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Biomechanical forces and signals operating in the ovary during folliculogenesis and their dysregulation: implications for fertility

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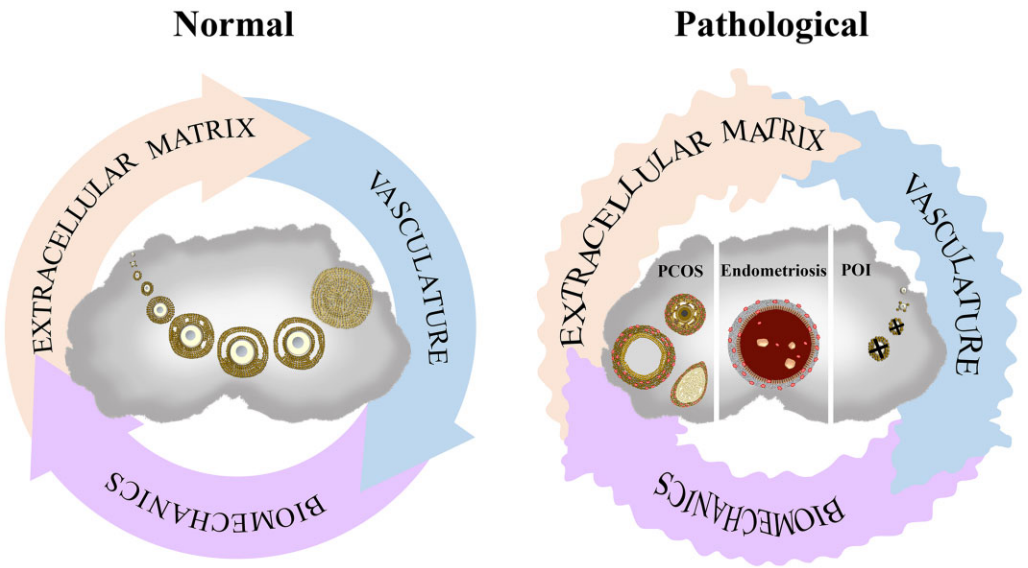
GRAPHICAL ABSTRACT



Alterations of the ovarian ECM, vasculature and biomechanical properties have implications for fertility.

BACKGROUND: Folliculogenesis occurs in the highly dynamic environment of the ovary. Follicle cyclic recruitment, neo-angiogenesis, spatial displacement, follicle atresia and ovulation stand out as major events resulting from the interplay between mechanical forces and molecular signals. Morphological and functional changes to the growing follicle and to the surrounding tissue are required to produce oocytes capable of supporting preimplantation development to the blastocyst stage.

OBJECTIVE AND RATIONALE: This review will summarize the ovarian morphological and functional context that contributes to follicle recruitment, growth and ovulation, as well as to the acquisition of oocyte developmental competence. We will describe the changes occurring during folliculogenesis to the ovarian extracellular matrix (ECM) and to the vasculature, their influence on the mechanical properties of the ovarian tissue, and, in turn, their influence on the regulation of signal transduction. Also, we will outline how their dysregulation might be associated with pathologies such as polycystic ovary syndrome (PCOS), endometriosis or premature ovarian insufficiency (POI). Finally, for each of these three pathologies, we will highlight therapeutic strategies attempting to correct the altered biomechanical context in order to restore fertility.

SEARCH METHODS: For each area discussed, a systematic bibliographical search was performed, without temporal limits, using PubMed Central, Web of Science and Scopus search engines employing the keywords extracellular matrix, mechanobiology, biomechanics, vasculature, angiogenesis or signalling pathway in combination with: ovary, oogenesis, oocyte, folliculogenesis, ovarian follicle, theca, granulosa, cumulus, follicular fluid, corpus luteum, meiosis, oocyte developmental competence, preimplantation, polycystic ovary syndrome, premature ovarian insufficiency or endometriosis.

OUTCOMES: Through search engines queries, we yielded a total of 37 368 papers that were further selected based on our focus on mammals and, specifically, on rodents, bovine, equine, ovine, primates and human, and also were trimmed around each specific topic of the review. After the elimination of duplicates, this selection process resulted in 628 papers, of which 287 were cited in the manuscript. Among these, 89.2% were published in the past 22 years, while the remaining 8.0%, 2.4% or 0.3% were published during the 1990s, 1980s or before, respectively. During folliculogenesis, changes occur to the ovarian ECM composition and organization that, together with vasculature modelling around the growing follicle, are aimed to sustain its recruitment and growth, and the maturation of the enclosed oocyte. These events define the scenario in which mechanical forces are key to the regulation of cascades of molecular signals. Alterations to this context determine impaired folliculogenesis and decreased oocyte developmental potential, as observed in pathological conditions which are causes of infertility, such as PCOS, endometriosis or POI.

WIDER IMPLICATIONS: The knowledge of these mechanisms and the rules that govern them lay a sound basis to explain how follicles recruitment and growth are modulated, and stimulate insights to develop, in clinical practice, strategies to improve follicular recruitment and oocyte competence, particularly for pathologies like PCOS, endometriosis and POI.

Key words: ovary / folliculogenesis / oocyte developmental competence / extracellular matrix / mechanobiology / neoangiogenesis / infertility / polycystic ovary syndrome / endometriosis / premature ovarian insufficiency

Introduction

Infertility affects 15% of couples worldwide and its incidence is increasing (Sun *et al.*, 2019). The main causes might be maternal (40%), paternal (40%) or attributed to both partners (20%) (Fidler and Bernstein, 1999; Tabong and Adongo, 2013). Maternal causes of infertility include women ageing, meiotic aneuploidies, endocrine disorders, polycystic ovary syndrome (PCOS), endometriosis and premature ovarian insufficiency (POI). Common to these conditions is a reduced availability or quality of oocytes with developmental potential (Farquhar *et al.*, 2019).

The acquisition of the oocyte's developmental capacity is a troubled odyssey (Zuccotti *et al.*, 2011; Conti and Franciosi, 2018). In brief, from an initial pool of thousands of primordial follicles localized in the cortex, a group undergoes important morphological and functional changes with proliferating follicle cells assuming a cuboidal shape and forming a single-layered epithelium enclosing the gamete (Gougeon and Chainy, 1987). Since gonadotrophin receptors are not yet present in primordial follicles, initial recruitment appears to be pituitary independent and controlled by biomechanical, autocrine and paracrine pathways (Hsueh *et al.*, 2015). From the primary follicle stage, an extracellular matrix (ECM), the zona pellucida (ZP), begins to be deposited (Wassarman and Litscher, 2018) and proximal stromal cells differentiate into a theca layer. Following FSH-dependent recruitment, secondary follicles are repositioned towards the nearby medulla and, once their growth in this region is terminated, they re-emerge into the cortex from where ovulation occurs (Fiorentino *et al.*, 2020). Early antral follicle growth is accompanied by the sprouting of a calix of new capillaries that surrounds each growing follicle and convoys it to full maturation (Fraser, 2006). During subsequent follicle growth, antral fluid production is intensified by increased vascularization and capillary fenestration (Fraser, 2006), while the oocyte reaches its final size preparing for the germinal vesicle (GV) to meiosis II (MII) transition and LH-induced ovulation. The great majority of follicles will never complete maturation, but, instead, will be eliminated together with their surrounding vasculature.

Follicle cyclic recruitment, neo-angiogenesis, spatial displacement, ovulation and atresia characterize the micro-anatomical context in which oocytes, as a result of an interplay between molecular signals and physical forces, gain competence.

This review will summarize the ovarian morphological and functional context that contributes to follicle recruitment, growth and ovulation, as well as to the acquisition of oocyte developmental competence. We will describe the changes occurring to the ovarian ECM and to the vasculature during folliculogenesis, considering their effects on the mechanical properties of the ovarian tissue, which, in turn, impact on the regulation of signal transduction. Furthermore, we will outline how their dysregulation might be associated with pathologies such as PCOS, endometriosis or POI. Finally, for each of these three pathologies, therapeutic strategies aimed at correcting the altered biomechanical milieu, in order to restore fertility, will be described.

Methods

For each area discussed, a systematic bibliographical search was performed, without temporal limits, using PubMed Central, Web of Science and Scopus search engines. The following keywords were included: extracellular matrix, mechanobiology, biomechanics, vasculature, angiogenesis, miRNA, signalling pathway, extracellular vesicle or maternal-effect gene in combination with: ovary, oogenesis, oocyte;, folliculogenesis, ovarian follicle, theca; granulosa, cumulus, follicular fluid, corpus luteum, meiosis, oocyte developmental competence, preimplantation, polycystic ovary syndrome, premature ovarian insufficiency/failure or endometriosis.

Through search engines queries, we yielded a total of 37 368 papers that were further filtered according to our focus on Mammals and, specifically, on rodents, bovines, equines, ovines, primates and humans, and eventually trimmed around each specific topic of the review. After manual elimination of duplicates, this selection process resulted in 628 papers, of which 287 were cited in the manuscript specifying, when needed, the corresponding species. Among these, 89.2% were published in the past 22 years, while the remaining 8.0%, 2.4% or 0.3% were published during the 1990s, 1980s or before, respectively.

Ovarian extracellular matrix: main components, their spatial localization and remodelling during folliculogenesis

The ECM, with its specific meshwork of macromolecules such as proteoglycans, non-proteoglycan polysaccharides and fibrous proteins, provides mechanical support to the ovary, and regulates osmotic exchange of fluids, soluble nutrients, hormones and other extracellular signals. These factors cross the follicular basal lamina (BL), guiding follicle growth and appropriate oocyte maturation (Berkholtz *et al.*, 2006). Changes to the ECM functional environment may cause derangements of local biochemical pathways, resulting in disturbed folliculogenesis and decreased fertility.

Both the ovarian cortex and medulla are characterized by follicular and stromal compartments in which ECM is observed.

Follicular ECM

During folliculogenesis, the ECM composition varies along with follicles growth and repositioning across cortex and medulla.

There are three main ECM components: the BL surrounding the granulosa layer of all follicle types, the ZP which begins to be deposited between the oolemma and the first layer of follicle cells in primary/secondary follicles (Litscher and Wassarman, 2020), and the follicular fluid secreted from the early antral stage onwards (Fig. 1).

