review

The state of the union: the cell biology of fertilization

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Look at songs, hidden in eggs. Carl Sandburg in *Prairie.*

Fertilization is the process by which sperm and egg unite. An expanded understanding of the mechanisms that underlie these events has provided insights into an important aspect of early development and also has proven to be a valuable model in which to study cellular function. In addition, many emerging strategies for contraception and for the treatment of infertility are based on the mechanism of gamete interaction. Here, we discuss the cell and molecular biology of mammalian fertilization, highlight selected recent breakthroughs and attempt to identify key unanswered questions.

Sexual reproduction occurs through fertilization, during which two haploid gametes unite to produce a genetically distinct individual. Advances in the understanding of fertilization are significant from several perspectives. Gametes have provided a valuable model system for the examination of cellular biology since the 19th century[1]. To cite several recent examples that underscore the continued utility of these cells, our understanding of cell division has been advanced by mitosis-promoting factor (MPF), by signal transduction through receptor guanylyl cyclases and soluble adenylyl cycles, and by the cellular interactions of ADAM (a disintegrin and metalloproteinase) proteins (see below); all molecules or activities initially described in gametes[2–5]. In addition, new strategies for the control of world population, which is now estimated to have surpassed 6.2 billion[6], as well as for the treatment of infertility, which affects more than 10% of couples, are likely to emerge from an expanded knowledge of the mechanisms of sperm–egg interaction. Finally, fertilization converts two terminally differentiated cells into a totipotent zygote that can form all of the cell types in the body. An appreciation of the mechanisms through which sperm initiate these events may be essential to utilize developmental processes, such as stem cell technology, within therapeutic contexts, such as the treatment of degenerative diseases. This review will focus on selected areas of fertilization research in which recent advances have been made or controversies persist. The reader may consult other reviews for additional information on these or other aspects of fertilization[7–9].

Overview of fertilization

Fertilization begins when free-swimming sperm approach eggs within the oviduct. These gametes are produced within the unique microenvironments of the ovarian follicle and the testicular seminiferous epithelium. The end-product of spermatogenesis is a highly polarized sperm, consisting of a head region that contains the nucleus and a single enlarged secretory granule, or acrosome, in the apical region, and a flagellum containing a 9 + 2 array of microtubules and associated sheath proteins. After release from the seminiferous epithelium in the testis, sperm are transported through the epididymis, where additional biochemical and functional modifications occur. They are then stored within the cauda epididymis, where they are in a functionally inactive state, immotile and incapable of interacting with eggs[8].

The mammalian egg complex that is ovulated and enters the oviduct consists of three components: first, the egg, arrested at metaphase of meiosis II in humans and most other mammals; second, the extracellular matrix of the egg, or zona pellucida, consisting of three glycoproteins (ZP1, ZP2 and ZP3) that are synthesized and secreted by the oocyte; third, the cumulus oophorus, consisting of several layers of ovarian follicular granulosa cells embedded in an extracellular matrix composed of hyaluronic acid (Fig. 1)[8,9].

Sperm acquire the ability to fertilize eggs through the process of capacitation, during migration through the female reproductive tract. Capacitated sperm penetrate the cumulus oophorus assisted by PH-20, a cell surface hyaluronidase (Fig. 1a)[10], contact the zona pellucida and undergo the acrosome reaction, a calcium-dependent exocytotic event (Fig. 1b). After completion of the acrosome reaction, sperm penetrate the zona pellucida, finally contacting and fusing with the plasma membrane of the egg (Fig. 1c,d). Gamete fusion results in egg activation, pronuclear formation and syngamy[8,9,11].

