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The mechanisms that control the preantral to early antral follicle transition and the strategies to have efficient culture systems to promote their growth *in vitro*

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Review Article

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Summary

Preantral to early antral follicles transition is a complex process regulated by endocrine and paracrine factors, as well as by a precise interaction among oocyte, granulosa cells and theca cells. Understanding the mechanisms that regulate this step of folliculogenesis is important to improve *in vitro* culture systems, and opens new perspectives to use oocytes from preantral follicles for assisted reproductive technologies. Therefore, this review aims to discuss the endocrine and paracrine mechanisms that control granulosa cell proliferation and differentiation, formation of the antral cavity, estradiol production, atresia, and follicular fluid production during the transition from preantral to early antral follicles. The strategies that promote *in vitro* growth of preantral follicles are also discussed.

Introduction

The transition from preantral (multilaminar follicles) to early antral follicles requires the action of local factors, hormones, and efficient bidirectional communication between granulosa cells and oocytes. Several attempts to promote *in vitro* growth of preantral follicles up to maturation have been reported in various domestic species (bovine: Paulino *et al*., 2018; Antonino *et al*., 2019; Bezerra *et al*., 2019a; Paulino *et al*., 2020; Vasconcelos *et al*., 2021; caprine: Ferreira *et al*., 2018; Soares-Costa *et al*., 2018; Pontes *et al*., 2019; ovine: Macedo *et al*., 2019; Mbemya *et al*., 2019; Barros *et al*., 2020; Silva *et al*., 2021; Gomes *et al*., 2022; and swine: de Lima *et al*., 2017; Kere *et al*., 2020). Different from mice (Eppig and O'Brien, 1996; O'Brien *et al*., 2003), embryo production from *in vitro* cultured preantral follicles from human and domestic animals has not yet, however, been reported in the literature (Wu and Tian, 2007; Arunakumari *et al*., 2010; de Figueiredo *et al*., 2018, 2020; Paulino *et al*., 2022). The maintenance of bidirectional communication between the oocyte and the granulosa cells in cultured follicles, as well as the large quantity of messenger RNA (mRNA) and proteins that the oocyte needs to synthesize during its growth make it difficult the complete their development *in vitro* (Alam and Miyano, 2020; de Figueiredo *et al*., 2020; Paulino *et al*., 2022).

The endocrine and paracrine control that regulates the transition from preantral to antral follicles is complex and involves a precise interaction of several factors (Araújo *et al*., 2014; de Figueiredo *et al*., 2018, 2020). Any interference with this control can lead to ovarian disorders such as polycystic ovary syndrome (Abdel Aziz *et al*., 2021; Asghari *et al*., 2021). The present review highlights the mechanisms involved in endocrine and paracrine control during the transition from preantral to early antral follicles, as well as the importance of granulosa cell proliferation, antral cavity formation, and estradiol production. The strategies to promote *in vitro* growth of preantral follicles are also discussed.

Endocrine and paracrine control during the transition from preantral to early antral follicles

The transition from preantral to early antral follicles is a step in which follicle development is regulated by intraovarian factors, but the follicles are responsive to gonadotropins. The slow growth of preantral and early antral follicles is gonadotropin independent, but progression to a late antral follicular state requires follicle-stimulating hormone (FSH; Iber and Geyter, 2013). FSH is the main endocrine regulator of follicle development (Paulino *et al*., 2018, 2020; Vasconcelos *et al*., 2021) and its receptors are detectable in granulosa cells of preantral follicles in various species (murine: Camp *et al*., 1991; human: Oktay *et al*., 1997; Méduri *et al*., 2002; porcine: Durlej *et al*., 2011; bubaline: Sharma *et al*., 2011; caprine: Saraiva *et al*., 2011; Barros

et al., 2013; Ferreira *et al.*, 2018). This hormone stimulates granulosa cell proliferation and promotes follicular growth and antrum formation (Ferreira *et al.*, 2018; de Figueiredo *et al.*, 2020; Fushii *et al.*, 2021). Other endocrine factors, such as melatonin have a functional role in preantral follicles by influencing their development, increasing the production of active mitochondria in oocytes and steroidogenesis in granulosa cells (Barros *et al.*, 2013; Riaz *et al.*, 2019; Barros *et al.*, 2020). It is well established that FSH stimulates the production of aromatase (Cyp19a1), which synthesizes 17β-estradiol, an important hormone for granulosa cell proliferation (Fitzpatrick and Richards, 1994; Bishonga *et al.*, 2001). Anti-Müllerian hormone (AMH) is also produced by granulosa cells of preantral and antral follicles (Umer *et al.*, 2019; Gautam *et al.*, 2021). It is already well established that this hormone prevents early depletion of follicles, but there is still much to elucidate in the role of this hormone during folliculogenesis. Rocha *et al.* (2021) have shown that there is an interaction between AMH and FSH, in which AMH reduces FSH-induced estradiol and progesterone production. Tanimoto *et al.* (2021) showed that, for the development of a viable follicle, blockage of AMH production by oestrogen is needed.

