Vertebrate Reproduction

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SUMMARY

Vertebrate reproduction requires a myriad of precisely orchestrated events—in particular, the maternal production of oocytes, the paternal production of sperm, successful fertilization, and initiation of early embryonic cell divisions. These processes are governed by a host of signaling pathways. Protein kinase and phosphatase signaling pathways involving Mos, CDK1, RSK, and PP2A regulate meiosis during maturation of the oocyte. Steroid signals—specifically testosterone—regulate spermatogenesis, as does signaling by G-protein-coupled hormone receptors. Finally, calcium signaling is essential for both sperm motility and fertilization. Altogether, this signaling symphony ensures the production of viable offspring, offering a chance of genetic immortality.

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1 INTRODUCTION

Mammalian reproduction depends on the proper development and maturation of both the female egg and the male sperm. These gametes fuse through a complex series of events, known as fertilization, that ensure the highest quality of offspring. Both gamete development and fertilization depend on numerous connected signaling pathways, a flaw in any of which can lead to infertility or birth defects.

The egg and sperm are haploid germ cells that, upon fertilization, reconstitute a diploid cell—the embryo. Production of haploid gametes from diploid precursors requires a modified cell cycle known as meiosis. Before meiosis, the full complement of parental chromosomes is first duplicated in S phase, to produce so-called sister chromatids (i.e., four copies of each chromosome per cell) and paternal and maternal chromosomes pair up. The homologous chromosomes from each chromosome pair are then separated in the first meiotic M phase (meiosis I, also known as MI). Subsequently, without further replication, the cells reenter M phase (meiosis II, also known as MII) to divide the sister chromatids equally into four haploid daughter cells. For male gametes, four mature sperm are generated, whereas in the female, a single final gamete (the egg) is produced together with three polar bodies. Movement through these stages of meiosis is carefully controlled by kinases, phosphatases, ubiquitin-dependent degradation of key regulators, and calcium flux.

Before acquiring the capacity to fertilize eggs, a sperm must reside in the female reproductive tract and undergo physiological changes that render it fertilization competent (Bedford 1970). The acquisition of fertilization competence and the biochemical, membrane, and enzymatic changes that underlie it are collectively known as capacitation (Austin 1951; Chang 1951). As with gamete development and maturation, capacitation and fertilization depend on careful regulation through signaling pathways. These include pathways involving gonadotropins, G-protein-coupled receptors (GPCRs), kinases, and calcium signaling (Salicioni et al. 2007).

2 OOCYTE MATURATION

Oocyte maturation has been most extensively studied in the frog *Xenopus laevis*, because its very large oocytes allow both physical manipulation of the cell (microinjection of proteins, RNAs, and antisense oligonucleotides) and observation of the progression through meiosis with the naked eye. Although some notable differences have been observed, genetic studies in mammals (primarily mouse) have revealed similar overall regulation of meiotic progression (Fig. 1).

Oocytes and sperm both begin life as primordial germ cells (PGCs) that migrate to the nascent gonads (ovaries in females, testes in males) in early embryonic development. Under the influence of a variety of cytokines and growth factors, PGCs that will become oocytes continue dividing mitotically within cell clusters. In oogenesis, the premeiotic S phase is followed by a prolonged arrest in prophase I of meiosis until sexual maturity. During this phase, the oocyte is maintained in a G₂-phase-arrested state through G-protein-coupled signaling (see below). When mitosis ceases, these oocytes each become surrounded by somatic granulosa and theca cells, which form the primordial follicles that serve as repositories of dormant oocytes for later ovulation. Oocytes nestled within the follicles grow and stockpile nutrients until they become competent to undergo maturation; upon receipt of appropriate hormonal signals, one follicle from the larger pool will mature fully during each menstrual cycle in the mammal. Stimulated by pituitary hormones (gonadotropins) and as a consequence of maturation-inducing steroid hormones (e.g., progesterone) synthesized by the ovarian follicle cells, the oocyte exits prophase arrest, and progresses through MI, transitioning promptly to MII without any intervening DNA replication. At MII, the oocyte arrests again awaiting fertilization.

The end product of oocyte maturation is a haploid egg capable of being fertilized. A strong MII arrest helps to prevent parthenogenesis, which is the aberrant entry of the haploid egg into the mitotic cell cycle in the absence of fertilization. In an effort to define the factors responsible for MII arrest, Masui and colleagues injected extract prepared from a mature M-phase-arrested frog egg into blastomeres formed after the first embryonic cell division (Masui and Markert 1971); injected cells remained arrested in M phase, whereas the uninjected cells continued to divide. These experiments helped to identify both maturation promoting factor (MPF), which drives entry into both MI and MII during oocyte development, and the cytostatic factor (CSF), which maintains MII arrest. We now know that MPF is equivalent to the complex of cyclin B and cyclin-dependent kinase 1 (cyclin-B–CDK1) that drives entry into mitosis in the somatic cell cycle (Dunphy et al. 1988; Labbe et al. 1989; Rhind and Russell 2012). CSF was shown to be the kinase Mos, the cellular counterpart of the viral oncoprotein v-Mos, which is expressed primarily in germ cells (Propst et al. 1987; Sagata et al. 1989). Proper maturation from MI entry through MII arrest depends on tightly controlled temporal regulation of both cyclin-B–CDK1 and Mos activity.