Most of our knowledge on the BL derives from studies on the bovine ovary, although a similar organization is observed in other mammals. Common BL components are laminins and collagen type IV and XVIII fibres (bovine: Rodgers and Irving-Rodgers, 2010c), whose concentration and interactions change during follicle growth, displaying a progressive decrease of collagens and increase of laminins (primate: Hornick *et al.*, 2012). More specifically, the BL of primordial follicles is a network of laminin α1, β2 and γ1 and a lattice-like matrix of collagen type IV α1, α2, α3, α4, α5 and α6 (Fig. 1A) (bovine: van Wezel *et al.*, 1998; Rodgers *et al.*, 1998; human: Heeren *et al.*, 2015). After follicle recruitment, at the preantral stage, BL composition changes with the production of nidogen, a sulphated monomeric glycoprotein, and of perlecan, a heparan sulphate proteoglycan, synthesized by both vascular endothelial and smooth muscle cells (Fig. 1B) (bovine: Rodgers *et al.*, 2003). The functional role of the highly conserved perlecan and nidogen in the ovary has not yet been elucidated. However, in other tissues, perlecan plays a regulatory function in the paracrine activity of fibroblast growth factor (FGF) pathway, by binding to both FGF and its receptor (FGFR), thus stabilizing the FGF-FGFR complex (Whitelock

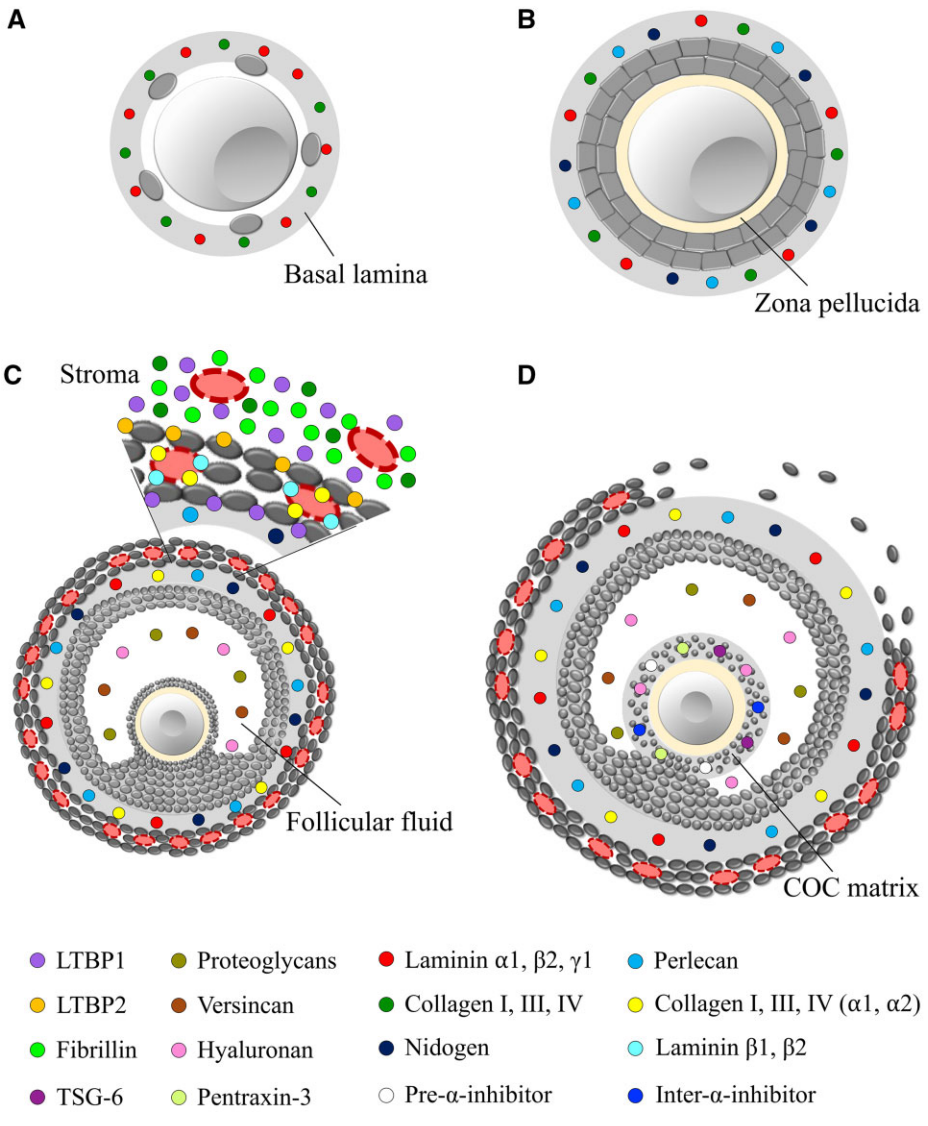


Figure 1. Ovarian extracellular matrix (ECM) components. Changes in follicular ECM molecules and their spatial localization during folliculogenesis, from the (**A**) primordial to the (**B**) secondary, (**C**) antral and (**D**) preovulatory follicle. The enlargement in (**C**) gives a detail of the theca and of the surrounding stroma. COC, cumulus–oocyte complex.

et al., 2008); the latter, as observed in human keratinocytes, plays a crucial role, through its central G2 domain, in the maintenance of a strong link between laminins, collagen IV and perlecan, thus preserving the BL structural integrity (Sage et al., 2012).

BL composition remains similar at the antral stage in bovines, with the exception of a loss of collagen type IV α3-α6, whose downstream effect is a marked reduction of BL stiffness. This is a biomechanical requirement for follicle size expansion and for preparation for ovulation (Fig. 1C) (bovine: van Wezel et al., 1998; Rodgers et al., 1998; McArthur et al., 2000).

Alterations to this ultrastructural organization with the addition of further BL layers result in a phenotype named 'loopy' BL, observed in both bovine and human follicles, which has been associated with oocytes with a lower developmental competence (bovine: Irving-Rodgers and Rodgers, 2000; Rodgers and Irving-Rodgers, 2010b). Experimental evidence showed that bovine oocytes with a normal BL display twice the capacity to reach the blastocyst stage, compared to those with a 'loopy' BL (bovine and human: Irving-Rodgers et al., 2009b).

A further interesting variation in the BL organization, described in mice (Zalewski et al., 2012), rats (Bagavandoss, 2014), bovines (Irving-Rodgers et al., 2004, 2006; Matti et al., 2010) and humans (Alexopoulos et al., 2000), and correlated to follicle quality, is represented by a specialized BL-type matrix which forms, at the time of dominant follicle selection, focal intra-epithelial aggregates (focimatrix) interposed amid granulosa cells. Typical focimatrix components are collagen type IV α1 and α2, laminin α1, β2 and γ1, perlecan and nidogen 1 and 2. Importantly, the volume density of focimatrix components is higher in follicles with high levels of molecular markers for dominance (bovine: Irving-Rodgers et al., 2009a).

A further follicular ECM component is the ZP, a layer of three/four main glycoproteins (e.g. ZP1-3 in the mouse or ZP1-4 in human) (mouse: Wassarman et al., 2004; mouse and human: Gupta et al., 2012), with a well-known role at the time of fertilization, when the oocyte encounters sperm and their binding with ZP3 prompts the acrosome reaction and subsequent crossing (mouse: Wassarman and Litscher, 2018). Just a few seconds after sperm-oocyte plasma membrane fusion, the oocyte's release of its enzymatic content of ∼4000 cortical granules (mouse: Ducibella et al., 1988) into the perivitelline space induces ZP hardening (ZPH), an evolutionary conserved mechanism that blocks polyspermy (Liu, 2011). Among the enzymes encased inside the cortical granules, ovostacin, an astacin-family metalloproteinase, cleaves ZP2, thus preventing further sperm binding (mouse: Burkart et al., 2012). On the contrary, the ovastacin-inhibitor, fetuin-B, released by the liver into the plasma, blocks the occurrence of ZPH prior to fertilization, thus maintaining oocytes' potential for fertilization (mouse: Dietzel et al., 2013). The timely occurrence of this process and the interplay between ovostacin and its inhibitor fetuin-B are considered of crucial relevance, on one side to avoid polyspermy, and, on the other, to allow correct fertilization (mouse: Korschgen et al., 2017).

Disturbances to this sequence of events leads to an early ZPH and, as a consequence, to infertility. Early ZPH has been observed during *in vitro* maturation of mouse (Larman et al., 2006), rat (Zhang et al., 1991) and human (Zhang et al., 1991; Schiewe et al., 1995) oocytes, but it has also been reported following cryopreservation of mouse (Carroll et al., 1990) and human oocytes (Coticchio et al., 2007; De Santis et al., 2020) and in mouse postovulatory aged oocytes (Xu et al., 1997). Lastly, worth mentioning is the case of the rare, but severe, genuine empty follicle syndrome, whereby mutations of *ZP1* and *ZP3* genes hamper ZP assembly, leading to human oocyte degeneration (Dai et al., 2019).

Parallel to the changes occurring to the BL organization and to the events involved in ZP deposition, during antral follicle growth, granulosa cells synthesize large ECM molecules such as proteoglycans, versican and hyaluronan (HA), whose relocation into the follicular antrum (Fig. 1C) induces an increase of osmotic pressure and, as a consequence, the recruitment of plasma fluid from the capillaries irrorating the theca layer (bovine: Clarke et al., 2006; Rodgers and Irving-Rodgers, 2010a). The follicular fluid contains other ECM components, such as laminin and fibronectin. High concentrations of fibronectin in antral preovulatory human follicles correlate positively with oocyte quality (Honda et al., 2004).