Oocyte-derived factors, such as growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP-15), and fibroblast growth factor-2 (FGF-2) are important regulators of preantral follicle growth by inducing granulosa cell proliferation and differentiation, and antral cavity formation (Reineri *et al.*, 2018; Monte *et al.*, 2019). Other factors, such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), influence the development of preantral follicles by expanding oocyte diameter and inducing granulosa cell proliferation (Bezerra *et al.*, 2019b; Paulino *et al.*, 2020). Vascular endothelial growth factor (VEGF) is an important angiogenic factor that induces granulosa cell proliferation, an essential characteristic for the transition from preantral to antral follicles, and improves oocyte maturation (Araújo *et al.*, 2011; da Silva *et al.*, 2015; Cadenas *et al.*, 2017). Activin is another intraovarian factor that accelerates the growth of preantral follicles, estradiol synthesis, and mRNA expression for FSH receptor in rat granulosa cells (Tanaka *et al.*, 2019). Conversely, in bovine species, activin decreases the FSH stimulating action in the bovine preantral follicle cultured *in vitro*, which was associated with decreased levels of transcripts for hyaluronan synthases (HAS-1, HAS-2) and proliferating cell nuclear antigen (PCNA; Silva *et al.*, 2014).

Granulosa cell proliferation and oocyte–granulosa cell interaction during the transition from preantral to early antral follicles

Granulosa cells form a favourable metabolic and hormonal environment for oocyte growth and maturation (Baumgarten and Stocco, 2018). Similarly, the oocyte can influence the proliferation of granulosa cells, providing follicular development through the production of growth factors such as BMP-15 and GDF-9 (de Figueiredo and de Lima, 2017; Baumgarten and Stocco, 2018). Orisaka *et al.* (2006) showed that GDF-9 controls follicular fate by promoting its survival and growth during the preantral to early antral transition, suppressing granulosa cell apoptosis and follicular atresia through PI3K/Akt activation.

During the development of the preantral follicle, GDF-9 and BMP-15 continue to stimulate the proliferation of granulosa cells, however, they decrease the production of progesterone and increase the expansion and reorganization of granulosa cells to form the antrum cavity. In addition, GDF-9 stimulates the expression of FSH receptors in granulosa cells, which become responsive to gonadotropins and influence the differentiation and recruitment of theca cells, which form a scaffold structure, supporting the vascular system (De Conto *et al.*, 2021). Theca cells begin to produce luteinizing hormone (LH) receptors, steroidogenic enzymes and androgens (Young and McNeilly, 2010), while LH stimulates the production of androgens by theca cells, which are used by granulosa cells to produce 17β-estradiol (Xavier and Freitas, 2021). 17β-Estradiol increases the sensitivity of granulosa cells to gonadotropins and IGF-1, which stimulates follicular steroidogenesis (Ginther *et al.*, 2001). BMP-4 and FSH also play an important regulatory role in the growth and steroidogenesis of preantral follicles. According to Sakaguchi *et al.* (2017), BMP-4 inhibits the luteinization of granulosa cells, while FSH increases their proliferation and the viability of the oocyte–cumulus–granulosa complex.

Granulosa cells produce several autocrine and paracrine factors that may be involved in the initiation of antrum formation such as kit ligand, activins, inhibins, hyaluronan, Versican, AMH and transforming growth factor α (TGFα). These factors also synchronize oocyte growth, granulosa cell proliferation and theca cell differentiation (Vasconcelos *et al.*, 2013; Dumesic *et al.*, 2015). In this sense, the interaction between the oocyte and granulosa cells, as well as the differentiation and proliferation of theca cells, can be determinants for the progress of preantral follicle growth, steroidogenesis and oocyte maturation (Chu *et al.*, 2018; Alam and Miyano, 2020). AMH plays an inhibitory role in modulating the responsiveness of granulosa and theca cells to gonadotrophic stimuli, thereby regulating follicular progression from the gonadotropin-responsive to the gonadotropin-dependent stage (Campbell *et al.*, 2012).

