Turathum and Sroyraya, Cell Dev Biol 2017, 6:3
DOI: 10.4172/2168-9296.1000189

Cell & Developmental Biology

Research Article Open Access

Protein Profile Involved in Mammalian Oocyte Maturation, Fertilization and Early Embryogenesis (Pre-Implantation)

Bongkoch Turathum[1] and Morakot Sroyraya[2,3]*

[1]Navamindradhiraj University, Bangkok, Thailand
[2]Department of Anatomy, Mahidol University, Bangkok 10400, Thailand
[3]Mahidol University, Nakhonsawan Campus, Nakhonsawan 60130, Thailand

Abstract

Proteomic analysis of oocytes can help identify proteins that are involved in female meiotic maturation and early embryonic development. Many proteins with well-defined functions have been identified during oocyte maturation. High levels of MPF, MAPK, Mos and low levels of cAMP play an essential role in the resumption of meiosis I. Following germinal vesicle breakdown, chromosome condensation and spindle formation occurred at metaphase I by assembly of the meiotic apparatus, which includes the proteins NuMA, γ-tubulin and Polo-like kinase 1. The metaphase II arrest is a result of high levels of MPF and MAPK. Proteins involved in the stress response and redox regulation, including peroxiredoxin, GST and HSF1, are also necessary for protection against oxidative stress. During fertilization, the sperm-egg interaction requires egg surface proteins, oocyte zona pellucida, molecular chaperones, GPI-anchored proteins and CD9 to recognize sperm proteins and prevent polyspermy. Following gamete fusion, resumption and complete of meiosis II is induced by GTP and CaM kinase II activation, which inactivates MPF and activation of the anaphase promoting complex/cyclosome results in sister chromatid separation. Decondensation of the sperm head begins after zona penetration and GSH and NPM2 are necessary for male pronuclear formation. MAPK inactivation is required for pronuclear formation. At the cleavage stage, the maternal effect proteins PADI6, FLOPED and FILIA are essential for embryonic progression past the two-cell stage. After cell adhesion, cell junctions and the cytoskeleton play an important role in compaction of the morula. Par6, Par3 and protein kinase C are components of the apical polarity complex and are important for formation of the blastocoel cavity. During the blastocyst stage, TEAD4 and CDX2 are required for trophoectoderm formation. This proteomic analysis of oocytes has improved our understanding of the molecular processes that regulate oocyte maturation, fertilization and pre-implantation in mammals.

Keywords: Proteomics; Oocyte maturation; Fertilization; Pre-implantation

Introduction

Mammalian oocytes are highly specialized cells with the ability to reconstruct sperm DNA and initiate zygotic development. During early fetal life, oogonia enlarge to form primary oocytes before birth. When a primary oocyte forms, connective tissue cells surround it and form a single layer of flattened, follicular epithelial cells. The follicular epithelial cells become cuboidal and columnar in shape, forming a primary follicle. Before birth, primary oocytes begin the first meiotic division. Oocytes remain at the diplotene stage in prophase of meiosis I after birth. During puberty, the primary oocyte increases in size and completes the first meiotic division to give rise to a secondary oocyte and the first polar body. The polar body is a small, nonfunctional cell that soon degenerates. At ovulation, meiotic division of the secondary oocyte begins, but arrests at metaphase II (MII). The mature MII oocytes are then ready for fertilization and to provide materials for early development of the embryo.

Development of the oocyte (meiotic maturation, fertilization and embryogenesis) is a complex process that involves the regulation of protein synthesis, phosphorylation and degradation. Cellular differentiation and maturation processes are identified by the expression of specific proteins. Moreover, protein activity can be affected by post-translational modifications. These modifications are controlled by specific enzymes, such as kinases and phosphatases. Post-translationally modified proteins are involved in many cellular processes, including cell differentiation, growth, meiosis and the cell cycle. The activation of some protein kinases plays an important role in meiotic maturation of oocytes. Recently, there have been several reports analyzing mammalian oocyte proteomics, including exploration of bovine, pig and mouse oocyte proteins. Recently, proteomics analysis was used to differentiate protein expression among the nuclear maturation, fertilization and early embryogenesis stages of oocytes [1-6]. This information will improve our overall understanding of oocyte meiosis and maturation.

The hallmarks of meiotic maturation in oocytes are (1) resumption of meiosis I, which includes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation, (2) the transition between meiosis I and II, which includes inhibition of S-phase and (3) arrest in MII as a result of cytostatic factor (CSF) activity. Meiosis II is completed after fertilization of the mature oocyte. Many proteins are upregulated at each stage of the oocyte. In this review, significant proteins upregulated during oocyte maturation are divided into three stages: GVBD, metaphase I (MI) and MII arrest. Proteins expressed during oocyte maturation (GVBD to MII) will be described below.

Materials and Methods

GVBD or resumption of the first meiosis

During the initiation of maturation, chromosomes begin to condense, the germinal vesicle breaks down and nucleoli disperse [7]. The processes of follicular development, meiotic resumption and subsequent ovulation in mammals are controlled by two pituitary-

***Corresponding author:** Morakot S, Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand, Tel: +66-2-2015418; Fax: 6623547168; E-mail: morakot.sry@mahidol.ac.th

Received November 22, 2017; **Accepted** December 07, 2017; **Published** December 14, 2017

Citation: Turathum B, Sroyraya M (2017) Protein Profile Involved in Mammalian Oocyte Maturation, Fertilization and Early Embryogenesis (Pre-Implantation). Cell Dev Biol 6: 189. doi:10.4172/2168-9296.1000189

derived glycoprotein gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Stimulation of meiotic resumption by gonadotropins occurs via their actions on the surrounding somatic cells rather than on the oocyte itself [8]. The actions of FSH and LH on mammalian oocyte meiotic resumption are believed to be mediated in large part by increasing the production of cyclic adenosine 3',5'-monophosphate (cAMP), which acts on the somatic follicle cells and promotes an increase in cAMP levels within the granulosa cell compartment and a decrease of cAMP in the oocyte, thus inducing the resumption of meiosis as well as cumulus expansion [9,10].

