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What does it take to make a developmentally competent mammalian egg?

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TABLE OF CONTENTS

BACKGROUND: A limitation to our ability to distinguish between developmentally competent and incompetent eggs is our still only partial knowledge of the critical features that are needed to make a good egg and when during oogenesis these specific characteristics are acquired. The main objective of this review is to summarize the results of areas of investigation that are contributing to our still inadequate understanding of the molecular aspects of making developmentally competent eggs.

METHODS: For each area discussed, a systematic search was made using PubMed. The search was without temporal limits but mainly yielded publications between 1982–1999 (23%) and 2000–2011 (77%).

RESULTS: Taking an oocyte-centred view, we describe throughout folliculogenesis: (i) the factors that regulate oocyte growth; (ii) the role of oocyte–cumulus cell dialogue; (iii) the epigenetic organization of the oocyte genome and (iv) the storage and regulation of maternal RNAs.

CONCLUSIONS: The multifaceted complex of factors involved in oocyte growth constitutes the backbone on which oocyte developmental competence is built up. Operating behind the expression of these factors is a specific epigenetic signature established during oogenesis, but our knowledge is only approximate and major efforts will be required for more accurate analyses at specific gene loci. The growing research on small silencing RNAs during oogenesis and early oocyte development is revealing these molecules' critical role in mRNA degradation. Our next challenge will be to dissect the complex interactions among the different molecular players identified and to establish the presence of functional links among these factors.

Key words: mammalian oocyte / developmental competence / gene expression / epigenetics / small silencing RNAs

Introduction

At the beginning of each reproductive cycle, a group of primordial follicles within the mammalian ovary is recruited into the growing phase. Oocytes contained in their follicles begin a journey that, in most cases, will be terminated with their elimination, as only one oocyte (in mono-ovulatory species such as cattle, sheep or humans) or a few oocytes (in poly-ovulatory species such as mice, pigs or rabbits) will complete their growth and be ovulated. As experienced by many couples with infertility problems, an ovulated metaphase II (MII) oocyte is not always a good egg, as it may resist fertilization or, when fertilized, may not be competent to sustain development. The quality of the female gamete has an impact on rates of preimplantation, implantation and clinical pregnancy. Thus, eggs have been studied with the aim of identifying non-invasive markers that would help the selection of the gametes to fertilize or the choice of preimplantation embryos to transfer.

A limitation to the ability to distinguish between developmentally competent and incompetent eggs is our incomplete knowledge of the critical features that are needed to make a good egg and when, during oogenesis, these specific characteristics are acquired. The identification of oocyte-specific molecular markers that could be used to predict the developmental competence of oocytes more precisely could be of help in establishing more objective criteria for the selection of oocytes (Patrizio *et al*., 2007). Although the understanding of the molecular processes that are involved, the genes that are activated or repressed, and the RNAs and proteins that are synthesized, degraded or stored during oogenesis is still rudimentary, with the use of animal models we are beginning to find links between specific molecular features and the oocyte's acquisition of developmental competence.

The main objective of this review is to summarize our knowledge on major areas of investigation of processes occuring during mammalian oocyte growth that could be involved in tailoring the characteristics of a developmentally competent female gamete. More specifically, we will describe, for the period of folliculogenesis: (i) the factors that regulate oocyte growth; (ii) the role of oocyte–cumulus cell dialogue; (iii) the epigenetic organization of the oocyte genome and (iv) the storage and regulation of maternal mRNAs.

Search methods

For each area discussed, a systematic search was made using PubMed. Over three-quarters of the studies described were performed during the past 10 years (2000–2011, 77.3%); more specifically, 23% of the studies were performed during the period 1982–1999, 32% during 2000–2004 and 45% in the period 2005–2011. A few articles mentioned were published during the period 1952–1981.

From oocytes to eggs: a journey full of hurdles

The difficulty of finding specific features that define a developmentally competent mammalian oocyte is probably intrinsic to the developmental history of the female gamete and may be explained by the complexity of the bidirectional interaction between oocytes and follicle cells (Gilchrist *et al*., 2008) and by the number of coordinated pathways, mutual signalling and regulatory loops that interact towards the formation of a fully-grown oocyte. Such an oocyte should be capable of resuming meiosis, accomplishing fertilization and sustaining development.

For the sake of simplicity, the process of folliculogenesis is divided here into three main phases: (i) the transition from a primordial to a primary follicle; (ii) follicle growth and (iii) ovulation. Figure 1 illustrates the main players that are involved in the regulation of oocyte growth during these three phases and that constitute the backdrop on which oocyte developmental competence is built up.

From primordial to primary follicles

Mammalian oogonia enter meiosis during fetal life (i.e. at 12.5 days *post-coitum* in mice or between 12–16 weeks in humans) and their development is soon arrested at the diplotene stage of the first meiotic prophase. They remain at this stage until puberty, when a surge of LH induces the resumption of meiosis and ovulation of eggs arrested at MII.

Oocytes control follicle formation through the activity of a specific transcription factor, factor in germ cell α, which regulates the initial organization of primordial follicles and modulates the survival of germ cells. In female mice lacking this gene, primordial follicles do not form and animals are sterile (Soyal *et al*., 2000). Primordial follicles can be identified within the ovary by their specific morphological characteristics. Oocytes have a diameter of 10–20 μm and are surrounded by a layer of squamous follicle cells. These represent the pool of follicles that, through various phases of recruitment in groups that vary in number in different species, initiate the growing phase. This transition is a highly co-ordinated process that involves a number of autocrine and paracrine factors whose exact role in the dynamics of these events is not yet fully understood. It must be emphasized that some of the factors described appear and function only during a specific transition period, while others continue to play a central role during the subsequent phases of folliculogenesis.

Figure 1 shows the factors that have been experimentally demonstrated to arrest or induce the recruitment of primordial follicles. *Inhibiting factors* include anti-Mullerian hormone (AMH; Durlinger *et al*., 2002), forkhead transcription factor O3 (FOXO3; Castrillon *et al*., 2003)—a downstream effector of the PTEN/PI3K/AKT signalling pathway of cell proliferation and survival (Cantley and Neel, 1999; Li *et al*., 2010)—and the chemokine (SDF-I) and its receptor (CXCR4) (Holt *et al*., 2006). The list of *activating factors* is longer and includes leukaemia inhibitory factor, which is produced by granulosa cells and induces these cells to express the kit ligand (KL) (Parrott and Skinner, 1999) that binds to its cognate receptor c-kit on the oocyte surface and regulates the expression of bone morphogenetic protein (BMP)-15 gene (*BMP-15*). Other factors are: basic fibroblast growth factor (Skinner, 2005), produced by the oocyte; BMP-4 (Nilsson and Skinner, 2003), a member of the transforming growth factor-β (TGF-β) family of growth factors, which is produced by the theca and stromal cells and also has a central role in follicle survival; keratinocyte growth factor, produced by precursor theca, theca and stromal cells (Kezele *et al*., 2005); BMP-7 (another member of the TGF-β family) also produced by the precursor theca and stromal cells (Lee *et al*., 2001); platelet-derived growth factor, expressed by

