Chapter 6

The Primordial to Primary Follicle Transition — A Reliable Marker of Ovarian Function

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Abstract

In many mammalian species, including humans, folliculogenesis begins in fetal life and progresses throughout adulthood. The growing follicles progress from a reserve of primordial follicles that constitute the pool of female gametes for the entire life. Primordial follicles may begin to grow either immediately after forming or at clearly defined species-specific gap. Alternatively, some follicles may become quiescent before they either degenerate or resume growth several months or years afterwards. The rate of follicular assembly and the primordial to primary follicle transition is a critical step in female fertility. Therefore, disturbed coordination of the formation of primordial follicles and activation of their growth may entail some reproductive disorders. A poor initial reserve or the precocious primordial follicle depletion will result in infertility that, in women, is escorted by a shortened reproductive lifespan and early menopause. Therefore, it seems necessary to reach a profounder understanding of the molecular and cellular mechanisms controlling follicular development during preantral transition. *In vitro* growth of isolated immature ovarian follicles (IVGF) appears as an emerging technology, allowing to expand the fertility options in particular ovarian disorders or after cancer treatment

Keywords: Preantral follicles, folliculogenesis, organ cultures, *in vitro*, growth of isolated immature ovarian follicles (IVGF)

1. Introduction

In the ovary of a mammalian female, the process of folliculogenesis begins during fetal life and proceeds until the end of reproductive capacity, which is manifested in cell proliferation and differentiation [1,2]. Folliculogenesis, involving growth and development of ovarian follicles from primordial to preovulatory stages, is a complex phenomenon requiring multidirectional

regulation. The ovarian follicle plays an essential role in securing optimal conditions for oocyte maturation and its release during ovulation, for which it will provide an appropriate microenvironment based on locally produced molecules, such as sex steroids and peptide hormones, growth factors, and cytokines, while also providing the appropriate communication among particular compartments of an ovarian follicle [3–5]. Sex steroids produced by follicular cells are known to play one of the main roles in the regulation of ovarian function [6]. These steroids present in the systemic circulation actively participate in the regulation of pituitary gonadotropin secretion. On the other side, sex steroids present in the ovarian microenvironment act as paracrine factors important for the maintenance of follicular development [3]. The majority of information about the role of sex steroids in ovarian functioning has been obtained in studies directed at the action of estrogens [7] and progestagens [8]. Nowadays, increasing attention is being devoted to the action of androgens because the activation of androgen receptors (ARs) located in follicular cells [9,10] modulates the expression and activity of many genes vital for the maintenance of follicular development [11–13].

From the initial pool of ovarian follicles recruited to grow, only a few reach a preovulatory stage. Less than 1% of follicles elude the process of atresia at various stages of development, and the preantral to early antral transition is the most susceptible to this process. The pool of primordial follicles established in fetal life constitutes a reserve that will not increase during the postnatal period. The initial stages of folliculogenesis, including the accumulation of primordial follicles, the recruitment of primordial follicles from the resting pool, and their transition into primary follicles, are crucial for the female reproduction regardless of the species [14]. Improper coordination of the formation of primordial follicles and activation of their growth may entail disturbed folliculogenesis in mature individuals manifested by a reduction of fertility. Recent research has revealed that primordial and primary follicles might not die by classical apoptosis. It is therefore possible that, in the immature ovary, other mechanisms are involved in follicular atresia [15].

The main factor determining the selection of follicles into the antral stage is their ability to respond to gonadotropins, especially follicle-stimulating hormone (FSH). Preantral follicles display an increase in the number of FSH receptors (FSHR) that, when activated, stimulate granulosa cell proliferation, antrum formation, and biosynthesis of estradiol after the activation of aromatase enzyme. There is quite ample evidence that follicle development is dependent on their granulosa layer, the functioning of which is influenced by endocrine, paracrine, and autocrine mechanisms. Granulosa cells are involved in the control of oocyte maturation and proper execution of ovulation and participate in early embryogenesis, maintenance of corpus luteum function, and production of chemotactic factors and those involved in angiogenesis [16]. Sustained oocyte growth depends on the effective communication and crosstalk between granulosa cells and the oocyte, because granulosa cells remain the major source of nutrients for the gamete through homologous and heterologous gap junctional contacts [17].