During GVBD, several proteins have been shown to regulate the resumption of meiosis, including maturation promoting factor (MPF), mitogen-activated protein kinase (MAPK), proto-oncogene serine/threonine-protein kinase mos (Mos), cAMP and protein kinase B (PKB) [3].

MPF is a hetero-dimeric protein composed of cyclin B and cyclin-dependent kinase (CDK1, also known as Cdc2 or p34 kinase) that stimulates the mitotic and meiotic cell cycles. This is an important activity that catalyzes entry into M-phase of meiosis I and II. While the function of MPF in promoting oocyte maturation is ubiquitous, MPF activation is stimulated by species-dependent differences in signaling pathways. MPF synthesis and degradation result in progression through the cell cycle. Nevertheless, MPF remains active and the cell is paused in metaphase if cyclin degradation is prevented [11]. When cyclin is degraded, the cell can return to S phase and begin DNA replication.

During oocyte maturation *in vitro*, MPF is important for chromatin condensation, nuclear membrane breakdown and spindle formation. MPF activation begins at GVBD, increases at MI, declines at the anaphase-telophase I transition and reaches a high level again at MII [3,12]. Murray et al. revealed that changes in MPF activity correspond with oocyte nuclear progression. They found that before the cell reaches MI, GVBD and chromosome condensation were induced by MPF activity and the reduction of MPF activity stimulates the metaphase-anaphase transition [11]. During oocyte maturation, the increase in MPF activity is controlled by active protein synthesis and phosphorylation. Phosphorylated MPF appears during meiotic resumption in cattle, sheep and mice and this phosphorylation plays a crucial role in the G2/M transition of mitosis and meiosis [13,14]. Activation of MPF is usually induced by dephosphorylation of Cdc2 and synthesis of cyclin B. These are present in an active complex in fully-grown pig oocytes [3]. In contrast, if MPF activation is prevented, GVBD is inhibited [3,15].

MAPK is involved in many signal transduction pathways and is activated during meiotic maturation in various species. MAPK could be involved in microtubule and chromatin organization during the transition between the two meiotic M-phases [16]. MAPK exists in an inactive form in the germinal vesicle (GV) stage and is activated at approximately the time of GVBD [3]. MPF and MAPK are stimulated at the time of GVBD and resumption of meiosis. Injection of MAPK into the GV oocyte markedly accelerates GVBD in a small proportion of oocytes [3,17]. Stojkovic et al. reported that MAPK mediates the maturation-inducing signal from the cytoplasm into the nucleus and induces re-initiation of meiosis and may play a role in nuclear envelope activity [18].

Moreover, it has been recognized that unidirectional communication from the cumulus cells to the oocyte mediated by gap junctions is a critical requirement for the initiation of oocyte maturation in response to gonadotropins [19]. Recent studies have shown that MAPK mediates LH-induced oocyte maturation by interrupting cell-to-cell communication through phosphorylation of connexin 43 within the ovarian follicle [20]. Breakdown of communication arrests the supply of cAMP from somatic cells to the oocyte, resulting in a decrease in the intra-oocyte concentration of this cyclic nucleotide and initiation of the first resumption of meiosis [21].

Proto-oncogene serine/threonine-protein kinase (Mos) is one of cytostatic factor (CSF). At ovulation, the cytoplasm of the oocyte contains CSF, which encourages meiotic arrest by maintaining MPF activity during MII [11]. In the MII mammalian oocyte, CSF and MPF activities remain at a high level, similar to GVBD. Mos play an important role in controlling meiotic arrest in oocytes, which is required for normal microtubule and chromatin organization during meiosis in mouse eggs [22,23]. During meiosis I and MII arrests of oocytes, MPF activation requires expression of the Mos protein [23]. The level of *mos* transcription in oocytes parallels the level of MAPK activation [16]. The Mos/MAPK pathway can promote early meiotic phenotypes in the absence of MPF and can activate and stabilize MPF [24]. The function of Mos/MAPK is characterized into two parts: the first part is to generate the second meiotic characteristics in cycling of the cell during suppression of S phase; the second is to produce an MII arrest with cdc2 so that the oocyte is competent for fertilization in bovine oocytes [25]. Mos is required for proper meiotic spindle formation.

cAMP is a second messenger important in many biological processes. During oocyte maturation, cAMP is transported into the oocyte from the cumulus via gap junctions, where it plays a crucial role in meiotic resumption. Generally, in the oocyte, the level of cAMP affects activity of the CDK/cyclin B protein complex. During the LH surge, oocytes maintain the meiotic arrest due to the high intracellular levels of cAMP. After the LH surge, the PKA signaling pathway is activated, resulting in low intracellular levels of cAMP and meiotic resumption [26]. Duckworth et al. revealed that phosphorylation and inactivation of CDK1 was controlled by high levels of cAMP in the oocyte [27]. A high level of cAMP is necessary for meiotic arrest within the oocyte. Inhibition of PKA, a cAMP-dependent protein kinase, also inhibits GVBD [3,28].