After antrum formation, granulosa cells are physically separated into mural granulosa cells, which organize along the follicle wall, and cumulus granulosa cells, which surround the oocyte (Baumgarten and Stocco, 2018; Zhang, 2018). Cumulus granulosa cells nourish the oocyte by providing specific amino acids, glycolysis products and cholesterol biosynthesis substrates through gap junctions (Baumgarten and Stocco, 2018). In addition, they prevent premature oocyte maturation and resumption of meiosis in the oocyte by maintaining high levels of cAMP in the oocyte (Russell and Robker, 2018; Zhang, 2018). The mechanisms that control the transition from preantral to early antral follicles are shown in Figure 1 and Table 1.

Mechanisms of antrum formation in preantral follicles

When the distance between the mural granulosa and the cumulus cells increases, the formation of the antral cavity occurs, marking the preantral to early antral follicles transition (Chu *et al.*, 2018). The antral cavity is formed between the granulosa cells and requires a fluid ingress from the vascularization of theca cells via membrane proteins such as aquaporins (AQPs; Kawashima and Kawamura, 2018; Paz *et al.*, 2018). Several AQPs, such as AQP5, AQP7, AQP8, and AQP9, are related to the influx of water through the follicle wall to form follicular fluid. These membrane proteins are expressed in the granulosa cells of different species (swine: Skowronski *et al.*, 2009; ovine: Sales *et al.*, 2015; bovine: Ishibashi *et al.*, 2009). Paz *et al.* (2018) also demonstrated the presence of AQP3 in granulosa cells, and is also involved in the expansion of the antral cavity during the transition from preantral to the antral follicle. Antrum formation depends on the stimulation of local

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Table 1. Role of growth factors in oocyte, granulosa and theca cells during the transition from preantral to early antral follicle

Origin	Target cells	References
Oocyte	**Granulosa cells**	
GDF-9	Stimulates granulosa cell proliferation, reduces apoptosis and increases expression of FSH receptors	Orisaka *et al.*, 2009a; Vasconcelos *et al.*, 2013; De Conto *et al.*, 2021
BMP-15	Promotes the secretion of follicular fluid and interacts with FGF-8 to stimulate glycolysis	Sugiura *et al.*, 2007; Celestino *et al.*, 2011; Passos *et al.*, 2013
BMP-6	Stimulates proliferation of granulosa cells and improves viability and increases the production of inhibin A, activin A and follistatin	Glister *et al.*, 2004; Frota *et al.*, 2009
FGF-10	Inhibits estradiol secretion	Buratini *et al.*, 2007
miRNAs	Regulate the expression of proteins	Assou *et al.*, 2013
Oocyte	Theca cells	
GDF-9	Promotes recruitment, differentiation, proliferation and androgen production in theca cells	Spicer *et al.*, 2008; Young and McNeilly, 2010
Granulosa cells	**Oocyte**	
cGMP	Prevents premature oocyte maturation	Ang *et al.*, 2022
cAMP	Prevents premature oocyte maturation	Baumgarten and Stocco, 2018
KL	Promotes oocyte growth and inhibits resumption of meiosis	Ismail *et al.*, 1997; Lima *et al.*, 2011
IGF-I	Stimulates oocyte growth and interacts with FSH increases oocyte metabolism	Adashi *et al.*, 1985; Zhou and Zhang 2005
EGF	Increases the expression of mRNA s for GDF-9	Assou *et al.*, 2013; Paulino *et al.* 2020
miRNAs	Regulate the expression of proteins	
Granulosa cells	**Theca cells**	
IGF-I	Increases gene expression for androstenedione, stimulates expression of LH receptors and acts synergistically with LH to increase expression of CYP11A1 and HSD3B	Magoffin and Weitsman, 1994; Huang *et al.*, 2001
KL	Stimulates the expression of FGF-7 and hepatocyte growth factor to and regulates the growth of theca cells	Parrott and Skinner, 2000
AMH	Inhibits the responsiveness of theca cells to gonadotrophic stimuli	Campbell *et al.*, 2012
Theca cells	**Granulosa cells**	
Androgens	Increases sensitivity to gonadotropins and IGF-I and it is involved in production of estradiol	Xavier and Freitas, 2021
BMP-4	Inhibits luteinization and apoptosis, promotes estradiol production and aromatase activity	Kayamori *et al.*, 2009; Sakaguchi *et al.* 2017
BMP-7	Promote granulosa cell proliferation and secretion of follicular fluid, regulates steroidogenesis and peptide secretion	Araújo *et al.*, 2010; Frota *et al.*, 2011
FGF-2	Increases DNA synthesis and proliferation	Nuttinck *et al.*, 1996
FGF-7	Interacts with hepatocyte growth factor to stimulate kit ligand production. Suppress apoptosis and increase expression of inhibin	Parrott and Skinner, 1998; McGee *et al.*, 1999

