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Review

# Molecular control of oogenesis ☆

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ABSTRACT

Oogenesis is a complex process regulated by a vast number of intra- and extra-ovarian factors. Oogonia, which originate from primordial germ cells, proliferate by mitosis and form primary oocytes that arrest at the prophase stage of the first meiotic division until they are fully-grown. Within primary oocytes, synthesis and accumulation of RNAs and proteins throughout oogenesis are essential for oocyte growth and maturation; and moreover, crucial for developing into a viable embryo after fertilization. Oocyte meiotic and developmental competence is gained in a gradual and sequential manner during folliculogenesis and is related to the fact that the oocyte grows in interaction with its companion somatic cells. Communication between oocyte and its surrounding granulosa cells is vital, both for oocyte development and for granulosa cells differentiation. Oocytes depend on differentiated cumulus cells, which provide them with nutrients and regulatory signals needed to promote oocyte nuclear and cytoplasmic maturation and consequently the acquisition of developmental competence.The purpose of this article is to summarize recent knowledge on the molecular aspects of oogenesis and oocyte maturation, and the crucial role of cumulus–cell interactions, highlighting the valuable contribution of experimental evidences obtained in animal models. This article is part of a Special Issue entitled: Molecular Genetics of Human Reproductive Failure.



## 1. Introduction

In the last decade, a substantial progress has been made in the elucidation of factors regulating oocyte and follicle growth and development, as well as oocyte maturation, through the study of the crucial roles of a large number of proteins expressed throughout oogenesis, in particular during the early stages of folliculogenesis. This has mainly been accomplished by assessing the lack of their gene products either by using knockout approaches and/or targeted deletion.

The application of molecular biology techniques for the quantification of gene expression, by using microarray analysis and real-time PCR technologies has provided new insights into the regulation of mRNAs in a stage-dependent manner during folliculogenesis, both in oocytes and cumulus cells. Likewise, proteomic approaches and a recent new genome-wide profiling of maternal mRNA in association with the polysome, have allowed to identify newly translated proteins during oocyte maturation, at a stage when transcription has already ceased.

In this sense, a huge progress in basic aspects of oocyte research has been made at the molecular level. The discovery of many molecular processes/pathways involved throughout oogenesis and ovulation, and the identification of potential markers of oocyte quality have been accomplished in the recent years.

The use of in vitro approaches in large animals, but mainly in the mouse model, has also become relevant in providing valuable information that could not be obtained in human for ethical reasons. Making use of molecular biology techniques alongside with in vitro preantral follicle culture systems and/or in vitro oocyte maturation, for instance, has enabled the study of the influence of a number of factors (such as hormones, recombinant proteins, and/or growth factors) on follicle development, survival, steroid production, oocyte growth and maturation, and developmental potential. Moreover, granulosa cell culture and oocyte co-culture with granulosa cells have become accepted models to study the interaction between oocytes and their surrounding somatic cells and to provide a focus on the potential of oocytes to regulate a variety of granulosa cell functions.

There has been an exponential increase in knowledge in the field of oocyte biology; the findings delivered from several studies massively contribute to the understanding of the molecular basis of the acquisition of oocyte meiotic and developmental competence. The present review has the ambition to provide information on the recent advances in the field, emphasizing the key factors involved in the regulation of oogenesis and oocyte maturation. A particular focus is provided on the crucial role of the oocyte–cumulus cells interactions and the oocyte control of granulosa cell function at different stages of follicle development and maturation.

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## 2. Early stages of oogenesis and folliculogenesis

### 2.1. Primordial germ cells and follicle formation

Oocytes originate from primordial germ cells (PGCs). Mouse PGCs originate as early as 7.5 days of embryonic development (E7.5) in the extra embryonic mesoderm and their development initially depends on signals derived from both the extra-embryonic ectoderm and visceral endoderm. Members of the TGFβ family such as bone morphogenetic proteins (BMPs), BMP4, BMP8b (ectoderm origin) and BMP2 (endoderm origin) are specific factors needed for PGC formation and regulation of gene expression [1–3] (Fig. 1).

PGCs migrate to the genital ridge at about E10.5 in the mouse, where they proliferate by mitosis and give rise to oogonia. Migration, proliferation and colonization of PGCs to the developing gonads are controlled by many factors and depend as well on the interaction of PGCs and their surrounding somatic cells. In vitro studies have shown that BMP2 and BMP4 increase the number of mouse PGCs in culture [4,5], whereas *Bmp7* mouse knockouts show a reduction in the number of germ cells around this period [6]. Activin has also been shown to increase the number of PGCs in human [7], although activin inhibits PGC proliferation in mouse [8]. The role of germ cell derived transcription factors at this stage has also been demonstrated by using knockout approaches and conditional deletions in mice. These include factors such as BLIMP1 and PRDM14, which are critical for PGC proliferation and migration [9–11] as well as OCT4, NANOG [11–13], which are essential for PGC survival. Several factors seem to be required for PGC survival, such as FIGα (factor in the germ line alpha), a factor responsible for the early expression of the glycoproteins that will form the zona pellucida [14], NANOS3 (nanos homolog 3; *Drosophila*) and DND1 (dead end homolog 1), two RNA binding proteins that protect PGCs from undergoing apoptosis, as well as the KIT/KIT ligand (KITL) pathway. Mutations in any of the genes coding for these factors lead to a deficiency in the formation of primordial follicles due to a depletion of germ cells [12–17].

Around the period of PGC migration into the genital ridges (E10.5) sex determination starts. Differentiation into ovaries seems to be the default pathway, since the XY genital ridges differentiate into testes under the influence of the Y-linked gene *Sry*. The XX gonads have no SRY, and therefore they develop into ovaries [18]. As soon as PGCs are formed, the initially bipotential gonad will continue its differentiation mostly under the influence of somatic cell derived transcription factors GATA4, FOXL2, LHX9, WT1, WNT4, and SF1 [19–22] (Fig. 1).

After colonization of the gonad (~E13.5), PGCs will undergo a phase of mitotic proliferation with an incomplete cytokinesis, leading to the formation of 'germ cell cysts' or 'germ cell nests' [23]. Following this event and before follicle formation mitotic divisions stop and germ cells initiate meiosis, become primary oocytes and commit to the female program of development [24].

Meiosis initiate with a prophase stage, a complex phase which is subdivided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Within the first period of the prophase, a series of crucial events occur, involving the pairing of homologous chromosomes, synapsis (close association between these chromosomes), and recombination or 'crossing over' (exchange of genetic material). Subsequently, oocytes progress to the diplotene stage where they enter into a prolonged resting phase called dictyate [25]. In mouse embryonic ovaries, initiation of the meiotic program is dependent on retinoic acid and *Stra8* (stimulated by retinoic acid gene 8) signaling. STRA8 is a cytoplasmic factor expressed by female germ cells just prior to entering the prophase of first meiotic division [26], and in response to retinoic acid (RA) [27–29]. In females *Stra8* is required for premeiotic DNA replication as well as for meiotic prophase events (i.e. chromosome condensation, cohesion, synapsis, and recombination) [30].

Oocytes remain at the dictyate stage of meiosis I throughout oogenesis, until LH induces final oocyte maturation (in most mammals). Prophase events are vital for germ cell survival and meiotic progression, and errors occurring along this stage, as well as throughout the consecutive phases of meiosis, may originate and/or contribute to female meiotic aneuploidies. Indeed, endocrine-disrupting chemicals, such as Bisphenol A (BPA), have been shown to cause disturbances in spindle formation, to interfere with microtubule polymerization and to induce multipolar spindles in mouse oocytes. In utero exposure to BPA appears to interfere with control of recombination in fetal prophase I oocytes and increasing

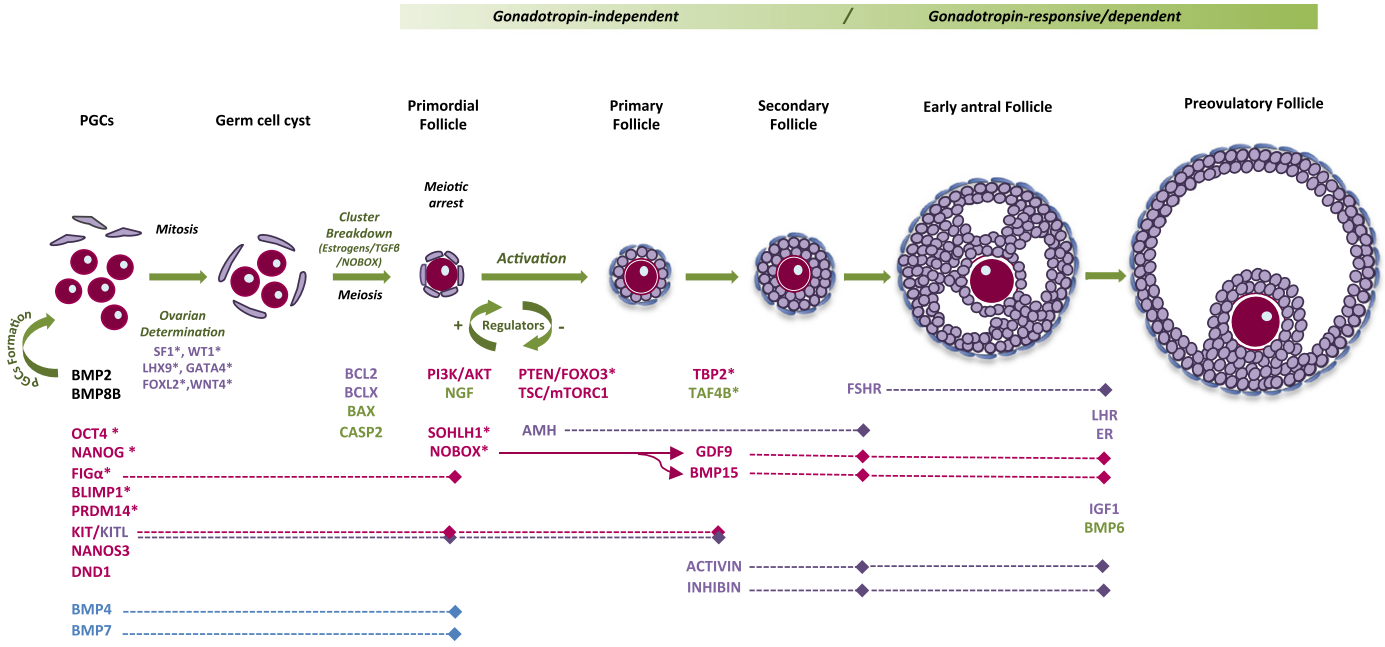


**Fig. 1.** Representative figure of the factors involved in primordial germ cell (PGC) formation, oogenesis and folliculogenesis. Ovarian factors produced by theca/stromal cells (in blue), somatic/granulosa cells (in purple), germ cells (in red) or in both germ cell and granulosa cell (green), participate and regulate oocyte and follicle development at each of the defined stages throughout folliculogenesis. Transcription factors involved are indicated with a star (*). Proteins from the extra embryonic ectoderm that participate in PGC formation are indicated in black.

the risks for errors in chromosome segregation in oocytes resuming maturation in adult females [31]. Moreover, in follicle-enclosed oocytes, continuous exposure to high levels (30 μM) of BPA during in vitro follicle development leads to an increase meiotic arrest in GV stage and an increase in aberrant meiosis I (most of which with unaligned chromosomes) and meiosis II spindles [32]. Thus, low doses exposure to BPA has been suggested to be harmful to humans by increasing oocyte aneuploidy [33]. Full-detailed information on the meiosis and the origin of meiotic aneuploidies are reviewed in the next chapter of the special issue on "Molecular Genetics of Human Reproductive Failure": Molecular Origin of Female Meiotic Aneuploidies: which factors determine risk for aneuploidy, what is the effect, what is specific about human.