At the time of ovulation (Fig. 1D), the LH surge initiates a cascade of events beginning with granulosa cell production of epidermal growth factor-like peptides, and oocyte synthesis of soluble oocyte-secreted factors [e.g. Bone morphogenetic protein 15 (BMP-15) and Growth differentiation factor 9 (GDF9), belonging to the transforming growth factor β (TGFβ) superfamily], all of which bind to their respective cumulus cells receptors and, via Mothers against decapentaplegic homolog 2/3 (SMAD2/3) or SMAD1/5/8, activate cumulus cells expression of hyaluronan synthase 2 and, in turn, the production of HA. HA accumulation, stabilized by proteins like pentraxin-3 (PTX3), Tumor necrosis factor-inducible gene 6 protein (TSG-6) and the heavy chains of inter-α-inhibitor and pre-α-inhibitor, produces a soft viscoelastic ECM which creates the physiological biomechanical condition for mouse cumulus–oocyte complex (COC) expansion (Chen et al., 2016; Lo et al., 2019). While these events are occurring, both BL and cortical stromal ECM are degraded at the follicle apex to facilitate COC ovulation (see below) (ovine: Murdoch, 1998; Mammals: Curry and Smith, 2006).

This process of ECM degradation determines an abrupt decrease in collagen type XVIII α1 in bovine developmentally competent COCs, as opposed to incompetent COCs which maintain higher collagen levels (bovine: Melo et al., 2017).

Stromal ECM

The stroma includes the tunica albuginea, the cortical and medullar stroma, and the theca externa and interna (mammals: Kinnear et al., 2020).

The main stromal components of ECM are collagen I, III and IV fibres, fibrillin 1 (FBN1) and latent TGF-β-Binding Proteins 1 and 2 (LTBP1, LTBP2) (Fig. 1C, enlargement) (human: Lind et al., 2006; Mammals: Kinnear et al., 2020). FBNs are glycoproteins with both structural and regulative roles. In association with elastin fibres or extracellular microfilaments, they form microfibrils (Ramirez and Pereira, 1999; Kielty et al., 2002); furthermore, they play a key role in TGFβ pathway regulation given their association with LTBPs. In epithelial cells of the ovarian stroma, TGFβ 1, 2 or 3 pro-peptides bind to LTBP1 forming large latent complexes (LLCs), which are then secreted into the ECM (bovine: Rodgers and Irving-Rodgers, 2010c). Once inside the ECM, the interaction of LLCs with the N-terminal domain of FBN1 inhibits the release of TGFβs (Hirani et al., 2007); instead, the

association of LTBP2 to the complex prompts TGFβ bioavailability (Mammals: Robertson et al., 2015).

The stromal compartment hosts a rich vasculature that may penetrate through to the bovine follicle theca layers. Its endothelium is surrounded by a BL made of laminin β1 and β2, collagen type IV α1 and α2 and collagen type XVIII α1 (Fig. 1C, enlargement) (bovine: Rodgers et al., 2003).

A dynamic network of blood vessels accompanies and supports follicle growth and oocyte maturation

Within the ovary, a further paramount regulative component is represented by a rich blood vasculature that, through a process of neo-angiogenesis, surrounds newly recruited follicles during the whole growth phase and, following ovulation, undergoes regression in the corpora lutea. Notably, physiological neo-angiogenesis is a remarkable and unique feature of the ovary, which has not been described in any other adult organ (Mammals: Fraser, 2006).

Before entering the ovary at the hilum site, the ovarian artery divides into uterine and ovarian branches. Once inside the ovary, the artery further divides into smaller arterioles which cross the medulla region and envelop the growing follicle within a meshwork of new capillaries (Fig. 2A) (rodents: Macchiarelli et al., 1992; Mammals: Redmer and Reynolds, 1996). Thanks to this abundant and dynamic network of blood vessels, follicles are delivered with oxygen, growth factors, gonadotrophins and steroid precursors, which are necessary to sustain their growth and to regulate their selection and acquisition of developmental competence (bovine: Kosaka et al., 2007; Mammals: Rizov et al., 2017). The vascular system is also made of a multitude of venules responsible for draining out of the follicles those by-products derived from their active metabolism. Although a flourishing vasculature is associated with the development of a healthy follicle, and a regressed vessels network is an early sign of atresia (hamster: Greenwald, 1989), the cause–effect relationship remains poorly understood. Similarly, the dominant follicle is characterized by a denser and more permeable vascular network (porcine: Barboni et al., 2000). In clinical practice, ultrasonographic assessment of high blood flow rate around single follicles is associated with both a higher developmental potential of the enclosed oocyte and higher chance of a viable pregnancy (human: Bhal et al., 1999; Borini et al., 2001; Costello et al., 2005).

At the beginning of folliculogenesis, a group of arterioles extends into the cortical region forming the stromal vasculature that will supply, by passive diffusion (Fig. 2B; wavy red arrows), all follicles from the primordial to the secondary with nutrients and oxygen (primate: Fraser and Duncan, 2005). The formation of new vasculature in the proximity of mouse primordial follicles determines an increase in blood supply which contributes to the activation of dormant primordial follicles (mouse: Komatsu and Masubuchi, 2020).

After recruitment, when follicles reach the pre-antral stage, FSH stimulates the surrounding theca cells to produce and release several pro-angiogenic factors outside the follicle into the stromal compartment (Mammals: Fraser, 2006). In the pig, these factors promote, within the thecal layers, the formation of two concentric anastomotic calyx-like networks of vessels surrounding the follicle (porcine: Reynolds et al., 1992). Theca cells generate a diffusion gradient of vascular endothelial growth factor A (VEGFA) which prompts endothelial cells migration towards the growing mouse follicles and capillaries sprouting (Zhang et al., 2019) (for a detailed description of the signalling pathways involved, see Fig. 2C).

Alongside these major events, two other crucial pathways favour the angiogenetic process. Key to a proper tip ECs migration is the concomitant matrix metalloproteinase (MMP)-mediated degradation of the surrounding ECM induced by local hypoxia via hypoxia-inducible factor 1α (Krock et al., 2011; mouse: Zhang et al., 2019). Also, both the ratio between the production of angiopoietin 1 (ANPT1) and 2 (ANPT2) by theca cells and the expression of VEGF regulate vessel stabilization or destabilization. High concentrations of VEGF, associated with an increased ANPT2:ANPT1 ratio, leads to the formation of new vessels; conversely, vessels destabilization is associated with low VEGF levels (bovine: Hayashi et al., 2003; primate: Hazzard et al., 1999; human: Wulff et al., 2000).

At the time of ovulation, the inner vessel network of the theca undergoes intensive sprouting towards the granulosa, whereas the BL, located in between, undergoes MMP-mediated fragmentation (ovine: Murdoch, 1998; Mammals: Curry and Smith, 2006). This remodelling facilitates the invasion of fenestrated capillaries from the theca to the granulosa and their subsequent extensive proliferation, a process that warrants the required supply of oxygen and metabolites to both the somatic and germ cell components of the porcine preovulatory follicle (porcine: Martelli et al., 2006). Interestingly, at least in the pig, this intensive capillary proliferation represents a mechanical force that might explain the observed change in follicular morphology, from the characteristic roundish to a more irregular shape. In addition to its trophic role, different regional contractions of the vasculature around the preovulatory follicle determine blood flow changes and contribute to apical rupture and subsequent oocyte ovulation (see below).

Immediately after ovulation, the corpus luteum is the site of a crescendo of blood vessel proliferation mediated by increased activity of the VEGFA-VEGFR2 signalling pathway (Mammals: Lu et al., 2019), needed for structural and endocrine functional differentiation of the corpus luteum (bovine: Shirasuna et al., 2012; human: Sugino et al., 2008; Mammals: Lu et al., 2019). A further key factor in the corpus luteum development is ANPT2, whose high levels allow for vasculature remodelling during the early stages of corpus luteum regression (human: Sugino et al., 2005; bovine: Mishra et al., 2016).

Mechanical properties of the ovarian tissue regulate signal transduction during folliculogenesis

Each histological context defines specific mechanical properties key to the regulation of cell maintenance, differentiation, growth, motility and apoptosis, or its deviance to a pathological state (Ingber et al., 2014; Ingber, 2018; Janmey et al., 2020). Growing follicles are subject to mechanical forces from their surrounding environment and exhibit variations in their own mechanical features during folliculogenesis. This was