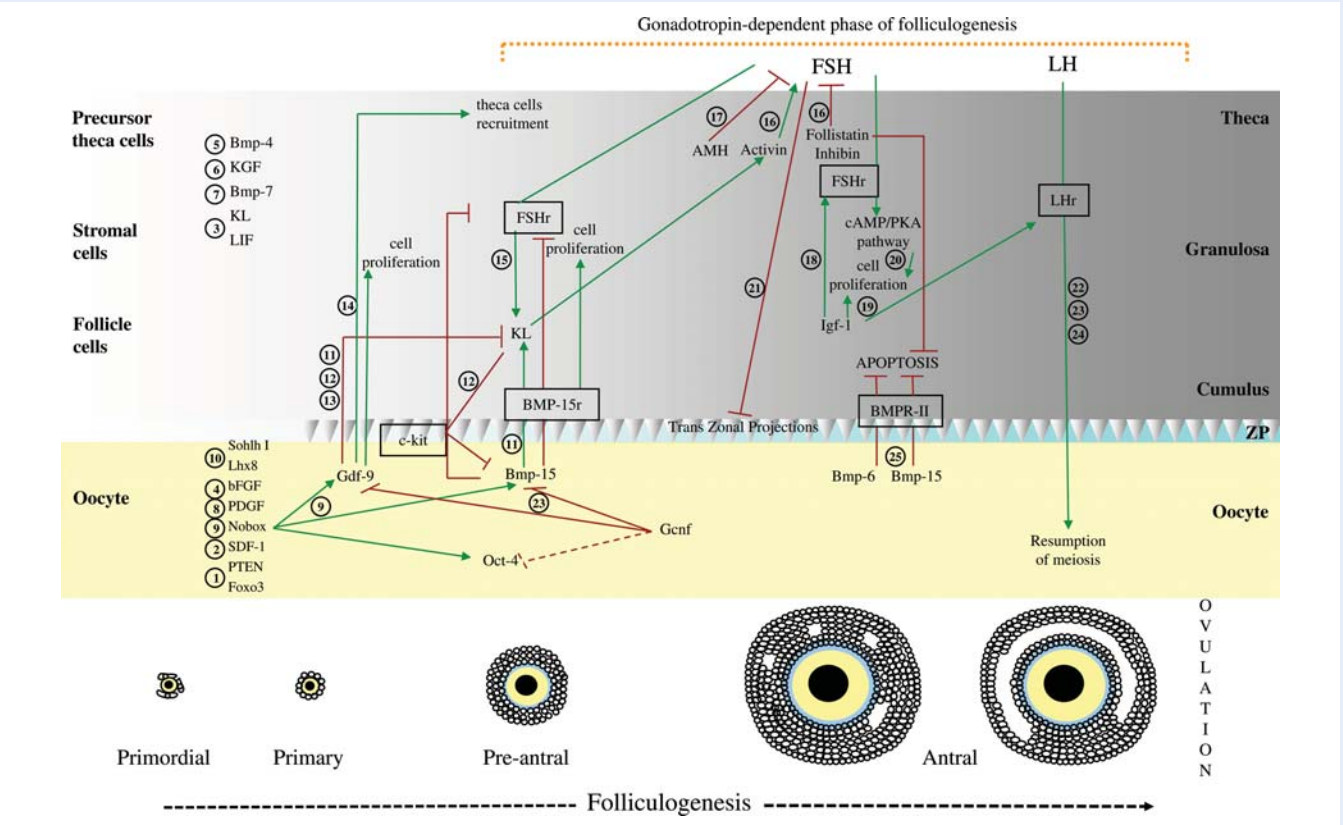

Figure 1 Summary of the main factors that regulate oocyte growth. Numbers within a circle refer to the following references: (1) Castrillon *et al.* (2003), (2) Holt *et al.* (2006), (3) Parrott and Skinner (1999), (4) Skinner (2005), (5) Nilsson and Skimmer (2003), (6) Kezele *et al.* (2005), (7) Lee *et al.* (2001), (8) Nilsson *et al.* (2006), (9) Rajkovic *et al.* (2004), (10) Pangas *et al.* (2006), (11) Otsuka and Shimasaki (2002), (12) Hutt *et al.* (2006), (13) Wu *et al.* (2004), (14) Dong *et al.* (1996), (15) Thomas *et al.* (2005), (16) Knight and Glister (2001), (17) Durlinger *et al.* (2001), (18) Zhou *et al.* (1997), (19) Magoffin and Weitsman (1994), (20) Richards (2001), (21) Combelles *et al.* (2004), (22) Mehlmann (2005), (23) Lan *et al.* (2003), (24) Ledan *et al.* (2001), (25) Perry and Verlhac (2008), Hussein *et al.* (2005).

the oocyte (Nilsson *et al.*, 2006); *Nobox* (newborn ovary homeobox-encoding gene) (Rajkovic *et al.*, 2004), *Sohlh 1* and *Lhx8* (Pangas *et al.*, 2006), which are three oocyte-specific genes whose lack of expression in deficient mice correlates with arrest at the transition from primordial to primary follicles; and FOXC1, the product of *Tgfβ-1* responsive gene, which regulates primordial germ cell migration, follicle formation and development beyond the preantral stage, as well as the responsiveness to BMP–TGFβ-related signals (Mattiske *et al.*, 2006).

The involvement of FSH in the recruitment of primordial follicles is a long-debated issue. An early study (Dierich *et al.*, 1998) showed that disruption of FSH receptor does not block the recruitment of primordial follicles into the growing pool, even though folliculogenesis is blocked before antral follicle formation. These data led to the idea that FSH is not involved at the beginning of oocyte growth. However, a number of studies demonstrated that FSH and its receptor make critical contributions to the transition of primordial follicles from the resting to the growing pool (Roy and Albee, 2000; Balla *et al.*, 2003; Thomas *et al.*, 2005). Although follicles do not have functional FSH receptors at this stage, pregranulosa cells and primordial follicles respond to activators of the cAMP pathway (forskolin and cAMP analogues) with increased expression of aromatase and FSH receptor (McNatty *et al.*, 2007).

The transition from primary to secondary follicles

When a primordial follicle leaves the resting pool, granulosa cells become cuboid, the oocyte increases in size and begins deposition of the zona pellucida, and stromal cells become organized into theca cell layers outside the basement membrane. At the time of follicle recruitment, growth differentiation factor 9 (GDF-9) and BMP-15 seem to have a co-operative function in regulating follicle cell proliferation (Edwards *et al.*, 2008), an activity performed mainly by GDF-9 (Dong *et al.*, 1996; Vitt *et al.*, 2000) during the early phases of folliculogenesis and by BMP-15 during more advanced phases (Galloway *et al.*, 2000; Yan *et al.*, 2001; Juengel *et al.*, 2002, 2004). The correct growth of the follicle is balanced through the regulation of KL expression, which is inhibited by GDF-9 and activated by BMP-15, this latter being itself inactivated by KL expression in a negative feedback loop (Otsuka and Shimasaki, 2002; Hutt *et al.*, 2006). KL seems to be the link that co-ordinates the growth of the oocyte and the proliferation of granulosa cells (Wu *et al.*, 2004). Later, in antral follicles, this factor up-regulates the expression of activin in granulosa and theca cells, which in turn positively regulates FSH secretion. FSH secretion is, in contrast, negatively regulated by both inhibin and

follistatin (Ying, 1988; Knight and Glister, 2001), this latter acting through affinity binding to activin which neutralizes its function (Knight and Glister, 2001). Insulin-like growth factor (IGF)-I has a role similar to that of activin, controlling FSH receptor expression in granulosa cells (Magoffin and Weitsman, 1994; Zhou et al., 1997).

Important structures that appear at this stage of development and that regulate the interactions between oocytes and the surrounding follicle cells, mainly those of the innermost layer bound to the zona pellucida, are transzonal projections that maintain the physical link between the oocyte and the somatic compartment of a follicle. The maintenance of a relationship between oocyte and follicle cells via transzonal projections is under FSH regulation (Combelles et al., 2004) and is required to ensure the growth of a healthy oocyte (Albertini et al., 2001; Eppig, 2001).

Antral development and ovulation

The transition from the pre-antral to the antral stage is under the control of both FSH and paracrine factors secreted by the oocyte. A recent study by Diaz et al. (2008) suggested that this transition is still controlled by TGF-β ligands, which might be processed differently depending on the presence of the convertase protein PCSK6 in granulosa cells. Since the levels of the expression of PCK6 protein, as well of GDF-9 and AMH, are high in the pre-antral stage but decrease during the transition to the antral stage, PCK6 could be considered an intra-ovarian regulator of GDF-9 and AMH activity.