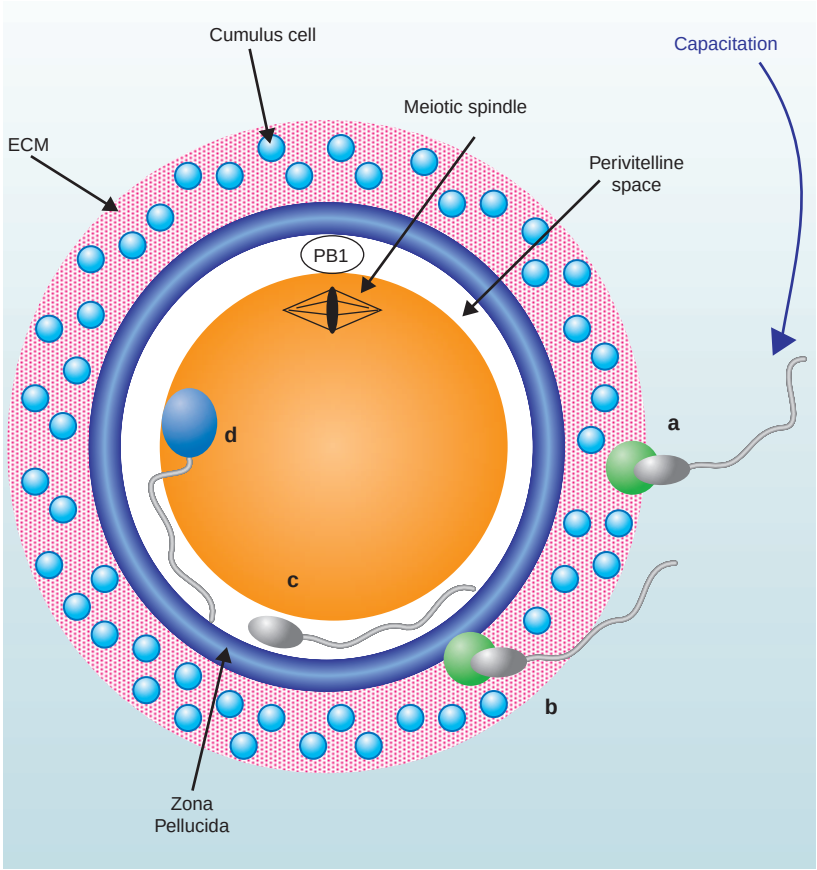

Figure 1 **The sequence of early events in mammalian fertilization.** A sperm–egg interaction begins after sperm capacitation. A sperm first penetrates the cumulus oophorus (**a**), consisting of cumulus cells (somatic cells from the ovarian follicle) embedded in an extracellular matrix (ECM). The sperm then contacts the zona pellucida (**b**), where the acrosome reaction is triggered by ZP3. Acrosome-reacted sperm penetrate the zona pellucida, enter the perivitelline space, then adhere to (**c**) and fuse with (**d**) the plasma membrane of the egg. The egg has extruded the first polar body (PB1) and progressed to metaphase II. In most mammals, sperm–egg fusion triggers the completion of meiosis. This model is based on *in vitro* studies of gamete interactions and is consistent with *in vivo* fertilization, which occurs in the oviduct.

Sperm capacitation

As noted above, mammalian sperm are unable to fertilize eggs until they complete functional reprogramming, or capacitation, within the female reproductive tract. Similarly, sperm of many non-mammalian species are activated by factors that are either associated with eggs or spawned with eggs[12]. Capacitation consists of a number of component processes, including: the functional coupling of the signal transducing pathways that regulate the initiation of acrosome reactions by ZP3; alterations in flagellar motility that may be required to penetrate the zona pellucida; development of the capacity to fuse with eggs. This is accompanied by alterations in metabolism, membrane biophysical characteristics, changes in protein phosphorylation state, elevations of intracellular pH and calcium levels, and hyperpolarization of membrane potential[9,13,14]. Much of our understanding of capacitation derives from *in vitro* studies, where a single set of incubation conditions initiate these events[13], although it is likely that a cluster of stimulatory molecules controls various aspects of capacitation *in vivo*.