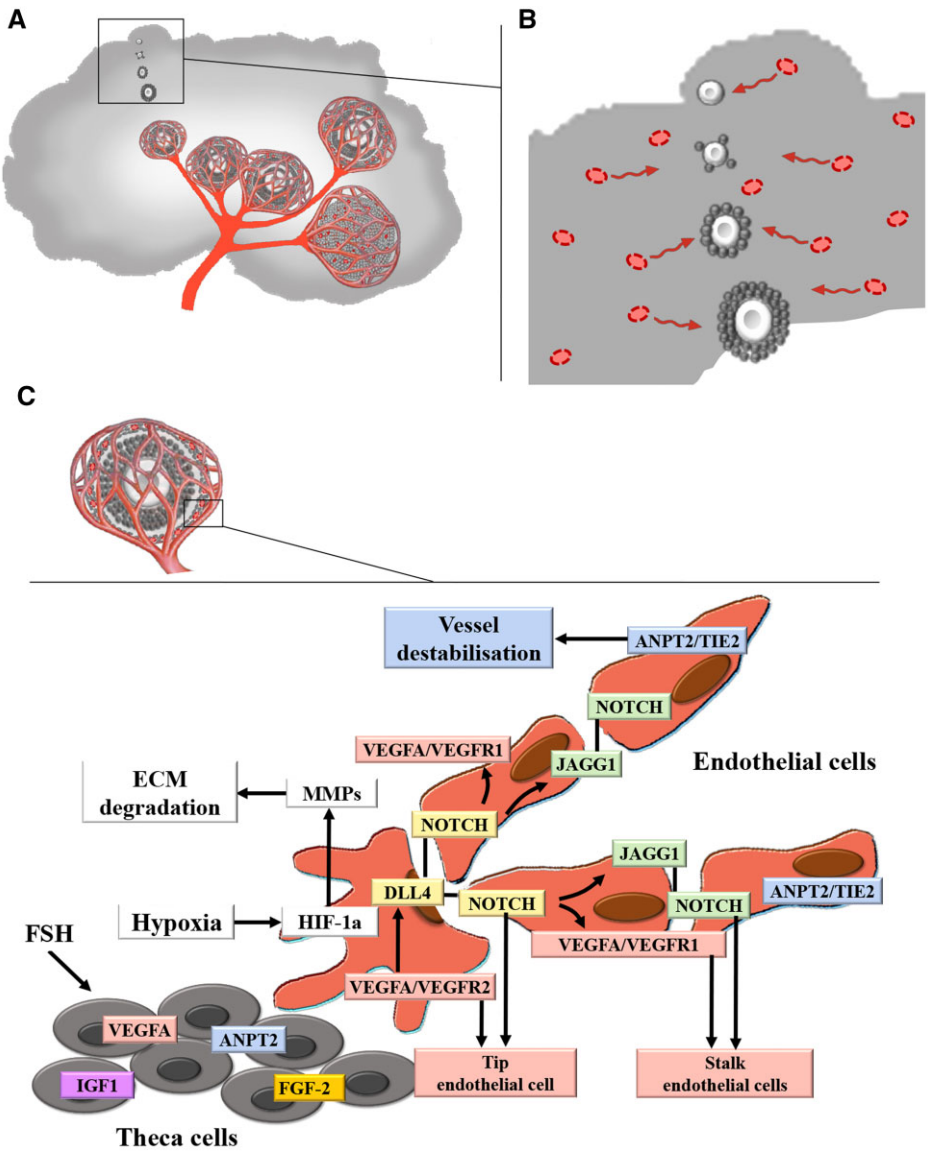


Figure 2. Remodelling of the blood vasculature during folliculogenesis. (**A**) A rich network of capillaries contributes to supply follicles with oxygen, nutrients and signalling molecules throughout folliculogenesis. (**B**) During the initial stages of follicle growth, from the primordial to secondary follicle, this contribution occurs through a passive diffusion mechanism (wavy arrows). (**C**) Then, after recruitment, a meshwork of capillaries, is generated thanks to a process of neo-angiogenesis, encapsulates each follicle. A diffusion gradient of vascular endothelial growth factor A (VEGFA) is sensed by the plasma membrane VEGFR2 receptor on tip endothelial cells (ECs), and triggers their amoeboid migration towards the growing follicles, and, in turn, promotes proliferation and reallocation of stalk ECs, contributing to capillary sprouting (Robinson *et al.*, 2009; Xie *et al.*, 2017). These early events lead to a signalling cascade that involves tip ECs expression of the transmembrane ligand Delta-like 4 (DLL4) and subsequent binding with the Neurogenic locus notch homolog protein 1 (NOTCH) on proximal stalk ECs (lateral inhibition). Then, stalk ECs are prevented from differentiating into tip ECs through the activation of two main pathways. The first pathway promotes the binding between VEGFR1 receptor and VEGFA, with the inactivation of the latter; the second, which involves the binding between the ligand Jagged 1 (JAGG1) and NOTCH on adjacent cells, replaces the DLL4-NOTCH interaction (lateral induction). ECM, extracellular matrix.

particularly clear when observing, in alginate hydrogel 3D follicle *in vitro* cultures, how follicle growth, theca development, antrum formation, hormone production and the overall oocyte quality are influenced by an interplay of physical forces and signalling pathways (mouse: Smith *et al.*, 2014; Tagler *et al.*, 2014; human: Laronda *et al.*, 2014; Yin *et al.*, 2016).

Inside the ovary, the most external cortex and the internal medulla, due to their cellular and extracellular composition, display decreasing inward stiffness (primate: Hornick *et al.*, 2012) and different overall mechanical properties (Mammals: Woodruff and Shea, 2011; mouse: Chan *et al.*, 2021) (Fig. 3A).

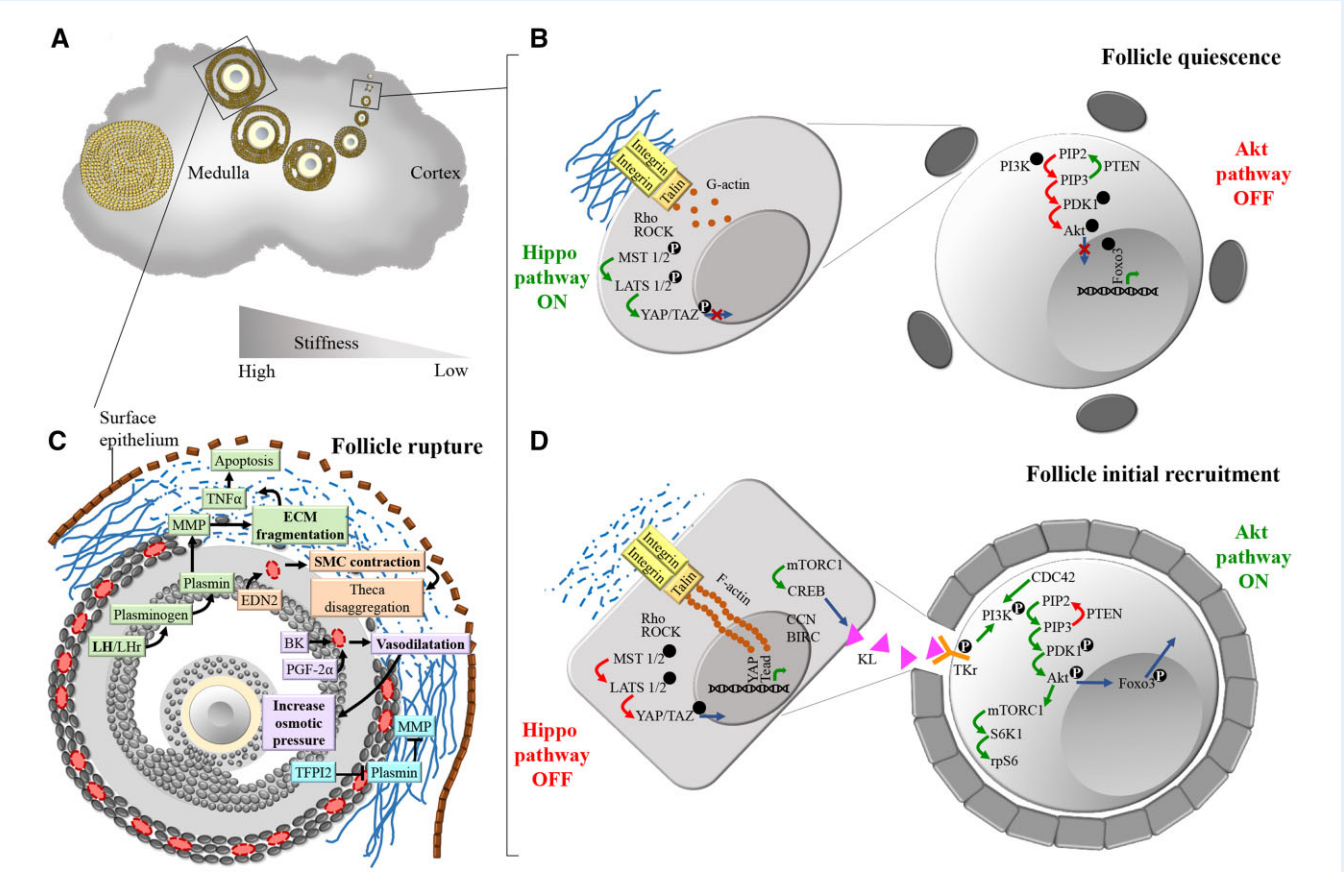


Figure 3. Biomechanical regulation of signalling pathways during folliculogenesis. (**A**) During folliculogenesis, follicles reposition between the cortical and medullar regions, histological environments with different degrees of stiffness. (**B**) Maintenance of primordial follicle quiescence. A higher degree of stiffness in the cortical ECM, sensed by integrin mechanoceptors on the granulosa cells plasma membrane, preserves the activity of Hippo signalling (enlargement, green arrows) and, in turn, an inactive Akt (red arrows) pathway in the oocyte. MST 1/2, serine/threonine protein kinases 1 and 2; LATS 1/2, serine/threonine-protein kinase LATS1 and 2; YAP, Yes-associated protein/Transcriptional coactivator; G-actin, globular actin; TAZ, PDZ-binding motif protein; CDC42, cell division control protein 42 homolog; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PDK1, 3-phosphoinositide-dependent protein kinase-1; Akt, protein kinase B; FOXO3, fork-head box O3. Black circle with P, phosphorylated form; black circle, unphosphorylated form. (**C**) A reduction of ECM stiffness, regulating the inactivation of Hippo (enlargement, red arrows) and the activation of Akt (green arrows) pathways, prompts the transition from the primordial to primary follicle. TEAD, transcriptional enhancer associate domain; F-actin, filamentous actin; CCN, cellular communication network; BIRC, baculoviral inhibitors of apoptosis repeats-containing protein; mTORC1, mammalian target of rapamycin complex 1; CREB, cyclic AMP response element-binding protein; KL, Kit ligand; TKr, tyrosine kinase receptor; S6K1, ribosomal protein S6 kinase beta-1; rpS6, ribosomal protein S6. (**D**) At the time of ovulation, follicle rupture occurs through the synergic activity of four main signalling cascades. The LH surge triggers a pathway that leads to matrix metalloproteinases (MMP)-mediated extracellular matrix (ECM) fragmentation and, as a consequence, to apoptosis of surface epithelial cells at the follicle apex (green boxes). Away from the follicle rupture region, the inhibition of the MMPs activity arrests ECM fragmentation (light blue boxes). To allow COC extrusion from the theca layers, endothelin 2 (EDN2) triggers the contraction of smooth muscle cells (SMCs) surrounding the vascular endothelium and theca disaggregation (orange boxes). Also, inside the theca, capillaries are stimulated by BK and PGF-2α to vasodilatation determining a downstream increase of osmotic pressure inside the antrum that prompts ovulation (violet boxes).

During folliculogenesis, while follicles are repositioning within these regions, they encounter different molecular microenvironments which determine the activation of local biomechanical signalling pathways, guiding further follicle growth and oocyte maturation (Mammals: Woodruff and Shea, 2011; Shah *et al.*, 2018). These differences may be critical to follicle recruitment which occurs right at the interface between the cortex and medulla; furthermore, the softer medulla environment is more propitious to accompanying follicle expansion (Mammals: Woodruff and Shea, 2011). In this context, the activities of Hippo and Akt, two mechano-transduction signalling pathways, are crucial at the time of initial follicle recruitment (human: Hsueh *et al.*, 2015) and, more generally, in the control of organ size (Halder and Johnson, 2011; Zhang, 2015).