Around the time of meiotic arrest, germ cell nests breakdown to initiate follicle formation. Oocytes become surrounded by somatic (pre-granulosa) cells and form primordial follicles. Follicle formation occurs before birth in human (during the second trimester of fetal development) and immediately after birth in the mouse [23].

The processes of germ cell cyst maintenance and breakdown are not fully understood (see for review, [34,35]). Studies in rodents point out to a role of estrogens in the maintenance of the germ cell nests [36–38]. Moreover, the work done by Jefferson et al. [39] suggests that estrogens maintain germ cell nests via the estrogen receptor (ER)-β, since ER-β knockout mice exposed to a genistein (an estrogenic compound) do not form multi-oocyte follicles (MOFs), whereas ER-α knockout or wild type mice do. MOFs are follicles containing two or more oocytes without a separating basement membrane. During nest breakdown, the formation of MOFs seems to occur as a result of an incomplete breakdown [35]. Besides steroids, members of the transforming growth factor beta (TGF-β) superfamily (such as GDF9 and BMP15) and other proteins such as FOXL2 and NOBOX also seem to be involved in this process. Lack of these genes or a reduced expression and function of the gene products affect the timing of nest breakdown and impair this process [40–44]. Although this model may apply in rodents, it is still not clear which signals induce germ cell nest breakdown in humans, although steroids also seem to play a role.

Throughout germ cell cyst breakdown, a substantial number of oocytes are lost. Many oocytes that are not surrounded by somatic cells undergo apoptosis [45]. Apoptosis is a crucial process, first determining the pool of primordial follicles, and later playing a role in follicular atresia. Many pro- and anti-apoptotic proteins have been demonstrated to regulate germ cell death. For instance, in the absence of BCL2, an anti-apoptotic member of the B cell lymphoma/leukemia (BCL) protein family, a reduced number of oocytes and primordial follicles but a normal number of primary follicles have been reported at an early age (6 weeks), suggesting that BCL2 may have an impact on follicle survival during the establishment of PGC and/or primordial follicle formation. A similar role has been suggested for BCLX [46,47]. On the contrary, the pro-apoptotic protein BAX, also member of the BCL family, promotes cell death. Loss of *Bax* shows an increased number of germ cells in E13.5; and despite some contradictory results, it has been reported that loss of *Bax* or loss of its regulator *Ahr* (aryl hydrocarbon receptor), results in an increase number of primordial follicles as observed at 6 weeks and postnatal day 4, respectively [48–50].

Likewise, the involvement of other pathways, such as the pathway activated by caspases, also leads to apoptosis. It has been demonstrated that in the absence of caspase 2 (casp2), an increased number of primordial follicles can be found [51].

### 2.2. Activation of primordial follicles

Primordial follicles constitute the total reservoir of germ cells available during the entire period of female reproductive life. They become activated and are continuously recruited in cohorts to initiate folliculogenesis, a process that takes around two weeks in mice and nearly six months in humans. Primordial follicle activation is a very dynamic and tightly controlled process, and despite the enormous progress that has been made, many molecular mechanisms are still not fully understood. The PTEN/PI3K signaling pathway, known to be involved in a different series of cellular processes such as regulation of cell proliferation and apoptosis [52] is a crucial pathway required for activation of primordial follicles. A functional PI3K (phosphatidylinositol 3 kinase) signaling pathway is present in oocytes from primordial and primary follicles [53]. Activation of the PI3K pathway leads to activation of its component AKT, a serine/threonine protein kinase that enhances cellular proliferation and survival, whereas PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a negative regulator of PI3K [52] (Fig. 2).

The absence of PTEN in oocytes leads to an increased PI3K activity and, as a result, an increased phosphorylation of AKT and FOXO3a (forkhead box O3), another component of the PI3K pathway [54]. FOXO3 is a transcription factor that leads to apoptosis and cell cycle arrest (Fig. 2). While phosphorylation activates AKT, it suppresses FOXO3 action. FOXO3 is expressed in mouse oocytes and is involved in the repression of primordial follicle activation, probably by inhibiting the transcription of genes essential during oogenesis and folliculogenesis. Mice lacking *Foxo3* show premature activation of primordial follicles and a further depletion of follicles at 18 weeks postnatally [55,56]. Similar to the phenotype of *Foxo3* null mutant ovaries, lack of *Pten* results in a depletion of the primordial follicle pool [54].

Manipulation of the PTEN/PI3K pathway, by in vitro treatment of mouse ovaries and human ovarian cortical tissue with a PTEN inhibitor and a PI3K activator, has enabled the induction of primordial follicle activation in vitro, generating preovulatory follicles that contain mature eggs, after transplantation of the ovarian tissue [57].

Arrest of primordial follicles, on the other hand, depends on the existence of another signaling pathway involving the tumor suppressor

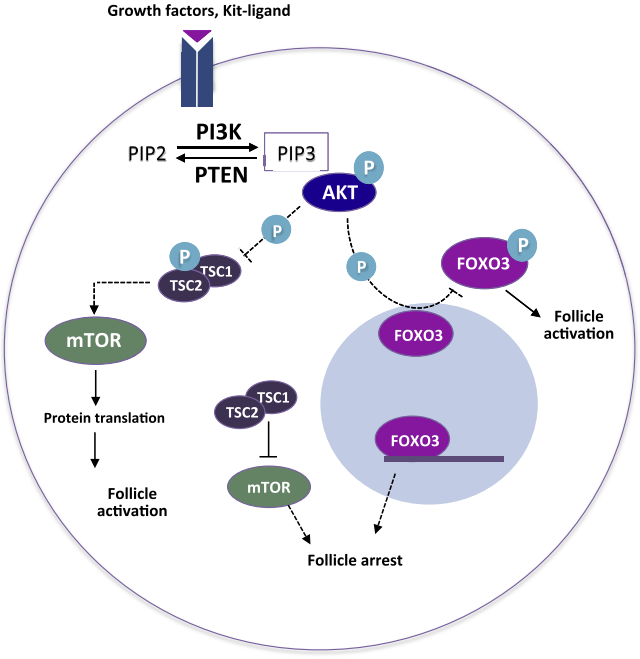


**Fig. 2.** Schematic representation of the involvement of PI3K/PTEN and TSC/mTOR pathways in primordial follicle activation and arrest. Activation of the PI3K pathway (i.e. by growth factors or Kit-Ligand) leads to phosphorylation and activation of AKT, which in turn phosphorylate FOXO3. FOXO3 is a transcription factor that induces the expression of cell cycle arrest genes. When FOXO3 becomes phosphorylated and translocates from the nucleus to the cytoplasm, it becomes inactivated, therefore allowing follicle development to progress. PTEN is a negative regulator of the PI3K pathway. In addition, via the AKT pathway, TSC, a negative regulator of mTOR, becomes phosphorylated and inactivated. As a result, mTOR pathway becomes activated leading to signals that regulate protein translation and follicle development to progress.

tuberous sclerosis complex 1 (TSC1) and the mammalian target of rapamycin complex 1 (mTORC1). In a mouse model, Tsc1 has been shown to negatively regulate mTORC1 and maintains quiescence of primordial follicles (Fig. 2). Null-mutation in oocytes lead to a premature activation of primordial follicles and to follicular depletion, and results in premature ovarian failure (POF) [58]. Thus, synergistic actions mediated by the Tsc/mTORC1 and PTEN/PI3K signaling pathways are crucial in the regulation of the resting and activation of the primordial follicle pool.

Interaction of KIT ligand and its receptor KIT, early expressed by somatic cells and oocytes, respectively, is also involved in the key events controlling initiation of follicular growth by promoting theca cell recruitment, proliferation of granulosa cells and stimulating oocyte growth [59]. In vitro studies have shown that treatment with KIT ligand regulates the PI3K/AKT pathway in oocytes, by activating AKT and repressing transcription factor FOXO3, as a result of an increased phosphorylation of both components [53].

Other important growth factors such as neurotrophins (which mainly play a role as part of the nervous system); have also been shown to regulate early folliculogenesis. For instance, nerve growth factor (*Ngf*) is expressed both in somatic cells and oocytes even prior to follicle formation and seems to play a role in primordial follicle activation [60,61]. Seven-day-old mice ovaries lacking *Ngf* show a decreased proliferation of somatic cells and contain few primary follicles, suggesting that NGF signaling is required for this transition [62].

Granulosa cell expression of anti-Mullerian hormone (AMH), a member of the TGFβ superfamily, is required to maintain a balance between the number of primordial follicles being activated and those that remain stay in the resting pool. As demonstrated in mice null mutant for the *Amh* gene, the absence of AMH led to an increased recruitment of primordial follicles and a rapid depletion of the pool of resting follicles. More preantral and small antral follicles were observed in the knockout compared to the wild type [63,64]. Thus, at least in mouse, AMH inhibits the recruitment of primordial follicles into the growing pool [65,66]. However, a positive effect on initiation of primordial follicle growth by treatment with AMH has been reported in the human model [67], which implies that further studies are required to understand this process in large mammals.