2.1 Meiosis I

During MI, oocytes are maintained in the G₂-arrested state by high levels of cytosolic cAMP. Constitutive signaling by

Figure 1. Regulation of oocyte maturation. Immature oocytes are held in G₂ arrest through the activity of PKA, which is stimulated by GPR3-dependent production of cAMP. Progesterone signaling leads to a loss of PKA activity, leading to disinhibition of the CDK1 activator Cdc25 and stimulation of the CDK1 inhibitor Wee1. Additionally, progesterone stimulates translation of both Mos and cyclin B proteins (the former also stimulating translation of the latter) through induction of Eg2, which phosphorylates and activates CPEB to unmask the messages and promote their polyadenylation. In parallel, progesterone stimulates translation of RINGO, which can activate CDK1 independently of cyclin and make it phosphorylate and inhibit the CDK1 inhibitor Myt1. All of these events result in activation of MPF, which drives the oocyte into MI. Maturation continues via the Mos-MEK-ERK pathway as shown. Blockade of APC (anaphase-promoting complex) activity by CSF and Emi2 holds the mature oocyte at a second arrest until the time of fertilization.

the GPCR GPR3 probably stimulates adenylyl cyclase to keep cAMP levels high. Indeed, overexpression of GPR3 in frog oocytes makes them resistant to the maturation effects of progesterone, and in mice lacking GPR3, oocytes mature in the absence of additional stimuli (Freudzon et al. 2005; Hinckley et al. 2005; Mehlmann 2005; Deng et al. 2008). Although sphingosine 1-phosphate and sphingosylphosphorylcholine have been proposed as GPR3 ligands, unliganded GPR3 appears to be able to stimulate adenylyl cyclase and thus the role of GPR3 in maintenance of G₂ arrest is not entirely clear (Eggerickx et al. 1995; Uhlenbrock et al. 2002; Hinckley et al. 2005). Progesterone stimulation seems to antagonize the GPR3 signal, triggering

a decrease in cAMP levels that is at least partly mediated by stimulation of phosphodiesterases that degrade cAMP (primarily PDE3 in the oocytes) (Tsafirri et al. 1996). This leads to a diminution of protein kinase A (PKA) activity. If PDE3 is artificially inhibited or cAMP synthesis is artificially stimulated, progesterone-induced maturation can be blocked. Conversely, injection of oocytes with the PKA inhibitor PKI can promote resumption of meiosis even without progesterone stimulation (Stanford et al. 2003).

As in the mitotic cell cycle, cyclin-B-CDK1 activity is controlled by phosphorylation of CDK1 on Y15 by Wee1-family kinases, which is opposed by the Cdc25 phosphatase (Watanabe et al. 1995; Berry and Gould 1996). The Wee1

relative Myt1 contributes to suppressing CDK1 phosphorylation, and the Cdc25c isoform mediates its subsequent dephosphorylation. In mice, this process is mediated by oocyte-specific isoforms: WEE1B and CDC25B. Loss of WEE1B irreversibly arrests oocytes in prophase (Han et al. 2005). In the G₂-arrested oocyte, PKA directly phosphorylates Cdc25 on S287 (*Xenopus* numbering), promoting the binding of the small acidic protein 14-3-3 (Duckworth et al. 2002). 14-3-3 interferes with the ability of Cdc25 to interact with and dephosphorylate cyclin-B-CDK1 and prevents its translocation into the nucleus, where it would promote rapid cyclin-B-CDK1 activation (Kumagai and Dunphy 1999; Lopez-Girona et al. 1999; Yang et al. 1999). Thus, the drop in PKA activity required for maturation promotes Cdc25 activation. PKA also phosphorylates and activates Wee1/Myt1 (Stanford and Ruderman 2005); so the drop in PKA also promotes CDK1 activation by alleviating its suppression by these kinases.

The formation and activity of MPF is also regulated by translation of cyclin B, whose mRNA is translationally dormant before the induction of oocyte maturation owing to its very short poly-A tail. At the time of oocyte maturation, *cis*-acting sequences within the 3' UTR of the cyclin B mRNA promote cytoplasmic polyadenylation, elongating the tail more than 100 nucleotides. These cytoplasmic polyadenylation elements (CPEs) within the 3' UTR of the mRNA are bound by CPE-binding protein (CPEB) (Hake and Richter, 1994). Through a process that is not entirely clear, the drop in PKA activity that heralds the onset of oocyte maturation also induces activation of a kinase, Eg2, which phosphorylates CPEB, activating it to both unmask the mRNA and recruit a poly(A) polymerase to elongate the poly(A) tail (Andresson and Ruderman 1998; Frank-Vaillant et al. 2000; Hodgman et al. 2001).

Note that in every species there is at least some pre-formed cyclin-B-CDK1 complex (known as pre-MPF) whose activity is suppressed by phosphorylation of CDK1 at T14 and Y15. Indeed, the initial discovery of MPF relied on the ability of the injected MPF to mobilize the pre-MPF pool through autoamplification (Masui and Markert 1971; Drury and Schorderet-Slatkine 1975; Wasserman and Masui 1975). Phosphorylation by cyclin-B-CDK1 suppresses Wee1/Myt1 and activates Cdc25, which promotes more conversion of pre-MPF to MPF. In species in which most of the CDK1 is bound to cyclin B in pre-MPF complexes, new cyclin B translation is not absolutely required for induction of oocyte maturation; however, in those species that have low amounts of pre-MPF and high levels of free CDK1, cyclin B synthesis is an obligate step in maturation (Jagiello 1969; Fulka et al. 1986; Moor and Crosby 1986; Hunter and Moor 1987; Gautier and Maller 1991; Mattioli et al. 1991).

In mammals, the signal emanating from a loss of PKA activity may be conveyed directly to CDK1 via Wee1B, as this appears to be a direct PKA target. For Myt1, there is evidence for indirect pathways of inhibition. First, soon after progesterone treatment, a non-cyclin alternative activator of CDK1 known as RINGO is translated (Ferby et al. 1999). This protein can bind to and activate CDK1, causing it to phosphorylate and suppress Myt1 (Ruiz et al. 2008). This, in turn, leads to activation of cyclin-B-CDK1 complexes. A second pathway is an oocyte-specific MAP kinase (MAPK) cascade involving the MAPKKK Mos, the MAPKK MEK, and the MAPK ERK. In frogs, the terminal effector in this pathway is RSK, which can phosphorylate and inhibit Myt1 (Palmer et al. 1998). RSK can also phosphorylate Cdc25, contributing to its activation. Accordingly, injection of activated RSK into *Xenopus* oocytes can induce meiotic maturation and RSK inhibition interferes with progesterone-induced maturation. In mice, alternative pathways must operate (e.g., the direct inhibition of WEE1B by PKA, as described above), because mice lacking all known isoforms of RSK do not show defects in oocyte maturation (Dumont et al. 2005).