The tool that allows studying the function of ovarian follicles irrespective of its complicated structure is the model of whole organ culture, which reflects the conditions and complicated interactions occurring *in vivo*. These kinds of cultures constitute very sensitive objects to test the biological activity of various factors; they allow to observe the responses to increased or

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decreased steroid hormone secretion, the induction or inhibition of cell proliferation, or the induction or inhibition of apoptosis. The technological revolution in reproductive biology that started with artificial insemination and embryo transfer technologies during the last 30 years has continued with oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF), or *in vitro* embryo culture (IVC), to name only a few. IVM has particular significance, providing the technology platform for the abundant supply of mature, good quality oocytes for diverse applications, such as reducing the generation interval in important species or studying *in vitro* human reproduction. Despite the convenience of IVM, we still do not understand the precise factors and conditions occurring *in vivo*, which yield the highest-quality mature oocytes for successful fertilization and embryo development outcomes; hence, we cannot completely imitate these conditions. Thus, *in vitro* growth of isolated immature ovarian follicles (IVGF) appears as an emerging technology allowing to expand the fertility options, particularly in young cancer patients [18–20], and may serve as a potential source of fertilizable gametes. Thus, assisted reproductive technologies allied to a profound understanding of granulosa/oocyte interactions can benefit from the capability to sustain primordial and primary follicle growth *in vitro* while supporting the acquisition of oocyte competence.

On this basis, the objective of this chapter is to review relevant data concerning the molecular factors crucial to the regulation of early stages of folliculogenesis and to provide basic information to the design of future culture strategies promoting the *in vitro* development of ovarian follicles.

2. Development of the primordial follicle

In the mammalian embryo, ovarian development begins between 3 and 6 weeks after conception. During this period, ovarian rudiment is massively colonized by mesonephric cells, which are regarded as the follicular cell precursors, and the primordial germ cells (PGC) migrate into the genital ridge; hence, other events take place, such as the differentiation of the gonads according to gender, proliferation, and apoptosis [14,21,22]. Oocyte development begins in the mammalian female fetus together with the differentiation of PGC. Proliferating PGC migrate towards the nascent genital ridges, where they differentiate into oogonia, before entering the first meiotic division to become primary oocytes [26].

Mammalian oocytes develop and reach ovulatory maturity inside the follicles where they are covered at first by pre-granulosa and then by granulosa cells [23] (Figure 1). Over the lengthy process of follicle development, granulosa cells proliferate and the theca layer is formed [24], allowing the follicle to take advantage of blood supply. Then, follicles pass through the succeeding stages of development before reaching full maturation and the ability to ovulate [25]. Primary oocytes, which are arrested at diplotene of the first meiotic prophase since late prenatal life in most mammal species, are the organizing centers of primordial follicles. The oocyte is considered to play the most important role in follicular organization during folliculogenesis. It is assumed that the oocyte controls both the proliferation and the differentiation of granulosa cells into cells capable of secreting steroids and various proteins. On the contrary, several oocyte features, such as growth, differentiation, meiosis, cytoplasmic maturation, or

control of transcriptional activity, are dependent on the presence and contact with granulosa cells [27]. Interestingly, when the oocyte reaches a certain size threshold, it secretes factors that inhibit the ability of granulosa cells to promote its own growth [4], which suggests that the oocyte may determine not only its own growth but also the growth of the whole follicle.

