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PRIMER

The cell biology of mammalian fertilization

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ABSTRACT

Fertilization is the process by which eggs and spermatozoa interact, achieve mutual recognition, and fuse to create a zygote, which then develops to form a new individual, thus allowing for the continuity of a species. Despite numerous studies on mammalian fertilization, the molecular mechanisms underpinning the fertilization event remain largely unknown. However, as I summarize here, recent work using both gene-manipulated animals and *in vitro* studies has begun to elucidate essential sperm and egg molecules and to establish predictive models of successful fertilization.

KEY WORDS: Fertilization, Izumo1, Acrosome reaction, Eggs, Hyperactivation, Spermatozoa

Introduction

Our individual body has a limited lifetime. However, through fertilization we are able to continue life as a species. The role of spermatozoa is to fertilize eggs. However, mammalian spermatozoa cannot accomplish this task when ejaculated. They must first undergo a physiological change called capacitation and a subsequent morphological change known as the acrosome reaction in the female reproductive tract. Spermatozoa also harbor the ability to migrate into the oviduct, where they interact with and subsequently fuse with the egg. A number of factors that contribute to sperm-egg interactions have been identified, based on observations using enzyme inhibitors and antibodies in *in vitro* fertilization systems (Box 1). This research led to the conclusion that various sperm enzymes within the acrosome dissolved the egg components and that various membrane proteins were used for binding with eggs. However, recent experiments using gene disruption of these factors did not result in an infertile phenotype, suggesting that they are not essential for fertilization, although they may indeed play a role during the fertilization event. By contrast, using *in vivo* gene-targeting experiments, a number of proteins have unexpectedly emerged as being essential factors for fertilization. In this Primer, I discuss the factors that have been implicated in the various stages of fertilization, ranging from sperm capacitation and migration to sperm-egg fusion. Newly arising views of mammalian fertilization are reviewed and compared with previously postulated models.

The nature of eggs

Ovaries are endowed at birth with a fixed number of oocytes enclosed in primordial follicles. This number declines as a result of ovulation and atresia during the reproductive life of the female (Faddy, 2000). Oocytes are arrested in the dictyate stage of first meiotic division. Over time, cohorts of oocytes enter into a growth phase and become among the largest cells in the body, their diameters reaching about 80 μm and 100 μm in mouse and human, respectively. During growth, eggs form an extracellular matrix called the zona pellucida (ZP) by secreting glycoproteins (Fig. 1A). The ZP of human eggs consists of four ZP glycoproteins (ZP1 to ZP4), whereas that of the mouse egg consists of three ZP proteins (ZP1 to ZP3; mouse *Zp4* is a pseudogene).

Full oocyte growth must be supported by surrounding granulosa cells, which proliferate and form multiple layers of cumulus cells that surround the ovulated egg (Fig. 1A). Cumulus cells support fertilization, and *in vitro* fertilization can be achieved more efficiently with them than without them (Jin et al., 2011; Tokuhiro et al., 2012). The molecular basis of this observation remains obscure; however, a few studies have addressed the function of cumulus cells (Oren-Benaroya et al., 2008; Shimada et al., 2008).

Following meiotic maturation, eggs are ovulated in preparation for fertilization. After ovulation, the eggs are picked up by adhesion between the extracellular matrix of the cumulus cells and oviductal cells of the oviductal fimbriae and are subsequently transferred into the oviduct (Talbot et al., 2003). The eggs are then transported to the ampulla portion of the oviduct, where they await spermatozoa for fertilization. However, there is only a short 'fertile window', which is less than a day after ovulation in humans (Wilcox et al., 1995) and a few hours in the mouse, during which fertilization can successfully occur.

The nature of spermatozoa

In human testes, ~1000 spermatozoa are produced per second (Amann and Howards, 1980), although the reason why mammalian males produce so many spermatozoa to fertilize so few eggs is not understood. Spermatozoa produced in the testes are transferred to epididymides, where they receive various proteins (Busso et al., 2007), probably in part through a structure called the epididymosome (Frenette et al., 2010). The spermatozoa are

Box 1. Studying fertilization *in vitro*

In vitro fertilization (IVF) requires different elements for different species. In mice, eggs are usually collected from the oviduct after treatment with hormones that induce super ovulation. The eggs are introduced into a tiny drop of IVF medium on a dish covered by paraffin oil and cultivated under 5% CO_2 in air. Spermatozoa from mice are normally prepared by squeezing them out from an opening made in the epididymis, followed by suspension in IVF medium; alternatively, ejaculated spermatozoa are utilized in larger animals after washing with medium. The spermatozoa are introduced into the egg culture drop at a final concentration of ~1×10^5 spermatozoa/ml (note that IVF requires a large number of spermatozoa compared with fertilization *in vivo*, in which only a few spermatozoa are required per egg).