Several factors may drive capacitation *in vitro*: first, cholesterol efflux from sperm membranes is mediated by sterol-binding proteins and initiates many aspects of capacitation[13]. The reorganization of the sperm membrane after cholesterol depletion may be an early step in the process of capacitation; second, an array of sperm proteins are tyrosine phosphorylated through a cAMP-dependent mechanism[13,15]. Mammalian sperm express a bicarbonate-sensitive, soluble form of adenylyl cyclase[4] that may control these phosphorylation events[15]. The involvement of this bicarbonate-sensitive adenylyl cyclase in capacitation may also account for the requirement for extracellular bicarbonate in capacitation; third, elevations of intracellular pH and bicarbonate levels, with the associated stimulation of cAMP production, may activate the cyclic nucleotide-gated channels that are present in sperm flagella and are linked to the control of flagellar motility[16]. This may account for the switch to the hyperactivated motility that is characteristic of the swimming patterns of capacitated sperm[17]; fourth, membrane potential hyperpolarization occurs during capacitation, when T-type calcium channels are released from voltage-dependent inactivation and thereby able to participate in ZP3 signal transduction (see below)[18]. Hyperpolarization is caused, at least in part, by an increased contribution of potassium channels in setting sperm membrane potential[14].

In vivo, multiple cooperative factors are likely to mediate capacitation. Sterol-binding proteins, such as high-density lipoprotein, are present in the oviduct and can accelerate cholesterol efflux from sperm[19]. In addition, progesterone may regulate some aspects of capacitation: this steroid is present in the oviductal milieu, derived both from follicular fluid and from secretion by cumulus oophorus cells associated with eggs[20]. A central goal for future studies is to extend our understanding of

capacitation from *in vitro* systems to the *in vivo* situation.

Sperm–zona pellucida adhesion

Capacitation is a prerequisite step for sperm to bind to the zona pellucida in a saturable, specific fashion. Initial sperm–zona pellucida adhesion is mediated by ZP3, a constituent glycoprotein of the zona pellucida that binds to receptors in the anterior head of acrosome-intact sperm. Isolated ZP3 has several of the anticipated characteristics of an adhesion molecule, including the ability to bind directly to sperm[21] and to function as a competitive inhibitor of adhesion[22]. By contrast, the other zona pellucida glycoproteins, ZP1 and ZP2, lack these activities[7]. Adhesion to the zona pellucida is based on protein–carbohydrate recognition processes, through the association of O-linked oligosaccharides (that is, carbohydrate chains attached to polypeptide by serinyl/threoninyl: N-acetylgalactosaminyl linkages) from ZP3 with a cognate receptor on sperm[23,24]. Sperm recognition oligosaccharides have been partially purified[23] and the application of sensitive mass spectrometric methods may soon provide a molecular definition of these sequences[25]. In addition, several high-affinity ZP3 binding proteins have been identified on sperm, including at least one carbohydrate-binding protein and another protein that displays species/order-selective binding to the zona pellucida[11,26,27]. However, a sperm receptor for ZP3 has not yet been identified unequivocally.

Triggering the acrosome reaction

ZP3 stimulation triggers acrosome reactions in sperm bound to the zona pellucida. ZP3 oligosaccharide chains account for adhesion, as discussed previously, but are not sufficient to drive acrosome reactions. ZP3 may be polyvalent with regard to adhesion oligosaccharide chains[28], and the resulting crosslinking and multimerization of sperm receptors initiates exocytosis. This model is supported by observations that adhesion oligosaccharide chains are conjugated to ZP3 at multiple sites[28]; that crosslinking β-1,4-galactosyltransferase, a sperm protein implicated in gamete interaction, triggers acrosome reactions[29,30]; and that polyvalent oligosaccharide structures may also trigger acrosome reactions in marine invertebrate sperm[31,32].