Primordial follicles will give rise to primary follicles in which the flattened granulosa cells will develop into single-layered cuboidal granulosa cells (Fig. 1). The early expression of two other transcription factors from oocyte origin, *Sohlh1* and *Nobox*, is decisive for the progression of primordial follicles to the next primary follicular stage. In ovaries lacking *Nobox* (NOBOX oogenesis homeobox) the majority of follicles are arrested at the primordial stage, the oocytes degenerate and do not develop beyond single-layered cuboidal primary follicles [43]. A deficiency in *Nobox* expression leads to downregulation of many important oocyte transcripts with roles at different stages of growth such as *Mos*, *Oct4*, *Rfpl4*, *Fgf8*, *Zar1*, *Dnmt1o*, *Gdf9*, *Bmp15*, and *H1oo*, indicating the essential role of *Nobox* as a regulator of oocyte and follicular development. Similarly, in null mutant mice for spermatogenesis and oogenesis basic helix–loop–helix transcription factor (*Sohlh1*$^{-/-}$), progression to the primary stage is disrupted and follicle growth is arrested at primordial follicle stage [68]. Moreover, a deficiency of this factor leads to downregulation of *Nobox* and, consequently, to the down-regulation of many genes regulated by the latter. This highlights the potential role of both *Sohlh1* and *Nobox* as key master regulators of early oogenesis and folliculogenesis.

Theca/stroma-derived factors, members of the TGFβ super-family, will also determine primordial follicle progression. In vitro exposure of neonatal rat ovaries to BMP4 enhanced the proportion of developing primary follicles and reduced the number of resting primordial follicles [69]. Similarly, an increase in the number of preantral and antral follicles was obtained after injection of recombinant BMP7 in rat ovarian bursa [70].

### 2.3. Progression from the primary to the secondary follicular stage

Early preantral follicles are independent of FSH for their initial growth, as evidenced by the fact that development to the primary and secondary stage can take place in the absence of hormones [71], although follicle stimulating hormone receptors (FSHR) are present on the granulosa cells (GC) of these early follicles, both in mouse and humans [72,73]. Primary to secondary follicle transition is rather driven by local intraovarian paracrine factors produced by oocytes, their companion granulosa cells and theca cells [74] (Fig. 1).

Two very well known members of the TGFβ superfamily, GDF9 and BMP15, have a role that starts during early stages and continues throughout folliculogenesis and ovulation. Female mice ovaries lacking *Gfd9* are able to form primordial follicles, but they do not progress beyond the primary follicle stage; as a consequence, these mice are not fertile. Concordantly, in vitro exposure of ovarian tissue to GDF9 supports a role of GDF9 in the promotion of follicle development beyond the primary stage [75,76]. Interestingly, GDF9 knockout mice ovaries develop abnormal granulosa cells with an increased expression of KitL and fail to acquire theca cells [77–79]. This evidences a role of GDF9 in the regulation of pre-granulosa cell function from very early stages onwards [79]. BMP15 plays a role in stimulating the proliferation of undifferentiated granulosa cells in an FSH-independent manner [80]. However, unlike GDF9, null mutant female mice lacking the *Bmp15* gene exhibit only minor fertility problems [81].

Progression of early follicle development also requires the expression and action of TATA-binding protein 2 (TBP2), an oocyte-specific transcription factor expressed along folliculogenesis. Mice ovaries deficient in *Tbp2* have a reduced number of secondary follicles and in addition they have an altered expression of oocyte-specific transcripts involved in early folliculogenesis; levels of *Gfd9*, *Bmp15* and *Zp3* are down-regulated, whereas levels of *Oct4* and *Nobox* were upregulated [82]. Thus, a potential role of TBP2 in the regulation of transcriptional control of mouse oogenesis has been suggested.

TAF4B, also known as TATA box binding protein (TBP)-associated factor is another transcription factor preferentially expressed in germ cells but is also found to be present in granulosa cells. Analysis of *Taf4b* null mutant female mice ovaries have revealed an important role of *Taf4b*, first at early folliculogenesis, documented by a reduced number of primordial and growing follicles and by compromised granulosa cell survival [83,84]. Interestingly, *Taf4b* null female mice have elevated levels of FSH, suggesting that their growing follicles are resistant to the FSH-induced granulosa cell proliferation [83,84]. This deficiency might be associated with a disruption of the AKT/FOXO pathway also observed in null-mutant ovaries [85]. At a later stage of development, oocyte meiotic resumption is impaired and there is a blockage around the two-cell stage of early embryo development [83].

The expression of maternal effect genes (i.e. *Mater*, *Zar1*, *Npm2*), essential for early embryogenesis to proceed properly is also initiated at the primary follicle stage and continues up to the antral stages [86–88].

### 2.4. Progression throughout preantral stages and to the early antral stage

Follicular development throughout the very early stages has been considered to be gonadotropin-independent and essentially driven by locally secreted factors. When follicles reach the preantral stages, development throughout this period and progression to the early antral stage still rely primarily on intraovarian factors; however, unlike in earlier stages, follicles express functional FSH and LH receptors and are able to respond to gonadotropins, as demonstrated both in vivo and in vitro.

Analysis of gene expression in vivo in growing oocytes in mouse has shown that during the transition from the primordial to the secondary follicular stage, the expression profile of some oogenesis genes was not altered after gonadotropin treatment. However, differential regulation of the gene expression between the gonadotropin-induced and

natural conditions was shown from the preantral to the antral stage [89]. Furthermore, in vitro studies have shown that cultured preantral follicles respond to gonadotropins [90], and exposure of early preantral follicles to FSH is favorable for follicle survival and required for in vitro antrum formation to occur [91–95].

Indeed, mice deficient in both FSH (FSH-β knockout) and FSHR (FSHR knockout) are infertile due to a block in follicle development prior to antral development [96,97]. However, due to the specific roles of gonadotropins throughout the antral stage, more details of gonadotropin response and knockout models will be explained in the next follicular stage.

Granulosa cells within preantral follicles proliferate at a very high rate, giving rise to a multi-layer preantral follicle, an increase in follicular size, followed by the appearance of an antral cavity (Fig. 1).

Many positive regulators during the transition from preantral to early antral development have been identified, especially those belonging to the TGFβ superfamily. The well-known GDF9 and BMP15 oocyte-secreted factors act in synergy to continue promoting granulosa cell proliferation. BMP15 is known to very potently promote mitosis in undifferentiated granulosa cell in a FSH-independent manner whereas at the same time it is involved in granulosa FSH-dependent cyto-differentiation at later stages [80]. *Bmp15*-null mutant female mice, though, show normal development up to the antral stages have reduced ovulation and fertilization rates and are subfertile [81]; in contrast, a natural mutation of the Bmp15 gene causes an increased ovulation rate in ewes [98]. The different nature of these species (mono-ovulatory versus the poly-ovulatory) may help to explain the observation of different phenotypes [99]. In humans a heterozygous inherited mutation of this gene is related with hypergonadotropic ovarian failure and decreased proliferation of granulosa cells [100]. Besides, other natural mutations of the BMP15 gene have been identified and might be associated with premature ovarian failure (POF). In fact, an association between these variants and a decreased production of the mature BMP15 protein form has been suggested, concordantly with a reduced biological effect of the protein [101]. However, other factors should be taken into account, such as ethnicity and/or some predisposing factors, and therefore more studies are needed to clarify a real association with POF and the potential impact of such mutations in ovarian functionality [102].

Comparable to the role of BMP15, GDF9 plays a crucial role as a potent granulosa cell mitogen. GDF9 induces the expression of FSHR in cultured preantral follicles [103]. Injection of GDF9-morpholino into oocytes from cultured preantral follicles induces apoptosis and suppressed preantral follicle growth. Thus, GDF9 may at least partly have a role in follicle growth in protecting the granulosa cells from undergoing apoptosis via activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway [103].

A role of androgens in preantral granulosa cell proliferation and survival has been indicated [104,105]. Indeed, in the porcine, an interaction between oocyte secreted factors and androgens seems to exist to promote follicle growth [106]. A more clear participation of androgens in follicle development, via the androgen receptor (AR), will be further detailed in the review of the next stage of follicle development.

Activins and inhibins, two closely related proteins with opposite roles belong to the TGFβ superfamily and also influence follicle development. Both protein complexes consist of two subunits that originate from the same family of related genes and proteins but differ in their composition [107]. Alongside an increase of follicular diameter, and granulosa cell differentiation, the activin tonus decreases whereas the inhibin expression increases. Inhibin-B ($\alpha\beta_B$) is predominantly expressed during early stages of folliculogenesis, a period of high proliferation of undifferentiated granulosa cells; however, after follicle selection and differentiation to become an antral follicle, Inhibin-A ($\alpha\beta_A$) turns out to be the most predominant inhibin [108,109].

Activin-A has been shown to induce proliferation in in-vitro cultured granulosa cells in mice and rats [110–112]. Likewise, mice null mutant for the Inhibin α subunit (*Inhα*) show an uncontrolled granulosa cell proliferation and ovarian tumor development, which might be the result of a strong increase in activin protein secretion in these mice [113].

A role of AMH as a negative regulator of follicle selection, by reducing follicle responsiveness to FSH to progress to the antral stages, has been suggested [66,114,115].

Communication among granulosa cells and between granulosa cells and the oocyte is crucial at all stages of folliculogenesis. The role of Gap junction proteins also known as connexins 43 and 37 (Cx43 and Cx37, respectively), play an important role in the maintenance of this communication. Cx43 is expressed by granulosa cells and required to forms gap junctions between granulosa cells [116–118], whereas Cx37, expressed in oocytes at all stages of follicle development, is crucial for an oocyte–granulosa cell gap junctional communication. In mice ovaries lacking Cx37, an arrest in folliculogenesis is evidenced at the early antral stage and oocyte meiotic competence is compromised [119,120]. Mice deficient in Cx43 die shortly after birth [121]. However, studies in which Cx43 knockout ovaries are prenatally removed and grown under in vitro conditions or in vivo (under the kidney capsule of wild-type mice) have demonstrated that lack of Cx43 leads to an arrest of follicle development at the primary stage [116,122].