In some species, including *Xenopus*, activation of the Mos-ERK pathway precedes completion of MI and breakdown of the nuclear envelope (known as germinal vesicle breakdown [GVBD] in oocytes). In others, it occurs after GVBD (because cyclin-B-CDK1 can actually activate ERK). Whether the Mos-MEK-ERK-RSK pathway is involved at MI depends on the organism, and Mos accumulation is controlled by multiple mechanisms (reviewed in Fan and Sun 2004). The 3' end of the Mos mRNA in the immature oocyte has a short poly(A) tail whose elongation (necessary for efficient translation) is masked through binding of CPEB. In addition to the Eg2-induced phosphorylation of CPEB, which unmasks and enhances the translation of Mos (Mendez et al. 2000), the stability of Mos protein is greatly enhanced by phosphorylation at S3 as both dephosphorylation of this residue and the presence of a proline at residue 2 are required for recognition by the ubiquitin-proteasome degradation system (Nishizawa et al. 1993). This site is phosphorylated in a positive-feedback loop by ERK, which stabilizes Mos. Where Mos accumulates only after GVBD, it is phosphorylated at the same site by cyclin-B-CDK1. Together, increased translation and stabilization promote Mos accumulation during maturation. Note that, in *Xenopus*, redundant pathways allow MI progression even when Mos is ablated; yet, the kinetics are delayed, indicating that Mos normally enhances meiotic progression in this species. Indeed, when either cyclin B synthesis or Mos synthesis is impaired, progesterone-induced GVBD can proceed, but ablation of both abolishes this.

2.2 MI-MII Transition

Mos appears to be more widely important for MII. For example, unlike RSK-knockout mice, Mos-knockout mice are sterile and oocytes fail to mature properly (Colledge et al. 1994; Hashimoto et al. 1994). To understand the role of Mos, we must first consider cyclin B dynamics in meiosis. At the time of exit from MI, cyclin B must be degraded by the anaphase-promoting complex (APC), a multisubunit E3 ubiquitin ligase also known as the cyclosome. However, because cyclin-B–CDK1 inhibits formation of prereplicative complexes necessary for DNA replication, complete loss of cyclin-B–CDK1 kinase activity (as occurs in a somatic mitosis) would result in reinitiation of S phase. Thus, cyclin B translation is ramped up immediately after GVBD. Moreover, there must be sufficiently rapid reaccumulation of cyclin B to drive MII. Mos participates in two ways: it helps to drive cyclin B synthesis, and it helps control cyclin B degradation. This allows partial but not complete loss of cyclin B at MI exit—a decrease sufficient to exit MI but not initiate S phase—followed by unimpeded cyclin B accumulation to drive MII.

A key effector of this pathway is an inhibitor of the APC known as Emi2 (reviewed in Wu and Kornbluth 2008). Emi2 binds directly to the APC, inhibiting its ability to ubiquitylate substrates and so cause their proteasomal degradation (e.g., cyclin B). Emi2 is also regulated at the level of protein stability and is a substrate of another multisubunit E3 ubiquitin ligase, SCF β –TrCP (Liu and Maller 2005; Rauh et al. 2005; Hansen et al. 2006). Recognition by this E3 ligase, which requires a phosphodegron in its targets, depends on phosphorylation of Emi2 at critical sites within the amino-terminal half of the protein, catalyzed by cyclin-B–CDK1. It is this feedback phosphorylation of Emi2 that helps to precisely control cyclin B levels. As cyclin B is synthesized, it binds to and activates its partner CDK1. Active cyclin-B–CDK1 complexes phosphorylate Emi2, promoting its degradation, thereby alleviating suppression of the APC to allow cyclin B degradation and MI exit. If Emi2 is artificially stabilized at this transition, it causes MI arrest by preventing cyclin B degradation (Fig. 2).

If cyclin-B–CDK1 phosphorylation of Emi2 were unopposed, then ultimately accumulation of cyclin B would eradicate Emi2, allowing complete, rather than the required partial, cyclin B degradation. However, the Mos-ERK pathway interferes at this point. Emi2 is phosphorylated directly by RSK, the kinase downstream from ERK. RSK-mediated phosphorylation of Emi2 promotes docking of the protein phosphatase PP2A on Emi2 (Wu et al. 2007b). PP2A, in turn, dephosphorylates the sites on Emi2 that are phosphorylated by cyclin-B–CDK1, thereby stabilizing Emi2 and restoring its inhibitory binding to the APC (Tang et al. 2008).

This allows the accumulation of cyclin B necessary for blocking S phase and, ultimately, for entry into MII. Mos may also promote inhibition of Myt1 and consequently T14 and Y15 dephosphorylation and activation of CDK1. This could help to activate cyclin-B–CDK1 as cyclin B accumulates. As MI is completed, cyclin B synthesis is markedly enhanced; eventually, this exceeds the ability of the APC to keep pace (even without Emi2 inhibition), cyclin B is degraded, and MII ensues. Although the general role of Emi2 appears to be conserved in mammals, the pathway appears to be somewhat different from that in frogs; because RSK is dispensable, another downstream target of Mos, MEK or ERK, probably phosphorylates Emi2 and recruits PP2A.

2.3 MII Arrest

Once MII is initiated, the oocyte arrests again, this time as a mature egg awaiting fertilization. Mos and Emi2 are also central to the activity that maintains this arrest (Kanki and Donoghue 1991; Hashimoto et al. 1994; Dupre et al. 2002; Madgwick et al. 2006; Ohe et al. 2007). The critical nature of Mos is clear because removal of Mos from egg extracts by immunodepletion destroys CSF activity (Daar et al. 1991), and eggs from mice or frogs lacking Mos fail to arrest in MII (Colledge et al. 1994; Hashimoto et al. 1994; Araki et al. 1996). The target of the Mos-MAPK cascade that produces the MII arrest is again the APC. Indeed, when radiolabeled cyclin B is injected into *Xenopus* eggs, treatment with the MEK inhibitor UO126 promotes cyclin B degradation because the APC inhibition mediated by the ERK MAPK pathway is lifted (Gross et al. 2000). Inhibition of the APC in a CSF-arrested egg requires Emi2, which is targeted by the ERK MAPK pathway during MI and MII (Tung et al. 2005). Loss of Emi2 in either frog or mouse eggs prevents MII arrest and allows parthenogenetic divisions.