PKB (as known as AKT) is a key downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway. PKB is likely involved in the maturation of oocytes, probably independent of MPF activation. A peak in PKB activity has been observed at approximately the time of GVBD and activity of PKB declined at MI in pig oocytes [29]. Kalous et al. [29] found that PKB is related to CDK1 activation and participates in resumption of meiosis in mouse oocytes. At GVBD, phosphorylated PKB has been observed on the centrosome. This result shows that one function of PKB is to initiate the activation of CDK1 [29]. Furthermore, while the mechanism of PKB activation in mouse oocytes is not understood, it may be linked to the ability of high levels of cAMP to inhibit PKB activity [4]. In mouse oocytes, the expression of PKB promotes oocyte maturation and this process requires cGMP-inhibited cAMP phosphodiesterase 3A (PDE3A). PDE3A inactivation is needed for PKB/Akt-mediated phosphorylation. The regulation of PKB/Akt and PDE3A are important in the regulation of cAMP in rodent and amphibian oocyte maturation [30].

Metaphase I: Assembly of the meiotic apparatus

During MI, chromosomes move together along the metaphase plate. DNA replication, spindle function and chromosome segregation are controlled by the spindle assembly checkpoint [31]. The spindle apparatus includes the spindle microtubules, associated proteins and

any centrosomes or asters present at the spindle poles. The dynamic lengthening and shortening of spindle microtubules determines to a large extent the shape of the mitotic spindle and promotes the proper alignment of chromosomes at the spindle midzone [32]. Microtubule-associated proteins associate with microtubules at the midzone and the spindle poles to regulate their dynamics. γ-tubulin is a specialized tubulin variant that assembles into a ring complex called the γ-tubulin ring complex (γ-TuRC), which nucleates the polymerization of α/β tubulin heterodimers into microtubules. Recruitment of γ-TuRC to the pericentrosomal region stabilizes microtubule minus-ends and anchors them near the microtubule-organizing center. Microtubule motor proteins and cytoplasmic dynein are involved in spindle pole organization. Cytoplasmic dynein, a minus-end-directed motor, is necessary to efficiently focus microtubule minus-ends at spindle poles in a variety of animal systems [33,34]. Upon nuclear envelope breakdown, nuclear mitotic apparatus protein (NuMA) associates with microtubules and is driven to microtubule minus-ends by the minus-end-directed motor activity of cytoplasmic dynein. Plk and in particular Plk1, has important roles in spindle maintenance by regulating microtubule dynamics. The completion of spindle formation is a crucial transition point in the cell cycle, which is regulated by the spindle assembly checkpoint. Checkpoints are regulatory mechanisms that control DNA replication, repair spindle function and chromosome segregation with high fidelity [31]. Meiotic cells have two surveillance checkpoints: the first ensures that meiotic recombination is finished before assembly of the meiotic spindle is complete and the second forces anaphase I to wait until all paired homologues is correctly attached to the spindle. If even a single chromosome is not properly attached to the mitotic spindle, the onset of anaphase will be delayed. The M-phase checkpoints regulate meiotic maturation and progression through anaphase and telophase. Mad2, a component of the spindle checkpoint, delays the onset of anaphase until all chromosomes are attached to the spindle. In this review, proteins involved in assembly of meiotic apparatus composed of NuMA, γ-tubulin and Plk1.

NuMA is an abundant component of interphase nuclei and an essential player in mitotic spindle assembly and maintenance. With its partner cytoplasmic dynein, NuMA uses its cross-linking properties to tether microtubules to spindle poles. After GVBD, NuMA aggregates near the chromosomes and γ-tubulin is also localized to the area surrounding the chromosomes. At MI, NuMA is observed at both poles of the MI spindle, while γ-tubulin is localized along the spindle microtubules. These proteins then become re-localized at the spindle midzone during anaphase I and telophase I. These data suggest that NuMA and γ-tubulin are involved in microtubule assembly and at meiotic spindle poles [3,35].

Plk1 accumulates at the GV stage, is detected on the spindle poles at MI and MII and is translocated to the middle region of the spindle at anaphase-telophase. Plk is also found at the MII spindle poles and spindle fibers at anaphase-telophase. Its activity is necessary for the maturation of centrosomes in early prophase and establishment of a bipolar spindle [18]. Plk1 can also promote kinetochore attachment to microtubules and the spindle assembly checkpoint [36].

Metaphase II arrest

The MII arrest is characterized by a high level of MAPK and MPF activity. MPF is composed of a heterodimer of cdc2 and cyclin B and its function is stabilized by CSF, which consists of Mos, MAPK and p90Rsk [37]. An oocyte-specific protein kinase, c-mos, plays an important role in upregulating the activity of MPF at various stages of final oocyte maturation. Several studies have suggested that the proper function of the c-mos-MPF system is associated with important features of the last stages of oocyte maturation such as the resumption of meiotic maturation, inhibition of DNA replication between meiosis I and II and maintenance of the MII arrest until the oocyte is fertilized.

In addition to MPF and CSF, many other proteins are upregulated at this stage, including nucleoplasmin 2 (NPM2), redox regulation proteins, myomegalin and cAMP-specific 3',5'-cyclic phosphodiesterase 4D (PDE4D). These proteins are involved in redox regulation and the cAMP dependent signaling pathway, both of which have been linked to oocyte maturation. It has been reported that the peroxiredoxin (PRDX) and GST proteins, which are involved in intracellular redox regulation and protection against oxidative stress, were among the most highly abundant oocyte proteins for oocyte cytoplasmic maturation. PDE4D and myomegalin are subcellular targeting of the components of the secondary messenger cAMP-dependent pathway is thought to be essential for the intracellular signaling that leads to oocyte maturation by controlling the level of cAMP.