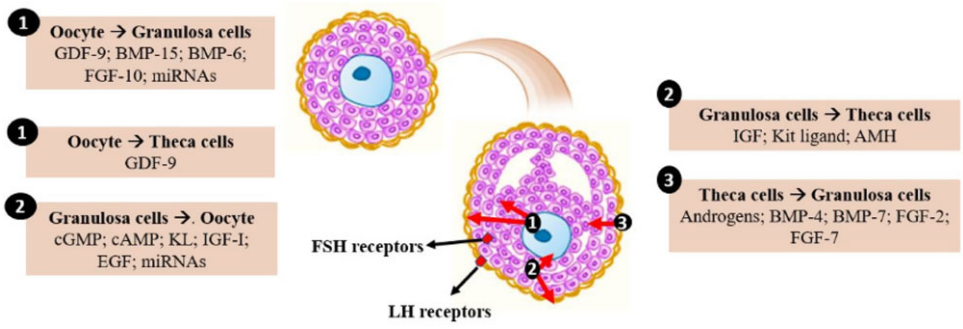

Figure 1. Factors that control the transition from preantral to early antral follicle.

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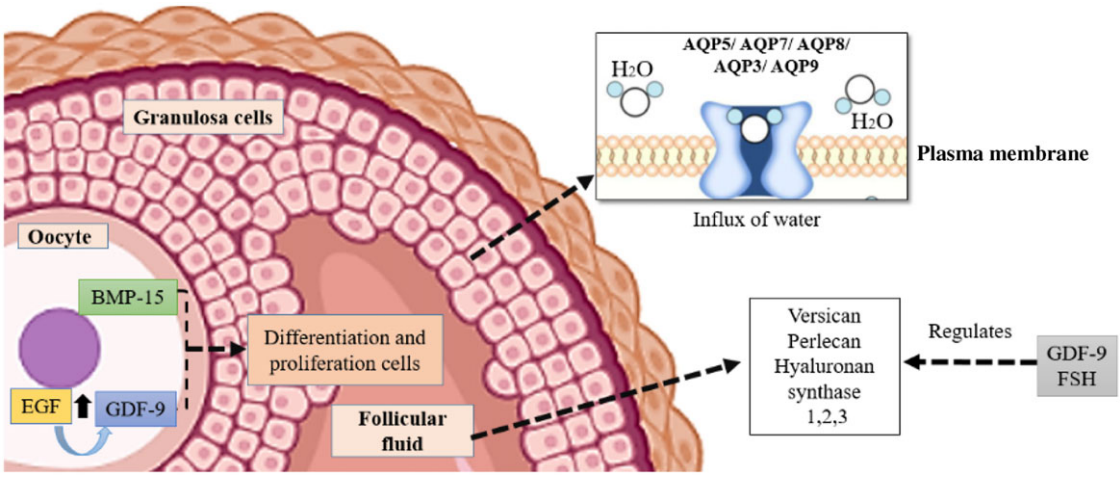

Figure 2. Hormones and growth factors that control the formation of the antral cavity during the transition from preantral to early antral follicles.

factors for the production and secretion of polysaccharides and proteins by the granulosa cells, which accumulate and generate an osmotic gradient that attracts fluid from the thecal vasculature (Baumgarten and Stocco, 2018). It is recognized that granulosa cells express the enzymes that synthesize hyaluronic acid, Versican and perlecan, which are responsible for the formation of follicular fluid (Schoenfelder and Einspanier, 2003; Clarke *et al.*, 2006; Vasconcelos *et al.*, 2013; Nagyova *et al.*, 2020).

The development of the antral cavity is intensified by granulosa cell activity. Alam *et al.* (2018) showed that, even without the presence of an oocyte, GDF-9 and BMP-15 influence the production of antrum-like structures. GDF-9 is known to stimulate Versican and perlecan expression and interacts favourably with FSH to increase hyaluronan synthetase 2 expression (Vasconcelos *et al.*, 2013; Silva *et al.*, 2014). Hyaluronan, proteoglycans and their glycosaminoglycan side chains are osmotic solutes that act to increase the osmotic pressure inside the follicle, resulting in fluid accumulation (Rodgers and Irving-Rodgers, 2010). Some studies have also demonstrated that EGF increases mRNA levels for GDF-9 in *in vitro* cultured preantral follicles (Alam *et al.*, 2018; Paulino *et al.*, 2020), showing that EGF acts via GDF-9 to promote antral cavity formation. The mechanisms involved in the formation of antral cavity formation are shown in Figure 2.