The antral stage is characterized by the appearance of a fluid-filled cavity, the antrum, which begins to form when follicles reach a critical size (from 180 to 300 μm, depending on the species) and a critical number of granulosa cells (about 2000 in the mouse) (Boland et al., 1994). The appearance of the antral cavity establishes the morphological and functional separation of granulosa cells into mural granulosa cells, which line the follicle wall, and the cumulus cells, which surround the oocyte. In mice, cumulus cells appear to be more closely related to pre-antral granulosa cells from large secondary follicles than to mural granulosa cells, because the oocyte can regulate a wide range of cumulus cell functions via paracrine control (Su et al., 2009). Although formation of the antrum is not fundamental for the acquisition of full developmental potential, the follicular fluid represents a microenvironment enriched in nutritional and regulatory molecules as well apoptotic factors. It is well known that high concentrations of estradiol and low concentrations of insulin-like growth-factor binding proteins (IGFBP-2, -4, and -5) in the follicular fluid are the hallmark of dominant and pre-ovulatory follicles (Fortune et al., 2004).

As mentioned earlier, the antral phase of follicular development is characterized by dependency on gonadotrophins, FSH and LH, which are cyclically secreted by the pituitary gland. FSH, binding to its receptor, activates the cAMP/protein kinase A pathway (Richards, 2001), thus promoting cell proliferation, the differentiation of follicle cells into cumulus and mural granulosa cells, and the acquisition of meiotic competence.

The final phase of folliculogenesis that leads to meiotic resumption and germinal vesicle break down is triggered by a surge of LH and results from the release from the inhibitory action exerted by the follicle cells surrounding the oocyte and the interruption of the action of cAMP or other inhibitory molecules on the oocyte (Mehlmann, 2005). However, germinal vesicle oocytes may resume meiosis spontaneously when they are released from the follicle.

The importance of LH at this stage is highlighted by the finding that while FSH-b knockout mice are infertile due to arrest at the preantral stage of follicle development (Kumar et al., 1997), LH-b knockout female mice are infertile because follicle development is arrested at the antral stage, with abnormal/degenerating follicles and lack of pre-ovulatory follicles and corpora lutea. It is noteworthy that theca recruitment occurs normally in these mice, as the expression of theca markers such as BMP-4 and LH receptor is unaffected, but the expression of the majority of steroidogenic enzymes is marvedly impaired (Zhang et al., 2001).

The M-phase promoting factor (MPF, Cdk1/cyclin B) and other cyclin-dependent kinases are key molecules in regulating cell cycle progression during both mitosis and meiosis. The mitogen-activated protein kinase (MAPK) cascade is another main regulatory pathway that acts parallel to, and interacts with, MPF in driving the meiotic progression of oocytes (Liang et al., 2007). The MOS/MEK1/MAPK/p90rsk signalling pathway regulates the cell cycle through a cascade of protein kinase phosphorylation. Resumption of meiosis in oocytes may be either MAPK-dependent or MAPK-independent. It is MAPK-dependent when resumption is induced by gonadotrophin (Su et al., 2002), overcoming the inhibitory effect of the follicle cells, whereas it is MAPK-independent in oocytes that are isolated from the ovary and released from the follicle (i.e. denuded oocytes) (Fan and Sun, 2004).

Following the LH surge, phosphodiesterase type 3A (PDE3A) is activated, the level of cAMP falls and protein kinase A is inactivated. As a consequence, Cdc25 phosphatase is activated and removes inhibitory phosphatases from the Cdk1 subunit of the MPF, chromosomes start to condense and germinal vesicle break down occurs. MPF activity regulates the entry and exit from meiosis I and II (Ledan et al., 2001; Perry and Verlhac, 2008).

In parallel to these signalling pathways, changes occur in the microtubule organization of the ooplasm. The first meiotic spindle forms, beginning from microtubule organizing centres around the chromosomes, and moves towards the cortex, inducing its differentiation with a local accumulation of actin filaments and loss of microvilli (Longo and Chen, 1985). The eccentric position occupied by the germinal vesicle or the MII plate limits the cleavage furrow and restricts the size of the first and second polar bodies, allowing the maintenance of most of the ooplasm, together with the maternal factors stored, within the egg (Verlhac and Dumont, 2008). The expression of a number of spindle assembly checkpoint proteins, which monitor chromosome attachment to microtubules and chromosome tension, is reduced during female ageing and this, together with dysfunction of the spindle and other cell organelles, increases errors in chromosome segregation and could be responsible for the augmented incidence of aneuploidy in ageing oocytes (Vogt et al., 2008).

The role of the oocyte–cumulus cell dialogue

The establishment of gap-junctional-mediated intercellular communications between the oocyte and companion somatic cells is critical for the development of both follicular compartments. In fact, although

the oocyte can autonomously take up some nutrients from the extracellular environment, the establishment of this bidirectional communication allows the production of developmentally competent germ cells (Cecconi *et al.*, 2004; Gilchrist *et al.*, 2008). Oocyte-follicular cell contacts should not be considered as permanent structures, but rather as specific 'devices' continuously adapting their morphology in response to the activity of both oocyte and cumulus cells.

In follicles, the physical contact between somatic cells is mediated by the presence of connexins (Cx), which are expressed from the early stage of development (Gittens and Kidder, 2005; Gittens *et al.*, 2005). In particular, Cx43 and Cx45 have been identified between granulosa cells, while communications between granulosa cells and the oocyte depend on the presence of Cx37. The fact that Cx43 and Cx37 channels have different permeability properties and that Cx43-positive and Cx37-positive plaques do not overlap suggest that each Cx could play a specific physiological role, e.g. the transfer of different signals between the different compartments of the developing follicles. Cx43 is detectable in pregranulosa cells of primordial follicles, probably mediating the relationship between somatic cells (Gittens *et al.*, 2005). The number of Cx43 gap junctions per granulosa cell increases concomitantly with follicle development and, in particular, during the transition from the preantral to the antral stage. In the absence of Cx43, gap junctions between somatic cells do not form and folliculogenesis arrests at the unilaminar stage (Gittens and Kidder, 2005). Mutation of Cx37 abolishes the production of mature Graafian follicles and fully grown oocytes (Carabatsos *et al.*, 2000).

The importance of metabolic co-operation between oocytes and cumulus cells is seen, for example, when glucose is metabolized into pyruvate by cumulus cells and then the latter transferred to the oocyte and used in energy-producing processes (Su *et al.*, 2009). Part of the cholesterol that is synthesized by cumulus cells is also transferred to oocytes, given the germ cell's inability to produce and take up this lipid from the external microenvironment (Su *et al.*, 2009). In other mammalian species, including humans, the potential role of this co-operation between cumulus cells and oocytes in the regulation of metabolic pathways (e.g. glycolysis and amino acid uptake) is still unknown.

Experiments in mice in which oocytes and granulosa cells at different stages of development were co-cultured, or oocytes microsurgically removed from cumulus cell-oocyte complexes (COC) established that oocytes play a leading role in the control of follicle development. Indeed, the oocyte controls granulosa cell proliferation (Joyce *et al.*, 1999; Cecconi and Rossi, 2001; Eppig *et al.*, 2002), induces the expression of a mural granulosa cell phenotype and promotes high levels of expression of specific mRNAs in cumulus cells (Su *et al.*, 2009). Cumulus expansion requires the presence of the oocyte; thus, isolated cumulus cells are unable to respond to FSH and form an expanded matrix. Genes such as *Ptgs2*, encoding prostaglandin-endoperoxide synthase 2 (COX-2), hyaluronan synthase 2 (HAS-2) and tumour necrosis factor-α-induced protein 6 (TNFAIP6/TSG-6), which play essential roles in this process, are all stimulated by the contemporary presence of gonadotrophins and oocytes, or oocyte-derived secreted factors (Dragovic *et al.*, 2005, 2007). More recently, Sugiura *et al.* (2010) demonstrated that GDF-9 and BMP-15, together with 17β-estradiol, co-ordinate cumulus cell development and expansion. In fact, in comparison with controls, COC cultured in the absence of 17β-estradiol exhibited low *Has2* mRNA levels and reduced cumulus expansion. Oocyte-dependent paracrine signalling acts on cumulus cells by stimulating the SMAD2/3 pathway that controls not only cumulus expansion (Dragovic *et al.*, 2007), but also *Egfr* and *Spry2* mRNA expression in both cumulus and mural granulosa cells (Sugiura *et al.*, 2009; Su *et al.*, 2010), which, in turn, elicits the synthesis of the EGF-like peptides amphiregulin (AREG), betacellulin and epiregulin. In large mammals, by contrast, cumulus expansion is regulated by factors produced by somatic cells rather than by the oocyte, as demonstrated for the porcine (Prochazka *et al.*, 1998; Liang *et al.*, 2005) and sheep (Cecconi *et al.*, 2008) COCs.