PRDX and GST proteins are involved in protection against oxidative stress and intracellular redox regulation and high levels of expression have been detected in oocytes [4]. In porcine oocytes, PRDX enzymes play a crucial role in maturation processes at the final stages [4]. GST is involved in anti-oxidative protection. In porcine oocytes, high level of GST protein expression at MII is required for oocyte maturation [4].

Myomegalin and PDE4D are important for cell signaling pathways that regulate the diffusion of cAMP and its ability affect PKA isoenzymes anchored to organelles [38].

Fertilization

During fertilization, sperm penetrate into the MII-arrested oocyte and trigger oocyte maturation by increasing intracellular calcium. High levels of intracellular calcium, which are essential for cellular signaling, lead to oocyte activation, resumption of meiosis, cortical reaction to block polyspermy and zygote development. Following egg activation and resumption of the cell cycle, sperm-egg fusion leads to sperm head decondensation and anaphase II plate formation, second polar body extrusion and recruitment and translation of maternal mRNAs [39]. The pronucleus (PN) is formed and its migration leads to the initiation of DNA synthesis during the one-cell stage of the embryo, which is the first time the oocyte will enter the meiotic prophase I of MI division [40]. Eventually, chromosomes are assembled at syngamy prior to mitosis and cytokinesis occurs to produce the two-cell stage.

Results and Discussion

Sperm-egg interaction

The zona pellucida of the egg binds the sperm. A glycoprotein in the zona pellucida, ZP3, was discovered to be responsible for egg/sperm adhesion in mice. Following the acrosome reaction, it is believed that the sperm remains bound to the zona pellucida through exposed ZP2 receptors. Fusion between the oocyte plasma membrane and the sperm follows, allowing entry of the sperm nucleus, centriole and flagellum, but not the mitochondria, into the oocyte. In mice, this fusion is likely mediated by the protein CD9 (the binding homolog). The egg "activates" itself upon fusion with a single sperm cell, thereby changing its cell membrane to prevent fusion with other sperm. Previous studies showed that the egg surface (oolemmal) proteins are involved in the sperm-egg interaction using two-dimensional polyacrylamide gel electrophoresis [3]. Important surface proteins identified in the oocyte include zona pellucida, molecular chaperones (calreticulin, calnexin and heat shock proteins [HSPs]), glycosyl-phophatidyniositol (GPI)-anchored proteins and CD9.

Oocyte zona pellucida proteins (ZPs) are first pointed on the oolemmal surface before moving to the zona pellucida matrix. In mice, the primary sperm receptor is ZP3, ZP2 is a secondary sperm receptor and ZP1 is a homologue of ZP2. The receptor galactosyltransferase (GalT) binds to the N-acetylglucosamine residues on ZP3 and is important for binding with the sperm and activating the acrosome reaction. In mammals, binding of the spermatozoon to GalT initiates the acrosome reaction. This process releases the enzyme hyaluronidase, which digests the matrix of hyaluronic acid in the vestments surrounding the oocyte. ZPs can be considered to act on zona pellucida as a secondary sperm binding receptor [41].

Molecular chaperones such as calreticulin, calnexin, HSP70 and HSP90 are present on the oocyte surface [42]. The molecular chaperones move from the endoplasmic reticulum (ER) to the surface of the cell and promote protein folding by binding nascent proteins in the ER that prevent misfolded and non-active protein aggregation. Calreticulin and calnexin are Ca^{2+}-binding chaperones in oocytes. In the guinea pig, calreticulin is localized in the cortical granules of oocytes. Tutuncu et al. (2004) reported that calreticulin plays a role in signal transduction and sperm egg activation following fertilization. HSPs are present in the zona pellucida during oocyte growth [43]. In *in vitro* fertilization of bovine oocytes, sperm-egg binding is reduced in the presence of anti-HSP70 and thus it has been suggested that HSP70 is involved in stimulating fertilization [42].

CD9 or motility-related protein-1 (MRP-1) is a member of the tetraspanin family, which is found on the oocyte surface. CD9 is essential for sperm-egg fusion. In assays of *in vitro* fertilization using Cd9 knockout (KO) oocytes, sperm could not penetrate and failed to fuse with the oocyte. Consequently, oocytes remain unfertilized and unfused sperm accumulated in the perivitelline space as a result of many sperm penetrating through the zona pellucida [17].

GPI-anchored proteins are present on the oocyte surface and involved in sperm-egg fusion. Removal of GPI-anchored proteins from the plasma membrane of oocytes resulted in highly decreased in rates of fertilization, but did not affect the binding of sperm and the zona pellucida. It has been reported that oocyte treatment with GPI inhibitors decreased sperm-oocyte binding and fusion [44].

Metaphase II-anaphase II transition: Resumption of the second meiosis

During resumption of the cell cycle, sister chromatids pass the anaphase-telophase transition and complete meiosis II by extrusion of the second polar body. After that, chromatin decondensation and formation of the pronuclei occurs. The important proteins involved in resumption of meiosis II are guanine nucleotide-binding regulatory proteins (G-proteins), Ca^{2+} calmodulin dependent protein kinase II (CaM kinase II), the anaphase promoting complex/cyclosome (APC/C) and separase.