Production of estradiol during the transition from preantral to early antral follicles

When preantral follicles reach six or seven layers of granulosa cells, the inner layer of theca becomes active and the formation of the antral cavity begins. Increased 17β-estradiol in follicular fluid is associated with increased mRNA expression for CYP19A1 in granulosa cells (García-Guerra *et al.*, 2018).

As a growing follicle acquires sufficient aromatase activity as a result of FSH stimulation, estradiol production suppresses FSH secretion below that necessary to support the development of less mature follicles, which consequently suffer atresia (Zeleznik, 2004). Therefore, estradiol biosynthesis is a tightly regulated molecular process, dependent on the expression of key steroidogenic enzymes by FSH and intraovarian signalling molecules, including beta-catenin, an essential co-transcription factor for maximal induction of the aromatase mRNA transcript and subsequent estradiol production (Forrest *et al.*, 2022). β-Catenin regulates the transformation of androgen to estradiol by increasing the transcription of CYP19A1 through functional interactions with steroidogenic factor-1 (SF1; Forrest *et al.*, 2022). Furthermore, LH promotes preantral to antral follicular transition by upregulating follicular androgen biosynthesis (Orisaka *et al.*, 2013).

Expression of luteinizing hormone/choriogonadotropin receptor (LHCGR) and cytochrome P450 family 17, subfamily A, member 1 (CYP17A1) mRNAs appear in large preantral follicles, concomitantly with theca differentiation. Followed by the expression of steroidogenic acute regulatory protein (StAR) in 1.0 mm antral follicles, granulosa cells from preantral and early antral follicles do not express StAR. Therefore, steroidogenesis in bovine follicles potentially begins in follicles $\geq$ 1.0 mm. Furthermore, the mRNA for CYP17A1 was located exclusively in theca internal cells, which indicates that the conversion of progestogens to androgens occurs only in the theca interna (Braw-Tal and Roth, 2005). Furthermore, the neonatal rat ovary is completely devoid of antral follicles at birth. By day 12 of age, small-sized to medium-sized antral follicles are present, in addition to follicles at all preceding stages of development. During the intervening period the ovary becomes steroidogenically active, and responsive to gonadotrophins on days 7–9 of age, suggesting that granulosa and theca cells become active at that time (Carson and Smith, 1986).

The expression of key enzymes involved in steroidogenesis is crucial for the proper development of the follicles. It has been observed that the mRNA encoding P450arom was not detectable until early antral cavity formation, in addition to being expressed only in granulosa cells (Yuan *et al.*, 2008). The mechanisms involved in the production of estradiol during the transition from preantral to early antral follicles are shown in Figure 2.

Follicle atresia during the transition from preantral to early antral follicles

Follicular atresia is characterized by morphological changes in the oocyte, granulosa, and theca cells that culminate with follicular death and, consequently, regulate the capacity of females to generate mature gametes during the fertile period (Makarevich *et al.*, 2018). This process promotes a drastic reduction in the ovarian follicle population during the reproductive lifespan. According to Zhang *et al.* (2018), crosstalk among cell apoptosis,

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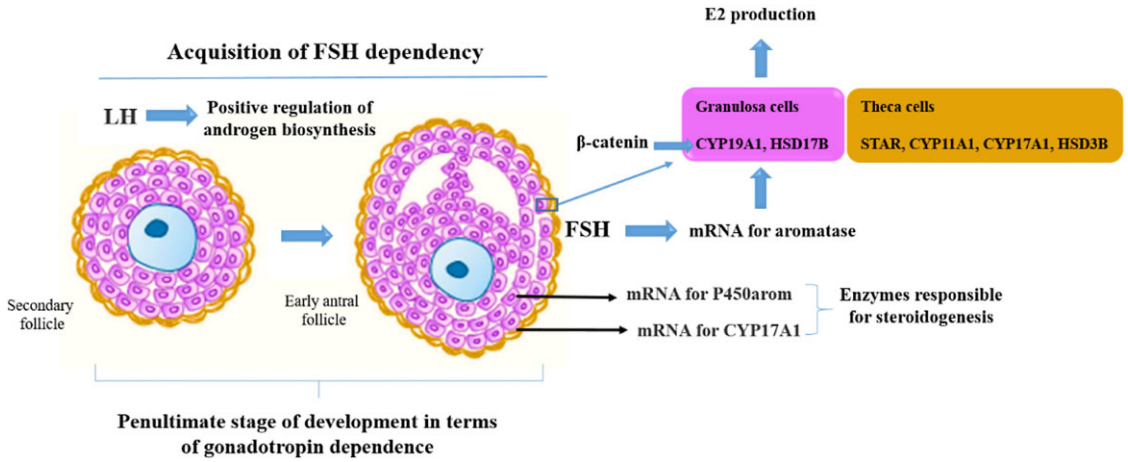