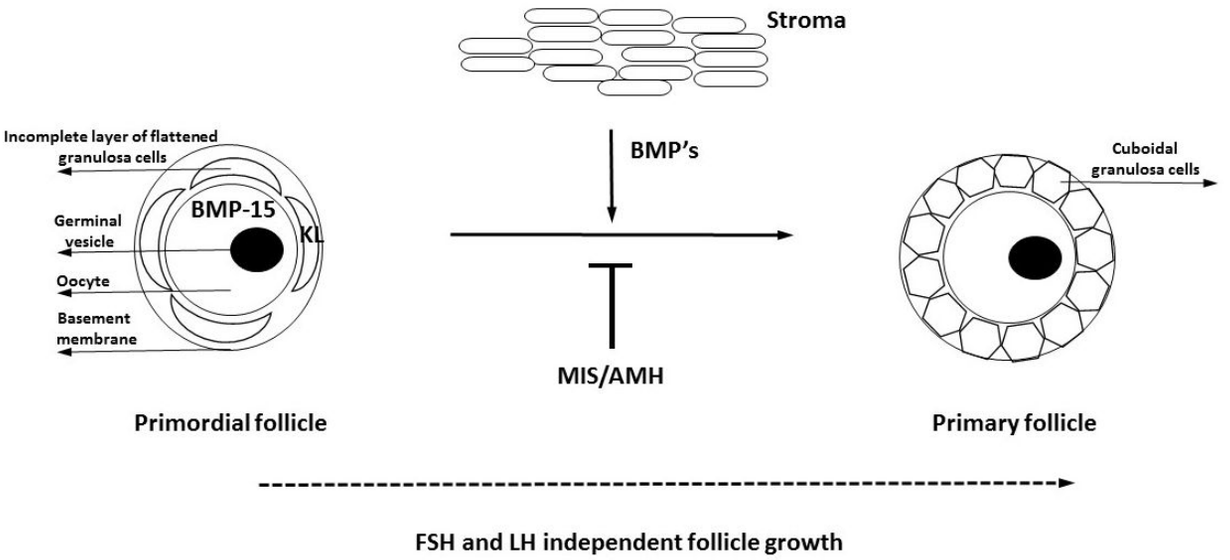

Figure 1. Simplified representation of early stages of mammalian folliculogenesis. KL, kit ligand; BMPs, bone morphogenic proteins; MIS/AMH, Müllerian inhibitory substance.

The assembly of primordial follicles, also described as the primordial follicle formation, demands for individual oocytes to associate with developing pre-granulosa cells, in a complex process that involves the breakdown of oocyte nests, oocyte separation, and subsequent recruitment of somatic (i.e. pre-granulosa) cells, which are regulated by circulating hormones and factors produced by the oocyte and somatic cells [28]. Newly created primordial follicles give rise to primary follicles that, in a series of transitions coordinated by gonadotropins, steroids, and other intraovarian factors, transform into preantral and then antral follicles and finally preovulatory follicles [6]. Among the signaling pathways that are important for primordial follicle assembly, apoptosis and autophagy are crucial in determining cellular fate. After nest separation, a large number of germ cells are lost by apoptosis; the mechanisms regulating cyst breakdown and germ cell death are still unclear. Indeed, much attention has been focused on germ cell elimination by apoptosis and the role of Bcl-2 gene family in regulating the balance between survival and death of oocytes before the formation of primordial follicles [29]. To date, autophagy has also been proposed to contribute in the mechanisms of prenatal and neonatal oocyte demise [30]. Increasing evidences showed that, in the immediate hours of the postnatal life, many tissues and organs evidence up-regulation of autophagy pathways, possibly acting as an adaptive response of the newborn organism to nutritional stress associated with the deprivation of placental nutrients [31]. The balance between quiescence and activation of the primordial follicle reserve seems to depend on a number of molecules. The phosphatidylinositol 3-kinase (PI3K) pathway was proposed as a key pathway playing a crucial integrative role by bridging the action of multiple factors in the balance

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between follicle growth suppression, activation, and the maintenance of healthy quiescence [32]. In mammalian ovaries, postnatal depletion of oocytes occurs also by atresia of follicles. Follicular atresia is directed by granulosa cell apoptosis and affects all stages of follicular development. Interestingly, recent evidence from studies on rats shows that autophagy of germinal cells is an alternative route to induce follicular atresia in the ovary [33]. This implies the importance of autophagy in cellular elimination within the ovary.