Understanding the effects exerted by gonadotrophin stimulation on gap-junctional-mediated intercellular communications (GJIC) is fundamental in order to increase our knowledge of the mechanisms regulating oocyte meiotic maturation. In mouse preovulatory oocytes, the acquisition of a mature chromatin organization (see below) requires the participation of unidentified paracrine signals released by companion somatic cells (De la Fuente, 2006). Following the LH surge, oocyte soluble factor(s) activate the production of a meiosis-inducing signal in cumulus cells and this signal is transferred back to the oocyte *via* gap junctions (Su *et al.*, 2009). At the same time, neuregulin 1, a potential ligand for the ERBB3 receptor, is produced by stimulated cumulus cells to enhance AREG-induced progesterone production in granulosa cells (Noma *et al.*, 2010). A recent study by Sasseville *et al.* (2009) provided new insights into the role played by GJIC in the control of gonadotrophin-stimulated meiotic maturation in porcine COC. These authors proposed that cumulus cells provide a still unidentified positive signal that is transferred to the oocyte via gap junctions, and co-operates in the acquisition of oocyte developmental competence by a gap-junctional-independent mechanism based on the modulation of oocyte PDE3A activity.

As the maintenance of GJIC is required not only for the oocyte to complete its growth, but also to acquire nuclear and cytoplasmic meiotic competence, it is obviously of great interest to understand how the disruption on of the oocyte–cumulus bidirectional communication during *in vitro* maturation (IVM) has an impact on subsequent embryonic and fetal development. It is well established that the addition of FSH and EGF to a maturation medium has positive effects on oocyte meiotic maturation, fertilization and pregnancy outcome. These effects are specifically mediated through the cumulus cells because these ligands stimulate oocyte developmental competence only in the presence of cumulus cells (Rossi *et al.*, 2006; Gilchrist *et al.*, 2008; Yeo *et al.*, 2009). In fact, oocytes denuded of their cumulus cells prior to IVM showed a lower incidence of first polar body extrusion and failed to develop into blastocysts. Moreover, removal of cumulus cells prior to IVM altered many cytoplasmic and molecular processes, which could be rescued by simply co-culturing the denuded oocytes with monolayers of cumulus cells (Ge *et al.*, 2008a, b). It has been proposed that FSH and EGF signalling contribute to the acquisition of oocyte developmental competence by prolonging gap-junctional communication, which mediates the exchange of factors necessary for optimal oocyte developmental competence and subsequent fetal development (Yeo *et al.*, 2009). In this context, it is not surprising that also addition of recombinant GDF-9 during mouse oocyte IVM significantly increases blastocyst quality and fetal survival (Yeo *et al.*, 2008). The novel IVM system proposed

by Albuz et al. (2010) for bovine and murine COCs, based on a rapid increase in cAMP concentration and on a prolonged IVM phase, might have positive implications also for the culture of human oocytes.

All these studies clearly highlight that GJIC mediate a complex network of metabolic and regulatory pathways essential for the development and function of both germ and somatic cell types.

The epigenetic organization of the oocyte genome

Within the context described, other important molecular changes that occur during folliculogenesis may define more precisely the oocyte determinants that contribute to the acquisition of its developmental competence. The establishment of specific epigenetic profiles during gametogenesis and their maintenance during early development is a key aspect to ensure correct and complete development (Reik, 2007).

There are several epigenetic mechanisms that regulate gene expression, with chromatin organization, DNA methylation and histone modifications being the best known.

Oocyte chromatin organization during folliculogenesis

During folliculogenesis, germinal vesicle oocytes undergo local chromatin remodelling at specific promoter regions, but also extensive chromatin changes that involve large parts of the genome (De La Fuente, 2006). In oocytes isolated from primordial and primary follicles, centromeres and chromocentres are predominantly localized at the periphery of the nucleus. During oocyte growth, centromeres and chromocentres are initially spread within the nucleus and then progressively cluster around the periphery of the nucleolus (Longo et al., 2003; Garagna et al., 2004). On the basis of their chromatin organization, germinal vesicle oocytes may be classified into two separate classes, termed SN (surrounded nucleolus) oocytes, with a ring of heterochromatin surrounding the nucleolus and NSN (not surrounded nucleolus) oocytes, with more dispersed chromatin not surrounding the nucleolus (Mattson and Albertini, 1990; Wickramasinghe et al., 1991; Debey et al., 1993; Zuccotti et al., 1995; for a review see Zuccotti et al., 2005; Tan et al., 2009; Fig. 2). Oocytes with a diameter between 10 and 40 μm (primordial to early growing) have an NSN-type of chromatin organization; later, at the time of follicular recruitment, some oocytes (~5%) acquire an SN chromatin organization, with the frequency reaching ~50% in fully matured antral oocytes (Zuccotti et al., 1995, 2005). Fully grown oocytes with an NSN-type of chromatin organization are considered an immature form that will acquire an SN chromatin organization just prior to ovulation.

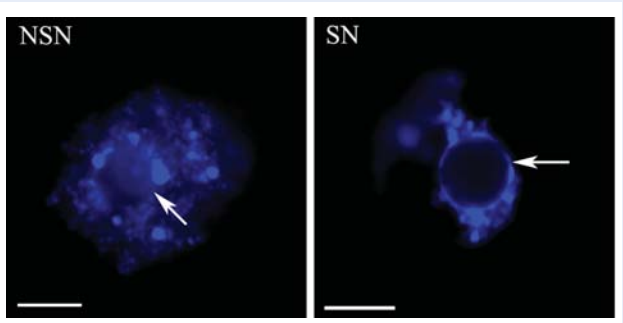

Figure 2 Hoechst staining of the nucleus of NSN and SN oocytes. The arrow indicates the nucleolus. Bar, 5 μm.

Oocytes possessing NSN or SN chromatin organization have also been found in rats (Mandl and Zuckerman, 1952), monkeys (Lefèvre et al., 1989), pigs (Crozet, 1983) and humans (Parfenov et al., 1989). Only goat (Sui et al., 2005) and equine (Hinrichs and Williams, 1997) oocytes seem to represent exceptions, as their germinal vesicles do not show the SN type of chromatin configuration. Tan et al. (2009) have provided an updated and detailed description of chromatin configuration during oocyte growth in all the mammalian species thus far studied. The morphological differences have biological relevance as they have been correlated with changes in transcription (Moore et al., 1974; Kaplan et al., 1982; Bouniol-Baly et al., 1999; Christians et al., 1999; Liu and Aoki, 2002; Miyara et al., 2003). NSN oocytes are transcriptionally active and produce all classes of RNA, whereas SN oocytes are transcriptionally inactive (Debey et al., 1993; Bouniol-Baly et al., 1999). Immunocytochemical analysis of the profiles of DNA methylation, histone acetylation and histone methylation showed that these are all higher in SN than in NSN oocytes (Kageyama et al., 2007).

Both SN and NSN oocytes mature *in vitro* to the MII phase (MII^{NSN} derived from antral NSN oocytes and MII^{SN} derived from SN oocytes), but, while MII^{SN} oocytes develop to term, the development of MII^{NSN} oocytes is arrested at the 2-cell stage (Zuccotti et al., 1998, 2002; Inoue et al., 2008).