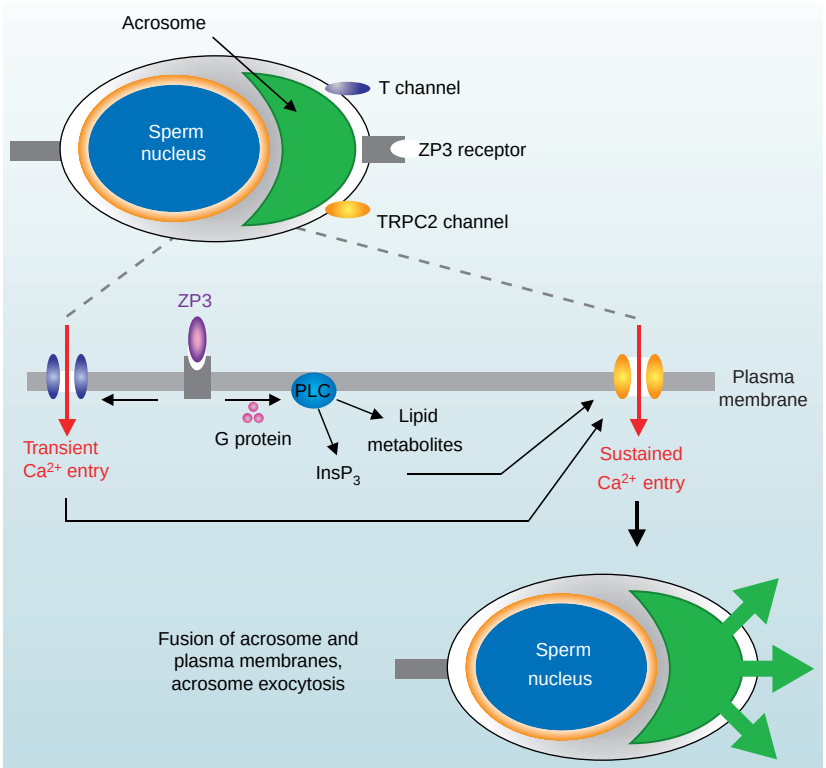

Figure 2 **Triggering of the acrosome reaction.** A model of the events involved in ZP3 signal transduction is shown. In the sperm head, TRPC2–ZP3 receptor activation during adhesion to the zona pellucida results in calcium entry through T-type channels, causing a transient elevation of cytosolic calcium concentration and activation of PLC through a G_{i1} and/or G_{i2} protein-mediated pathway, resulting in the production of $InsP_3$ and diacylglycerol. PLC and the transient calcium elevation function in concert to produce persistent calcium entry through a TRPC2 channel that directly drives the acrosome reaction, triggering fusion of the outer acrosome membrane with the plasma membrane and releasing acrosomal contents. See text for details.

The early events of ZP3 signal transduction in sperm include the opening of T-type, low voltage-activated calcium channels[33], resulting in a transient calcium influx and the activation of the heterotrimeric G proteins, G_{i1} and G_{i2} (Fig. 2)[34]. These initial responses produce an activation of phospholipase C (PLC)[35–37] and the elevation of intracellular pH[38,39], resulting in a sustained calcium influx that directly drives exocytosis[38,40].

Recent studies have focused on identifying the channel that mediates the sustained phase of ZP3-evoked calcium entry. The *Drosophila melanogaster transient receptor potential* (*TRP*) gene encodes a light-activated cation channel in photoreceptor cells[41]. The seven members of the classical mammalian TRP (TRPC) family are homologues of this dipteran gene and are candidate subunits of PLC-dependent calcium entry channels[41]. The gating mechanisms that link

PLC action to the opening of TRPC channels have not yet been resolved. Alternative models have been proposed, either based on lipid products or PLC hydrolysis, or on the generation of inositol-1,4,5-trisphosphate ($InsP_3$) and the activation of a calcium-store depletion-operated entry pathway[41]. A number of *TRPC* genes are expressed in the mammalian male germ lineage[42–44] and *TRPC2* has specifically been shown to be a subunit of the sustained calcium entry channel in mouse sperm that is activated by ZP3 (ref. 43). In this regard, $InsP_3$ receptors are present in the sperm acrosome[45] and may participate in the activation of TRPC2 channels by ZP3. However, *TRPC2* is a pseudogene in humans[46,47], and also possibly in bovine systems[42]. Therefore, this role in ZP3 signal transduction must be assumed by another ion channel in the sperm of those species. Particular attention will focus on the role of other TRPC family members in mediating ZP3 signal transduction in species that lack TRPC2. A number of soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins are present in the acrosomal region of mammalian and sea urchin sperm[48–50], and these may couple calcium entry to exocytosis.