G-proteins are a family of proteins involved in the transmission of chemical signals outside the cell as a result of intracellular changes. G-proteins can be activated by G-protein-coupled receptors which are transmembrane receptors. Microinjection of mRNA encoding a G-protein-coupled receptor in mature oocytes showed that the G-protein-coupled signal transduction system exists in pig oocytes. This pathway can stimulate a series of intracellular changes that trigger resumption of meiosis [45]. Inhibition of G-proteins leads to inhibition of sperm-induced calcium release and activation of the oocyte [46]. Moreover, previous studies have reported that overexpression of G-protein-coupled receptors stimulate phospholipase C, resulting in calcium oscillations and resumption of meiosis in mice and pig oocytes [46].

CaM kinase II is a multi-functional protein kinase localized in specific regions inside the cell. Tatone et al. reported that the present of CaM kinase II could be observed at the MII spindle and in the cortex of mouse oocytes [47]. Following *in vitro* fertilization, the activation of CaM kinase II transiently increased, reached its highest level at 1 h and remained at this high level 30 min later when most of oocytes had extruded the second polar body. In contrast, inhibition of CaM kinase II results in negatively affected inactivation of MPF, resumption of the cell cycle and exocytosis of cortical granules in fertilized oocytes [47].

The anaphasepromoting complex/cyclosome APC/C is an E3 ubiquitin ligase that targets cell cycle proteins for degradation. Before fertilization, mammalian oocytes are arrested in MII by CSF. The APC/C is inhibited by CSF to prevent the metaphase-anaphase transition and complete meiosis II. Cyclin B and CDK2 control the establishment of CSF and the Mos signaling pathway and are also involved in controlling CSF activity. Activation of the APC/C at metaphase targets destruction-box containing substrates, including securin and cyclin B1, allowing for completion of meiosis II.

Separase is a cysteine protease. It stimulates anaphase in meiosis II by hydrolyzing cohesin, the protein responsible for binding sister chromatids together during metaphase. The securin/separase/cohesin pathway regulates chromosome segregation during the meiotic metaphase-anaphase transition in meiosis II. Separase activation is required before degradation of securin, it's associated inhibitor. After degradation of securin, Separase activation cleaves cohesin, which is followed by separation of sister chromatids and initiation of anaphase [48].

Pronuclear formation

After activation of the oocyte by sperm, the second polar body is extruded [49]. The sperm head enlarges in the ooplasm and chromosome decondensation occurs. Decondensation of the sperm head occurs after zona penetration. Male pronuclear formation occurs at the same time as the nuclear membrane disappears. Chromosome decondensation and pronuclear membrane reformation from the oocyte ER is supported by the action of growth factors. Glutathione (GSH) and NPM2 promote male pronuclear formation. After fertilization, one of the roles of maternal factors is processing of the male genome, which is necessary for its involvement in embryogenesis. At the pronuclear stage, zygote arrest 1 (ZAR1) is a maternal factor involved in the egg-embryonic transition. Thus, the important proteins involved in the pronuclear stage are GSH, NPM2 and ZAR1.

In the oocyte cytoplasm, GSH reduces disulfide bonds and leads to sperm chromatin uncoiling. Thus, GSH may play a role in decondensation of sperm chromatin [50].

Nucleoplasmin 2 (NPM2) plays an important role in sperm decondensation during fertilization. After fertilization, NPM2 is found in pronuclei and in the embryo nuclei and then drop down after embryos reach the eight-cell embryos. Sun and Nagai [3] reported that the expression of NPM2 is to maintain phosphorylation during the pronuclear stage and de-phosphorylation in the two-cell embryo. In addition to hyperphosphorylation of NPM2 during maturation of the oocyte, this process activates its nuclear to cytoplasmic targeting along with histone replacement in the sperm and decondensation.

ZAR1 is an oocyte specific cytoplasmic protein of unknown function. Absence of ZAR1 in genetically ablated females affects pronuclear syngamy (one-cell stage), a process that is important for embryo progression into the one-cell stage [15]. It has recently been

shown that ZAR1 KO females are infertile and produce distinct pronuclei [15].

Pre-implantation

During pre-implantation development, there are cleavage divisions of the zygote, which form the two-, four- and eight-cell stage embryos, followed by the morula and finally the blastocyst. Early embryonic development is characterized by a series of defined periods of gene expression, including: degradation of maternal transcripts, activation of the zygotic genome, activation of the mid-preimplantation genome, morula compaction and blastocyst cavitation. Important proteins play essential roles during early embryo development, which is divided into three stages: cleavage, morula and blastocyst.

Cleavage stage

Cleavage is the first of three cell divisions in embryonic development prior to compaction. A maternal effect occurs when the phenotype of the progeny is determined by the genotype of the mother. Maternal effect proteins are synthesized by maternal effect genes and are important in early embryonic development. Beyond the effect of individual maternal effect proteins, a subcortical maternal complex assembles during oocyte growth and is essential for embryonic progression past the two-cell stage. Other maternal proteins also involved in the early two-cell stage are FILIA, factor located in oocyte permitting development (FLOPED) and peptidyl arginine deiminase, type VI (PADI6).

It was first demonstrated in mice that the protein level of FILIA was reduced in Mater KO oocytes. Previous studies have described FILIA function and oocytes derived from FILIA KO mice exhibited a 50% decrease in fertility. This was shown to be due to a defect in development during pre-implantation, which delayed progression of the cell cycle in the absence of FILIA. Moreover, FILIA is important in the regulation of mitotic kinase activity, activation of the spindle assembly checkpoint and chromosome stability [51].