Accumulation of cyclin B must be carefully regulated to maintain MII arrest. Cyclin B is synthesized continuously. If unopposed, this would make it difficult to achieve the rapid degradation of cyclin B required for a sharp cell-cycle transition upon fertilization. Again, tight regulation of Emi2 occurs through a negative-feedback loop involving its phosphorylation by cyclin-B–CDK1 and RSK. The carboxy-terminal Emi2 phosphorylations impede association of Emi2 with the APC; although the precise manner in which Emi2 inhibits the APC is not yet clear, the physical association of Emi2 with the APC is critical for APC inhibition and the amino-terminal phosphorylations dissociate Emi2 from the APC, allowing cyclin B degradation (Wu et al. 2007a). Thus, the Mos-ERK-RSK pathway maintains the CSF arrest, restricting cyclin B levels within a narrow limit. Although the precise sites of phosphorylation do not appear to be conserved from *Xenopus* to mammals,

Figure 2. Regulation of Emi2 and the APC during the MI–MII transition. Phosphorylation controls Emi2 stability during oocyte maturation. At MI, CDK1 phosphorylates four amino-terminal sites (S213, T239, T252, and T267) on Emi2; this triggers Emi2 degradation, required for MI exit. At MI anaphase, cyclin B is degraded, leading to a drop in CDK1 activity. Emi2 is stabilized by dephosphorylation triggered by Mos signaling. Emi accumulates, resulting in APC inhibition, critical for S phase block and MII entry. CDK1 activity is low in MII relative to MI. Emi2 is stable in MII, as required for CSF arrest. At fertilization, Emi2 is quickly degraded through a CaMKII-mediated pathway, allowing activation of the APC and exit from MII. At the onset of MI anaphase, APC-mediated cyclin B degradation results in decreased CDK1 activity. With the Mos-PP2A pathway predominant, dephosphorylated and stabilized Emi2 protein prevents complete ubiquitylation of cyclin B by the APC. This is essential for the inhibition of S phase between MI and MII. (From Tang et al. 2008; adapted, with permission.)

the overall mode of regulation may be conserved through phosphorylation of alternative sites.

3 SPERM MATURATION

Spermatogenesis occurs over the course of several weeks and encompasses three successive phases (Sharpe 1994): proliferation, meiosis, and differentiation. During proliferation, spermatogonial stem cells (SSCs) differentiate into spermatogonia. These undergo several mitotic divisions, giving rise to spermatocytes. After two meiotic divisions, spermatocytes form haploid spermatids. The final transformation of spermatids into mature sperm entails a major physical and structural reorganization of the cell that is known as spermiogenesis (Fig. 3).

As oocyte maturation is supported by follicular cells, so is spermatogenesis supported by nongermline cells in and

around the seminiferous tubules. These include Sertoli, Leydig, and myoid cells (Hermo et al. 2010). The Sertoli cells perform a plethora of functions to sustain germ cells through all stages of development. They express receptors for growth factors and hormones and secrete many essential regulatory factors required for spermatogenesis. Lying outside the tubules in the interstitial space and in close proximity to blood vessels, Leydig cells are responsible for the regulated production of androgens in the testis. Lastly, in the peritubular tissue, along with extracellular matrix, myoid cells support the seminiferous tubules (Yoshida et al. 2007; de Rooij 2009). These cells create a microenvironment that allows SSCs to self-renew and/or differentiate, as is required for continual fertility (Nalam and Matzuk 2010).

Complete spermatogenesis requires the coordinated action of peptide and steroid hormones, which are important

Figure 3. Regulation of sperm maturation. (A) Spermatogenesis is a continuous process that starts after puberty. This is possible because a subset of spermatogonium, spermatogonial stem cells (SSCs), are capable of self-renewal. Glial-cell-line-derived neurotrophic factor (GDNF) and the receptor complex composed of Ret protooncoprotein and the GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) are key signaling events for self-renewal. (From Hofmann 2008; adapted, with permission.) (B) Spermatocytes undergo a series of maturation steps before differentiating into sperm. These are tightly regulated by interaction with Sertoli cells and testis factors such as GDNF, ERM, KitL, and retinoic acid (RA). (C) Sperm must additionally undergo capacitation before they are capable of fertilization. This includes both fast and slow events. The fast events stimulate flagellar activity through a calcium flux that is controlled by the adenylyl cyclase SACY and mediated by the CatSper and sodium/bicarbonate (NBC) channels. Slow events increase motility and introduce changes that prepare sperm for fertilization. These include increases in both intracellular calcium levels and tyrosine phosphorylation of PKA substrates. cAMP levels are also controlled by endogenous phosphodiesterase (PDE). Unknown cholesterol acceptors present in uterine/oviduct fluids mediate cholesterol efflux during capacitation. (From Visconti 2009; adapted, with permission.)

regulators of seminiferous tubule function (McLachlan et al. 2002). Only somatic cells in the testis express hormone receptors; therefore these cells (i.e., Sertoli cells) are the exclusive mediators of hormone activity in spermatogenesis. Of the two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which also play important roles during oogenesis (Richards and Pangas 2010), FSH has broader involvement in spermatogenesis whereas LH functions primarily in the regulation of testosterone production by Leydig cells (Ruwanpura et al. 2010). However, both gonadotropins exert their biological effects by activating cognate GPCRs. In mice, following formation of the seminiferous cords, gonocytes, the precursors of SSCs, proliferate until day E15–16, when they become quiescent, which is coincident with changes in the expression of several cell-cycle proteins (van den Ham et al. 2003). Formation and proliferation of SSCs is observed during the first postnatal week and continues with the synchronous first wave of spermatogenesis that extends in the prepubertal testis over the first 35 days after birth (Itman et al. 2006).