An approach using chimeric mice ovaries that combines either Cx37 or 43 KO oocytes with WT granulosa cells, and vice-versa, has revealed important differential roles for connexins 37 and 43 in either cell type. Follicles lacking Cx43 only in the oocyte, or Cx37 only in granulosa cells, are able to grow normally with oocytes capable of resuming meiosis and being fertilized. However, a lack of Cx43 in granulosa cells mimics the early arrest in folliculogenesis observed in Cx43 KO ovaries, whereas oocytes lacking the expression of Cx37 have an impaired meiotic resumption and are not able to be fertilized, similar to what is observed in Cx37 KO ovaries [123,124].

Interestingly, although the phenotype observed in ovaries lacking Cx37 suggests a critical and unique role of Cx37 in oocytes, a transgenic mouse model has revealed that these adverse effects can be restored when growing oocytes lacking Cx37 ectopically express Cx43. In this transgenic model, oocyte–granulosa-cell communication, oocyte growth, and oocyte potential to mature and be fertilized were restored [125].

### 2.5. Antral development — role of gonadotropins

Antral development starts with antrum formation (Fig. 1) and the differentiation of granulosa cells into the cumulus and mural cell compartments, which confers to the oocyte the competence to resume meiosis. However, although the oocyte has become meiotically competent, the acquisition of developmental competence will occur along these later stages (see also Oocyte maturation).

The progression throughout the antral stages and ovulation has been considered to be dependent on pituitary-secreted gonadotropin (FSH and LH) support. FSH is the essential driver of in vivo antral development. FSH induces luteinizing hormone receptor (*Lhcgr*) mRNA expression in mural cells, which will be required for follicles to respond to LH, the latter being crucial for triggering the ovulatory process.

Action of both gonadotropins in the ovary is mediated by binding and activation of their receptors (LH receptor, LHR and FSH receptor, FSHR). Knockout mouse models of each of these receptors have shown the relevant role of gonadotropin signaling within the ovary.

In LHR knockout (LuRKO) mice, follicle development does not progress beyond the antral stage; these mice are infertile due to the low estrogen production and anovulation [126]. Moreover, high doses of FSH are not capable of inducing final follicular development and

ovulation when LHR is not present [127]. Therefore, expression of LHR is essential not only for ovulation but also for follicle maturation prior to ovulation.

Mice deficient in FSH (FSH-β knockouts) are infertile due to a block of the follicle development prior to the antral stage. Within these follicles, granulosa cells express increased levels of FSH-R transcripts, but decreased levels of aromatase, and inhibin/activin subunits. Moreover, they fail to express normal levels of LH receptors [97]. *Fshr*-null mutant female mice, in which all forms of the FSHR have been eliminated (also known as FORKO — follitropin receptor knockout mice), display an atrophic uterus and are also infertile due to a block in folliculogenesis before antral formation [96]. In these mutants, the largest follicles observed have no more than four layers of granulosa cells. However, in spite of a normal expression of aromatase mRNA and protein in *Fshr*-null mutant mice, there is a complete loss of estrogen production by the ovary, leading to metabolic alterations such as obesity and skeletal abnormality (some of which can be reversed by treatment with estradiol-17beta). This may suggest that signaling through the FSHR may be involved in the activation of the aromatase enzyme [128].

Whether the block in follicle development in both FSH-β and FSHR knockouts is due to altered granulosa cell proliferation or due to increased apoptosis is not fully understood. Although the precise role of cyclin D2 (a gene responsive to FSH and involved in granulosa cell proliferation) in this process has not been identified, FSH-β knockout mice have a modest decrease in the expression of cyclin D2, whereas this has not been clearly demonstrated in FORKO mice [96].

These studies are relevant for human reproduction since phenotypes similar to those reported in mice have been found in human with mutations in FSH-β and FSHR [129]. Moreover, a number of menopausal symptoms in women, as well as features of hypergonadotropic-hypogonadism that can be observed in infertile women are also mimicked by the phenotype of FORKO mice.

By in vitro approaches, studies have demonstrated that in the absence of FSH cultured follicles are arrested in their development, do not support antrum formation and exhibit apoptosis, all of which can be prevented by FSH supplementation [91,92]. In spite of this, research conducted by our group has demonstrated that once the antrum formation is established, follicle development to the preovulatory stage under decreased FSH concentrations induce a better cumulus cells differentiation [130,131]. Accordingly, low FSH levels applied in follicle culture after antrum formation appear to promote development of primate follicles and oocytes achieve larger diameters [95]. On the contrary, exposure to supraphysiologically high FSH levels during antral stages or throughout development from preantral to antral stages in vitro perturbs oocyte control of granulosa cell differentiation, as well as cumulus cell function, in mouse and primate follicle cultures [95,130]. Thus, although gonadotropins are essential during antral stages in vitro, a dose-fine-tuning is critical to obtain an appropriate antral development.

Under the influence of gonadotropins, follicles synthesize steroid hormones such as androgens and estrogens, which contribute to follicular development, by inducing granulosa cell proliferation and differentiation via the androgen receptor (AR) and estrogen receptor (ER), respectively [109,132,133]. Through the well-known two-cell, two-gonadotropin model, theca cells produce androgens under influence of LH stimuli, whereas granulosa cells produce estrogens using androgens as a substrate, under influence of FSH [108,134].

Androgens, have been shown to participate in granulosa cell proliferation and survival via the androgen receptor (AR). AR-deficient female mice are subfertile, and show a reduced number of antral follicles and ovulated oocytes, and a high rate of granulosa cells apoptosis; they eventually develop premature ovarian failure [135,136]. Most of the characteristics observed in these *Ar*-null mice seem to be the result of a lack of AR expression more specifically in the granulosa cells, as demonstrated by a model in which specific knockouts for the AR in either granulosa cells or oocytes were made. Therefore, granulosa cell-specific AR appear to be essential for follicle development and survival [137].

Estradiol is the predominant estrogen in terms of estrogenic activity. Indeed, one of the major functions of preovulatory granulosa cells is the synthesis of estradiol. Inside the follicle, estradiol is produced via the enzyme aromatase, and it enhances the response of granulosa cells to the gonadotropins [138].

Knockout models for the two different estrogen receptors, ERα and ERβ have lead to the elucidation of the role of estrogens in follicular development. Knockout mice for the ERα, also known as ERKO mice, have increased levels of estradiol and LH, whereas FSH levels are normal. Ovaries from ERKO mice show an arrest at the early antral stage and are infertile [139,140]; this demonstrates the essential role of estradiol in gonadotropin-induced follicle differentiation. On the contrary, in ERβ knockout (BERCO) mice follicles develop to the antral stages and these mice are fertile, but they have a diminished ovulatory response to hCG stimuli [140–142].

Another approach involved the targeted disruption of the *Cyp19* (aromatase) gene in mice (ArKO), which causes a deficiency in estradiol synthesis. These mice have elevated levels of circulating gonadotropins and testosterone and are infertile. At adulthood, female ArKO mice ovaries may contain large antral follicles, but after one year folliculogenesis is severely affected and secondary follicles are no longer found [143,144], which suggests an important role of estradiol in follicular development. Moreover, the ArKO phenotype seems to result not only from the lack of estrogens but also from the high levels of circulating gonadotropins, mainly LH [145].

Members of the insulin growth factor (IGF) family (i.e. IGF1, IGF2) also cooperate with gonadotropins to determine follicle selection and the further progression through the antral stages. In rodents, IGF1 and IGF2 are the predominant forms in granulosa and theca cells, respectively, where they co-operate with FSH and LH action in each cell type [146]. Moreover, in IGF1 deficient mice, follicle development does not progress beyond the small antral stage. In these mice there is reduced expression of FSHr, and decreased synthesis of estradiol and reduced granulosa cell proliferation [147,148].

Besides IGFs, follicular development throughout the antral stages is clearly dependent on many other intraovarian factors. Similar to earlier stages, activins and inhibins produced by granulosa cells play essential paracrine roles by regulating the LH-induced androgen synthesis produced by theca cells [149–151], and therefore, they ensure the estradiol supply. In mice lacking *Acvr2b* (the activin type-IIB receptor), progression beyond the early antral stages fails [152]. The activin–inhibin system does not only regulate granulosa cell proliferation but also granulosa cell differentiation and oocyte maturation, the latter being accelerated by activin A action [153–155].

Oocyte-secreted GDF9 and BMP15 also play a critical role during antral stages. GDF9 and BMP15 regulate cumulus cell function (*more detailed explanation is given in the next section*). As evidenced in rat and human, these oocyte factors alone, or in combination, attenuate FSH effects and stimulate granulosa cell proliferation and differentiation. GDF9 suppresses FSH-stimulated estradiol secretion by repressing aromatase activity and also suppresses FSH-induced LH receptor formation in differentiated cumulus cells. BMP15 is believed to reduce cumulus cell apoptosis and to suppress FSH receptor expression [79,156,157]. Both BMP15 and GDF9, together with BMP6, have been demonstrated to inhibit FSH-induced progesterone production, which may be considered important for follicle survival and prevention of premature luteinization.

In preovulatory follicles, a critical role of GDF9 and BMP15 in the induction of cumulus mucification/expansion and the regulation of genes involved in this process has been demonstrated and will be further explained hereafter [79,158,159].

Other factors such as theca-derived factors BMP4 and BMP7 are potential paracrine regulators of granulosa cell function. In the rat, these two factors attenuate FSH-induced progesterone secretion whilst they enhance FSH-induced estradiol secretion [70,160].

Finally, preovulatory follicles containing fully-grown oocytes are ready to undergo ovulation, which is induced by the preovulatory surge of gonadotropins. Ovulation is characterized by the rupture of the follicle wall and the release of the cumulus–oocyte complex; at this time the oocyte has resumed meiosis and has progressed to the metaphase II stage of meiosis (Fig. 1) (*oocyte maturation is described in detail in the following sections*).

After ovulation, granulosa and theca cells become luteal cells and are responsible for the production of estradiol and progesterone, the latter is predominantly expressed in the corpus luteum [161].

## 3. Regulation of gene expression and mRNA storage during oogenesis

During oogenesis, oocytes increase in size (approximately 20–80 μm in mouse and 35–120 μm in human) and in volume (~100-fold) [162,163]. Throughout this period, oocytes synthesize and accumulate RNAs and proteins that are vital for their appropriate growth and maturation, and indispensable for the development into a viable embryo [164–166].