FLOPED is present in the cortical region of the oocyte and early embryo and then disappears after the blastocyst stage. FLOPED KO mice primarily arrest at as two-cell embryos, with a few embryos developing into the four-cell stage [52]. The embryos of FLOPED KO females arrest at the two-cell stage with progressive degeneration thereafter [53]. The first cleavage in embryos derived from FLOPED KO females was delayed and asymmetrical, resulting in blastomeres of unequal sizes. These data demonstrated that FLOPED plays a key role in development of the zygote.

The expression of PADI6 is generally restricted to oocyte and early embryonic development. PADI6 expression begins in the primodial follicle and persists, at the protein level, throughout development of the embryo to the blastocyst stage [54]. In mouse, it has been shown that inactivation of PADI6 leads to infertility in female mice, with an arrest at the two-cell stage. This result suggested that PADI6 contributes to development of the early embryo.

Morula

In mammalian development, the process of first cell differentiation and compaction appear in the late eight-cell stage. Loose cells become more adhesive and develop into the strictly organized cell mass of the morula. Within the morula, blastomeres gain a tighter contact with each other by an increase in intercellular adhesions and adopt a more flattened morphology, a process known as compaction. Several proteins, including cell adhesion, cell junction and cytoskeletal proteins have been shown to be upregulated in the morula stage compared with the four-cell stage. Cell adhesion, cell junction and cytoskeleton proteins play an important role in the process of compaction, but most of the molecules involved in this developmental stage have not yet been characterized. However, it is already known from the mouse model that E-cadherin is a major component of adherens junctions, involved in regions of cell-cell contact. During morula compaction, tight junction (TJ) proteins attach to the outer cells of the epithelial differentiation in the trophoectoderm (TE) lineage [55]. TJs and adjacent TE cell attachment is required for the integrity of epithelial cells and blastocoelic cavity production by processing of transepithelial ion transport. Various cytoskeletal, cell adhesion and junction proteins have been shown to be upregulated during the morula stage compared with the four-cell stage. These proteins are involved in compaction and early developmental processes. Par6 is the most interesting cell junction protein expressed at the morula stage, which may provide additional information about the early processes of development and compaction. During blastocyst formation, the inner cell mass (ICM) and the TE generates the embryonic-abembryonic polarity. In the mouse, it is known that eight-cell stage embryos start to compact and begin asymmetric distribution of the Par6/aPKC complex. High levels of Par6 are involved in establishment of the cell's polarity during compaction.

E-caherin switches to regions of cell-cell contact at the eight-cell stage due to protein kinase C (PKC) and other signaling molecules. E-cadherin is involved in compaction and is linked to the cytoskeleton and is localized in areas of cell contact. Maternal E-cadherin is involved in the initial compaction of the morula but not in further preimplantation development. Oocytes lacking E-cadherin give rise to embryos whose blastomeres do not adhere during compaction at the morula stage and the zona pellucida serves to maintain blastomere proximity [56].

Partitioning defective proteins (Par3 and Par6) and PKC are components of an apical polarity complex that has been shown to influence TE/ICM fate choice. Par3 and Par6 are cell junction proteins that are upregulated during the morula stage. This apical-basal polarity can be seen in compaction of the eight-cell stage embryo and through the localization of known polarity markers from other organisms and developmental contexts, including Par3, Par6 and PKC [57]. In the mouse embryo, Par3 and aPKC homologs regulate the orientation of cell cleavage planes as well as cell polarity and adhesion, which together can influence the allocation of blastomeres to an outer or inner position in the blastocyst. Par6 is essential for normal blastocyst formation in the mouse embryo [58]. Par6 gene knockdown resulted in cavitation failure without compromising blastomere cleavage or compaction by abnormal epithelial junctions, which then lead to defective paracellular permeability sealing in the outer cells of the embryo. Increased Par6 expression is involved in establishing a cell's polarity during compaction.

Blastocyst

During blastocyst development, the outer cells develop into TE cells, which are progenitor cells of the placenta, while the inner cells form the ICM from which the embryo develops. Furthermore, a fluid-filled cavity, the blastocoel, is formed by the merging of intercellular spaces and water movement into the embryo. Attachment of TJs and adjacent TE cells is essential for the integrity of epithelial cells and production of the blastocoelic cavity by processing of transepithelial ion transport. Formation and expansion of the blastocoel depends on aggregation of membrane/ion transporters to both the apical and basolateral domain. In addition to membrane aggregation, the maintenance of epithelium integrity also requires mature junctional complexes [59]. Many proteins are upregulated during blastocyst formation compared with

the morula stage, including membrane traffic, ion channel, electron transporter and transfer/carrier proteins. Cytoskeleton and cell adhesion protein assembly combined with TJ formation is restricted to the outer cells. Blastocoelic cavity generation by processing of transepithelial ion transport depends on TJs. Water osmotic transport across the epithelium is thought to be regulated by Na^+/K^+-ATPase [60]. It has been reported that active K^+ transport occurs primarily at the apical membrane. Moreover, electron transporters such as NADH dehydrogenase subunits have been characterized, which may be related to mitochondrial function. Electron transporters are required to maintain mitochondrial activity during blastocyst formation. Glucose transporter 3 (GLUT3) is essential for the transport of maternal glucose across the apical membrane of the TE and is involved in blastocelic cavity formation. In addition to TE and ICM separation, TEAD4 and Caudal-related homeobox 2 (Cdx2) are important for TE formation at the blastocyst stage. Thus, several proteins are important at the blastocyst stage, including Na^+/K^+-ATPase, GLUT3, TEAD4 and Cdx2.