In conclusion, the acrosome reaction is a critical functional switch. Before exocytosis, capacitated sperm can penetrate the cumulus oophorus and adhere selectively to the zona pellucida[51], but the acrosome reaction is a necessary prerequisite step for the sperm to be able to fuse with the egg plasma membrane[9,14].

Sperm–egg adhesion and membrane fusion

After penetration of the zona pellucida, sperm adhere to and fuse with the plasma membrane of the egg (Fig. 3). The involvement of sperm fertilin-α (also known as a disintegrin and a metalloprotease domain 1 (ADAM1)), fertilin-β (ADAM2) and cyritestin (ADAM3), as well as CRISP1 (cysteine-rich secretory protein 1), in sperm–egg adhesion are indicated by studies using antibodies, peptides, and native or recombinant proteins[52,53]. In addition, sperm from fertilin-β and cyritestin knockout mice show greatly reduced abilities to adhere to the egg membrane, although some of the few sperm that adhere can go on to fuse with the egg[54,55]. Structure–function studies have identified specific functional motifs in the fertilin-β and cyritestin disintegrin domains[56–58]. Integrins found on the surface of eggs are thought to be receptors for sperm ADAMs, although which member(s) of the integrin family remains to be definitively identified. Initial evidence suggested that $\alpha_6\beta_1$ integrin was an egg receptor for fertilin-β (refs 59–61), although the demonstration that eggs from α_6 knockout mice can be fertilized[62], and other data[63–65], suggest that $\alpha_6\beta_1$ integrin is not required for fertilization or fertilin-β-mediated adhesion. Recent evidence implicates $\alpha_9\beta_1$ integrin as a binding partner for fertilin-β (refs 64,65), and other egg integrins may also be involved[63].

> "...the acrosome reaction is a necessary prerequisite step for the sperm to be able to fuse with the egg plasma membrane."

These adhesion-mediating proteins on both gametes are likely to function within the context of multimeric complexes at the plasma membranes. In the egg, an integrin-associated protein, the tetraspanin CD9, is clearly important for sperm–egg interactions. Sperm do not fuse with eggs from CD9 knockout mice[66–68], a recombinant form of an extracellular portion of CD9 inhibits sperm–egg fusion[69], and anti-CD9 antibodies inhibit sperm–egg fusion and the binding of sperm and sperm ADAMs to eggs[58,59,62,64,70]. Other integrin-associated proteins may also facilitate sperm adhesion[58]. This is consistent with models in which tetraspanins and other accessory membrane and cytosolic proteins modulate the function of cell adhesion molecules[71,72]. In sperm, insights into the importance of multimeric membrane protein complexes have come from analyses of protein expression profiles of sperm from the fertilin-β and cyritestin knockout mice. Sperm from these mice have greatly reduced amounts of other ADAM proteins[55] and these molecular deficiencies are likely to contribute to the defects in gamete membrane interactions.

After adhesion, sperm fuse with the egg plasma membrane. However, the molecular basis of this intercellular fusion process remains elusive. Contrary to initial hypotheses[73], fertilin-α is not thought to be involved in membrane fusion[55,74]. Similarly, CRISP1 was thought to mediate gamete fusion, but is now thought to function before membrane fusion[53]. CD9 is implicated in certain types of membrane fusion[71,72], but it is not known whether CD9 in the egg facilitates gamete fusion (either directly or indirectly), or instead functions upstream by enhancing sperm adhesion or making the egg membrane environment 'fusion-competent'. Significant inroads in the membrane fusion process have been made from studies of virus–cell fusion and the fusion of intracellular vesicles. Although less is known about cell–cell fusion events, new insights are emerging from studies of sea urchin and abalone sperm[75,76].