3. Primordial to primary follicle transition

The concept of primordial follicle activation refers to the process by which primordial follicles gradually exit the nongrowing follicle pool and enter the intermediate or primary follicle stage [23]. The clarification of the mechanisms that regulate primordial follicle activation is an important issue for the success of assisted reproduction [4]. Whereas the primordial follicle activation seems to depend mainly on signals originating in the ovary, pituitary and metabolism-related hormones are required for folliculogenesis to proceed past the primary or secondary stage [34]. In the ovary, the crosstalk between oocytes and somatic cells (i.e. granulosa or theca cells) occurs at an early stage of follicular development [4]. The activation of primordial follicles is associated with oocyte growth, and simultaneous differentiation of the adjacent pre-granulosa cells occurs. During the transition into primary follicles (showing a complete layer of cuboidal granulosa cells), pre-granulosa cells change into a cuboidal shape [23], and in the process, they form an intermediate form of follicles presenting both cuboidal and flattened pre-granulosa cells [35]. The proliferation of granulosa cells allows to originate multiple layers of cells, and follicle develops to secondary, antral, and further advanced follicle stages [23,36,37].

Recent research revealed that factors secreted by the oocytes regulate the initiation of primordial follicle growth [38] (Figure 2). The tyrosine kinase receptor Kit (c-Kit) and two different isoforms of its ligand (kit ligand, KL), localized in oocytes and granulosa cells, stimulate oocyte growth and maintain it in meiotic arrest depending on FSHR levels. The up-regulated expression of KL, triggered by low concentrations of FSH, promotes a reduction in the ratio of KL/c-Kit and stimulates oocyte growth, whereas high concentrations of FSH enhance follicle development but impair oocyte growth [5]. Other important regulators of follicle growth are activin [39] and oocyte-derived growth differentiation factor-9 (GDF-9) [14,40]. GDF-9 promotes follicular survival and growth during transition due to suppression of granulosa cell apoptosis and follicular atresia, whereas activin promotes FSH release, antral cavity formation, and granulosa cell proliferation. Bone morphogenetic protein-15 (BMP15) has been shown to promote granulosa cell growth by stimulation of the proliferation of undifferentiated granulosa cells in an FSH-independent manner. It was shown that two markers of proliferation, Ki-67 and proliferating cell nuclear antigen (PCNA), are regulated by these oocyte-derived factors (for review, see Ref. [41]). It was also suggested that PCNA could act as a key regulator of the development of ovarian follicles. The time expression of PCNA in oocytes is coincident with the initiation of primordial follicle formation. By promoting the apoptosis of oocytes, PCNA can also regulate primordial follicle assembly in neonatal mouse ovaries [42]. Moreover,

proliferation of granulosa cells is increased by insulin-like growth factor-I (IGF-I), which was also associated with the regulation of follicular growth from the primordial stage [43]. The anti-Müllerian hormone (AMH) is synthesized early in the follicle formation, by the cuboidal granulosa cells of primordial follicles. This factor, subsequently produced by preantral and antral follicles, inhibits the initial recruitment of primordial ones as well as their further FSH-dependent growth [44].