The distinct chromatin organizations described earlier are morphological markers that underlie molecular differences. Using micromanipulation techniques, Inoue et al. (2008) performed reciprocal germinal vesicle transfer between SN and NSN antral oocytes and followed the meiotic and developmental competence of the reconstructed gametes in culture. While few reconstructed SN/NSN (nucleus/cytoplasm) oocytes reached the MII stage, 88% of the NSN/SN oocytes were capable of meiotic resumption, even though they could not reach the blastocyst stage following IVF. When MII plates of NSN/SN oocytes were transferred to enucleated ovulated MII oocytes, most of them completed preimplantation development and some of them, following embryo transfer into pseudo-pregnant females, reached full term. These results indicate that factors within the ooplasm of the mouse germinal vesicle oocyte are involved in determining the oocyte's meiotic competence, whereas factors present in the nucleus itself are associated with the oocyte's developmental competence beyond the 2-cell stage.

In search of maternal factors crucial for early development, recent studies have shown a differential expression of maternal-effect genes and proteins in NSN and SN oocytes. Maternal-effect transcripts are stored in the egg and sustain the very early stages of preimplantation development through a timely controlled translation while gametic genome reprogramming and zygotic genome activation occur. The correct expression of maternal-effect genes such as *Zar1*, *Npm2*, *Stella* (*Dppa3*), *Smarca4* (*Brg1*) and *Oct4* is crucial for preimplantation development since lack or faulty expression of one of these genes results in developmental arrest at the time of zygotic genome activation (Burns et al., 2003; Payer et al., 2003; Wu et al., 2003; Bultman et al., 2006;

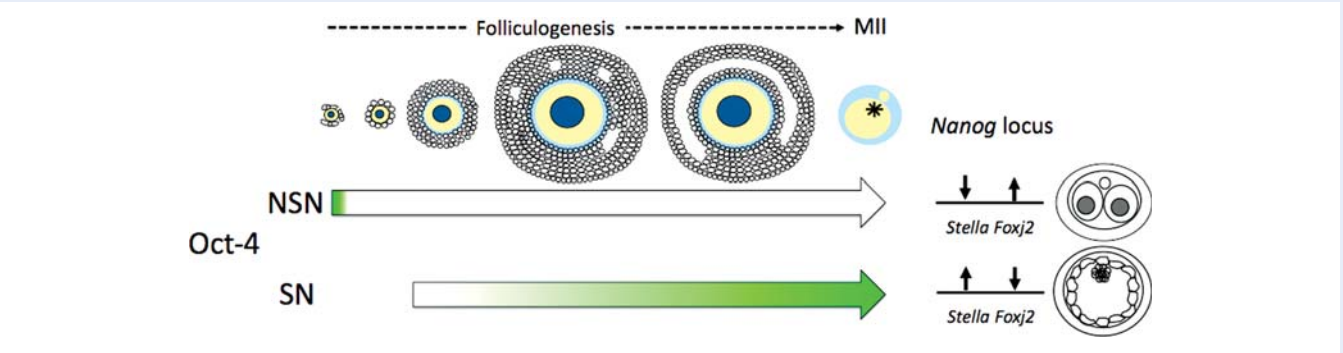

Figure 3 Oocytes of primordial follicles possess an NSN-type of chromatin organization and express the Oct4 protein (green). In oocytes of primary follicles Oct4 is down-regulated and reappears again at the beginning of oocyte growth, but only in SN oocytes and in their derived MII^{SN} eggs. On the basis of earlier evidence on the role played by Oct4 in the regulation of the expression of genes within the *Nanog* locus (Levasseur *et al*., 2008), we hypothesize that, in MII^{NSN} eggs, Oct4 down-regulation explains the inactivation of *Stella* gene expression and the up-regulation of *Foxj2*. In contrast, Oct4 expression in MII^{SN} eggs governs the expression of *Stella* and down-regulates *Foxj2*. Down-regulation of the maternal-effect factor Stella and up-regulation of Foxj2 are detrimental to embryo survival as the development of most embryos with this pattern of expression is interrupted at the 2-cell stage (Payer *et al*., 2003; Martín-de-Lara *et al*., 2008).

Foygel *et al*., 2008), when a novel set of genes and proteins, expressed by the embryonic genome, becomes essential for the continuation of development. Alteration of the correct expression of zygotic genes is believed to cause the loss of a high percentage of IVF embryos during human preimplantation development.

Among the several maternal-effect genes whose expression is required to govern the early stages of development, Oct4 may also play a role in the acquisition of oocyte developmental competence (Zuccotti *et al*., 2008, 2009). Oct4 is expressed in oogonia and, in the adult mouse ovary, in primordial oocytes; then, with the beginning of folliculogenesis, it is down-regulated. When follicles are recruited to begin growth, the transcription factor is up-regulated, but only in oocytes with an SN-type of chromatin organization (Monti and Redi, 2009; Zuccotti *et al*., 2009). This pattern of Oct4 expression is maintained until SN oocytes reach full maturation and in their derived MII^{SN} oocytes. In contrast, Oct4 is always down-regulated in NSN oocytes throughout their growth.

An interesting function of Oct4 is its involvement in the regulation of chromatin modelling and chromatin-mediated transcription regulation (Campbell *et al*., 2007). In mouse embryonic stem cells, Oct4 has been found to influence chromatin organization at the *Nanog* locus, which contains *Stella*, *Nanog*, *Foxj2* and other genes important for the maintenance of pluripotency (Levasseur *et al*., 2008). As in embryonic stem cells, in oocytes the down-regulation of Oct4 expression correlates with the down-regulation of Stella protein and up-regulation of *Foxj2* (Zuccotti *et al*., 2009), an expression profile that explains the developmental block at the 2-cell stage that MII^{NSN} oocytes encounter following fertilization (Fig. 3). In fact, the down-regulation of Stella (Payer *et al*., 2003) and the up-regulation of Foxj2 (Martín-de-Lara *et al*., 2008) in oocytes is incompatible with preimplantation embryonic development, as the development of most of the embryos is arrested at the 2-cell stage. While the molecular function of Foxj2 is still unclear, that of Stella is to act as a protector against demethylation of the maternal genome and of some paternal imprinted genes during the very early stages of development (Nakamura *et al*., 2007).

The effect of Oct4 down-regulation in developmentally incompetent MII^{NSN} oocytes goes beyond the control of gene activity within the *Nanog* locus, and is extended to the activation of known Oct4-regulated genes (Boyer *et al*., 2005; Loh *et al*., 2006) involved in the induction of adverse pathways such as mitochondrial dysfunction and apoptosis, as shown by microarray analysis (Zuccotti *et al*., 2008).

From these studies Oct4 emerges as a potential regulator of the acquisition of oocyte developmental competence and, since it is up-regulated in developmentally competent (SN) and down-regulated in incompetent (NSN) oocytes, as a molecular marker of oocyte quality.

Histone modifications

Phosphorylation, acetylation, methylation, poly(ADP) ribosylation and ubiquitination are post-translational modifications at specific amino-acid residues of histone proteins that play a central role in the regulation of the changes that occur in chromatin organization and, ultimately, in gene expression. They participate in the establishment of stable and heritable epigenetic modifications (Michelotti *et al*., 1997; Turner, 2000) and may occur simultaneously during development and cell differentiation (Jenuwein and Allis, 2001).

Histone acetylation is associated with enhanced transcriptional activity, whereas histone deacetylation is correlated with repression of gene expression (Berger, 2007). The dynamic nature of histone acetylation/deacetylation provides the cell with an epigenetic mechanism for controlling gene expression over a genome-wide scale.

The analysis of the levels of acetylation at K9 and K18 on histone H3 (H3K9ac and H3K18ac) and K5 and K12 on histone H4 (H4K5ac and H4K12ac) showed that they all increased, with similar profiles (except for H4K5ac), during oocyte growth (Kim *et al*., 2003; De La Fuente *et al*., 2004; Kageyama *et al*., 2007; Fig. 4).