Egg activation

Egg activation occurs after fertilization and initiates embryonic development[77]. In all animals and plants where this has been investigated, an early event in egg activation involves an increase in the cytosolic calcium concentration to approximately 1 μM. This increase has distinct spatial and temporal features in each species. The increase in cytosolic calcium concentration seems to occur between several seconds and a few minutes after the establishment of membrane continuity between gametes, and often occurs as a 'wave' that travels across the egg[77,78]. In mammals, a low-frequency oscillation in cytosolic calcium concentration (approximately

1–2 min spike duration and 1 spike per 10 min) is generated by $InsP_3$, through activation of $InsP_3$ receptors in the endoplasmic reticulum and the subsequent release of sequestered calcium (Fig. 3). Oscillations persist for several hours preceding the time of entry into the first embryonic mitosis[79]. The increase in cytosolic calcium concentration induces exit from meiotic arrest and progression into mitosis, and the exocytosis of cortical granules, the contents of which modify the zona pellucida to prevent fertilization by additional sperm[9,80]. These two responses differ in the 'dose' of calcium oscillations they require[81]. Later egg activation responses include the recruitment of maternal mRNAs for translation, changes in protein synthesis, and zygotic genome activation[9,80].

In non-mammalian species, this cytosolic calcium concentration response may be mediated by PLC-γ. This PLC isoform is typically activated by tyrosine phosphorylation, often through an interaction of PLC-γ Src homology 2 (SH2) domains with a tyrosine kinase or adaptor proteins. Candidate Src family tyrosine kinases that may be involved in this pathway have been identified in echinoderm and ascidian eggs[82–86]. Furthermore, injection of recombinant PLC-γ SH2 domains into these eggs inhibits calcium release and egg activation responses[86–88]. However, recombinant PLC-γ SH2 domains and phosphatidylinositol-3-OH kinase inhibitors have no effect on sperm-induced egg activation in mouse eggs[89,90]. The alternative hypothesis that PLC-β mediate egg activation is unlikely. This isoform is typically stimulated through a heterotrimeric G protein-dependent pathway; however, the perturbation of G protein function does not seem to inhibit the activation of mammalian eggs by sperm[91,92].

Research in egg activation has focused on the mechanism by which sperm evoke oscillations of cytosolic calcium concentrations in the egg, and has largely examined two models. In one case, the binding of a sperm ligand to an egg receptor may initiate a signal transduction cascade that results in calcium release. Sperm ADAMs and egg integrins have been considered candidate effectors. However, although there are reports that ligand-mimicking synthetic peptides can induce calcium release in bovine and frog eggs[93–95], no other purified sperm ligands that bind to eggs have been shown to evoke egg activation.

An alternative model suggests that fusion of the gametes introduces a factor(s) from the sperm into the egg that initiates egg activation[77]. The injection of sperm lysate into eggs induces fertilization-like oscillations of intracellular calcium concentrations. The use of intracytoplasmic sperm injection to fertilize eggs also demonstrates that extracellular sperm–egg contact is not necessary to activate eggs, and sufficient controls and analyses have been performed to demonstrate that it is not simply the act of injection or the introduction of calcium from the media that initiates egg activation[96]. Egg-activating activity seems to be associated with the nucleus of the sperm[97], possibly in the perinuclear theca[98], and develops during spermatogenesis[96]. Several candidates have been proposed, including oscillin (glucosamine-6-phosphate isomerase)[99]