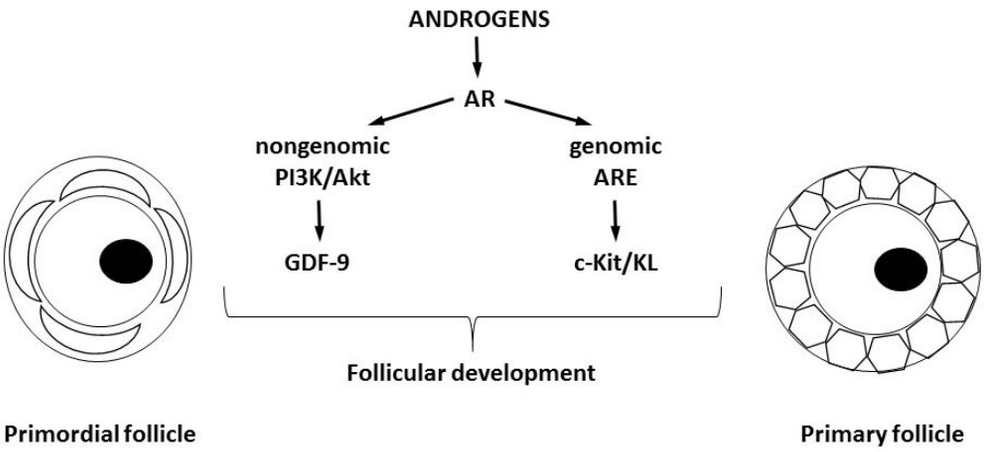

Figure 2. Mechanisms of androgen actions in follicular development. Physiological functions of androgens during primordial follicle recruitment are mediated through androgen response element (ARE)-dependent genomic actions and/or via PI3K/Akt nongenomic signaling pathway.GDF-9, growth differentiation factor-9; AR, androgen receptor.

The idea that androgens might regulate follicular development initially started with studies indicating AR expression in the different compartments of follicles throughout most stages of folliculogenesis [45–48]. However, AR expression pattern may differ between cell types, and in most species, AR is abundant in the preantral/antral stages of follicular development but declines as a follicle matures to the preovulatory stage [49–51]. Based on these observations, it was suggested that androgens might differentially regulate various stages of follicular development through an autocrine and/or paracrine way. It is generally accepted that androgens primarily affect preantral follicles and that their activities are important for preantral follicle growth and prevention of follicular atresia. Moreover, it seems possible that androgens are involved in the activation of primordial follicles [52–54] (Figure 2). How androgens influence primordial follicle recruitment and whether this is a primary or secondary response to androgens are still open-ended questions needing further investigation.

The mechanisms of primordial follicle activation can be studied using *in vitro* culture methods. However, until now, the success of primordial follicles culture as a method of oocyte growth has been limited to mice. Eppig and O'Brien [55] were the first to obtain mouse offspring derived from oocytes acquired from cultured primordial follicles. As to other species, several studies carried out in farm animals and primates showed that the transition of primordial into primary follicles in culture of cortical strips from caprine [56], bovine [57], baboon [58], and human [59] ovaries is possible. A confirmation of the normality of follicle development *in vitro* was obtained through the changes in follicle morphology and cell number as well as from

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the stage-specific follicular responsiveness to above-mentioned factors or the development of steroidogenic capacity.

4. Primordial and primary follicle isolation

In vitro follicle growth is a promising fertility preservation strategy [19,60] despite that, in some mammalian species, including humans and pigs, the success has been limited when the process started with primordial follicles. This could be explained by the fact that adequate isolation methods and culture strategies have not yet been fully established, thereby impairing the ability to obtain mature gametes from the culture of isolated primordial follicles in those species. The manipulation of primordial follicles is a challenge due to their small size and the existing physical connections between the oocyte and the surrounding squamous granulosa cells, which are also poorly studied. Conversely, the conditions that support their activation and growth are not well defined. Several studies have indicated that primordial and primary follicles rapidly degenerate in cultures carried under multiple conditions [61–63]. For example, primordial follicles isolated from human ovarian tissue using collagenase digestion and subsequently cultured in collagen gels resulted in the degeneration of the follicles within 24 hours [64].