Synthesis of transcripts is highest in the earliest phases of development, which coincides with active proliferation of follicular cells; however, by the end of growth (antral stages) and at the time of oocyte maturation, silencing of transcriptional activity and degradation of some mRNA will be the predominant processes [167–169] (Fig. 3).

Fully-grown and meiotically competent murine oocytes have been estimated to contain ~6 ng of total RNA which is almost ~200 times the amount of RNA found in a typical somatic cell [170,171]. Approximately 10–15% of all RNA produced by a fully-grown oocyte comprises heterogenous RNA, whereas the majority, ~65% comprises ribosomal RNA [171].

The fate of oocyte mRNA depends, to a large part, on its association with different types of proteins that regulate the accessibility to initiation factors and ribosomes [166]. After transcription, polyadenylation occurs by default, i.e. a poly-A tail (>150 residues) is added at the 3′ end of mRNAs. After polyadenylation, the association with cap-binding and initiation factors is a crucial step determining the initiation of translational activation. Subsequently, a more complex process involving association with ribosomal subunits and the participation of a high number of factors will allow the stage-dependent translation of mRNAs [166,172] (Fig. 3).

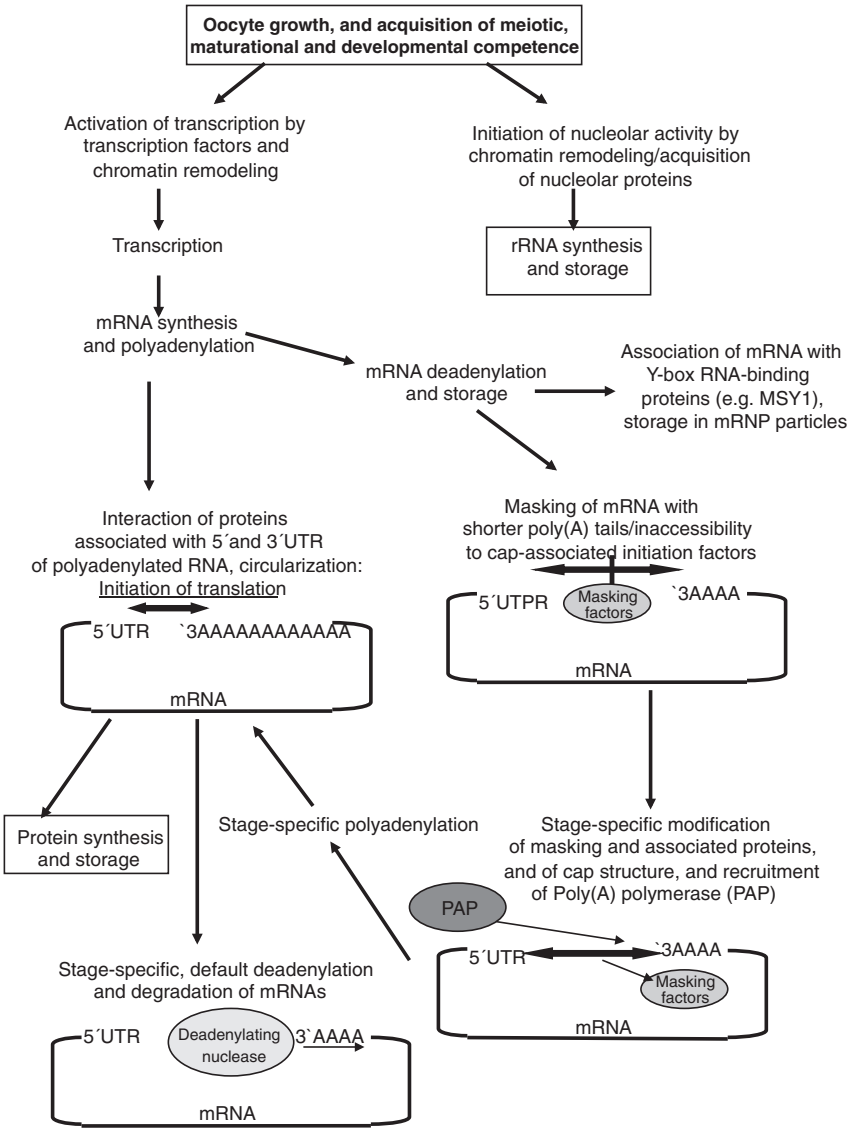


**Fig. 3.** Representation of some general events involved in the regulation of expression in the oocyte during growth and maturation. As explained in the text, the fate of mRNAs can vary considerably. After transcription, some mRNAs undergo translation immediately after polyadenylation, whereas others undergo deadenylation and are stored to be further translated at specific stages according to oocyte's needs. Accumulation of oocyte transcripts and proteins is crucial for oocyte acquisition of meiotic and developmental competence. Reproduced with permission of Oxford University Press, from Eichenlaub-Ritter and Peschke [166].

Regulation of gene expression in the oocyte is not only dependent on polyadenylation but also on the presence of highly conserved sequences at the 3′ and 5′ untranslated regions (UTRs) of RNA which are implicated in processes such as polyadenylation, initiation of translation, masking of RNAs and the disposition of mRNA to undergo deadenylation and degradation [166,173]. Regulation of transcripts occurs in a well-orchestrated and stage-specific manner. While some transcripts are synthesized for immediate use, other transcripts will be deadenylated (by shortening of the poly-A tail, which confers more stability) to be either degraded or stored in the ooplasm in ribonuclear particles (RNPs) to be used at further stages. Transcripts stored in RNPs do not associate with polyribosomes but rather associate with masking factors, and therefore are not translated (Fig. 3).

Active regulation of transcripts in the oocyte occurs predominantly during the transition from primordial to the primary follicular stage [174], where the majority of genes related to cell proliferation, cell cycle and transcription are up-regulated. Likewise, the transition from primary to secondary follicular stage is characterized by an active up-regulation of many oocyte genes (i.e. transcripts involved in cell-cycle, biosynthesis, and macromolecular metabolism). From the secondary to antral stage, genes involved in basal transcription from Polymerase II promoters are down-regulated, and may account for the large-scale transcriptional silencing towards the end of oocyte growth [174]. A selective degradation of transcripts occurs during oocyte maturation in mouse [167–169,175] and human [176]. In mouse, a decrease of ~30% in total RNA has been described to occur during maturation [175]. However, although oocytes do not synthesize RNA de novo at this stage, post-transcriptional modifications are crucial in the regulation of protein expression at this stage [167,177]. In the next section (Oocyte maturation) more information on the crucial transcripts regulated during this stage are given.

Gene regulation at the post-transcriptional level, is also induced by the interaction of transcripts with small RNAs. Small RNAs are non-coding RNAs of a short length: 19–31 nucleotides (nt), whose main role is gene silencing of target mRNAs. Three major classes of small non-coding RNAs have been identified that play essential roles in mammalian development: endogenous small-interfering RNAs (siRNAs), micro RNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) (see for review, [178,179]).

Endogenous siRNAs and miRNAs are originally a long transcripts of approximately 200 nt. siRNAs are initially double strand (ds) RNAs which are directly cut by Dicer, a cytoplasmic RNase III, into a shorter length of 19–25 nt. miRNAs are transcribed as long primary-miRNAs, which are first recognized and cleaved by the complex formed by Drosha, an RNase III enzyme, and the RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8) into a pre-miRNAs, and later cleaved by Dicer into an ~22-nt miRNA [180,181]. Contrary to siRNAs and miRNAs, piRNAs do not require Dicer for their processing; however, piRNAs biogenesis is still poorly characterized [179].

After being processed by Dicer, both miRNAs and siRNAs are incorporated as a single strand into the RNA-induced silencing complex (RISC), and act through this complex to silence target mRNAs. Silencing of gene expression by siRNAs is induced by cleavage of target mRNAs. The Argonaute 2 (AGO2) is a key component of the siRISC since it possesses an endonuclease activity and is responsible for the cleavage of target mRNAs [178].

Differently to siRNAs, the role of miRNAs in repression of mRNA translation is specified by alternative mechanisms [178]. Translation can be either suppressed at the initiation-stage or after translation has been initiated [182]. miRNAs accelerate mRNA degradation by, for instance, triggering destabilization of mRNAs by deadenylation; or they sequester mRNAs and transport them to a storage compartment where they are degraded, and finally interfering with gene expression [178,183].

The regulation of reproduction function by miRNAs has not been extensively studied. Recently, in vitro studies have demonstrated that miR-224 is expressed in granulosa cells at different stages of follicle development and its levels are up-regulated by TGFβ1 and activin A. Interestingly, *Smad4* seems to be a potential target of miR-224, and by negative regulation of SMAD4 protein expression, miR-224 might be involved in TGFβ1-mediated granulosa cell proliferation and function [184].

With regard to the expression of miRNAs in oocytes, an analysis of the expression pattern of miRNAs suggests that miRNAs are maternally inherited from the oocyte to the zygote, whereas de novo expression of miRNAs may occur beyond the 2-cell stage of embryo development. Among the existing maternal miRNAs, the Let-7 family has been shown to undergo a dynamic regulation during oocyte growth and later in early embryo development (as shown by an abundant increase) [185].

Interestingly, an essential role for siRNA in oocyte maturation has been implied from mice deficient in either *Dicer* or *Dgcr8*. Loss of *Dicer* in mutant oocytes causes a depletion of most miRNAs in the oocyte and more critically, thousands of mRNAs are dysregulated (i.e. expression of two oocyte transcripts, *Mos* and *H2ax*, have been shown to be over-expressed compared to control oocytes). Furthermore, mutant oocytes have an aberrant spindle organization and chromosomal segregation, resulting in meiotic arrest. On the contrary, when *Dgcr8* is absent in mouse oocytes, mRNA levels remained unchanged and there is no adverse effect in oocyte maturation. From these findings it was inferred that effects observed in the Dicer mutant oocytes are determined by the loss of siRNAs rather than miRNAs, and therefore miRNAs are not required for oocyte maturation but, as mentioned before, for later events of embryo development [185–187].