3.1 Stem Cell Proliferation and Maintenance

The predominant role of FSH is regulating Sertoli cell proliferation during prepubertal development (Holdcraft and Braun 2004). Stimulation of the FSH receptor on Sertoli cells activates several downstream signaling cascades, including those involving cAMP, calcium release, ERK, PI3K, and phospholipases A2 and C. These activate the cAMP-responsive transcription factor CREB, leading to stimulation of gene expression (Ruwanpura et al. 2010). Testosterone produced in response to LH mostly functions via the nuclear androgen receptor (AR) and its subsequent stimulation of gene expression (Wang et al. 2009; Sever and Glass 2013). It may also act via alternative mechanisms, which also result in phosphorylation of CREB. These “nongenomic” pathways are thought to involve the activation of several kinases, including Src (Cheng et al. 2007). Importantly for proliferation of both Sertoli cells and SSCs, FSH stimulation increases expression and secretion of glial-cell-line-derived neurotrophic factor (GDNF) (Hu et al. 1999), a distant member of the transforming growth factor β (TGF β) family superfamily that regulates the proliferation of uncommitted SSCs (Meng et al. 2000; Hermo et al. 2010).

GDNF promotes the activation and expression of many signaling molecules, including the transcription factors Fos and BCL6. GDNF also stimulates Ras, Akt, and Src-family kinases; Akt activation, specifically, is important for preventing apoptosis of SSCs (Sariola and Saarma 2003; Hermo et al. 2010; Oatley and Brinster 2012). These signals are

initiated following its engagement of a receptor complex composed of the Ret proto-oncoprotein (McGuinness et al. 1996) and the GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) protein (Meng et al. 2000). GFR $\alpha 1$ appears to be expressed only by a subpopulation of SSCs, possibly a marker of true “stemness.” This is consistent with the effects of its down-regulation, which causes widespread inhibition of SSC proliferation and differentiation (reviewed in Nalam and Matzuk 2010). SSCs lacking GFR $\alpha 1$ display reduced phosphorylation of Ret at Y1062, a known binding site for several of the downstream targets of this pathway. Additionally, loss of either member of the receptor complex leads to defective SSC proliferation and differentiation (Naughton et al. 2006). The GDNF pathway is thus essential for the maintenance of uncommitted SSCs.

SSC renewal requires the transcription factor Ets variant gene 5 (ERM) a product of the Sertoli cells. Deletion of ERM in mice results in inhibition of spermatogenesis following the first wave (Chen et al. 2005). Thus, although GDNF plays a critical role in spermatogenesis during the perinatal period, ERM does so at puberty. Expression of ERM and GDNF is increased in Sertoli cells by addition of the fibroblast growth factor (FGF) 2 and activation of the FGF2 receptor, which stimulates ERK and PI3K signaling. Therefore, FGFs may also play an important role in regulating the functions of Sertoli cells that control the establishment of the SSC pool (Simon et al. 2007).

Another factor regulating proliferation and differentiation of SSCs is the protooncoprotein Kit, a receptor tyrosine kinase expressed at high levels in differentiating spermatogonia and early spermatocytes (Sorrentino et al. 1991). In undifferentiated spermatogonia, Kit expression is suppressed by the transcription factor Plzf. Plzf is necessary for the maintenance of undifferentiated SSCs. Plzf-null mice display accumulation of Kit-positive cells and depletion of germ cells (Buaas et al. 2004; Costoya et al. 2004), and dominant white spotting (W) mutants affecting the Kit locus inhibit spermatogonia differentiation without affecting either mitosis of undifferentiated SSCs or initiation of meiosis in spermatocytes (Yoshinaga et al. 1991). Furthermore, in the postnatal testis, expression of the Kit ligand (KitL, also known as stem cell factor [SCF]), in Sertoli cells is critical for spermatogenesis (Flanagan et al. 1991), and mutations in KitL phenocopy the spermatogenic defects observed in W mutant mice (Bedell and Mahakali Zama 2004). The binding of KitL to Kit causes receptor dimerization and phosphorylation of cytoplasmic tyrosine residues. These phosphorylated residues become anchoring sites for SH2-domain containing proteins such as phospholipase C γ (PLC γ), Src, and PI3K. Consequent induction of calcium release, phosphorylation of target proteins such as p70S6K (Feng et al. 2000), and gene expression

leads to proliferation and differentiation of spermatogonia (Sette et al. 2000). Note that spermatogonia from Kit-null testes can nevertheless undergo spermatogenesis, which indicates other parallel pathways must also operate (Nalam and Matzuk 2010).

3.2 Spermatocyte Meiosis and Release

After differentiation, spermatogonia undergo a few mitotic divisions before entering meiosis, which is delayed until puberty. Retinoic acid (RA) (see Sever and Glass 2013) is a key signaling molecule in meiotic initiation (Vernet et al. 2006), and Sertoli cells are believed to be the main source of RA in the postnatal testes. Importantly, although RA is generated in Sertoli cells by the enzyme aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1), male gonads also express an enzyme responsible for RA degradation, cytochrome P450, family 26, subfamily b polypeptide 1 (CYP26B1) (Bowles et al. 2006). This limits the availability of RA and postpones meiosis in the male.

Hormonal signaling plays an important role in control of meiosis and production of functional spermatids. Suppression of FSH production and signaling leads to reduced numbers of pachytene spermatocytes (Matthiesson et al. 2006) and compromises the release of sperm, which suggests that FSH regulates adhesion between Sertoli cells and spermatids (Saito et al. 2000; Ruwanpura et al. 2010). Spermatocyte meiosis is arguably even more dependent on testosterone signaling. In mice lacking AR in Sertoli cells spermatogenesis is arrested at the late spermatocyte stage and spermiation, which is the release of the mature spermatids into the lumen of the seminiferous tubules, fails (Chang et al. 2004; De Gendt et al. 2004). Moreover, in adult mice that lack both gonadotropins owing to a defect in the *GnRH* gene, testosterone supplementation alone can restore full spermatogenesis (Haywood et al. 2003). The processes modulated by testosterone during spermiogenesis include adhesion between Sertoli cells and different stage spermatids. Testosterone is thought to regulate expression and/or function of proteins required for the assembly and/or disassembly of adhesion junctions, including α and β integrins, focal adhesion kinases, and Src kinases (Ruwanpura et al. 2008, 2010; Shupe et al. 2011).