Figure 3. Mechanisms that control production of estradiol (E2) during the transition from preantral to early antral follicles.

autophagy and ferroptosis is involved in the control of atresia in ovarian follicles. In preantral follicles, degeneration initially occurs in the oocyte and, subsequently, in granulosa cells (Meng *et al.*, 2018). The first signs of atresia in the oocyte are the retraction of nuclear chromatin and oocyte fragmentation, which triggers the process of irreversible elimination of ovarian follicles at this stage of development. In the granulosa cells of preantral follicles, changes are rarely observed.

Zoheir *et al.* (2021) showed that cellular DNA fragmentation is an important biochemical marker of apoptosis during follicular atresia. Other evidence confirmed that apoptosis is not the only death pathway active in the ovary. Gannon *et al.* (2012) reported a decrease in preantral follicle numbers without a concomitant increase in apoptosis, and no change in apoptosis markers caspase 3 and TUNEL. Recent studies have demonstrated that, in granulosa cells from cultured preantral follicles of buffaloes, transmembrane protein AQP8 is involved in the regulation of cycle progression and apoptosis (Cao *et al.*, 2021). Furthermore, miRNAs have been shown to control several fundamental biological processes, including follicular atresia through their target genes and signalling pathways, and play a central role in the regulation of autophagy (Ma *et al.*, 2020). Gannon *et al.* (2012) showed that degeneration of preantral follicles is associated with the activation of the autophagy cascade. Meng *et al.* (2018) reported that the standard pathway of degeneration in preantral follicles is through autophagy, and that the activation of this pathway occurs under normal physiological conditions. Considering that, *in vivo*, follicular atresia during preantral to early antral follicles transition is controlled by various intra-follicular regulators such as growth factors, cytokines, and steroids (Orisaka *et al.*, 2009a), the development of strategies to interrupt atresia is a difficult task.

Despite early antral follicles (~2.0 mm in diameter – human species) are still not dependent on gonadotropins, Orisaka *et al.* (2009b) showed that they are susceptible to apoptotic signals. In these follicles, with the progression of atresia, there is a reduction in the number of layers of granulosa cells, and invasion of fibroblasts and macrophages (Seneda *et al.*, 2021). Meng *et al.* (2018) reported that atresia in these follicles was associated with the activation of the autophagy cascade. Increasing the levels of microtubule-associated light chain protein 3 (LC3) and of autophagy homeostasis-associated protein BECLIN1 is linked with the death of preantral and early antral follicles by the autophagy cascade (Gordy and He, 2012). Activation of this pathway occurs under normal physiological conditions and the presence of LC3, sequestosome 1 (SQSTM1/P62) and autophagy-related protein 7 (ATG7) are markers for autophagy (Gannon *et al.*, 2012). During atresia of those follicles, Meng *et al.* (2018) also reported that a loss in mitochondrial antioxidant capacity in granulosa cells led to the activation of AMP-activated protein kinase alpha 1 (AMPK-α1) and AMPK-α2, while at the same time the expression of protein kinase B (AKT) and mammalian target survival of the rapamycin complex 1 (mTORC1) was decreased. AMPK is an important regulator of metabolism, an inhibitor of the mTORC1 complex, and a direct activator of autophagy (Emerling *et al.*, 2009; Egan *et al.*, 2011; Kim *et al.*, 2011). Oxidative stress is able to reduce superoxide dismutase (SOD) expression in granulosa cells of preantral follicles, and to activate AMPK, which leads to the activation of autophagy. The mechanisms that regulate the preantral and early antral follicles atresia are shown in Figure 3.

Strategies to promote *in vitro* growth of preantral follicles

Preantral follicles represent 90% of the ovarian follicle population and, therefore, the development of effective culture systems to promote their growth *in vitro* is an interesting field of study in regard to avoiding the process of atresia that naturally occurs *in vivo* during the late antral stages of development. Follicular growth, antrum formation and acquisition of oocyte competence have been obtained in some species through the addition of different substances such as hormones, growth factors and cytokines to the culture medium in species that require short culture periods, such as murine (O'Brien *et al.*, 2003). However, in human and large animal species, the development of primordial to preovulatory follicles is a very long process (~6 months; Paulino *et al.*, 2022) and various researchers have been working to develop efficient culture systems.