Together with hormonal signals, the local composition of the ECM surrounding early follicles plays a role in the transition from quiescence

(Fig. 3B) to recruitment (Fig. 3C). A higher degree of stiffness in the cortical region maintains the basal physiological state and follicle quiescence: mechanical forces of the cortex ECM are sensed by integrin mechanoceptors on the surface of primordial oocytes, determining the activation and inactivation of Hippo and Akt pathways, respectively (Fig. 3B). On one side, the Hippo pathway cascade induces, through negative growth factors (MST 1/2 and LATS 1/2) and via Rho-GTPase and Rho-associated protein kinase (ROCK), the phosphorylation of the Yes-associated protein/Transcriptional coactivator with PDZ-binding motif complex (YAP/TAZ) (mouse: Abbassi *et al.*, 2016; human: Lunding *et al.*, 2020). On the other side, the activation of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) maintains the phosphorylated and inactive form of the Akt pathway molecules favouring, thanks to Fork-head box O3 (FOXO3), the transcription of follicular quiescence regulators (mouse: Castrillon *et al.*, 2003; John *et al.*, 2008; ovine: Adib *et al.*, 2019; primate: Ting and Zelinski, 2017; human: McLaughlin *et al.*, 2014; Ernst *et al.*, 2017; Albamonte *et al.*, 2020).

The opposite situation occurs at the passage from the cortex to medulla (Fig. 3C). Integrins on granulosa cells surface sense a reduced collagen concentration (lower ECM stiffness) and activate a mechano-transduction cascade leading to actin polymerization and Hippo pathway inactivation (human: Lunding *et al.*, 2020; Hsueh and Kawamura, 2020). Specifically, the dephosphorylation of Hippo intermediates induces the migration of YAP/TAZ into the nucleus, its association with transcriptional enhancer associate domain (TEAD), and the transcription of CCN growth factors and baculoviral inhibitors of apoptosis repeats-containing (BIRC) proteins, that promote follicle initial activation for growth. In parallel, granulosa cells activate the mTOR pathway and, via the cyclic AMP response element-binding protein (CREB), the secretion of KIT ligand, which binds to its tyrosine kinase receptor (TKr) on the oolemma (mouse: Zhang *et al.*, 2014; Li *et al.*, 2020). This signal triggers the phosphorylation of Akt signalling factors inducing Akt-dependent FOXO3 phosphorylation and its exit from the nucleus. This process ends with the initiation of oocyte maturation and BMP-15 and GDF9 synthesis (mouse and human: Kawamura *et al.*, 2013; human: Grosbois and Demeestere, 2018; mammals: Kawashima and Kawamura, 2018). Meanwhile, by inhibiting the Tuberous sclerosis complex 1/2 (TSC1/2), Akt intermediates activate the mTORC1-S6K1-rpS6 signalling cascade, essential for protein translation and follicle activation (mouse: Tong *et al.*, 2013; human: Yorino and Kawamura, 2020).

A very recent study of the ECM surrounding human primordial to primary follicles, before and after puberty, described higher collagen levels in the former age compared to the latter, thus explaining the non-permissive environment for follicle activation and growth of pre-pubertal ovaries (Ouni *et al.*, 2020).

Beside the ECM differences described for cortical and medullar regions, important biomechanical events occur locally in the ECM surrounding single growing follicles, capable of inducing the activation of other signalling pathways. This is well described in preovulatory follicles at the time of ovulation, when follicle rupture occurs through the synergic activity of four main signalling cascades (Fig. 3D). The LH surge stimulates ovine granulosa cells production of plasminogen that, after its activation into plasmin, induces theca cells MMPs production and release into the surrounding ECM. Here, the enzymatic activity of MMPs degrades filaments (human: Vos *et al.*, 2014; McCord *et al.*, 2012) and triggers the release of the tumour necrosis factor-alpha (TNFα) ligand that promotes apoptosis of apical superficial epithelial cells (Fig. 3D, green boxes) (ovine: Murdoch, 1998; Mammals: Curry and Smith, 2006).

Distant from the area of follicle rupture, the tissue factor pathway inhibitor 2 (TFPI2) inhibits rodents granulosa cells plasmin production contributing to the maintenance of the ECM integrity (Fig. 3D, light blue boxes) (rat and human: Puttabyatappa *et al.*, 2017). Concurrently, granulosa cells synthesize a number of factors that contribute to ovulation. Endothelin 2 (EDN2), a mechano-transduction protein, triggers the contraction of smooth muscle cells surrounding the vascular endothelium, initiating a process of theca disaggregation which will lead to COC extrusion (Fig. 3D, orange boxes) (mouse: Ko *et al.*, 2006). Furthermore, inside the theca, bradykinin and Placenta growth factor 2α (PGF-2α) induce capillary vasodilatation, a downstream increase of the osmotic pressure inside the antrum, and ovulation (Fig. 3D, violet boxes) (rat: Matousek *et al.*, 2001; Mammals: Jorge *et al.*, 2014).

With ageing, both mouse and human ovaries display an increased collagen and decreased HA deposition, which correlates with augmented stiffness and fibrosis in the stroma (mouse and human: Amargant *et al.*, 2020; human: Ouni *et al.*, 2020).

Alongside the mechanical forces acting at the level of the different functional compartments, the growing female gamete itself exhibits specific mechanical properties reacting to the changes occurring during maturation. The measurement of these changes during the GV-to-MII transition showed an overall reduction of human (Yanez *et al.*, 2016) and mouse (Larson *et al.*, 2010) oocytes stiffness; using microfluidic devices, microelectromechanical analyses showed a 2.8-fold reduction of ZP rigidity and a 6-fold decrease of oocyte cortical tension, modifications which could both favour the fertilization process. Combining time-lapse imaging with particle image velocimetry, measurements of local cytoplasmic visco-elasticity highlighted profiles of cytoplasmic movements during the GV-to-MII transition and at the time of fertilization, correlating with mouse oocytes developmental competence (Ajduk *et al.*, 2011; Bui *et al.*, 2017; Cavalera *et al.*, 2018).

Alterations of ovarian ECM, vasculature and mechanical properties associated with infertility-related pathologies and therapeutic approaches

Alterations of the ovarian morpho-functional context are associated with some of the most frequent causes of infertility-related pathologies. We will review some changes in ECM composition, biomechanical properties and vascular network, which have been described in PCOS, endometriosis and POI. For each of these pathologies, potential therapeutic strategies, aimed at correcting an altered biomechanical context and at restoring physiological folliculogenesis, will be also reviewed.

Polycystic ovary syndrome

PCOS is the most common endocrine disorder in women of reproductive age, with a prevalence between 6% and 15%, depending on

the diagnostic criteria applied (Teede et al., 2018). PCOS also represents one recognized cause of female infertility, mainly due to chronic anovulation (Teede et al., 2010). Whilst the pathophysiology remains poorly understood, the main clinical features of PCOS, occurring in various combinations, are polycystic ovarian morphology at ultrasound examination, chronic anovulation resulting in irregular menstrual patterns, and clinical and/or biochemical hyperandrogenism (human: Shorakae et al., 2018; Teede et al., 2018). Furthermore, a significant proportion of women with PCOS display signs and symptoms related to the metabolic syndrome, such as upper body obesity, insulin resistance, hyperlipidaemia, hypertension and many suffer from type II diabetes and cardiovascular disease (human: Anagnostis et al., 2018). These metabolic disturbances influence ovarian steroidogenesis and function and may aggravate the phenotypic expression of the syndrome (human: de Medeiros et al., 2021).

Whilst women with PCOS show hypothalamic–pituitary abnormalities (Liao, Qiao and Pang, 2021), which often relate to metabolic aberrations and to pro-inflammatory cytokine and chemokine profiles (human: Rudnicka et al., 2021), intrinsic alterations of ovarian function are considered the main culprit of the syndrome (human: Gilling-Smith et al., 1997; Chang and Cook-Andersen, 2013). The distinct morphology of polycystic ovaries is characterized by an increased number of preantral and antral follicles, which tend to arrest their growth at the mid-antral stage. This in turn leads to the formation of cystic follicles, which exhibit thin granulosa cell layers and theca cell hyperplasia (human: Chang, 2007). Furthermore, polycystic ovaries display an increased number of atretic follicles and a reduced number of corpora lutea (Fig. 4). In extreme cases of PCOS, stromal hyperplasia and hyperthecosis (nests of luteinized theca cells within ovarian stroma) can be observed (human Meczekalski et al., 2021). A further feature of polycystic ovaries is disturbed angiogenesis, characterized by increased vascularization of the ovarian stroma. Indeed, increased concentrations of several angiogenic factors, including VEGF, are found in both plasma and follicular fluid of women with PCOS (Di Pietro et al., 2018).

As described above (Fig. 1), the structural organization and function of the ovarian ECM is regulated by a large number of proteoglycans, non-proteoglycan polysaccharides and fibrous proteins, which are critical to correct follicle maturation, and whose localization and amount change during folliculogenesis.