3.3 Sperm Capacitation and Calcium Channels

After entering the oviduct, most mammalian sperm associate with epithelial cells in the isthmus creating a sperm reservoir. Sperm are sporadically released from this reservoir and migrate to the ampulla, which is the site of fertilization (Suarez 2008b). The mechanisms that facilitate this release are unclear but they involve the loss of BSPs (orig-

inally isolated from the cow, so named bovine seminal plasma proteins) and other extrinsic proteins from the sperm; these events are seemingly precipitated by the progression of capacitation events, including hyperactivation (a change in sperm motility) (Suarez 2008a). Unfolding over several hours, sperm capacitation begins as sperm come in contact with the female reproductive tract and involves a series of sequential and simultaneous processes. Sperm acquire motility immediately after their release from the cauda epididymis (for reviews see Yanagimachi 1994; Salicioni et al. 2007). These subsequent changes involve both modification of the plasma membrane phospholipid composition and increases in intracellular calcium concentration. Early in capacitation, activation of soluble adenylyl cyclase (SACY) (Chen et al. 2000) causes an increase in cAMP levels, which activates PKA containing a unique catalytic subunit (C α 2 or Cs). These events are initiated by an increase in the intracellular concentration of bicarbonate, which activates the enzyme by promoting closure of the catalytic active site and metal recruitment (Steegborn et al. 2005). This is itself triggered by the high concentrations of bicarbonate in the seminal fluid, which enters via the Na⁺/HCO₃⁻ cotransporter. PKA activation coincides with phosphorylation of a number of proteins, although its targets remain mostly unknown (Signorelli et al. 2012) except for FSCB, a 270-kDa protein involved in the biogenesis of the fibrous sheath (Li et al. 2007), a cytoskeletal structure in the mammalian sperm flagellum. The ongoing alkalization, which activates the downstream sperm-specific plasma membrane calcium channel CatSper (Ren et al. 2001), also increases intracellular calcium levels (Carlson et al. 2003). Together, this increase and the PKA-regulated phosphorylation of proteins on tyrosine residues, including A-kinase-anchoring proteins (AKAPs) (Ficarro et al. 2003), drive the activation of flagellar motility that is necessary for both sperm migration and residence in the female reproductive tract.

Additional aspects of capacitation that are more protracted are required for sperm to fertilize the egg. These occur closer to the site of the ovulated egg(s) and include a general increase in protein phosphorylation, loss of cholesterol from the plasma membrane, hyperactivation (acquisition of an asymmetrical, nonlinear motility pattern), and increased susceptibility to stimuli that promote the acrosome reaction. These slow processes are also regulated by bicarbonate; however, they additionally require the transfer of cholesterol from the plasma membrane to unknown cholesterol acceptors present in the uterine/oviduct fluids. Also, despite the dominant role of PKA in sperm capacitation, research suggests that other kinases, such as Src-family kinases and PKC, and phosphatases such as PP1 α , PP1 γ 2, PP2A, and PP2B may play a role regulating

the rapid and slow events of capacitation (reviewed in Visconti et al. 2011; Signorelli et al. 2012).

Hyperactivated sperm show increased flagellar bend amplitudes (Yanagimachi 1970), which is most often observed in the oviduct. This amplification generates enough power for the sperm to make its way through the oviductal mucus, cumulus matrix, and the zona pellucida (ZP) surrounding the egg (Chang and Suarez 2010; Hung and Suarez 2010). CATSPER channels and calcium are critical for hyperactivation; either the absence of external calcium or the presence of mutations in any of the four CATSPER subunits results in a failure to hyperactivate (Carlson et al. 2003), and CATSPER-defective sperm cannot leave the oviduct sperm reservoir and are incapable of penetrating the ZP. The mechanism of CATSPER regulation in the oviduct remains unclear. Progesterone is a possible regulator (Lishko et al. 2011; Strunker et al. 2011) produced by cumulus cells and is present in the follicular fluid (Sun et al. 2005). It may act as the long-sought chemoattractant. In this regard, hyperactivation could promote sperm chemotaxis (Chang and Suarez 2010). Indeed, progesterone can induce increases in intracellular calcium near the base of the sperm head—the site where the calcium stores are located (Fukami et al. 2001). Note that in marine species such as the sea urchin *Arbacia punctulata*, in which sperm migrate toward the oocyte along a chemoattractant gradient, motility is regulated by incremental increases in intracellular calcium concentration caused by the binding of the chemoattractant resact to the sperm (Bohmer et al. 2005). Progesterone could act similarly in mammals.

4 FERTILIZATION

4.1 The Acrosome Reaction in Sperm

Before fusing with the egg's plasma membrane, sperm must undergo the acrosome reaction (Yanagimachi and Mahi 1976). The acrosome is a Golgi-derived organelle that lies above the tip of the sperm head. Its contents are released following fusion between the outer acrosomal membrane and the plasma membrane (Kim et al. 2011), and this reaction is critical for interaction with the ZP of the egg. Only capacitated sperm are capable of undergoing the acrosome reaction. Progesterone produced by the cumulus cells surrounding the oocyte has been proposed as the possible inducer of this reaction (Osman et al. 1989; Jin et al. 2011). Upon breaching the egg's plasma membrane, the sperm induces the initiation of embryonic development by evoking an increase in the intracellular concentration of free calcium, a signaling mechanism that regulates numerous cellular processes (Fig. 4) (Berridge et al. 2000b; Bootman 2012).

The signaling mechanisms leading to the acrosome reaction are well characterized (Arnoult et al. 1996; Evans and

Florman 2002). Activation of PLC δ 4 by a calcium influx and the consequent generation of inositol 1,4,5-trisphosphate (IP $_3$) (Distelhorst and Bootman 2011) causes a second influx of calcium (Fukami et al. 2001). This promotes exocytosis by stimulating the reorganization of a SNARE protein in the sperm membranes (Mayorga et al. 2007). This reorganization is facilitated by the NSF and α -SNAP proteins and the calcium sensor synaptotagmin. Release of proteolytic enzymes and hyaluronidase from the acrosome aids the penetration of the cumulus cells and ZP by the sperm (Kim et al. 2008).