Na^+/K^+-ATPase is an ion transport protein that plays an important role in mediating blastocyst formation. In the rabbit, Na^+/K^+-ATPase is synthesized at a high level during preimplantation development at a period characterized by rapid accumulation of fluid in the blastocyst. The β1 subunit of the Na^+/K^+-ATPase is essential for formation of the blastocyst and is also required to maintain normal Na^+/K^+-ATPase localization and distribution of TJ-associated polypeptides during preimplantation development. Deletion of this β1 subunit gene has suggested that the Na^+/K^+-ATPase is linked to abnormal blastocyst formation *in vitro* and likely peri-implantation lethality *in vivo* and also indicated that Na^+/K^+-ATPase regulates the formation and function of TE tight junctions [60].

GLUT3 has been shown to localize on the apical membranes of polarized cells of the morula and the apical membranes of TE cells of the blastocyst, where it has access to maternal glucose [61]. Inhibition of GLUT3 using antisense oligodeoxynucleotides confirmed that GLUT3 is essential for the transport of maternal glucose on the apical surface. The expression of GLUT3 is necessary for blastocyst formation and primary differentiation in mammalian development. There is evidence that GLUT3 is involved in the apical glucose transporter as partial ablation of GLUT3 during the blastocyst stage results in a reduction of more than 40% of glucose uptake. This result suggested that GLUT3 is the functional transporter of glucose by the apical TE. Loss of GLUT3 expression inhibited blastocyst formation while the expression of GLUT3 was sufficient to permit blastocyst formation in mouse embryos. Thus, the secondary function of GLUT3 is important for blastocyst formation, which is necessary for normal embryonic development.

TEAD4 is a ubiquitously expressed transcription factor. TEAD4 is found in blastocysts from rats, cattle, rhesus monkeys and humans. TEAD proteins control various developmental processes, such as skeletal muscle regeneration, skeletal and cardiac muscle development, notochord development and neural crest development. TE Na^+/K^+-ATPase development requires TEAD4 activation, in a manner that suggests it functions upstream of Cdx2, separating the ICM from the TE. **Tead4** KO embryos resulted in defects specifically in TE lineage delineation and the embryos died at early stages without forming the blastocoel cavity [62].

Cdx2 appears to play an essential role in TE specification and its expression may be regulated by the transcriptional regulator TEAD4 [62]. Cdx2 is found only in the TE. Interestingly, in the absence of *Cdx2*, TE cell identity could not be sustained in the blastocyst. In mice, KO of the *Cdx2* gene allows compaction, but TE epithelial integrity cannot be established at the late blastocyst stage. *Cdx2* KO embryos exhibit increased evidence of programmed cell death during the blastocyst stage by blastocoel formation is occurred in *Cdx2* mutants but epithelial integrity is not retained and the embryos fail to implant. Loss of *Cdx2* showed failure to downregulate *Nanog* and Oct 4 in outer cells of the blastocyst, following which cell death was observed. Hence, *Cdx2* is necessary for separation of the TE and ICM lineages at the blastocyst stage [63].

During pre-implantation, epigenetic profiling of the embryo is also dramatically changed. Histone modifications, including methylation, acetylation, phosphorylation and ubiquitylation are involved in the regulation of gene transcription [64]. X chromosome

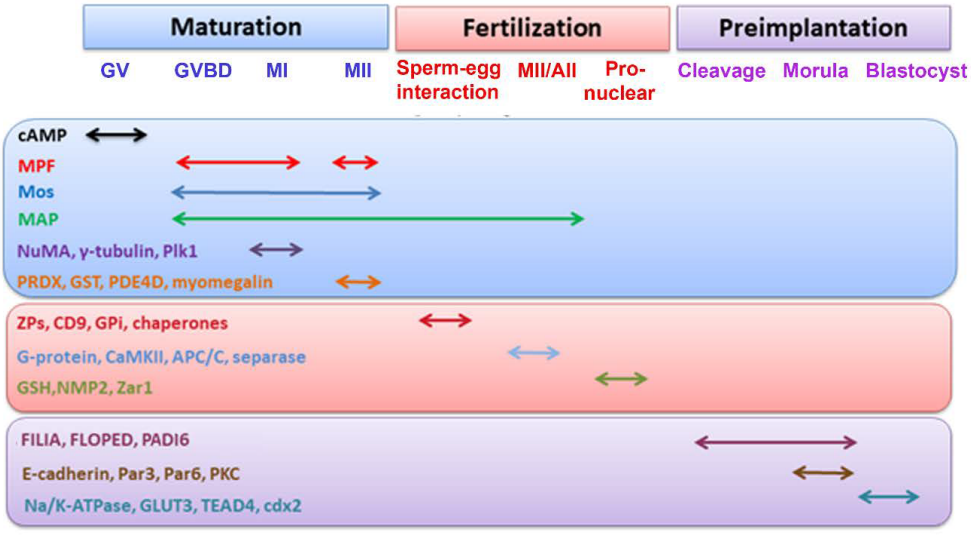

Figure 1: Schematic of proteins whose expression is increased to control oocyte maturation, fertilization, and early embryo development.
GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; MI: Metaphase I; MII: Metaphase II; AII: Anaphase II