To date, three major classes of histone deacetylases have been described (Thiagalingam *et al*., 2003); however, the nature of the oocyte-specific histone deacetylases remains unknown. What is known is that genome-wide histone deacetylation participates in the

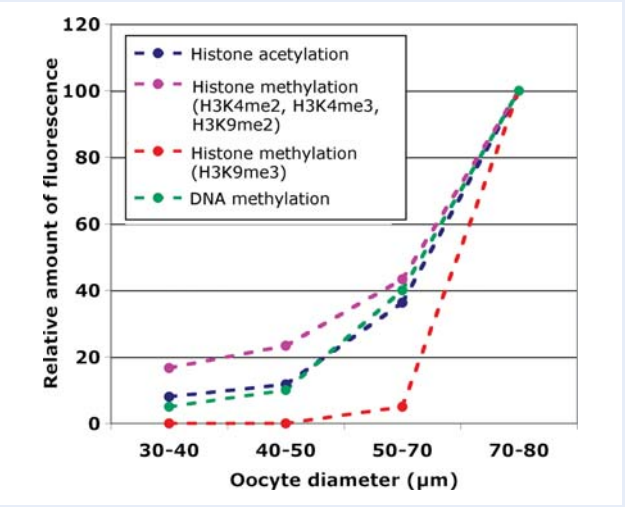

Figure 4 Profiles of histone acetylation and methylation and DNA methylation during oocyte growth.

maintenance of the SN configuration in mouse germinal vesicle oocytes (De La Fuente *et al*., 2004; Meglicki *et al*., 2008) and it occurs at several lysine residues upon meiosis resumption, when gene expression is down-regulated (Kim *et al*., 2003; De La Fuente *et al*., 2004; Sarmento *et al*., 2004; Endo *et al*., 2005). The process of histone deacetylation involves the histone variant MacroH2A that recruits histone deacetylases (Chakravarthy *et al*., 2005) and inhibits nucleosome remodelling (Angelov *et al*., 2003). Deacetylation reaches its peak in MII oocytes (Akiyama *et al*., 2004; Kim *et al*., 2003; Spinaci *et al*., 2004) and is necessary for the binding of a chromatin remodelling protein (ATRX) to the centromeric heterochromatin, an essential step for the correct alignment of the chromosomes, since disrupting the binding of ATRX by using an histone deacetylation inhibitor (i.e. trichostatin A) results in abnormal chromosomal alignment at the meiotic spindle (De La Fuente *et al*., 2004). Histone deacetylation during gametogenesis is thought to be a process of erasure of gamete epigenetic memory. Soon after fertilization, the zygote removes the histone variant MacroH2A (Chang *et al*., 2005) and gradually re-establishes the levels of acetylation in both male (faster) and female pronuclei (Adenot *et al*., 1997; Kim *et al*., 2003). These same remodelling mechanisms are also utilized following somatic nuclear transfer experiments (Chang *et al*., 2010), although the deacetylation feature seems to have an adverse effect on nuclear reprogramming as inhibition of the removal process by trichostatin A treatment during oocyte activation and nuclear remodelling improves preimplantation and, albeit slightly, full-term development (Rybouchkin *et al*., 2006).

Compared with histone acetylation, histone methylation is a more stable process that, together with DNA methylation (see below), contributes to the establishment of an imprinted pattern of gene expression during oogenesis and its maintenance following fertilization.

The profile of histone methylation during oocyte growth is similar to that described for the acetylation process (Fig. 4). The di- and trimethylation of H3K4 (H3K4me2 and H3K4me3) and H3K9 (H3K9me2 and H3K9me3), which are known to be involved in the activation and suppression of gene expression, respectively, increases during folliculogenesis, but with significantly different patterns. While the levels of H3K4me2, H3K4me3 and H3K9me2 methylation increase in oocytes of 30–60 μm diameter, and then increases significantly in oocytes of 50–80 μm, the level of H3K9me3 methylation remains low until oocytes reach the diameter of 60 μm, then it rises abruptly in fully grown antral oocytes (Kageyama *et al*., 2007) and localizes exclusively in pericentric heterochromatin (Meglicki *et al*., 2008).

Another non-histone chromosomal protein that is important not only in heterochromatin formation and gene silencing, but also in telomere stability (Minc *et al*., 1999; Song *et al*., 2001) and in positive regulation of gene expression in *Drosophila* is the heterochromatin protein 1 (HP1) (Piacentini *et al*., 2003; for a review see Fanti and Pimpinelli, 2008). Heterochromatic regions contain two isoforms named HP1α and HP1β (Furuta *et al*., 1997; Minc *et al*., 1999; Guenatri *et al*., 2004); a third HP1γ localizes outside these chromatin regions (Minc *et al*., 1999). HP1 selectively binds to nucleosomes that have di- or tri-methylated H3K9 (Bannister *et al*., 2001; Lachner *et al*., 2001; Fischle *et al*., 2005). HP1α is present in oogonia, but disappears when these enter meiosis. The nuclei of primordial oocytes show the presence of only HP1β, whereas HP1α reappears at the beginning of oocyte growth and only in heterochromatic regions. With growth and at the transition from NSN to SN oocytes, HP1α diffuses from pericentric regions to the entire nucleus. In contrast, HP1β localizes on the chromatin of fully grown SN oocytes, dissociates at the time of germinal vesicle break down and remains, but only for a very short time, around the MII plate (Meglicki *et al*., 2008).

DNA methylation

A mammalian zygote inherits one haploid genome complement from each parent. Although the great majority of the inherited alleles are transcriptionally equivalent, some of the genes behave differently depending on their parent-of-origin. These genes are named imprinted and their expression is regulated by DNA epigenetic modifications mainly established during gametogenesis (Reik, 2007). Thus, at the end of spermatogenesis or oogenesis, the paternal or maternal allele, respectively, will be differentially marked.

The addition of a methyl group CH_3 at the cytosine of CpG sites is an epigenetic modification of the DNA sequence found in the genomes of vertebrates, plants, fungi and in some species of invertebrates and bacteria. CpG methylation regulates the expression of imprinted and non-imprinted sequences.

The majority of the about 90 imprinted genes identified to date in mammals (www.geneimprint.com) are maternally imprinted; i.e. their sequences are epigenetically modified during oogenesis. Maternally methylated regions usually extend over the promoters of imprinted genes, whereas paternally methylated regions may be positioned many kilobases away from the imprinted gene (Lewis and Reik, 2006).

The precise timing of the acquisition of maternal imprinting during mouse oogenesis is still unclear and the small amount of information available has been derived from a restricted number of genes. Using the bisulphite method (for a description of this technique see Warnecke *et al*., 1998; Lucifero *et al*., 2002), recent studies have suggested that methylation imprinting is acquired after birth during the growth phase of diplotene-arrested oocytes, that it is asynchronously scheduled in

different genes and that it may be governed by distinct mechanisms (for a summary of the profile of DNA methylation during oocyte growth, see Fig. 4). In one study, parthenotes were reconstructed by injecting a mouse MII oocyte with the genome of an oocyte taken at increasing growing phases, to demonstrate that genes are imprinted at different, specific time-points during oogenesis. The genes *Snrpn*, *Znfl27* and *Ndn* were found to be imprinted in oocytes during the passage from primordial to primary follicles; *Peg3*, *Igf2r* and *p57^{kip2}* in secondary follicles, *Peg1* in tertiary to early antral follicles and *Impact* in antral follicles (Obata and Kono, 2002). A subsequent study found similar results for the same genes and determined the mechanistic basis of the asynchronous imprinting (Lucifero *et al*., 2004). *Snrpn* was found to be differentially methylated in the two alleles: namely, the methylation imprint was established first in preantral early growing oocytes on the maternally inherited allele and then, at the antral follicle stage, on the paternal allele. These findings indicate that *Snrpn* alleles are not equivalent and maintain some sort of memory of their parental origin; while methylation imprints are erased in primordial germ cells, other epigenetic modifications (e.g. a specific chromatin structure) are perhaps maintained (Lucifero *et al*., 2004).