The limitations of *in vitro* culture of preantral follicles come from the difficulty of maintaining the three-dimensional structure of follicles, and the many signals necessary to coordinate the stimulation of follicular growth (Paulino *et al.*, 2022). Although bi-dimensional (2D) follicle culture has been successfully performed in many studies (da Cunha *et al.*, 2018; Paulino *et al.*, 2018, 2020; Vasconcelos *et al.*, 2021), a major limitation of these systems is their inability to maintain follicle architecture, with the oocyte surrounded by granulosa cells. This is particularly problematic

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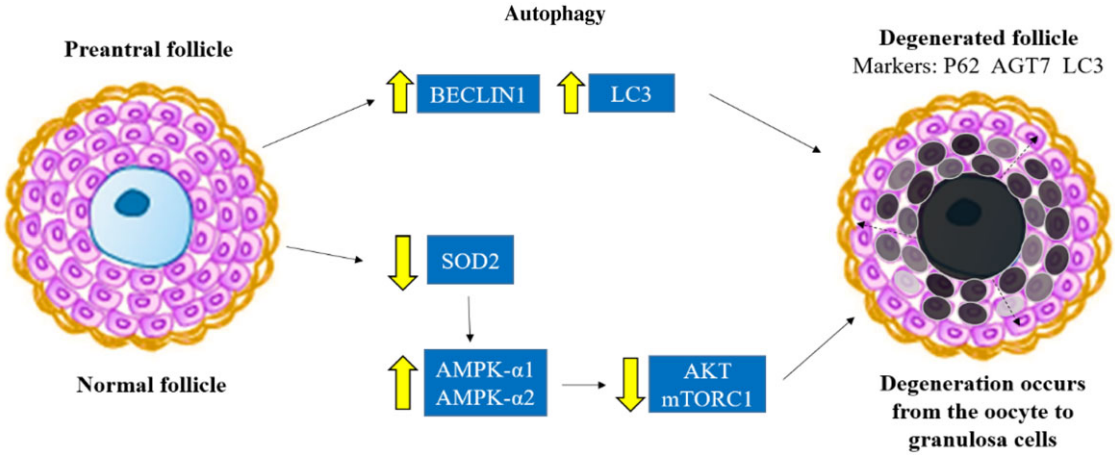

Figure 4. Strategies to promote *in vitro* growth of preantral follicles.

with follicles from large mammalian species that require longer term cultures (Simon *et al.*, 2020). Given the importance of maintaining the follicle complex architecture, three-dimensional (3D) culture systems can help to maintain follicle complex architecture (Simon *et al.*, 2020). For this system, various types of matrices are used to encapsulate follicles to maintain follicle architecture and cell–cell interactions, thereby creating a micro-environment similar to that of the *in vivo* ovary, improving somatic cell proliferation and oocyte growth (Belli *et al.*, 2012). In general, matrices have been engineered from natural components such as collagen and alginate (Healy *et al.*, 2021), or Matrigel (Hao *et al.*, 2020), or from synthetic components such as polyethylene glycol (Green and Shikanov, 2016; Tomaszewski *et al.*, 2021). Maintaining the follicular shape along with regulating the physical and chemical features of the microenvironment can be used as a tool to understand the underlying biology of follicle growth and maturation (Ghorbani *et al.*, 2022; Khunmanee and Park, 2022).

Preantral follicles cultured in the 3D system had greater homogeneity of daily growth, higher rates of viability and antrum formation, as well as low rates of degeneration (Antonino *et al.*, 2019; Panta *et al.*, 2019). Preantral follicles from other species have been successfully cultured in a three-dimensional culture system using alginate and fibrin (monkey: Xu *et al.*, 2013; Bulgarelli *et al.*, 2018; cat: Chansaenroj *et al.*, 2019; human: Chiti *et al.*, 2017).

Multistep culture systems have also been developed to further mimic the physiologic environment of developing follicles (Green and Shikanov, 2016; Simon *et al.*, 2020). These systems have been used for culturing early preantral follicles. The multistep method starts with the culture of ovarian cortical tissue for 3 weeks to initiate primordial follicle activation and to support follicle growth to the preantral stage. At the end of ovarian tissue culture, preantral follicles are isolated mechanically and cultured individually or in a group for 6 weeks (Xu *et al.*, 2021). In this system, Xu *et al.* (2021) demonstrated that ~50% of human follicles survived for 6 weeks. Most surviving follicles grew to the antral stage and produced the ovarian steroid hormones estradiol and progesterone, in addition to paracrine factors such as activin A, IGF-2 and VEGF. In addition, the cultured preantral follicles exhibited morphology similar to that of human follicles developed *in vivo*.