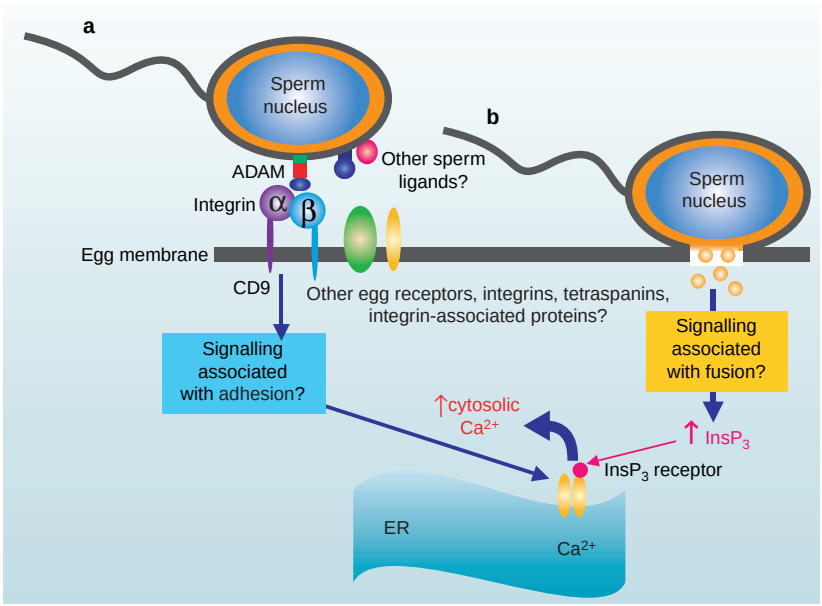

Figure 3 **Gamete membrane interactions and egg activation a**, Adhesion is mediated by a sperm ADAM interacting with egg integrins through its disintegrin domain (dark blue). At least three ADAMs can participate in murine gamete adhesion (fertilin-α, fertilin-β and cyritestin). Additional sperm ligands, including CRISP1, may be present. Integrin-associated proteins (including CD9, perhaps other tetraspanins and CD98) are present on eggs, where they seem to facilitate gamete membrane interactions. **b**, Egg activation is initiated after the establishment of membrane continuity between the gametes. A key event in the activation mechanism is the generation of $InsP_3$, resulting in the release of calcium from intracellular stores. The mechanism through which $InsP_3$ is generated remains unresolved and may involve the introduction of a PLC stimulatory factor(s) from the sperm into the egg during gamete fusion, or the stimulation of egg PLC through signal transduction pathways (see text for details). $InsP_3$ then binds to the $InsP_3$ receptor on the endoplasmic reticulum, inducing calcium release into the egg cytoplasm. In echinoderm and ascidian eggs, $InsP_3$ is generated through the activation of a PLC, a process that is possibly mediated through tyrosine phosphorylation by a Src family tyrosine kinase (not shown). This mechanism has not been observed in mammalian eggs.

and a truncated form of the tyrosine kinase c-Kit[100], but there are also data that cast doubt on the involvement of these molecules in egg activation[89,101,102]. Nitric oxide synthase has been proposed to be involved in nitric oxide-mediated calcium release in sea urchin eggs[103], although this is an unlikely model to explain how calcium release is initiated in ascidian and mammalian eggs[104]. A sperm PLC may be introduced into the egg during gamete fusion. Mammalian sperm lysates have $InsP_3$–generating activity[105]; however, none of the known PLC family members cofractionate with this activity[106,107], and it is unclear if there is sufficient PLC activity in a single sperm to initiate calcium release in eggs[90,105]. PLC-δ4 is not a candidate, as sperm from PLC-δ4 knockout mice can activate eggs[36], although other isoforms are present in sperm and may function in this fashion. The overall mechanism through which a sperm activates an egg still remains largely unknown and highly controversial.

Unanswered questions and future directions

This review attempts to provide a condensed synopsis of our understanding of mammalian fertilization, with particular emphasis on current hotspots and controversies. In the interests of conciseness, we were therefore not able to address many interesting aspects of gamete biology. In addition, there are numerous unanswered questions and unresolved controversies in the topics that were reviewed here. Many of the critical molecular players on gametes still need to be identified: these include the sperm receptors for ZP3, the egg receptors for sperm membrane ligands, and the fusion-mediating and sperm-borne egg-activating factors. The mechanistic roles of multimeric complexes in sperm and egg membranes in adhesion and signalling are only just beginning to be examined. With these fundamental biological questions remaining to be answered and with the broad applications of gamete cell biology, this will be a fertile area of research for some time to come. ❑

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