Local quantitative alterations of collagen type IV, fibrillin-3, perlecan and PTX3 are correlated with the polycystic ovary phenotype (Fig. 4). More specifically, increased collagen deposition in the ovarian stroma, resulting in partial fibrosis, has been for many years considered a hallmark of the syndrome (human: Hughesdon, 1982; Papachroni et al., 2010). Although the mechanisms behind the increased collagen deposition are not clearly elucidated, the evidence points to a key role of fibrillins and of the TGFβ signalling pathway in the aetiology of the disease. Among the three fibrillins found in the ECM, the ubiquitously distributed fibrillin 1 and 2, localized around antral and lytic follicles, are quantitatively similar in control and PCOS ovaries. Instead, fibrillin 3, whose distribution is restricted to the perifollicular stroma at the primordial to primary follicles transition, is less represented in human PCOS ovaries (Jordan et al., 2010) (Fig. 4, left-hand side). A decrease in fibrillin 3, observed in both foetal and adult PCOS ovaries

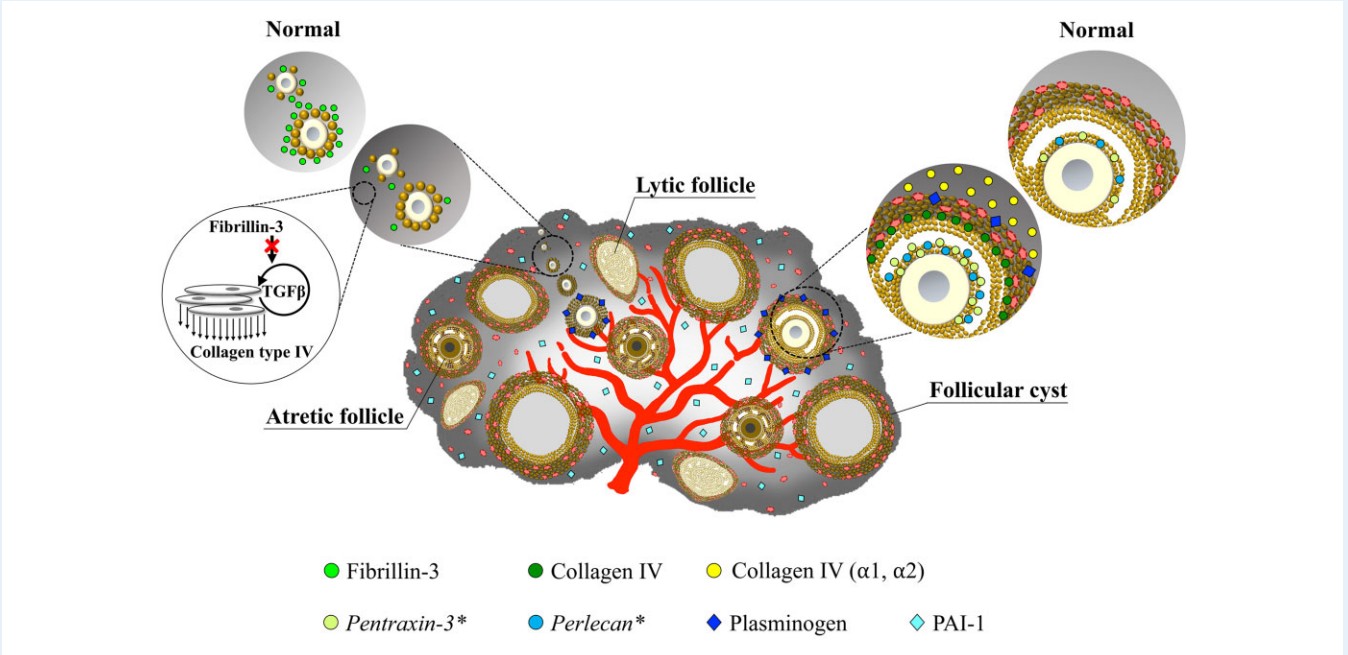


Figure 4. Main alterations observed in the PCOS ovary. Clearly observable are follicular cysts, enveloped by a collagen fibrous capsule, thickened theca cell layers and thin granulosa cell layers, an increased number of atretic small antral follicles, lytic follicles, the absence of corpora lutea, an increased vascular density, and altered levels of extracellular matrix (ECM) proteins or transcripts (*). PAI-1, plasminogen activator inhibitor-1; TGFβ, transforming growth factor β.

(Mammals: Raja-Khan *et al.*, 2014), is associated with an increased TGFβ bioavailability, which, as in many other fibrotic organs, may stimulate an increased fibroblast production and collagen deposition (rodents: LeClair and Lindner, 2007; Mammals: Chung *et al.*, 2021). This condition results in a 50%, 30% and 20% thicker tunica albuginea, external cortical stroma and deep cortical or medullary stroma, respectively (human: Hughesdon, 1982). Evidence for a key role of the TGFβ pathway has been obtained by using a TGF-βRI inhibitor in a PCOS rat model. The inhibitor significantly reduced the expression of pro-fibrotic TGFβ, while increasing anti-fibrotic MMP2, thus preventing collagen deposition (rat: Wang *et al.*, 2018).

A further alteration in the ECM composition has been described for the proteoglycan molecule perlecan, whose *HSPG2* transcript in cumulus cells of preovulatory follicles is more abundant in women with PCOS than in controls. When co-detected with *ADAMTS1* transcript level, the ratio *ADAMTS1/HSPG2* is directly related to the oocyte's developmental competence (mouse and human: Ma *et al.*, 2020).

PTX3 stabilizes the ECM of the cumulus oophorus, preventing proteolytic degradation (mouse: Salustri *et al.*, 2004), and, thus, playing a crucial role for correct cumulus expansion and fertilization. The lack of PTX3 in homozygous null-PTX3 mice leads to disruption of the structural integrity of the cumulus complex and to subfertility (mouse: Varani *et al.*, 2002). On the other hand, an excessive expression of PTX3 in both human follicle cells and follicular fluid of PCOS patients has been associated with the pro-inflammatory profile often found in these women (Pan *et al.*, 2018, 2021).

The impairment of ECM remodelling is among the potential mechanisms behind oligo/anovulation in women with PCOS (Fig. 3D). The ECM of these patients shows down-regulation of key genes (*ECM1*, *CTNNA1*, *ITGA5*, *LAMA3*, *LAMB1*, *FN1* and *ITGA7*) and the absence of MMPs (Tarkun *et al.*, 2004; Gomes *et al.*, 2011; Hassani *et al.*, 2019). Studies in animal models suggest that a further potential cause of disturbed ovulation involves both the distribution and quantity of molecules of the plasminogen system, i.e. plasminogen activator inhibitor-1 (PAI-1) and plasminogen (Fig. 3D, right-hand side). The former is distributed throughout the PCOS mouse ovary, but its presence is limited to the cortex in the normal gonad; the latter is detected from the late preantral to the fully-grown preovulatory follicles in PCOS, whereas it is restricted to the preovulatory phase in control ovaries (mouse: Burchall *et al.*, 2019). The changes in amount and localization of these proteins suggest the involvement of the proteolytic/fibrinolytic plasminogen system in PCOS ovaries not only at the time of follicle rupture but also during the initial phases of follicle growth. Whilst immunohistochemical studies have not yet been performed on human ovaries, it has been known for some time that PAI-1 concentrations are increased in the plasma of women with PCOS (Koiou *et al.*, 2012; Koiou *et al.*, 2013; Mammals: Cesari *et al.*, 2010).

All the evidence listed so far supports the notion that increased density of collagen fibres within the ovary, augmented cortical stiffness and altered ECM composition determine a biomechanically non-permissive framework for follicle recruitment and growth (human: Takahashi *et al.*, 1994; Swanson *et al.*, 1981; Azziz *et al.*, 2009; Mammals: Woodruff and Shea, 2011).

A further distinct feature of PCOS ovaries is an increased capillary density within the extrafollicular tissue, a condition which decreases cortical stiffness and may contribute to the lower recruitment rate of primordial and primary follicles (human: Delgado-Rosas *et al.*, 2009) (Fig. 4). Due to the larger stromal vascularization, the ovarian blood flow is significantly increased in patients with PCOS compared to that in control women (Zaidi *et al.*, 1998; Battaglia *et al.*, 2008; Dwivedi *et al.*, 2020).

These anatomic and functional changes are matched by changes at a molecular level. Modifications in the concentration and activity of proangiogenic factors belonging to the VEGF family (Fig. 3C) are associated with PCOS (Mammals: Tal *et al.*, 2015). Several VEGF gene polymorphisms have been described in association with either increased (human: Zhao *et al.*, 2020) or decreased (human: Liu *et al.*, 2021) PCOS susceptibility. In addition, there is a general consensus that both VEGF follicular production and bioavailability are increased in PCOS ovaries (human: Artini *et al.*, 2009; Scotti *et al.*, 2014; Tal *et al.*, 2014). From a clinical standpoint, up-regulation of VEGF-related factors, both in serum and within the ovary, likely contributes to the dysregulation of the early stages of folliculogenesis in women with PCOS (Agrawal *et al.*, 2002; Abd El Aal *et al.*, 2005; Artini *et al.*, 2006; Patil *et al.*, 2021).

Therapeutic strategies

The first-line approach for women with PCOS seeking pregnancy is induction of ovulation. This is usually obtained by administering either antioestrogens (clomifene citrate), aromatase inhibitors (letrozole) or exogenous gonadotrophins (Teede *et al.*, 2018). While ovulation can be accomplished in a significant proportion of cases, some women with PCOS will either resist the therapy or display a multifollicular response, thus incurring the risk of multiple pregnancies and of ovarian hyperstimulation syndrome (OHSS). In order to overcome these problems, an alternative approach is represented by ovarian laparoscopic drilling (LOD), a surgical procedure aimed at correcting the altered biomechanical context of the PCOS ovary. LOD consists of multiple punctures on the ovarian surface, performed by either unipolar diathermy or laser vaporization (Bordewijk *et al.*, 2020). Although he efficacy of LOD in terms of increased ovulatory cycles is established, the true impact on live birth rate is still a matter of debate and the exact mechanisms by which ovulation is restored are not fully elucidated (human: Bordewijk *et al.*, 2020). Clearly, LOD implies destruction of the ovarian tissue, in particular within the thickened cortical and subcortical stroma (Kinnear *et al.*, 2020).

Clinical studies have shown that androgen serum concentrations decrease dramatically after LOD, and, as a consequence, conversion of androgens to oestrogens is also decreased (human: Flyckt and Goldberg, 2011). Thus, one potential mechanism of LOD could be the partial normalization of the hypothalamic–pituitary–ovarian (HPO) axis.

A second mechanism, which could explain the efficacy of LOD, is normalization of ovarian angiogenesis and vasculature; in fact, studies in women with PCOS have shown decreased VEGF serum concentrations (Amin *et al.*, 2003; El Behery *et al.*, 2011) and decreased ovarian blood flow after LOD (Giampaolino *et al.*, 2017).

A further alternative mechanism that improves ovarian function after LOD is that activated by a decreased Anti-Müllerian hormone (AMH) serum level, reported after this procedure, which contributes to increased follicle recruitment (human: Garg and Tal, 2016).