This pattern of acquisition of CpG methylation is extended to non-imprinted sequences such as the 5′ long-term repeat within the intracisternal A particle elements, although in these sequences methylation is targeted slightly earlier during oocyte growth (Lucifero *et al*., 2007).

DNA methyltransferases (DNMTs) are the enzymes that catalyse the addition of a methyl group to cytosines within CpG sites (Bestor, 2000). The oocyte-specific DNMT1o is an alternatively spliced form of DNMT1 that, like this major methyltransferase, plays a crucial role in the maintenance of CpG methylation in oocytes and early embryos. In contrast, DNMT3a and DNMT3b are involved in the transfer of methyl groups to hemimethylated and unmethylated DNA (Kaneda *et al*., 2004). The activity of these two enzymes is catalysed by another methyltransferase, DNMT3L, which changes their conformation favouring their binding to the target sequence (Hata *et al*., 2002). In DNMT3L null female mice, the establishment of maternal imprinting in the oocytes is precluded (Bourc'his *et al*., 2001); thus heterozygous offspring show biallelic expression of genes that should be maternally methylated and inactivated, causing the death of the embryo by mid-gestation, even though there seems to be no effects on the profile of methylation of retrotrasposons of the long interspersed elements (LINE-1) and intracisternal A particle elements (Bourc'his *et al*., 2001). These results suggest that although both are necessary, the methylation of imprinted genes and that of transposons follow different regulatory mechanisms and perhaps involve different DNMTs.

The expression of *Dnmt3a*, *Dnmt3b*, *Dnmt3L* and *Dnmt1o* genes increases with oocyte diameter (Lucifero *et al*., 2007). DNMT1o accumulates in the ooplasm and moves to the nuclei of 8-cell stage embryos to maintain specific patterns of methylation (Howell *et al*., 2001).

Storage and regulation of maternal RNAs

A vast number of transcripts are expressed by the oocyte, many of which are used for its maturation, while others are stored and play important roles during the early stages of development. Almost half of the 85 pg mRNA stored during oocyte maturation is degraded during meiosis resumption and by the MII stage the oocyte carries about 35 pg mRNA; of this, about half undergoes stabilization through selective deadenylation of the poly(A) tail at the 3′ region (Paynton *et al*., 1988). For example, transcripts important for germinal vesicle block and for the following stages of meiosis resumption, such as oxidative phosphorylation, energy production, protein synthesis and metabolism, are eliminated. In contrast, transcripts associated with the maintenance of MII oocyte features (such as those involved in protein kinase pathways) (Su *et al*., 2007) or maternal-effect transcripts (such as *Stella*, *Mater* and *Zar1*), whose function is crucial in early development (Thélie *et al*., 2007) are stabilized through deadenylation.

Soon after fertilization and by the time the embryonic genome is first expressed, translation of maternal mRNAs (e.g. maternal-effect transcripts), necessary for the early phases of development, is begun through the polyadenylation of the 3′ untranslated region operated by embryonic *cis* regulatory cytoplasmic polyadenylation elements (Mendez and Richter, 2001; Racki and Richter, 2006). During these early cleavage stages, 90% of the maternal transcripts are inactivated or degraded through co-ordinated post-transcriptional regulation. Translation of these latter maternal mRNAs is repressed by processes of deadenylation (Huarte *et al*., 1992) and association with RNA-binding proteins such as the Y-box protein MSY2 (Gu *et al*., 1998; Davies *et al*., 2000), which may prepare RNAs for their elimination. The oocyte-specific histone *H1oo*, *c-mos* (crucial in regulating meiosis), the tissue type plasminogen activator gene (*tPA*) and *GDF-9* are the examples of transcripts that are rapidly degraded after fertilization (Alizadeh *et al*., 2005) because they are unnecessary for, or even detrimental to, development (e.g. injection of the c-mos protein in mouse embryos causes a cleavage block; Sagata *et al*., 1989).

Accumulating evidence indicates that a class of small silencing RNAs (ssRNAs) is implicated in the elimination of maternal mRNAs. ssRNAs are short length (20–30 nucleotides) RNAs that associate with proteins of the Argonaute family to form a ribonucleic complex that binds to the 3′ untranslated region of target mRNAs to degrade them or repress their translation (for a review, see Rana, 2007). Three main classes of microRNAs have been studied so far, although new classes and sub-classes continue to be discovered. Small interfering RNAs (siRNAs or endo-siRNAs) are double-stranded RNAs (the guide strand directs the silencing, the other strand, the passenger, is eventually eliminated), Piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs) are single-stranded RNAs. These ssRNAs have been extensively studied in *Drosophila* and *Caenorhabditis elegans* since the late 1990s, but they have also emerged as critical players in translational regulation in mammalian cells. The biogenesis of these different types of ssRNAs is similar: once they have been transcribed they are translocated to the cytoplasm, processed by Dicer (an RNase III-like enzyme) and the resulting mature silencing RNA is bound to the Argonaute protein to form an RNA-induced silencing complex (RISC) that binds to complementary RNAs and proceeds to their degradation. siRNAs have the function to finely tune the level of protein production. Mammalian cells possess a number of Argonaute-like genes, but only Ago2 is required for RNA cleavage (Liu *et al*., 2004a, b). Ago2 is maternally expressed and plays an essential role in the degradation of maternal mRNAs in the early stages of

mouse embryogenesis, regulating maternal-to-embryo transition and allowing development beyond the 2-cell stage (Lykke-Andersen *et al.*, 2008).

So far, very few studies have analysed the expression of and the role played by miRNAs during oocyte growth and preimplantation development. The average relative amount of mRNAs does not change during oocyte maturation, although single miRNAs may vary consistently during oocyte growth (Tang *et al.*, 2007). Following ovulation, miRNAs are increased 3-fold in the egg compared with that in growing oocytes, with the amount of mRNA remaining unchanged in the zygote, suggesting maternal inheritance of these miRNAs. The paternal miRNAs, brought into the zygote by the sperm, do not seem to contribute significantly to the total miRNAs in the zygote (Amanai *et al.*, 2006). Among the maternal miRNAs analysed at this stage of development, the most abundant are those belonging to the let-7 family, particularly the miR-17–92 cluster, previously demonstrated to be involved in cell proliferation (He *et al.*, 2005; O'Donnell *et al.*, 2005). The total amount of miRNA is down-regulated by 60% between 1-cell and 2-cell embryos, suggesting an active process of degradation that coincides with a global RNA degradation occurring at this time of development (Hamatani *et al.*, 2004); miRNAs are then expressed *de novo* during the passage from the 2- to the 4- and 8-cell stages, with an average increase of 2.2 times, but with 15-fold (4-cell embryos) and 24-fold (8-cell embryos) peaks for the miR-290 and miR-295 clusters. The single blastomeres of a 2-cell or a 4-cell embryo have the same miRNA expression profile. To investigate the role of these miRNAs during oocyte growth and preimplantation development, Tang *et al.* (2007) used mice with a deleted *Dicer* gene. Oocytes lacking the *Dicer* allele lost most of their miRNAs during folliculogenesis and although the females produced mature and morphologically normal eggs, most of them, after fertilization, failed to go through the first cell division. Furthermore, the expression of *C-mos* and *H2Ax* genes was higher in eggs and transcripts of *H1foo* and *SCP3* genes were up-regulated in 1-cell embryos, highlighting the effects on mRNA determined by incorrect expression of miRNAs. Loss of *Dicer* and miRNAs also affected the spindle organization and these oocytes were unable to complete meiosis (Murchison *et al.*, 2007). Another class of siRNAs, referred to as pseudogene-derived siRNAs, has been shown to regulate gene expression in mouse oocytes (Tam *et al.*, 2008; Watanabe *et al.*, 2008).