Many efforts have been made to elucidate the mechanisms involved in the growth of preantral follicles, as reviewed recently by Paulino *et al.* (2022). Although comprehension of the molecular regulation and composition of the microenvironment coordinating the events in preantral follicles remains incomplete, over time many studies have been conducted to optimize culture systems to support follicular growth (Healy *et al.*, 2021). Preantral follicles from human and animal species require the development of a tightly regulated culture system with adequate nutrients, cytokines, growth factors, and developmental stage-dependent hormones to support cell survival and proliferation, as well as cellular function, which changes as follicles grow and oocytes mature (Simon *et al.*, 2020; Paulino *et al.*, 2022). Therefore, understanding the influence of several compounds for supplementation of culture medium for preantral follicles is fundamental. In this context, melatonin increases follicular and oocyte diameters, forms the antral cavity and preserves high rates of morphologically intact sheep preantral follicles for up to 18 days of culture (Barros *et al.*, 2020).

Another alternative to improve follicular development *in vitro* is to supplement the culture medium with EGF, whose signalling regulates many cellular processes associated with survival (Sabbah *et al.*, 2020). EGF has an important role in folliculogenesis, by promoting several processes, such as granulosa and theca cell proliferation (Jachter *et al.*, 2022). The effects of adding EGF to the culture medium are directly associated with improved survival of bovine preantral follicles. In general, EGF-treated follicles reach a larger diameter at the end of the culture period (Paulino *et al.*, 2020; Jachter *et al.*, 2022). Hormones, such as progesterone, and cytokines, such as TNF-α, are able to maintain healthy follicle morphology and positively influence follicular growth and antrum formation in cattle (Paulino *et al.*, 2018, 2020). Also in cattle, preantral follicles cultured in the presence of BMP-2 or BMP-4 showed a significant increase in follicular diameter and greater average daily growth (da Cunha *et al.*, 2018).

Understanding the mRNA transcription of preantral follicles can provide important insights to detect follicular gene expression at several critical stages of its development under different culture conditions. Therefore, the identification of genes differentially expressed at each of growth and follicular stage development can be used to elucidate the processes involved in follicular growth (Paulini *et al.*, 2022). In bovine species, an overexpression of mRNA for oocyte maturation factor Mos (c-*MOS*) and GDF-9 was observed when oocytes from preantral follicles were cultured in the presence of EGF. Furthermore, higher levels of cyclin B1 and GDF9 mRNA were observed in oocytes from follicles cultured with progesterone (Paulino *et al.*, 2020). In addition, bovine preantral

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follicles cultured in the presence of alpha-lipoic acid expressed higher levels of transcripts for the FSH receptor, LHCGR, IGF-1, BMPR1a, TGFβ1, TGFβR1, ActRIIB, GDF9, and activin. The expression of pro-apoptotic genes *BCL2* associated X (BAX) and c-*Myc* were also downregulated (Zoheir *et al.*, 2017).

To mitigate the effects of oxidative stress induced by culture conditions, such as lower quality oocytes, follicular cell death, inactivation of antioxidant enzymes, and mitochondrial damage, there has been an increasing interest in the antioxidant potential of natural compounds (Lins *et al.*, 2017). Preantral follicles from sheep cultured in medium supplemented with kaempferol showed high percentages of follicular survival, antrum formation, and greater follicular diameter. In addition, kaempferol preserves higher levels of metabolically active mitochondria (Santos *et al.*, 2019). In cattle, the presence of eugenol in a culture medium promoted higher daily growth rates of bovine preantral follicles, in addition to stimulating the expression of mRNA for glutathione peroxidase 1 (GPX1; Vasconcelos *et al.*, 2021). The strategies to promote *in vitro* growth of preantral follicles are represented in Figure 4.

Final considerations

The transition from preantral to antral requires intense and precise granulosa–oocyte interaction, any dysregulation can interfere with the acquisition of oocyte competence. Endocrine, paracrine and autocrine factors are essential for follicular growth, antral cavity formation, granulosa cell proliferation, differentiation, and future gonadotropin dependence. *In vitro* studies have contributed to a better understanding of the roles of hormones and growth factors during follicular development, addressing molecular events and bidirectional communications between the oocyte and surrounding granulosa cells.

Data availability. All data searched in this study are included in this publication.

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Conflict of interest. The authors declare that they have no competing interests.

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