How the sperm interacts with ZP has been a point of some dispute. Early studies implicated several sperm-surface enzymes as critical for the interaction of the sperm with the egg coat (Lu and Shur 1997; Ikawa et al. 2010), but more recent work supports the involvement of a disintegrin and metalloproteinase family member, ADAM3 (Shamsadin et al. 1999). On the egg side, recent studies point to the amino-terminal domain of ZP2, which is cleaved after fertilization by the egg cortical granule component ovastacin, as the ZP ligand (reviewed in Avella et al. 2013). After binding, sperm must penetrate the ZP, probably using sperm-associated serine proteases such as acrosin and testisin (also known as PRSS21 or TESP5) (Baba et al. 1994; Yamashita et al. 2008).

4.2 Fusion and Egg Activation Leading to Fertilization

Soon after negotiating the ZP, the sperm fuses with the egg plasma membrane (Evans and Florman 2002). Early studies implicated the proteases ADAM1b and ADAM2, together known as fertilin, but gene knockout studies showed that fertilin is not required for fusion (Inoue et al. 2007). Subsequent work identified IZUMO, a member of the immunoglobulin superfamily, as the protein that appears to mediate fusion (Inoue et al. 2005; Sosnik et al. 2009). In the egg, the tetraspanin protein CD9 has been shown to be critical for fusion (Le Naour et al. 2000; Miyado et al. 2000). Whether CD9 and IZUMO directly interact is not clear.

Fusion of the gametes produces a calcium signal in the egg. A variety of calcium signals are required for egg activation; these reflect both the plasticity of the signaling machinery as well as the distinct requirements for egg activation in different species. Species typically either display a single increase in calcium concentration (e.g., in sea urchins, starfish, frogs, and fish) or show calcium oscillations (e.g., in nemertian worms, ascidians, and mammals) (Stricker 1999; Stricker and Whitaker 1999; Miyazaki and Ito 2006). The mechanism(s) that mediate calcium influx remains poorly characterized in mammalian eggs. Cells use several calcium influx mechanisms, including

Figure 4. Fertilization events. Both egg and sperm must undergo complex changes before fertilization can occur. Upon interaction with the zona pellicula, sperm undergo a calcium-flux-driven reorganization of SNARE fusion proteins, called the acrosome reaction. Fusion with the egg membrane releases factors from the sperm into the cytoplasm. These factors stimulate calcium release from the egg ER and subsequent activation of CaMKII and calcineurin (CN). The consequences of CaMKII activation include phosphorylation of Emi2, which promotes its Plx1 and SCF β -TrCP-dependent degradation and consequent reactivation of the APC. CN activation promotes dephosphorylation of Cdc20 and also the Cdc27 subunit of the APC. At the same time, dephosphorylation of M phase phosphoproteins is promoted by inactivation of the Greatwall kinase (Gwl), thereby alleviating inhibition of PP2A, allowing it to dephosphorylate M phase CDK1 substrates.

receptor-operated channels (ROCs) and voltage-operated calcium channels (VOCCs) (Berridge et al. 2000a; Tosti and Boni 2004; Smyth et al. 2006). Calcium influx may be attained, at least in part, by store-operated calcium entry (SOCE), a mechanism regulated by ER calcium levels and driven by store-operated calcium channels (SOCs) (Park et al. 2009). Stromal interaction molecule (STIM1) in the ER acts as a calcium sensor (Liou et al. 2005; Roos et al. 2005) and causes opening of Orai1, a channel partner protein in the plasma membrane to replenish stores (Feske et al. 2006; Vig et al. 2006).

The IP₃ receptor (IP₃R) on the ER is the main intracellular calcium-release channel in many mammalian cell types (reviewed in Berridge et al. 2000b; Bootman et al. 2001; Bootman 2012). Although mammalian oocytes, eggs, and the surrounding cells express all three IP₃R isoforms (reviewed in Fissore et al. 1999a; Berridge et al. 2000b; Díaz-Muñoz et al. 2008), oocytes and eggs overwhelmingly express the type I IP₃R isoform (Kume et al. 1997; Fissore et al. 1999b; Jellert et al. 2000; Tokmakov

et al. 2002), which requires binding by both calcium and IP₃ for activation and is stimulated at low calcium levels and inhibited at high calcium levels (Iino 1990a,b; Finch et al. 1991). This makes it especially suited to support long-lasting oscillations. The importance of IP₃R1 in fertilization is supported by studies showing injection of an anti-IP₃R1 antibody blocks sperm-initiated calcium oscillations (Miyazaki et al. 1992) and egg activation in mice (Xu et al. 1994) and works in the same manner in other vertebrates (Parys et al. 1994; Thomas et al. 1998; Yoshida et al. 1998; Runft et al. 1999; Goud et al. 2002; Iwasaki et al. 2002).

The signals that initiate the calcium increase at fertilization have proven elusive (Whitaker 2006; Parrington et al. 2007). Nonetheless, in some species, especially those with a single calcium transient at fertilization, Src-family kinases and PLC γ have been shown to lead to production of IP₃ during fertilization (Giusti et al. 1999; Sato et al. 2000). The receptor responsible for recruiting and activating the kinases remains to be identified (Mahbub Hasan et al. 2005). Similarly, it is unclear how sperm induce calcium

oscillations in mammals. Calcium responses can be initiated in eggs from various species, including mammals by the same agonists that cause calcium release in somatic cells (Katayama et al. 1993; Miyazaki and Ito 2006). However, these fail to replicate the pattern of calcium oscillations associated with fertilization; other mechanism(s) might, therefore, be at play. Studies of sea urchin, ascidian eggs, and later, mammalian eggs showed that injection of sperm extracts or whole sperm can replicate fertilization-like responses in these species (Stice and Robl 1990; Swann 1990; Tesarik and Testart 1994; Nakano et al. 1997; Stricker 1997; Swann and Lai 1997; Wu et al. 1997; Kurokawa and Fissore 2003; Malcuit et al. 2006; Swann et al. 2006) strengthening the notion of a sperm cytosolic factor (SF). The mechanism may involve the release of SF into the ooplasm after fusion of the gametes. Importantly, the SF is not IP_3 or calcium but an uncharacterized protein moiety (Swann 1990; Wu et al. 1997; Kyojuka et al. 1998; Harada et al. 2007). Once initiated, calcium oscillations trigger all events of egg activation (Schultz and Kopf 1995). The presence of oscillations ensures the persistent degradation of Emi2 and the stepwise progression of activation, as events such as cortical granule exocytosis exit require fewer calcium increases than exit from MII or recruitment of maternal RNAs (Ducibella et al. 2002).