The regulation of miRNAs by gonadotropins has not been fully investigated. So far, the expression of three miRNAs within the mouse ovary, miR-21, miR-132 and miR-212 has been found to be increased in mural granulosa cells after the LH surge [188]. Also, the expression of mir-143, let-7a and mir-15b during folliculogenesis has been suggested to be negatively regulated by FSH [189].

Therefore, regulation of gene expression in the oocyte throughout oogenesis at the transcriptional and post-transcriptional level is a crucial process that is tightly controlled in a stage-dependent manner, and this process ultimately ensures that the oocyte will mature and acquire full developmental competence.

## 4. Oocyte maturation

Oocytes gradually acquire nuclear and cytoplasmic maturation during growth. Meiotic competence, which is the capacity of the oocyte to resume meiosis and become nuclearly matured, is acquired during folliculogenesis and coincides with antrum formation, when oocytes have reached approximately 80% of their final size (in mice [190]; and human [191]). Developmental competence is related to cytoplasmic maturity of the oocyte and refers to the capacity of the oocyte to be fertilized and develop into a healthy embryo capable of continuing its development to term and producing a live birth. Cytoplasmic maturation is acquired after the oocyte becomes meiotically competent and involves an accumulation of transcripts and other factors described in more detail in the following pages.

A clear example of acquisition of both nuclear and cytoplasmic maturation on time is that despite oocytes isolated from early antral follicles are able to resume meiosis, they do not progress further than the metaphase I (MI) stage [192]. Furthermore, if these same small antral follicles are matured and fertilized in vitro they are able to progress to the MII stage and fertilize, however, different to oocytes isolated from large antral stages, the early embryo development is compromised [193,194]. Therefore, an oocyte that has acquired meiotic competence has not necessarily acquired cytoplasmic maturity.

### 4.1. Meiotic maturation

Since oocyte meiotic competence is acquired at an earlier stage, when oocytes are still developmentally incompetent, oocytes have

to be maintained at the germinal vesicle stage until they complete their full maturation.

Within the oocyte, elevated levels of cyclic adenosine monophosphate (cAMP), produced by adenylyl cyclase, are crucial in maintaining oocytes under meiotic arrest (in rodents [195]; in humans [196]) (Fig. 4, upper panel). In oocytes, the source of cAMP has been suggested to be the product of the influx of cAMP through gap junction communication from the cumulus cells to the oocyte [197] and/or endogenous production of cAMP within the oocyte, induced by activation of G-protein coupled receptors 3 and 12 (GPR3, 12) [198,199].

Recently, it has been proposed that the inflow of cyclic guanosine monophosphate (cGMP) from the cumulus cells to the oocyte (also via gap junction communication), prevents the activation of oocyte cAMP-phosphodiesterase (PDE3A), the enzyme that is responsible for the degradation of cAMP and by doing so, circumvents meiotic resumption [200] (Fig. 4, upper panel).

Notably, the oocytes themselves, and through the interaction with their surrounding cumulus cells, have been shown to contribute to their own meiotic arrest. Oocytes promote cumulus cell expression of *Npr2* (natriuretic peptide receptor 2). NPR2 is activated by its ligand CNP (C-type natriuretic peptide) to produce cGMP, which as mentioned earlier diffuses into the oocyte to inhibit PDE3A activity [200–202]. Recently, in vitro experiments have revealed a direct role of oocyte-secreted factors, among which GDF9, BMP15 and FGF8B have been identified and a role of estradiol in the regulation of the expression of *Nrp2* in cumulus cells [203]. This highlights a unique role of estradiol in preovulatory follicles in the control of oocyte meiotic arrest before ovulation.

Overall, these findings contribute to the understanding of the mechanisms involved in meiotic arrest maintenance, and may be of particular importance when designing in vitro maturation (IVM) techniques to improve developmental competence.

Oocyte meiotic maturation involves a cascade of processes that is initiated with the preovulatory LH surge, leading to the progression of the oocyte (by that time suspended in prophase I of the first meiotic division) to the metaphase II stage and ending with the extrusion of the first polar body.

After the LH surge, an event that is essential for the oocyte to resume meiosis is the mucification/expansion of cumulus cells, which is caused by the production of hyaluronic acid produced by the cumulus cells in response to gonadotropins. Cumulus expansion is dependent on the stimulation of LH-induced epidermal growth factor (EGF)-like peptides, via the activation of protein kinase A (PKA) and in response to high levels of cAMP produced by the outer layers of the granulosa cell compartment. Under these stimuli, levels of transcripts involved in mucification/expansion such as *Has2*, *Ptx3* and *Tnfaip6* [204–207] are increased in cumulus cells (Fig. 4, lower panel).

Induction of this response by EGF-like factors is mediated by the activation of the ERK1/2 pathway, which in turn stimulates the production of prostaglandin E2 (PGE2) via the induction of both *Ptgs2* mRNA and protein expression. PGE2 has been shown to additionally induce granulosa cell production of EGF-like factors, which enhances the LH signal [208]. As depicted in Fig. 4 (lower panel), both granulosa cell types (mural and cumulus cells) respond to and contribute to EGFR-mediated signaling, although expansion (and expression of cumulus expansion-related genes) is a unique response of cumulus cells.

Alternatively, cumulus expansion is also induced by cumulus-expansion enabling factors (CEEFs) produced by the oocyte [158,209]. Oocyte regulation of cumulus expansion will be further discussed in the following section (Oocyte–cumulus interaction) (Fig. 4).

Within the oocyte, LH stimulated meiotic resumption is initiated by a drastic drop in cAMP levels. After LH-triggered signaling, PDE3A in the oocytes becomes activated [210] and degrades cAMP. Alternatively, a limited diffusion of cGMP and/or cAMP from the cumulus cells to the oocyte also appears to contribute to meiotic resumption. This is supported by the fact that following LH trigger, levels of *Nppc* mRNA (encoding CNP) decrease, leading to a limited production and diffusion of cGMP into the oocyte, which enables PDE3 to actively degrade cAMP [211]. Moreover, closure of the gap junctions may also contribute to this process, as a result of the activation of the ERK/MAPK signaling pathway induced after EGFR activation [212,213] (Fig. 4, lower panel).

#### 4.1.1. Regulation of oocyte transcripts required for meiotic maturation

Oocytes participate actively to the process of meiotic maturation, thereby ensuring their progression to the mature stage. As mentioned earlier, a predominant massive degradation of oocyte transcripts occurs during maturation; however, this event appears to be a selective process [169]. Evidently, transcripts and/or proteins associated with oocyte meiotic arrest at the germinal vesicle (GV) stage are degraded or have drastically decreased expression levels. For instance, translation of CDH1 – a co-activator of the anaphase-promoting complex/cyclosome (APC/C) which represses cyclin B1 levels through ubiquinylation maintaining meiotic arrest – is reduced by 70% [214,215].

On the other hand, transcripts involved in signaling pathways essential for the regulation of oocyte meiosis and the maintenance of meiotic arrest at MII, such as ERK/MAPK and PI3/AKT are retained [169,216]. While some maternal-effect transcripts have been shown to undergo degradation during this transition, some other transcripts appear to be stable.

Aberrant degradation or maintenance of some transcripts during oocyte maturation and/or fertilization may be deleterious to oocyte quality and may compromise developmental competence [217].

Although most transcripts within the oocyte are degraded during maturation, many other transcripts undergo polyadenylation and associate with the polysomes to undergo translation [177,215]. Through wide-genome profiling of maternal mRNAs, a recent study has revealed different patterns of oocyte transcripts in association with the polysomes, indicating active translational regulation during maturation. By this approach, approximately 7600 transcripts have been shown to be actively translated during oocyte maturation, whereas translation of many others is repressed [215].

Interestingly, there appear to be two different mechanisms of translational repression during oocyte maturation: transcript degradation, as previously demonstrated [169,216]; and the other seem to be independent of degradation. In this latter case, translation seems to be repressed by the translocation of transcripts that remain as stable, from the polysome to the subpolysome/RNP fraction [215].

Among the transcripts undergoing active translation during GV–MII transition, well-established cell cycle regulators (*Ccnb1* and *Mos*) have been identified, as well as components of the anaphase-promoting complex and components of the spindle assembly checkpoint (*Mad2*, *Bub1b*, and *Sogl2*). This class also includes a set of transcripts coding for transcriptional regulators and chromatin remodelers [215].

CPEB and DAZL have recently been identified as two crucial modulators of translation during oocyte maturation. CPEB promotes *Dazl* mRNA translation during the transition to the MI stage; DAZL protein subsequently induces translation of its own mRNA, thereby establishing a positive regulatory loop. Although a role of DAZL as a translational regulator during oocyte maturation has been proposed earlier [218,219], Chen et al. [215] have recently demonstrated a clear and crucial role of DAZL during this late stage. Oocytes in which DAZL has been down-regulated by injection of specific antisense morpholino oligonucleotides (MOs) have been shown to be delayed in meiotic resumption and the few oocytes that extruded a polar body had a mostly defective spindle [215]. Moreover, DAZL has been shown to regulate the translation of transcripts necessary for spindle assembly and MI–MII transition (i.e. *Tex19.1*, *Tpx2* and *Dazl* itself).