The species and the reproductive age of the ovarian tissue affect preantral follicle yield in the ovary because of the existence of a larger number of follicles and the easiness of the isolation method in neonatal and prepubertal ovaries compared with mature ovaries [65,66]. The success of either culture or transplantation of isolated follicles depends on the high quality of retrieved follicles. That is why an effective method for retrieving viable, preantral follicles is an essential condition. Different methods are currently available to isolate follicles for preantral follicle culture. The mechanical isolation methods include the use of fine-gauge needles or forceps to isolate follicles from mice [67], rats [68], pigs [69], cattle [70], and humans [64]; the combination of ovarian dissociation methods, such as grating or mincing, with sieving [71]; and the follicular dissection from the ovarian cortex using a skin-grafting knife and/or small scalpel blades [34,70]. The mechanical isolation methods have a main advantage, as they allow retrieving intact follicles, surrounded by the basement membrane and theca layers, although they are slow and laborious techniques that typically yield only a small number of follicles [72]. These technical problems can be avoided by the use of enzymes to aid follicle recovery. The incubation of ovarian tissue in collagenase and/or DNAse (e.g. Refs. [55,73]) softens and disaggregates the tissue matrix and allow detaching follicles from the surrounding stroma with the aid of needles. However, the degradation of the basement membrane and the absence of theca cell layers are the most common undesirable consequences of the use of enzymes in follicle isolation [74], as they foster the spontaneous loss of granulosa cells from the follicles in culture. Nevertheless, the time of enzyme exposure can be controlled to minimize the damage [75].

The ovarian stroma is dense and fibrous; thereby, it is more efficiently isolated using a combination of mechanical and enzymatic procedures that have been shown to preserve follicle viability [64,76–78]. Dolmans et al. [77] developed a new isolation protocol using Liberase Blendzyme 3. This blend of purified enzymes allowed the isolation of a high number

of preantral follicles, which were viable as well as morphologically and ultrastructurally normal. However, this type of Liberase is no longer produced. Therefore, the second generation of Liberase DH (Dispase High) Research Grade has been successfully tested for the preparation of human ovarian follicles [79]. However, the efficiency of the mixture may vary with the species. Our group recommends the use of Liberase TH (Thermolysin High) Research Grade to obtain a high number of fully isolated primordial follicles from porcine ovarian cortex [80], as it presents a really fibrous tissue. Using prepubertal gilt ovaries, we applied different types of Liberase (DH, TM, and TH) Research Grade and treatment protocols to isolate primordial follicles (Figure 3). The quality of the isolated follicles was evaluated by their general morphology and viability upon routine hematoxylin and eosin (H&E) and fluorescent staining, whereas their ultrastructure was assessed by electron microscopy. Additionally, to determine the purity of isolated follicles, a germ cell-specific protein, MSY2, was used to recognize oocytes. Liberase TH Research Grade was the mixture presenting a very high proportion of retrieved viable follicles whose majority exhibited good morphology with a complete granulosa cell layer. In addition, primordial follicles stained with either Hoechst 33342 or H&E indicated that Liberase TH Research Grade only occasionally induced atresia. This was supported by ultrastructural studies revealing that the oolemma-follicular cell interface was well preserved, which would allow the complex to express the correct metabolic profile (Figure 4). The results obtained in those experiments also showed that almost all of the Liberase TH Research Grade-isolated primordial follicles were MSY2 positive. As shown in the literature [81,82], primordial and primary follicles may rapidly degenerate after isolation because of the loss of critical connections between the oocyte and the granulosa cells. It seems that Liberase is a promising alternative to collagenase treatment, allowing the use of isolated primordial follicles for further reproductive studies.