Cytosolic preparations from mammalian sperm possess high PLC activity (Parrington et al. 1999; Jones et al. 2000; Rice et al. 2000), and this is highly sensitive to calcium and could therefore be (Rice et al. 2000) the oscillation initiator. Indeed, a novel sperm-specific PLC ζ (Saunders et al. 2002) has been identified (Fujimoto et al. 2004; Kouchi et al. 2004) and can evoke appropriate oscillations in mouse (Saunders et al. 2002), rat (Ito et al. 2008), human (Rogers et al. 2004), bovine (Malcuit et al. 2005; Ross et al. 2008), porcine (Yoneda et al. 2006), and equine (Bedford-Guaus et al. 2008) eggs. The findings that aberrant PLC ζ expression is associated with infertility, and sperm from patients with repeated fertilization failure after intracytoplasmic sperm injection (ICSI), which also fail to initiate calcium oscillations (Yoon et al. 2008; Heytens et al. 2009), supports the idea that PLC ζ released from the sperm triggers oscillations in fertilized eggs. Nevertheless, questions remain regarding its expression during spermatogenesis and storage, the means by which release into the ooplasm occurs, and how activation occurs upon egg entry.

5 FROM EGG TO ZYGOTE

Once fertilization has occurred, interphase nuclei form separately around the paternal (sperm) and maternal (Eggerickx et al. 1995) genomes in structures known as pronuclei. The paternal genome undergoes a series of

transformations that result in both exchange of spermatogenic chromatin packaging proteins (protamines) for maternal histones and demethylation of many repressed paternal genes. Upon completion of DNA replication, the pronuclear envelopes break down and the maternal and paternal chromosomes come together on a common mitotic spindle in the zygote. In this transition to the zygote, maternal mRNA transcripts are largely degraded; many are replaced by zygote-specific transcripts both to take over the function of maternal housekeeping messages and to allow expression of new proteins that will build the early embryo.

At fertilization, the egg exits its MII arrest, in part as a consequence of events set in motion by the calcium response, which inactivates CSF through degradation of Mos and reactivates the APC, leading to cyclin degradation. Reactivation of the APC appears to be mediated in part by the calcium-dependent phosphatase calcineurin (also known as PP2B). Inhibition of calcineurin by cyclosporine A halts destruction of cyclin B. Not only has a core component of the APC, Cdc27, been shown to serve as a calcineurin substrate, but the APC-activating subunit Cdc20 is also a substrate of calcineurin. To exit meiosis, a host of CDK1–cyclin-B substrates must be dephosphorylated. This is accomplished largely by PP2A (as described above). Premature inactivation of CDK1–cyclin-B substrates would prohibit entry into or maintenance of MII. Inappropriate PP2A activation is therefore prevented through binding of a small peptide called Arpp19 and α endosulfine. Phosphorylation of Arpp19 by the conserved mitotic kinase Greatwall, which is activated by CDK1 at M phase entry, allows it to inhibit the PP2A isoform B55 δ . At M phase exit, Greatwall is inactivated, which alleviates inhibition of PP2A and consequently allows the requisite dephosphorylation of M phase substrates. Additionally, rapid destruction of Emi2 alleviates inhibition of the APC, leading to loss of CDK1 activity (through degradation of cyclin B). In *Xenopus* eggs, this is triggered by calcium via a calcium-calmodulin-dependent kinase (CaMKII). CaMKII phosphorylates Emi2 protein on T195. This creates a docking site on Emi2 for another kinase, Plx1 in *Xenopus*, which then phosphorylates Emi2 within a sequence (the phosphodegron) required for recognition by the SCF β –TrCP ubiquitin ligase. This series of events leads to ubiquitylation and degradation of Emi2, liberation of the APC, and degradation of substrates required for M phase exit. Note the Plx1-phosphorylation site does not appear to be conserved in mammals, and it is not known whether the ability of CaMKII to trigger Emi2 degradation in mammalian eggs is also triggered by recruitment Plx1 (the mammalian Plx1 ortholog). The culmination of these events allows the fertilized egg to exit arrest and proceed with cell division.

6 CONCLUDING REMARKS

In gametes of both sexes, development, maturation, and fertilization require the careful coordination of complex processes. From hormone-initiated and kinase/phosphatase-controlled maturation, to calcium-induced capacitation and fertilization, regulatory mechanisms ensure that reproduction occurs only under conditions in which they are best poised for success. For males, the signals that regulate spermatogenesis are at first contained within the testis, but following spermiation and ejaculation, proper sperm function depends on factors outside of the male reproductive tract: for external fertilizers, factors in the outside environment; and for internal fertilizers, the milieu of the female reproductive tract. Taking into account these potentially harsh environments, male reproduction relies on the production of large quantities of sperm, with progenitor cells retaining mitotic capacity into adulthood. For female internal fertilizers, the production of eggs is more contained, restricted to the follicle until ovulation and to the female ducts until fertilization and beyond, relying on the production of few gametes; in contrast, externally fertilizing females, like their male counterparts, produce large numbers of gametes. Even for these organisms, though, oocytes and eggs are self-contained developmental units capable of sustaining the early stages of development whose progenitors have generally entered meiosis and have lost the capacity to regenerate.

In every species, regardless of the site of fertilization, cascades of protein modifications regulate cell-cycle transitions to guarantee that oocytes can be fertilized only at an appropriate time. Hormonal regulation and calcium signaling promote capacitation of sperm only in the correct environment for fertilization. Furthermore, multiple overlapping pathways provide the checks and balances that are necessary to prevent defective reproduction.

Although great progress has been made over the last 50 years in detailing the molecular events underlying most aspects of vertebrate fertilization, there are still aspects of gamete development and fertilization whose precise regulation by cell signaling events remain to be determined. Elucidation of these events is likely to have important implications for the continued development of reproductive technologies and for maximizing the health of gametes, and thus of progeny.

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