### 4.2. Cytoplasmic maturation

Many different aspects determine oocyte cytoplasmic maturation. Synthesis and accumulation/storage of transcripts during oocyte growth and large-scale transcriptional silencing at the end of oocyte

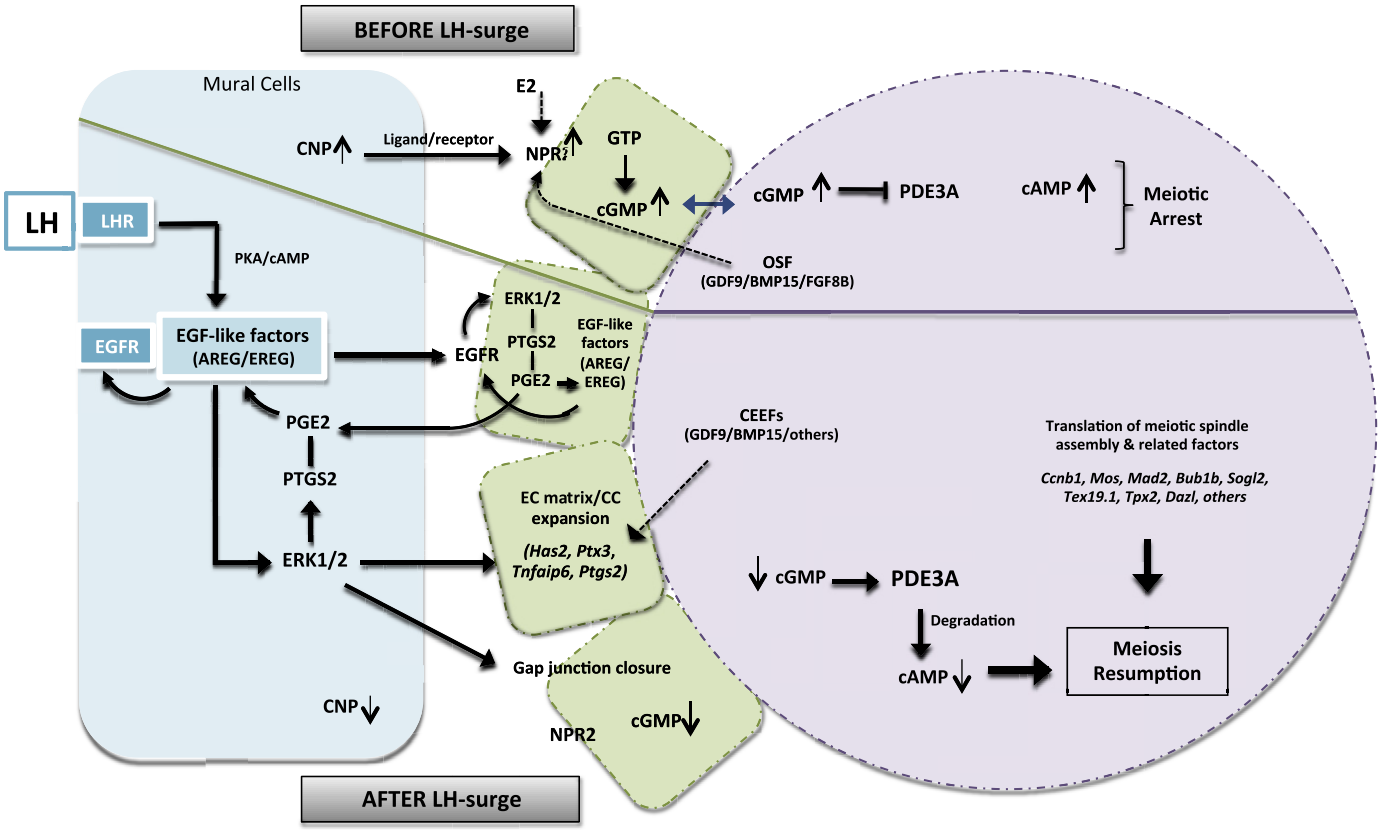


**Fig. 4.** Schematic representation of LH-induced oocyte maturation. The upper panel (above the lines) shows the maintenance of oocyte meiotic arrest before the LHsurge. Oocytes are kept in meiotic arrest due to the intracellular high levels of cAMP. Interaction of CNP produced by the mural cells and its receptor NPR2, expressed in cumulus cells (and up-regulated by OSF and E2) induces the conversion of GTP into cGMP, which is then transferred into the oocytes via gap junctions. cGMP prevents PDE3 activation within the oocyte and cAMP is then maintained at high levels. After the LH-surge (below the lines), the PKA signaling pathway is activated and induces the production of the EGF-like factors (i.e. AREG/EREG). EGF-like factors, in turn, will activate a cascade of events mediated by the EGFR (both in mural and cumulus cells). As a result, ERK1/2 will induce other main events: 1) the up-regulation of transcripts responsible of cumulus expansion *Has2*, *Tnfaip*, *Ptx3* and *Ptgs2* (which are also positively regulated by the CEEFs derived from the oocyte); 2) production of PTGS2, which enhance the response to LH stimuli through the production of PGE2 and by inducing the production of more EGF-like factors and activation of the subsequent cascade; and, 3) the closure of the gap junctions, which avoids the transfer of cGMP into the oocyte. In parallel, CNP and NPR2 levels decrease and also contribute to the low production of cGMP. Within the oocyte, some transcripts crucial for the transition from the GV to the MII stage are actively translated.

growth are essential to support early stages of embryo development (*as reviewed in the previous section*). Indeed, a contribution of granulosa cells in oocyte global transcriptional silencing in pre-ovulatory mouse oocytes, and therefore in acquisition of oocyte cytoplasmic maturation, has been suggested [220].

Global transcriptional silencing in the oocyte is associated with – although not dependent on – another key event that also determines oocyte developmental competence, chromatin condensation into the surrounded nucleolus configuration [221]. Mattson and Albertini [222] initially demonstrated that the time of antrum formation in mouse coincides with the appearance of two different oocyte chromatin configurations, the 'surrounded nucleolus' (SN) configuration, in which a ring of chromatin can be distinguished around the nucleolus, and the 'non-surrounded nucleolus' (NSN), in which the chromatin is dispersed throughout the nucleolus. While NSN oocytes are transcriptionally active and have a poor developmental competence, due to a block at the two-cell stage, oocytes in the SN configuration are transcriptionally inactive and have a better developmental competence [223–225].

Furthermore, a microarray analysis has revealed that in-vitro matured MII oocytes derived from either oocytes at the SN or NSN stage showed different patterns of gene expression. OCT4 (or POU5F1), has been shown to have an important role as a potent key regulator of molecular events governing the establishment of developmental competence of mouse oocytes [226]. Transcript levels of the transcriptional regulator *Oct4* and of *Stella*, an oocyte transcript regulated by OCT4, with an essential role during early embryo development, have been found to be downregulated, and their proteins have been shown to be absent in antral and MII oocytes with a NSN configuration.

There is increasing evidence suggesting that factors present in oocytes at the SN stage but absent in the NSN configuration determine oocyte competence, as reported by Zuccotti et al. [226]. Their findings have also been supported by results of micromanipulation experiments of the germinal vesicle of fully grown oocytes (SN and NSN), which strongly indicate that factor(s) present in cytoplasm of MII SN oocytes (after GVBD occurs), determine developmental competence and are not present in oocytes with the NSN configuration [227].

DAZL is another crucial protein not only essential for meiotic maturation but also required for acquisition of oocyte developmental competence. Down-regulation of DAZL in GV oocytes does not only compromise meiosis progression but also fertilization. Moreover, down-regulation of maternal-derived DAZL in early zygotes causes a block at the two-cell stage. Therefore, an important role of DAZL as a translational regulator in early embryo development and as a determinant of developmental competence has also been proposed [215].

## 5. Oocyte–cumulus cell interaction

In the recent years, remarkable progress has been made in the study of oocyte–granulosa cell interaction and of oocyte regulation of granulosa cell function [228–233].

In concert with the correct gene expression patterns in the oocyte, oocyte–granulosa cell interaction mediated by either paracrine signals and/or by gap junctional communication from early stages of development determines the rate of follicle growth and differentiation. Furthermore, after differentiation, oocyte–cumulus cell interaction is essential to promote oocyte nuclear and cytoplasmic maturation, which determine the capacity of the oocyte to support early embryo development [192,220,234].

Oocyte–granulosa cell interaction and oocyte control of granulosa cell function are essential before and after LH surge. Indeed, in antral follicles, fully grown GV oocytes have been shown to be the most potent regulators of cumulus cell function [235]. Before the LH surge, oocytes do not only influence granulosa cell proliferation [233,236] and differentiation [231,232], but very importantly, oocytes regulate metabolic activity of cumulus cells within the COC (aminoacid uptake, glycolysis and cholesterol biosynthesis) [229,237,238]. After the LH surge, oocytes regulate the expression of cumulus genes responsible for the mucification/expansion process.

Overall, knowledge of oocyte control of the status of cumulus differentiation and function has been achieved by oocyte co-culture experiments, generation of knockout mice, and by molecular biology techniques including quantification analysis of mRNAs and RNA regulation, for instance through RNA interference.

### 5.1. Cumulus–oocyte interaction before the LH surge

#### 5.1.1. Regulation of cumulus cell differentiation

At the time of antrum formation, granulosa cells differentiate into two compartments: the cumulus granulosa cells remain close to the oocyte, whereas the mural granulosa cells remain in the outer part of the follicle. In vitro studies have provided evidences that within the follicle, mural and cumulus granulosa cells are regulated differently by the opposing intrafollicular effects of gonadotropins (FSH) and oocytes, respectively, determining their phenotype and functionality [231,232].

Oocytes promote the expression of cumulus cells transcripts whereas they suppress mural cell transcripts [79,231,232,239]. When fully grown GV oocytes are microsurgically removed from the cumulus–oocyte complexes (a procedure known as oocytectomy — OOX), cumulus cells tend to dedifferentiate, as shown by down-regulation of the expression of cumulus cell markers (i.e. *Amh*, *Slc38a1*) [232,239] and, under this condition, FSH is capable to induce mural transcripts (i.e. *Lhcgr*, *Cyp11a1*) in these cells. Oocyte co-culture with OOX cumulus cells reverts this effect.

As mentioned earlier, high FSH levels during follicle culture lead to an altered expression of cumulus transcripts, as evidenced by decreased levels of *Amh* and high levels of functional *Lhcgr* [130]. This atypical phenotype caused by high doses of FSH during culture is apparently also reflected in oocytes, which under this conditions express significantly increased levels of *Gdf9* and *Bmp15* mRNA compared to oocytes cultured under lower doses of FSH [130]. Given the regulation of *Lhcgr* and *Amh* by oocyte transcripts, the increased levels of *Gdf9* and *Bmp15* mRNA are believed to reflect an oocyte response to compensate for effects that occur in cumulus cells.

Oocytes have the potential to induce the expression of cumulus cell transcripts in mural cells, even in the presence of FSH. This clearly demonstrates that within the follicle, cumulus cell features are mainly influenced by their close association with the oocyte, which can potently buffer the continuous effects of FSH in order to maintain cumulus cells differentiated [232,240]. Interestingly, strong evidence suggests that these actions are mediated through the activation of SMAD2/3, thus identifying GDF9, which acts through this pathway, as one of the ideal candidates among the oocyte-secreted factors exerting such effects [232,233].