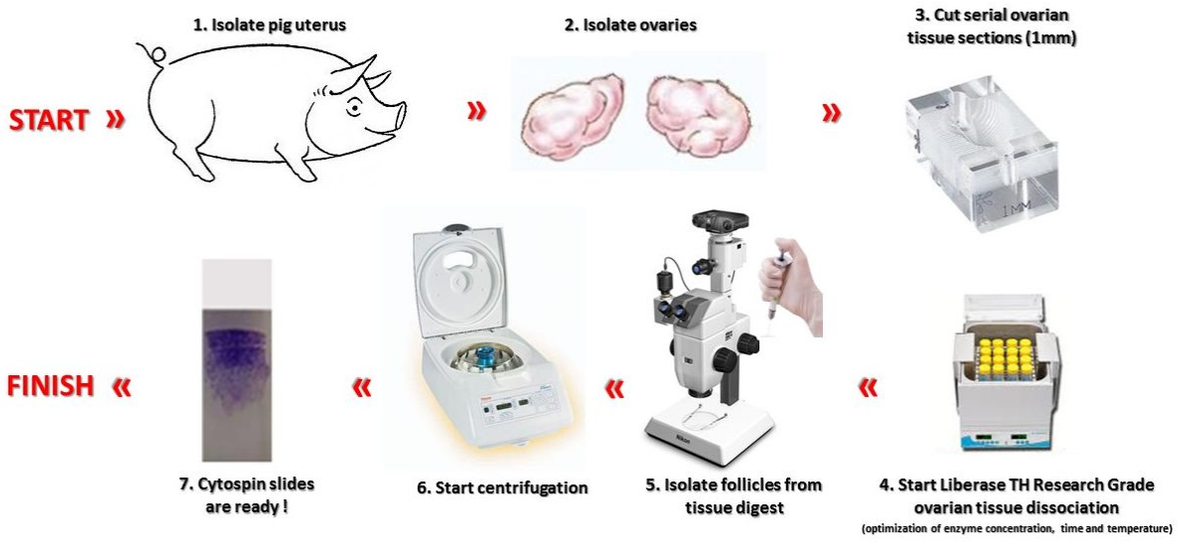

Figure 3. Pig ovarian medulla collection and preantral follicle isolation protocol. 1–3: Ovarian medulla collection (4- to 5-month-old prepubertal gilts); 4–6: isolation of primordial and primary follicles using different types of collagenase (types I, II, and IV) and Liberase (DH, TM, and TH); 7: evaluation of preantral follicles morphology (H&E staining). For details, see Ref. [80].

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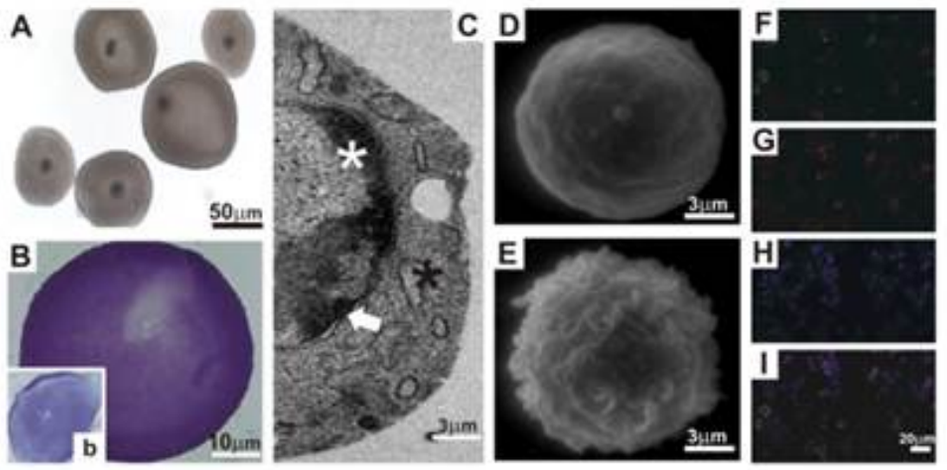

Figure 4. Morphology and ultrastructure of primordial and primary pig follicles isolated from ovarian medulla using Liberase TH-Research Grade. (A) Morphology of Liberase TH-treated pre-antral follicles (light microscopy), (B) morphology of Liberase TH-isolated pre-antral follicles stained with hematoxylin and eosin; (b) interrupted granulosa cells layer in pre-antral pig follicles isolated with collagenase (type II); (C) Transmission electron microscopy (TEM) showed a single uninterrupted layer of cuboid follicular cells (black asterisk) surrounding the oocyte (white asterisk), which was bordered by a continuous basal lamina (arrow). Scanning electron microscopy (SEM) of primordial (D) and primary (E) follicles isolated using Liberase TH. A continuous layer of cuboid follicular cells surrounds the oocyte in the primary follicle (E) while in the primordial follicle a flattened layer of cells covers the oocyte; immunoconfocal images recorded from three selected areas of centrifuged ovarian digest: follicles were stained for actin (F), MS2Y (G) and with DAPI (H), merged images (I).