Conclusions

During the past 10 years, numerous studies have tried to identify non-invasive prognostic markers of oocyte or embryo developmental competence. Specific morphological features of the oocyte, the concentration in the follicular fluid of inhibin B, myo-inositol, AMH, estradiol, leptin, insulin growth factor binding (IGFB) proteins, caspase 3, lactoferrin or progesterone receptor, the level of expression of cumulus cell-specific genes and telomere length are some of the most investigated candidate quality markers.

The concentration of inhibin B (a member of the TGF-β family whose expression is stimulated by FSH) or myo-inositol (a serum trophic factor that promotes *in vitro* preimplantation development) in the follicular fluid has been correlated with and used as a predictor of human preimplantation embryo quality (Chang *et al.*, 2002; Chiu *et al.*, 2002). Other studies have been more critical with respect to the predictive value of these two markers and have compared their prognostic capability with that of AMH, suggesting that this latter is a better predictor, being associated with higher oocyte fertilizability (Takahashi *et al.*, 2008) and pregnancy rates (Hazout *et al.*, 2004). In contrast, another work described opposite results, indicating that while AMH is a good predictor of ovarian responsiveness, it is not a good marker of embryo quality or pregnancy (Smeenk *et al.*, 2007).

Various studies have reported that high levels of estradiol on the day of human chorionic gonadotrophin administration are associated with lower pregnancy rates (Chenette *et al.*, 1990; Sharara and McClamrock, 1999), whereas others found that this hormone has no effect on final pregnancy outcome (Simon *et al.*, 1995; Pellicer *et al.*, 1996). Instead, more recently, it was shown that the highest implantation and pregnancy rates are correlated with the concerted action, at specific concentrations, of both estradiol and leptin in serum and follicular fluid (Anifandis *et al.*, 2005).

The amounts of the low-molecular-weight IGFB proteins in the follicular fluid, when associated with the analysis of caspase 3 activity in cumulus cells (Nicholas *et al.*, 2005) or high concentrations of lactoferrin in the follicular fluid (Yanaihara *et al.*, 2007), were correlated with fertilization and preimplantation development success rate. A reduction of progesterone receptor protein and transcripts in human cumulus cells at the time of oocyte collection is also associated with morphologically good oocytes (Hasegawa *et al.*, 2005).

Another group of studies has investigated the expression of genes and proteins in the oocyte companion mural granulosa and cumulus cells. The oocyte-specific GDF-9 is one of the factors that cause cumulus cell expansion (Elvin *et al.*, 1999) by regulating the expression of some key cumulus genes involved in this process (Pangas and Matzuk, 2005). Higher levels of expression of *PTGS2*, *HAS2*, *GREM1* and *PTX3* cumulus cell-specific genes have been correlated with better oocyte developmental competence for fertilization and *in vitro* development (McKenzie *et al.*, 2004; Zhang *et al.*, 2005; Cillo *et al.*, 2007), although no correlation was found with final pregnancy outcome. The levels of expression of another group of cumulus genes (*GPX3*, *CXCR4*, *CCND2* and *CTNND1*) have been inversely correlated with human preimplantation embryo quality (van Montfoort *et al.*, 2008) and pregnancy outcome (Assou *et al.*, 2008). Along this line of investigation, a recent microarray analysis of the whole transcriptome of mural granulosa and cumulus cells isolated from single aspired follicles that resulted in a pregnancy and from follicles that led to embryos whose development was arrested at the preimplantation stage, identified a wide range of putative quality marker genes that will need further study (Hamel *et al.*, 2008).

In summary, different studies have reached contrasting and sometime even opposite conclusions and, so far, none of these quality marker candidates has been selected as a single, reliable predictor of oocyte and embryo developmental competence.

Apart from these studies, an interesting theory has recently been proposed linking the telomere length in eggs with developmental competence and IVF outcome. In most cells, telomeres are progressively shortened through cell divisions, resulting, when beyond a critical short length, in chromosome instability and cell senescence (Allsopp *et al.*, 1992). Telomere shortening, which depends on a number of variables including oxidative stress and mitochondrial dysfunction, also occurs in mammalian oocytes during female ageing, while they are arrested in prophase I. Oocytes of older women have shorter

telomeres than those of younger women (Keefe *et al*., 2005), a predisposition to aneuploidy (Hassold and Hunt, 2001), to form fewer chiasmata (Liu *et al*., 2004a, b) and to undergo apoptosis and cytosplasm fragmentation during preimplantation development (Keefe *et al*., 2005). Following fertilization, telomeres undergo telomerase-independent lengthening and the length of elongation may depend on the initial telomere length in oocytes (Liu *et al*., 2007).

With respect to the egg morphology, under a light microscope a human egg is classified as normal when it has an evident circular zona pellucida, a narrow perivitelline space, a single first polar body and cytoplasm with little granularity (Ubaldi and Rienzi, 2008). Several cytoplasmic dysmorphisms, such as the presence of vacuoles, a centrally located granular area, smooth endoplasmic reticulum clustering or refractile bodies, have been described and correlated with reduced egg fertilizability and developmental competence. However, the use of these morphological prognostic markers has not been free of discussion and criticism, suggesting that a phenotypic characteristic does not strictly and always correlate with the developmental competence of an egg (De Sutter *et al*., 1996; Xia, 1997; Balaban *et al*., 1998; Balaban and Urman, 2006; Ebner *et al*., 2006, 2008; Ubaldi and Rienzi, 2008).

In the search for oocyte-specific molecular markers, in this review we have taken an oocyte-centred view, with an emphasis on those molecular changes that occur in the female gamete during folliculogenesis and that represent the backbone on which its developmental competence is built up. The framework is still unclear, but interesting factors are emerging and are worth further investigation. These include some that are relevant to the growth of the oocyte itself, while others may be more specific to the acquisition of oocyte developmental competence, even though it is still difficult to draw a clear functional separation, as some of them may act at both levels. Our next challenge will be to dissect the complex interactions of the multifaceted scenario described and establish the presence of functional links among these factors. The use of animals lacking specific genes has already provided valuable information as to when during folliculogenesis the function of these genes is first exerted, other putative interconnected genes, and the gene expression networks and biochemical pathways altered, and will continue to be an indispensable tool for this type of studies. However, this method is insufficient when a given gene has an alternating pattern of expression during folliculogenesis (e.g. it is expressed, then inactivated and later expressed again) and/or when its protein plays distinct roles at different stages during oocyte growth, as may be the case for some of the genes and factors described in this review. Another strategy would be to interfere with the functionality of a gene at chosen time points during folliculogenesis, while preserving the integrity of the oocyte-follicle structure during its growth. To this end, an approach that we think will give an important contribution is a combination of follicle culture and micromanipulation procedures. Follicle-enclosed oocytes (isolated from the ovaries of model animals or humans) could be cultured *in vitro* (Gosden *et al*., 2002; Thomas *et al*., 2003; Picton *et al*., 2008) and, using micromanipulation techniques (as pioneered by Laurinda Jaffe's group; Jaffe *et al*., 2009), injected for example with plasmids containing specific gene sequences (to induce the expression of a specific gene), siRNAs (to inactivate RNA translation), antibodies (to inactivate the activity of proteins), signalling proteins (to interfere with signalling pathways), demethylating substances such as 5′-azacytidine or supravital fluorochromes (to follow changes of particular cellular or nuclear structures). Although with the limitations of an *in vitro* system, this approach could be used to follow and keep records of each single follicle/oocyte, which could, hypothetically, even be injected repeatedly during its growth. As an example of the use of this experimental approach, in our laboratory we are testing the hypothesis that Oct4 plays a functional role in the acquisition of oocyte developmental competence by micromanipulating its expression at different stages of oocyte growth, i.e. inactivating the activity of its transcripts in early growing SN oocytes and inducing the expression of the gene in NSN oocytes.

These functional experiments will help us to build up a sound molecular basis of the factors that are required to govern the acquisition of oocyte developmental competence.

Authors' roles

M.Z., V.M., S.C., C.A.R. and S.G. have contributed to conception, design, acquisition and interpretation of data. Drafting and revising the article critically, they all have given final approval of the version to be published.

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