Of major interest in the context of the present review is the notion that LOD could induce ovulation by mechanical disruption of the Hippo signalling, in turn resulting in the promotion of follicle growth

(human: Farquhar et al., 2012; Abu Hashim, 2015). This mechanism is supported by *in vitro* studies of entire murine ovaries and human ovarian cortical strips. Actin polymerization-promoting drugs, such as jasplakinolide and sphingosine-1-phosphate, increase the conversion of actin from a globular to a filamentous form, promoting YAP translocation into the nucleus, and stimulate CCN expression and follicle growth (mouse: Cheng et al., 2015). On the basis of these observations, alternative future pharmacological treatments of anovulation in PCOS can be foreseen.

Due to the high prevalence of metabolic aberrations and insulin resistance in women with PCOS, hypoglycaemic drugs have been used in the last 30 years to induce ovulation directly or, at least, to facilitate clomiphene/gonadotropin-induced ovulation. The most used is metformin (MET), a biguanide originally introduced for the treatment of type II diabetes (Notaro and Neto, 2022), and currently used in various pathologies, in particular as anticancer drugs for its antiangiogenic activity (human: Fujita and Inagaki, 2017; Yang et al., 2021; Park et al., 2021). When administered to women with PCOS, MET reduces both ovarian volume and blood flow, improving hormonal profiles (Makled et al., 2014). Indirect evidence of an effect of MET on ovarian vasculature and blood flow is the reduction of OHSS after ovarian stimulation for IVF (human: Palomba et al., 2013). In a rat model of PCOS, MET restored the ovarian endothelium and normalized VEGF and ANPT1 protein levels, decreasing follicular cyst formation, and improving follicle maturation and ovulation (Di Pietro et al., 2015).

Accumulating evidence for the role of ovarian angiogenesis and blood flow in the pathogenesis of PCOS anovulation points to VEGF production and biological activity as a potential target for future pharmacological therapies. Studies in PCOS model animals have shown that local injection of the platelet-derived growth factor B decreased intraovarian concentrations of VEGF, and partially re-established the number of small follicles, limiting cystic follicle formation (rat: Di Pietro et al., 2016).

Ovarian endometriosis

Endometriosis is a hormone-dependent gynaecologic disease affecting up to 5–10% of women of reproductive age (Bulun, 2009; Macer and Taylor, 2012). It is characterized by the presence of endometrial tissue outside the uterine cavity, most frequently in the pelvis (i.e. peritoneum, rectovaginal septum, fallopian tubes and ovaries), and occasionally outside the pelvis (i.e. colon, liver, lungs) (human: Rock and Markham, 1992; Giudice, 2010; Vercellini et al., 2014). Hypotheses on the origin, the pathogenesis and the classification of the disease are still controversial. The most credited theory is based on the concept of retrograde menstruation. The core of this theory is based on the observation of physical displacement of endometrial fragments (retrograde transportation of menstrual debris via fallopian tubes), which would escape immune clearance, invading the surrounding tissues and establishing local vascularization (human: Sampson, 1927).

In the ovary, endometrioma is a common lesion that affects 30–50% women diagnosed with endometriosis (Macer and Taylor, 2012). Endometriomas have a major negative impact on the reserve of dormant primordial follicles. This is aggravated when surgical interventions are performed in order to remove the lesions. The reduction of ovarian reserve becomes particularly evident in patients with endometriomas undergoing assisted reproductive technologies. In fact, whilst the proportion of good quality embryos is maintained, the overall number of oocytes retrieved and those at the MII stage is significantly lower than that in healthy women (Yland et al., 2020; Alshehre et al., 2021; Bishop et al., 2021; Vaiarelli et al., 2021).

Similarly to other endometriotic lesions, endometriomas may originate from fragments of endometrial tissue reaching the ovarian cortex (Fig. 5, left-hand side). Fragments localize immediately beneath the surface epithelium and develop into cysts surrounded by stroma and a single layer of columnar endometrial epithelial cells. These implants maintain responsiveness to oestrogenic stimulation, and display cyclic proliferation, shedding and bleeding, similarly to the eutopic endometrium (human: Brosens et al., 1994).

The implants grow and gradually accumulate cytokines, TGFβs, MMPs, factors of the plasminogen system and VEGF, together with menstrual debris (human: Exacoustos et al., 2018). At some point, the epithelial wall of the endometrioma outbreaks, releasing its content in the surrounding stroma. The release of cytokines stimulates chronic inflammation, which in concert with VEGF, intensifies the process of neo-angiogenesis around the endometrial cyst, supporting its survival and further growth (human: Cooper and Rathbone, 1990; McLaren et al., 1996; Mahnke et al., 2000; Gazvani and Templeton, 2002; Goteri et al., 2004; Ceyhan et al., 2008; Goteri et al., 2010). At the same time, TGFβ1 induces fibrosis via the TGFβ1/Smad signalling pathway (human: Shi et al., 2017; Zeng et al., 2018), whereas the enzymatic activity of MMPs contributes to tearing endometrial tissue, favouring new implants and spreading of the disease (human: Kokorine et al., 1997; Chung et al., 2001; Luddi et al., 2020). Aberrations in the plasminogen system are a characteristic common to all types of endometriotic lesions (human: Gilabert-Estellés et al., 2003; Alotaibi et al., 2019). Since PAI-1 regulates the activity of plasmin and plasmin-dependent MMPs in the ECM remodelling (human: Silva et al., 2013), the invasiveness and pro-fibrotic properties of endometriomas may be further explained by the higher concentration of PAI-1 found in both cyst fluid and inner epithelium (human: Boss et al., 2002; Alotaibi et al., 2019).

In summary, the physical invasiveness of the cyst in combination with the release of its stored factors and an inflammation outburst are the main aspects that concur to reduce the reserve of dormant primordial follicles (human: Kitajima et al., 2011; Kitajima et al., 2014; Carrillo et al., 2016). Importantly, as shown in non-endometriotic benign ovarian cysts (human: Kuroda et al., 2012), the growing endometrioma is not able to alter the biomechanical properties of the cortex per se and determine a reduction in primordial follicles. In endometriosis, the latter is also induced by the local activity of (i) TGF-βs, MMPs and PAI-1 factors, which determine a radical perturbation of the major ECM components (a cause of subsequent fibrosis), (ii) VEGF, which contributes to a flourishing of capillaries and (iii) cytokines, which stimulate an inflammatory response. Although the molecular mechanisms behind the follicle reduction are still unknown, some have observed an accelerated follicular recruitment (human: Kitajima et al., 2014), that, perhaps, could be explained by a local inactivation of the Hippo pathway (Fig. 3C), and, concurrently, by increased follicles atresia (human: Kitajima et al., 2014).

Therapeutic strategies

In patients with endometrioma, milder forms of the disease can be treated with pharmacological therapies targeted at reducing both pain

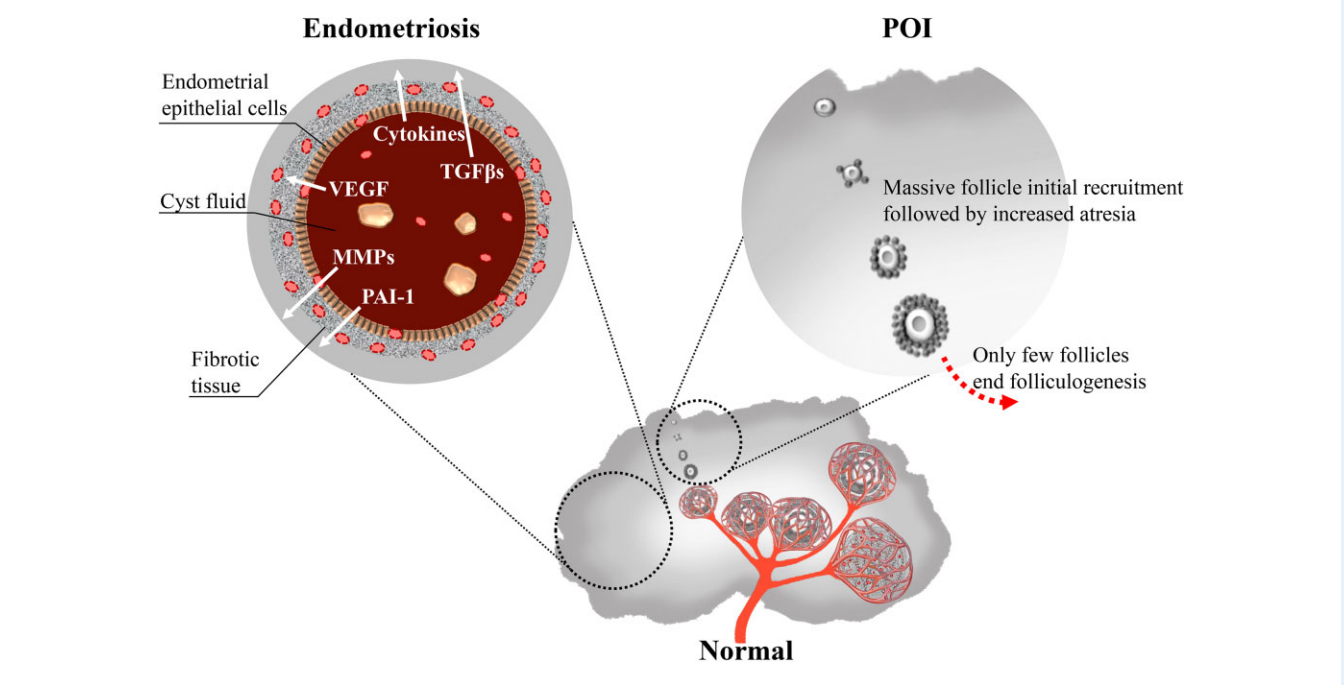


Figure 5. Main alterations observed in the endometriotic or POI ovary. The endometrioma is surrounded by stroma and a single layer of columnar endometrial epithelial cells. These cysts keep their receptivity to oestrogens, cyclic proliferation, shedding and bleeding. The release of cytokines, VEGF, TGFβs, MMPs and PAI-1 and an inflammation outburst cause local reduction of the primordial follicles reserve. Premature ovarian insufficiency is mainly characterized by a massive and uncontrolled initial follicle recruitment, followed by increased atresia, and terminating with an overall depauperated follicular pool. POI, primary ovarian insufficiency; VEGF, vascular endothelial growth factor; TGFβ, transforming growth factor β; MMPs, matrix metalloproteinases; PAI-1, plasminogen activator inhibitor-1.

and the size of the cysts. Contraceptive pills, progesterone and progestins, GnRH agonists (e.g. leuprolide) or androgen-derived compounds (e.g. danazol) are the approaches most frequently used (human: Guzick *et al.*, 2011; Hull *et al.*, 1987; Uludag *et al.*, 2021). Common to all these therapies is the inhibition of the HPO axis, whereas some of them (e.g. progestins or danazol) exert also a modulation of oestradiol receptor function and of oestrogen synthesis, resulting in a reduction of both the oestrogenic stimulation and oestrogen sensitivity of endometriotic tissues.