Protein	Meiotic maturation				Fertilization			Pre-implantation		
	GV	*GVBD*	*MI*	*MII*	*Sperm-egg interaction*	*MII/AII transition*	*Pro-nuclear*	*Cleavage*	*Morula*	*Blastocyst*
TACC3	++	+	+	+						
HSP70	++	+	+	+						
HSP90	++	+	+	+						
HSP105	++	+	+	+						
STI1	++	+	+	+						
LCN1	++	+	+	+						
Lysozyme1	++	+	+	+						
CTPs	++	+	+	+						
MPF	+	+++	+	+++						
MAP	+	+++	+++	+++						
Mos	+	+++	+++	+++						
cAMP	++	+	+	++						
UCHL1	+	++	++	++						
H1K	+	++	+++	+++						
HSF1	+	++	++	++						
PKB	+	+++	+	+						
NuMA	+	+	++	++						
γ-tubulin	+	+	++	++						
Plk1	+	+	++	++						
NPM2	+	+	+	+++			+++			
PRDXs	+			+++						
GST	+			+++						
Myomegalin	+			+++						
PDE4D	+			+++						
PRKA	+			+++						
ZPs				+++	+++					
Calreticunin				++	++					
Calnexin				++	++					
CD9				++	++					
GPI-anchor				++	++					
CaMKII				+	++	++	+			
APC/C				+	+	+++				
Separase				+	++	+++				
G-protein				+	++	+++				
GSH						+	+++			
ZAR1							+++			
MATER				+	+	+	+	+++		
FILIA				+	+	+	+	+++		
FLOPED							++	+++		
PEDI6				++				+++		
TCL1								+++		
E-caherin								+	+++	
PKC								+	+++	
Par3								+	+++	
Par6								+	+++	
Na/KATPase								+	+	+++
NADH								+	+	+++
GLUT3								+	+	+++
TEAD4								+	+	+++
CDX2								+	+	+++

Table 1: Summary of protein expression during meiotic maturation, fertilization and pre-implantation.
GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; MI: Metaphase I; MII: Metaphase II; AII: Anaphase II

activity is changed dramatically during pre-implantation and it's inactivated by combined with other epigenetic events such as DNA methylation and histone modifications [65]. During pre-implantation stages in embryogenesis, DNA methylation is also dynamically changes. In the mouse embryo, after fertilization paternal and maternal pronuclei (PN) are formed. The paternal PN are demethylated, while DNA is active demethylation during the later cleavage stages [66]. The embryo continue the cell differentiation process, accompanied by DNA methylation, which cause stable silencing of genes involved the maintenance of pluripotency [66].

Conclusion

Fundamental discoveries of oocyte biology have provided insight on the regulation of molecular pathways controlling oocyte maturation, fertilization and early embryonic development. A summary of protein expression during meiotic maturation, fertilization and preimplantation is shown in Figure 1 and Table 1. Meiotic maturation of mammalian oocytes is the process of functional connection and interaction of many proteins important for fertilization and early embryonic function. During meiotic maturation (GV to MII), resumption of meiosis I, which includes GVBD, is the first evidence to drive oocyte at prophase I arrest

reach to metaphase I stage. High levels of MPF, MAPK and Mos and low levels of cAMP play an essential role in the resumption of meiosis I. The level of cAMP is regulated by its components, myomegalin, PDE4D and PRKA. Following GVBD, chromosome condensation and spindle formation occurred at MI by assembly of the meiotic apparatus, which includes NuMA, γ-tubulin and Plk1. Immature oocytes then pass to the meiosis I/meiosis II transition and become mature at MII with the first polar body and await fertilization. High levels of MPF and MAPK cause the MII arrest. In addition to these proteins important for meiotic maturation, proteins involved in the stress response and redox regulation are also necessary to protect against oxidative stress, including peroxiredoxin, GST and HSF1.

During fertilization, the fusion of two gametes is required for the generation of a new organism. Sperm-egg interaction is the first step of fertilization that requires egg surface proteins, oocyte zona pellucida, molecular chaperones, GPI-anchored proteins and CD9, which recognize sperm proteins and prevent polyspermy. After gamete fusion, resumption and completion of meiosis II is induced by GTP activation, which results in a transient increase in Ca^{2+} and CaM kinase II activation, which then results in inactivation of MPF and activation of the APC/C and sister chromatid separation. Decondensation of the sperm head begins after zona penetration and GSH and NPM2 are necessary for male pronuclear formation. MAPK inactivation is required for pronuclear formation. At the pronuclear stage, ZAR1 is a maternal factor that is involved in the egg-embryonic transition.

At the cleavage stage, the maternal effect proteins, MATER, PADI6, FLOPED, TCL1 and FILIA are essential for embryonic progression past the two-cell stage. Following the cleavage stage, cell adhesion, cell junction and cytoskeletal proteins play an important role during compaction of the morula. Apical-basal polarity can be seen in compaction of the morula and Par6, Par3 and PKC are components of the apical polarity complex and are important in formation of the blastocoel cavity. During the blastocyst stage, TEAD4 and CDX2 are required for TE formation. Membrane traffic, ion channel, electron transporter and transfer/carrier protein are important for formation of the blastocoel cavity.

This knowledge will be useful for the reprogramming of stem cells in regenerative medicine. In patients with fertility disorders such as oocyte maturation arrest, premature ovarian failure and especially infertility conditions with unknown reasons, the identification and association of alterations of the patterns of specific factors in these particular conditions may help to initiate new treatments. Finally, the assessment of protein expression may be a potential use for the generation of drugs to improve fertility. On the other hand, because *in vitro* generation of a large source of mature eggs for treatment of infertile women with reduced ovarian reserves or cancer patients with cryo-preserved ovaries remains an obvious challenge, more data on *in vitro* regulation are acquired. Future studies might aid in improving culture protocols for *in vivo* culture of mammalian oocytes and embryos to be used for assisted reproduction.

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