A1, STAR, and/or CYP17A1[16]; some TCF21, COL1A2, STAR[17]	Cellular identification and ontology, regionality, steroid production, common and differentiating markers
Extracellular Matrix Components	Collagens, glycoproteins, proteoglycans, ECM-affiliated proteins, ECM regulators, secreted factors	Stiff cortex with radially aligned collagen fibers and less dense medulla	Scaffold, direct and indirect signaling, dynamically remodeled, stiffness involved in follicular dormancy[18]	Extracellular	ECM composition and structure not adjacent to follicles

[1](http://docs.abcam.com/pdf/immunology/immune-cell-markers-poster.pdf, https://media.cellsignal.com/www/pdfs/science/pathways/Immune-Cell-Markers-Human.pdf, accessed March 2020);

[2](https://www.rndsystems.com/research-area/endothelial-progenitor-and-endothelial-cell-markers, accessed March 2020; Rensen *et al.*, 2007; Kizuka-Shibuya *et al.*, 2014);

[3](Uchida, 2015),

[4](https://docs.abcam.com/pdf/neuroscience/neural-markers-guide-web.pdf, accessed March 2020);

[5](Brown *et al.*, 2010);

[6](Kong *et al.*, 2017);

[7](Hummitzsch *et al.*, 2013; Hartanti *et al.*, 2020);

[8](Svingen *et al.*, 2012; Liu *et al.*, 2015);

[9](Russo *et al.*, 2000);

[10](Neilson *et al.*, 1970; Erickson *et al.*, 1985);

[11](Hummitzsch *et al.*, 2015);

[12](Reeves, 1971);

[13](Hummitzsch *et al.*, 2013);

[14] (Rotgers *et al.*, 2018);

[15] (Fan *et al.*, 2019);

[16] (Wagner *et al.*, 2020);

[17] (Wang *et al.*, 2020);

[18] (Irving-Rodgers and Rodgers, 2006; Kawamura *et al.*, 2013)

[†] Example cellular markers are presented for general components and possible cellular markers are presented for ovary-specific components