More recently, a number of VEGF antagonists have been identified for their ability to inhibit angiogenesis and, thus, reduce the vascular network that supplies the cysts, preventing their growth. In a murine model of endometriosis obtained by implanting human endometrium into nude mice, two VEGFA antagonists, a truncated inhibitory receptor and an antibody, inhibited the growth of endometriotic implants (Hull *et al.*, 2003).

In addition to synthetic drugs, natural compounds have been also considered. Epigallocatechin-3-gallate (EGCG), is a pleiotropic polyphenol, found in large concentrations in green tea, which is currently studied for its potential application in several fields of medicine (Chu *et al.*, 2017). EGCG suppresses VEGF expression in cultured endometrial cells. *In vivo*, 14 days of EGCG administration to hamsters selectively inhibited angiogenesis around the cysts without affecting blood vessel development in ovarian follicles. Eventually, the treatment reduced the volume of endometriotic lesions (Laschke *et al.*, 2008).

When endometriomas are very large and/or rapidly expanding, surgical drainage and excision might be more indicated (human: Hayasaka *et al.*, 2011). However, there is a long-lasting debate about the consequences of such procedures on the ovarian reserve. Although several studies suggest that cystectomy provides good results in terms of recurrence, pain symptoms and subsequent spontaneous pregnancy, the traditional approach of cyst stripping leads to a reduction of the ovarian reserve caused by an unintentional excision of the surrounding ovarian cortex (human: Muzii *et al.*, 2005, 2016; Rius *et al.*, 2020). Furthermore, the use of energy-based technologies to guarantee haemostasis during surgery, negatively affects ovarian blood supply, thus worsening the risk of follicle loss (human: Reich and Abrao, 2006; Peters *et al.*, 2017). As an alternative, the use of CO_2 lasers and plasma energy devices is associated with a minimal interference with ovarian biomechanics and a reduced loss of primordial follicles (human: Wyns and Donnez, 2003; Chen *et al.*, 2021).

Premature ovarian insufficiency

POI, also known as premature ovarian failure, is a degenerative disease of the ovary that affects up to 1% of women under 40 years of age and causes infertility (Coulam, 1982; Cohen *et al.*, 2015; Gleicher *et al.*, 2015; Tucker *et al.*, 2016). POI occurs when the pool of primordial follicles is depleted or inhibited in activation (human: Kawashima and Kawamura, 2017). The aetiological and pathophysiological

mechanisms underlying spontaneous POI have not yet been determined, whereas chemo- and radiotherapy represent known causes of iatrogenic POI (human: Bao et al., 2018; Blumenfeld, 2019). Clinically, POI is associated with amenorrhoea, increased gonadotropin production and low serum concentrations of ovarian steroids (mouse: Wang et al., 2013; human: Sullivan et al., 2016; Kozub et al., 2017; Ishizuka et al., 2021).

One of the putative mechanisms involved in the pathogenesis of POI is compromised vascularity. Recent evidence shows that some allelic variants of the pro-angiogenic *VEGFA* gene, are associated with low serum levels of VEGFA and with the risk of POI (human: Li et al., 2021).

Of relevance for the present review, POI correlates also with biomechanical variations of the cortex that affect the early stages of folliculogenesis and lead to a diminished follicle pool (human: de Koning et al., 2008). Disturbances in the Hippo and Akt mechanotransduction pathways, which regulate follicle quiescence/recruitment (Fig. 3B and C), have been indicated as the molecular context in which POI develops. One example comes from studies in a mouse model of POI, in which animals lacking a major negative regulator of phosphatidylinositol 3-kinase (PI3K), display Akt hyperactivation in primordial follicles. As a consequence, follicles undergo massive and uncontrolled initial recruitment, which is followed by increased atresia, eventually leading to an overall diminished follicular pool (Fig. 5, right-hand side) (mouse: Reddy et al., 2008).

Therapeutic strategies

Traditionally, the treatment for women with POI is aimed at restoring physiological concentrations of sex hormones, by exogenous hormone replacement. While this treatment alleviates the symptoms and prevents several long-term systemic complications related to oestrogen depletion, it does not reconstitute the ovarian reserve (human: Webber et al., 2017; Kozub et al., 2017). Expanding knowledge about the physiology of biomechanics and of paracrine pathways within the ovary has paved the way for recent attempts to rescue the small number of primordial follicles still remaining in the ovaries of POI patients.

One of the methods most explored is *in vitro* activation (IVA) of primordial dormant follicles (human: Li et al., 2010). In recent clinical studies, the surgical isolation of ovarian fragments, followed by *in vitro* culture with Akt stimulators, led to the disruption of the Hippo signalling pathway by conversion of the G-actin into F-actin, which resulted in activation of primordial follicles. When the ovarian fragments were auto-transplanted in POI patients, follicular growth was restored, mature oocytes were recovered, and healthy babies were delivered following IVF (human: Kawamura et al., 2013, Suzuki et al., 2015; Zhai et al., 2016).

As a further development, the possibility of a drug-free IVA has been successfully explored. Laparoscopic removal of part of the ovarian cortex, simply cut in small cubes and immediately repositioned as autograft within either the tubal serosa or between the ovarian cortex and medulla, proved to be sufficient to disrupt the Hippo signalling, activating follicle recruitment and growth in POI patients (Tanaka et al., 2020) and in women with diminished ovarian reserve (DOR) (Lunding et al., 2019; Kawamura et al., 2020; Tanaka et al., 2020). This allowed either spontaneous pregnancy or successful IVF in a proportion of treated women. In some cases of POI, the procedure might result in resumption of spontaneous ovulation even for several months post-surgery. The relevance of biomechanics in ovarian follicle dynamics is further supported by the results of a clinical study performed in women with a syndrome of ovarian resistance to exogenous gonadotropins (Kawamura et al., 2013). A simple laparoscopic ovarian incision resulted in improved follicular responsiveness and growth in 7 out of 10 women (human: Tanaka et al., 2020). Although these surgical techniques have not yet reached the stage for general clinical applicability, and attitudes towards such an approach are controversial (Steiner, 2019), these studies nevertheless represent a proof-of-concept for future therapies focused on ovarian biomechanics.

To date, follicle activation has been obtained in clinical settings by surgery and mechanical injury to the ovary. However, studies in rodents demonstrated the ability of actin polymerization-enhancing drugs to promote YAP nuclear translocation and follicle growth (Cheng et al., 2015). Basis on these results, future studies might evaluate the efficacy of intraovarian injection of drugs for Hippo signalling disruption in human ovarian pathologies.

An alternative line of research is focused on the ovarian vascular network and on the putative role of growth factors within the ovary. Following intraovarian injection of either platelet-rich plasma or mesenchymal stem cells, recent studies reported successful stimulation of folliculogenesis and induction of ovulation via remodelling of the ovarian microvasculature (rat: Vural et al., 2019; Ahmadian et al., 2020; Cho et al., 2021; mouse: Feng et al., 2019; human: Park et al., 2019). This approach has also been used in patients with DOR or poor ovarian response to FSH, but so far with conflicting results (Sills et al., 2018; Atkinson et al., 2021; Hajipour et al., 2021).

Conclusions

Our understanding of the biomechanical interactions occurring within the ovary is far from exhaustive. However, the evidence collected so far support the notion of a crucial role of the ovarian mechanical environment in both the development of the gonad and its function during the reproductive years (Ouni et al., 2020). Indeed, dynamic is the most appropriate adjective to describe the adult mammalian ovary, as it portrays an organ in continuous histological re-organization. The changes occurring during folliculogenesis to the ovarian ECM and the vasculature remodelling around the growing follicle are aimed to sustain its recruitment and growth, together with the maturation of the enclosed companion oocyte. These events define the scenario in which mechanical forces are key to the regulation of cascades of known, or still poorly known, molecular signals. Alterations to this context determine impair folliculogenesis and oocyte developmental potential, as observed in pathological conditions like PCOS, endometriosis or POI that lead to infertility.

Improving our knowledge in this specific field of ovarian physiology has implications for future clinical applications. As an example, a better understanding of the biomechanical properties of follicles could help to refine ART. Applying certain mechanical inputs to oocytes cultured *in vitro* might improve the quality of the embryos obtained, thus increasing pregnancy rates (He, 2017; Yanez and Camarillo, 2017). Furthermore, expanding our knowledge on the mechanisms behind follicular activation and on the modulation of the Hippo signalling pathway, might help developing new clinical protocols for fertility-sparing treatments in patients with POI or in women who face gonadotoxic

therapies. To date, fertility preservation in pre-pubertal women is performed by ovarian tissue freezing. This procedure has been available over the last two decades, and the technique has been progressively improved. However, when ovarian fragments are thawed and transplanted, it is estimated that 50–90% of primordial follicles are lost (Celik *et al.*, 2020). Protocols using either pharmacological or mechanical follicular activation might overcome this crucial problem. Similarly, these techniques could help in the clinical management of women with large and/or rapidly growing endometriomas, who face the same risk of ovarian depletion. Even wider applications in reproductive medicine can be predicted, considering the rapidly increasing number of women of advanced reproductive age and DOR, who resort to ART (Choi *et al.*, 2022). Finally, since the same conditions apply to PCOS, alternative, less invasive and more efficient strategies for ovulation induction in women unresponsive to conventional therapies, including ovarian drilling, might be foreseen.

Data availability

No new data were generated or analysed in support of this research.

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Conflict of interest

The authors have no conflicts of interested related to this review.

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