5. Primordial and primary follicle culture

The clinical application of IVFG is still at the investigational stage, in a laboratory setting, although it stands a robust approach to study the basic biology of the ovary or the follicle under a controlled yet adjustable environment. Multiple culture systems have been developed to support the development of isolated preantral follicles [69,72,83], each one with its own advantages and providing useful insights into the follicle physiology. By this time, hydrogel-based follicle culture systems have been well characterized. The oocyte and the surrounding granulosa cells interact with each other and the environment, maintaining the same spatial location, connections, and dimensionality as in the intact ovary. The *in vitro* growth and development of mouse preantral follicles was successfully supported by alginate-based hydrogels, a substrate that was also applied to several large mammalian species, including dogs [84], rhesus monkeys [19], and humans [85], resulting in stage IV oocytes (human) [86], meiosis II (MII)-arrested eggs, and fertilized two-cell embryos (rhesus macaque) [87]. This developmental stage has not been reached in other systems.

It is commonly agreed that early follicular growth is largely independent of a gonadotropin stimulus; instead, it seems that it is controlled by paracrine and autocrine signals originating from several sources in the ovary, including stromal cells, macrophages, and other follicles [38]. Recent studies showed that these local factors may also play an important role in *in*

vitro culture, supporting the growth of isolated preantral follicles: isolated primary ovarian follicles survived and grew when cocultured with purified ovarian stroma including theca-interstitial cells and macrophages [88] or with mouse embryonic fibroblasts (MEFs) as a feeder cell layer [89]. Coculture with MEFs resulted in an increased follicle survival, growth, and differentiation until antral follicles contained meiotically competent oocytes capable of reaching metaphase II in response to adequate hormone stimulation [89]. As suggested in those studies, individual primary follicles require factors beyond the standard culture media additives, including insulin, transferrin, selenium, fetuin, bovine serum albumin, and FSH [90], which can be supplied *in vitro* by coculture with stromal cells or MEFs; nevertheless, *in vivo* observations also suggest that follicles themselves may have a stimulatory effect on IVFG, because, in the mammalian ovarian cortex, the distinctive architecture and follicle distribution may influence follicle development: primordial and primary follicles are located close to the rigid, collagen-dense cortical stroma, whereas larger, growing follicles are typically closer to the interior medulla, which presents a less rigid stroma [91]. It has been shown, in a study examining the spatial relationship of follicles within ovaries, that follicles surrounded by growing follicles are more likely to be growing, suggesting the existence of an *in vivo* stimulatory effect of other follicles [92] that could be exerted by signals originating from both the oocyte and the growing follicles, which enhance the differentiation of preantral follicles.

6. Summary

In summary, the ability to sustain preantral follicle growth *in vitro* while supporting the acquisition of oocyte competence is of great scientific interest. This relies on supplying oocytes for assisted reproductive technologies and broadening our understanding of somatic cell/oocyte interactions in species characterizing by prolonged follicular growth, such as humans and pigs. IVGF is becoming a useful tool to assess follicular development, offering also the potential to preserve reproductive options in cases of polycystic ovarian syndrome (PCOS), premature ovarian failure, or definitive sterility (post-oncotherapy). In addition, it is known that certain ovarian dysfunctions, such as PCOS and gonadotropin poor responsiveness, are consequences of deregulated follicle growth at this transitional stage. Therefore, the elucidation of molecular and cellular mechanisms involved in the control of follicular development during transition from preantral to early antral stage may provide an important insight into the pathophysiology and rational treatment of these disorders.

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Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Author's contributions

Malgorzata Duda and Malgorzata Grzesiak prepared the paper, Malgorzata Knet-Seweryn collected articles for this chapter, and Tabarowski Zbigniew reviewed the paper. All authors read and approved the final manuscript version.

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