#### 5.1.2. Metabolic cooperativity

Paracrine signals mediated by oocyte-secreted factors are probably among the most essential, but not exclusive, mechanisms mediating cumulus–oocyte interactions. Gap junctions are highly specialized intercellular connections that facilitate communication between the oocyte and its surrounding cumulus cells. Notably, given that oocytes and cumulus cells are physically separated by the zona pellucida, gap-junctional communication can occur thanks to the specialized transzonal cytoplasmic projections (TZP) developed by cumulus cells. These projections are able to penetrate through the zona pellucida reaching the oocyte membrane and allow the formation of gap junctions [241].

Gap junctions play a crucial role in the bidirectional communication between oocytes and cumulus cells, allowing the passage of molecules of different types (i.e. aminoacids and metabolites such as pyruvate) from the cumulus to the oocyte [228]. Indeed expression of gap junction proteins Cx43 and Cx37 in granulosa cells and oocytes, respectively, has been shown to be of vital importance in guaranteeing oocyte and follicle development [123,124].

In the recent years, oocytes and cumulus cells have been shown to cooperate in more than one metabolic process. For instance, oocytes, by themselves, are not capable of metabolizing glucose [242], whereas cumulus cells very efficiently do so, as evidenced by measurement of the glycolytic activity of cultured COCs compared to denuded oocytes [229,230]. Since oocytes require products obtained from metabolic processes as a source of energy to support their growth and maturation, cumulus cells are required to metabolize glucose for oocytes [242,243].

Regulation of the glycolytic activity of cumulus cells by oocytes has been evidenced through the analysis of transcripts in the cumulus cells involved in this process. Sugiura et al. [229] demonstrated that the transcripts coding for glycolytic enzymes involved in glucose metabolism such as *Eno1*, *Pkm2*, *Ldh1* and *Pfkp* were down-regulated in cultured OOX cumulus cells compared to intact COCs controls, and this was accompanied by a reduction of the glycolytic activity measured in these cells. However, co-culture of OOX cumulus cells with oocytes resulted in the recovery to normal transcript levels and normal glycolytic activity [229].

A very interesting study has pointed towards a direct role of oocyte-secreted factors in the regulation of glycolytic transcripts and glycolysis in cumulus cells. Cumulus cells from *Bmp15*$^{-/-}$ or double mutant (DM) *Bmp15*$^{-/-}$*Gdf9*$^{+/-}$ mice were defective in promoting the expression of transcripts involved in glycolysis in cumulus cells and were also defective in metabolizing glucose [230]. Furthermore, notably, only the combined effect of BMP15 and fibroblast growth factor 8 (FGF8B) administered exogenously to cultured OOX cumulus cells was able to restore the normal expression of glycolytic enzymes and restore glycolytic activity [230].

A similar metabolic cooperation appears to exist between oocytes and cumulus cells with regard to cholesterol biosynthesis. Mouse oocytes and embryos appear to require cholesterol to support pre-implantation development [244,245]. Oocytes are not able to synthesize cholesterol since the enzymes required for this process (such as *MvK*, *Pmvk*, *Fdps*, *Sqle*, *Cyp51*, *Sc4mol* and *Ebp*) are barely detected or absent in oocytes, whereas cumulus cells actively and highly express these enzymes [238]. As shown by culture of intact COCs and denuded oocytes, the ability of oocytes to convert acetate to cholesterol has been shown to be very poor, which supports the idea that cumulus cells provide the oocytes with products of this pathway [238].

The involvement of GDF9 and BMP15 as factors mediating these actions has been evidenced by a similar approach to the one mentioned earlier. Mutant cumulus cells derived from *Bmp15*$^{-/-}$ or DM *Bmp15*$^{-/-}$ *Gdf9*$^{+/-}$ mice were not able to produce cholesterol de-novo at similar rates to control wild type (WT) cumulus cells, whereas in OOX cumulus cells this deficiency was even larger when compared to WT COCs. Likewise, co-culture of OOX with fully-grown denuded oocytes restored the normal rates of sterol biosynthesis [238].

A third well-known example is related to amino acid uptake. Oocytes, compared to cumulus cells, appear to have a poor ability of amino acid uptake. A clear example is the low amount of radiolabeled L-alanine present in denuded oocytes after culture when compared to the very high amounts found in COCs [237,246]. L-alanine has been shown to be a substrate preferentially transported by the amino acid transporter SLC38A1 (solute carrier family 38, member 3). *Slc38a1* transcripts are not expressed in oocytes although *Slc38a1* is highly expressed in cumulus cells [237]. The involvement of oocytes in the amino acid uptake of cumulus cells has been elucidated by culturing OOX cumulus cells, by showing that in the absence of oocytes not only a dramatically reduced incorporation of L-alanine is observed, but also an important reduction of *Slc38a1* transcript levels in cumulus cells. Co-culture with fully-grown oocytes can restore the normal transcript levels in cumulus cells and, at the same time, the incorporation of L-alanine [237]. Once more, this demonstrates that oocytes regulate a different metabolic activity in cumulus cells.

Overall, these studies have demonstrated the cooperation between oocytes and their surrounding cumulus cells, and indicate that the metabolic activity of cumulus cells is controlled by the oocyte. Products of cumulus cell metabolism are most likely transferred to the oocytes via gap junctions and will compensate for the metabolic disability of oocytes.

To a large extent, these processes seem to be mediated by oocyte-secreted factors involving the GDF9, BMP15 and FGF8 signaling pathways. Whether these factors are the only regulators of these processes, and whether other processes depend on the cooperation of oocytes and cumulus cells before the LH surge, requires further investigation.

### 5.2. Cumulus–oocyte interaction after the LH surge

#### 5.2.1. Cumulus expansion

As reviewed in the previous section, one evident response to the LH surge is cumulus cells expansion, which is crucial for ovulation.

Oocytes participates in cumulus expansion as illustrated by the observation that OOX cumulus cells do not expand in response to FSH, whereas co-culture with fully grown oocytes restores the capacity of these cells to undergo expansion [234]. Through the secretion of cumulus expansion enabling factors (CEEFs), oocytes promote the expression of cumulus cell transcripts, such as *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6*, which are responsible for cumulus expansion [79,158,231,247].

In a similar way, the involvement of oocyte factors (such as GDF9, TGFβ1, BMP15 and activin A) in cumulus expansion has been established through exogenous addition of these recombinant proteins in culture. Induction of expansion of OOX cumulus cells and, furthermore, the promotion of the expression of *Has2*, *Tnfaip6*, *Ptgs2*, and *Ptx3* in OOX cumulus cells and/or isolated granulosa cells in culture has been shown to be reproduced by the addition of these oocyte factors, which suggests that they are potential components of the CEEFs [79,158,159,209,248].

These and other observations provide clear evidence that GDF9 and BMP15 are important elements of the CEEFs. By an RNA interference approach, the generation of *Gdf9* knockdown oocytes has been shown to compromise expansion of surrounding cumulus cells [249], whereas this is not the case in *Bmp15* knockdown oocytes. However, studies in *Bmp15*$^{-/-}$ or in double mutant (*Bmp15*$^{-/-}$ *Gdf9*$^{+/-}$) knockout mice have demonstrated that cumulus cells from these mutant mice fail to expand [250]. Moreover, oocytes from these mice are not able to restore the ability of the cumulus to expand in OOX wild type (WT) cumulus cells, and mutant cumulus cells fail to expand even in the presence of WT oocytes [250]. These studies highlight the importance of both GDF9 and BMP15 in the induction of cumulus expansion and suggest that their presence before the LH surge might be crucial for proper cumulus cell differentiation, and to confer to the cumulus cells the capacity to support expansion. In addition, recombinant BMP15 has been shown to induce the expression of the EGF-like peptides, epiregulin, amphiregulin and betacellulin, which points towards a more important role of BMP15 after the induction of ovulation by LH [159].

After GDF9 and BMP15 interact with their respective receptors ALK5 and ALK6, they act through downstream-signaling molecules called receptor-regulated molecules 'SMADs'. GDF9 signals through SMAD2/3, whereas BMP15 activates SMAD1/5/8 [233,251,252]. The use of a blocker of the SMAD2 phosphorylation (SB-431542) has been shown to neutralize both GDF9 and oocyte induction of expansion in OOX cumulus cells [209]. Moreover, Diaz et al. [232] have demonstrated that oocyte-induced *Ptx3* expression in cumulus cells requires the activation of both SMAD2 and SMAD3, whereas expression of *Ptgs2* and *Has2* transcripts only depends on the activation of SMAD2. Therefore, these studies strongly indicate GDF9 as a crucial factor regulating cumulus expansion. Despite this, GDF9 neutralizing antibody has been shown not to be able to counteract oocyte-induced *Has2* expression in cumulus cells [158], suggesting that GDF9 is not the only OSF involved in this process.

## 6. Conclusions

Fundamental discoveries in basic aspects of oocyte and follicle biology have provided insights on the regulation of molecular pathways controlling early stages of oogenesis, folliculogenesis and oocyte maturation, which are determined by complex activation and interplay of many factors acting in a stage-specific manner. From the earliest stage of oogenesis in the fetal ovary, growth factors (i.e. many members of the TGFβ superfamily) and the interplay between the somatic environment and the germ cell drive oocyte development and primordial follicle formation.

Over the last fifteen years, many studies have contributed in pointing out to the significance of the interaction between oocytes and their surrounding cumulus cells; the oocyte has been revealed to be the orchestrator in the promotion of follicle development and in assuring its own developmental competence by regulating granulosa cell function.

This information is of particular interest since it contributes to the establishment of the molecular basis that determines the acquisition of oocyte competence; furthermore, it reinforces the nexus that should exist between basic science and clinical application.

Because the in vitro generation of a large source of mature eggs for treatment of infertile women with reduced ovarian reserve or cancer patients with cryopreserved ovaries remains an obvious challenge, more data on in vitro regulation have to be acquired. The achievement of a precise view on the regulation of transcripts under certain in vitro conditions and/or under a specific stimulus could tremendously contribute to the refinement of culture methodologies for in vitro follicle culture and in vitro maturation techniques.

In patients with fertility disorders such as oocyte maturation arrest, premature ovarian failure, PCOS, and especially those infertility conditions with unknown etiology, the identification and association of alterations of the patterns of specific genes in these particular conditions may help to initiate new treatment modalities. Finally, the assessment of certain genes and gene products may be of potential use for the generation of drugs that may improve their fertility.

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