The success of fertilization can be assessed by observing: (1) spermatozoa inside the ZP; (2) the formation of pronuclei; or (3) the formation of two-cell embryos. The eggs fertilized by IVF can also be transferred into oviducts of pseudo-pregnant females to assess the developmental outcome of these embryos. When antibodies or inhibitors added to the IVF medium successfully inhibit fertilization, the factors that these antibodies and inhibitors target have traditionally have been viewed as fertilization-related factors.

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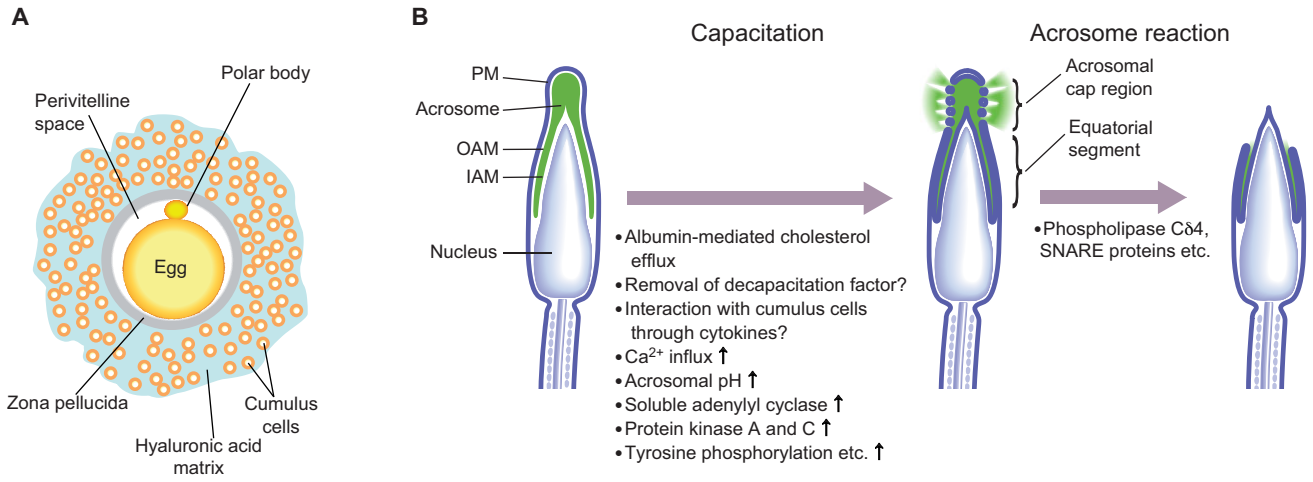

Fig. 1. The features of mammalian eggs and spermatozoa. (A) Ovulated eggs (yellow) are surrounded by small cumulus cells embedded in extracellular material (blue) that consists mainly of hyaluronic acid. The cumulus layer is separated from the egg by the zona pellucida (gray), which is made up of zona pellucida proteins (ZP1-3 in mouse and ZP1-4 in human). (B) Physiological and morphological changes in spermatozoa required for fertilization. When spermatozoa are deposited in the female reproductive tract or suspended in *in vitro* fertilization medium, a number of changes start to occur that involve the phosphorylation of various proteins, activation of PKA and PKC, removal of cholesterol from the membrane and elevation of the intracellular Ca^{2+} level. It is not known whether interaction with cumulus cells is beneficial for some of these changes or if the removal of an inhibitory or 'decapacitation' factor is involved. Sperm also undergo a morphological change called the acrosome reaction, during which various enzymes and proteins are released from the acrosome. Only acrosome-reacted spermatozoa can penetrate and fuse with eggs. The acrosomal vesicle (green) is surrounded by the outer acrosomal membrane (OAM) and the inner acrosomal membrane (IAM). The acrosome contains both instantly and slowly released proteins and its contents are exocytosed during the acrosome reaction in which the sperm plasma membrane (PM) and the OAM fuse in multiple places within the acrosomal cap area. The acrosome reaction is thought to involve phospholipase C (PLCδ4) and SNARE proteins and is generally considered to be a good indicator for the completion of capacitation. Once capacitated, spermatozoa demonstrate a vigorous swimming pattern called hyperactivation, which involves CatSper ion channels.

mature (Fig. 1B), yet they remain unable to fertilize eggs. According to Yanagimachi, the sperm plasma membrane remains 'biologically frozen' until spermatozoa leave the male's body and begin the 'defrosting' process, known as capacitation, which is necessary for spermatozoa to be fertilization competent (Yanagimachi, 1994).

During capacitation, various physiological changes occur in the head, acrosome and tail of spermatozoa, which biologically 'defrost' spermatozoa and prepare them for fertilization (Fig. 1B). It is known that spermatozoa in suspension are heterogeneous and, as such, when a sperm suspension is judged to be capacitated, a certain percentage of spermatozoa in the same suspension are already degrading. Numerous papers discuss the phosphorylation of sperm proteins during capacitation, but no clear mechanisms leading to capacitation have been elucidated (Bailey, 2010; Visconti et al., 2011). This may be due, at least in part, to the heterogeneity of spermatozoa.

Glucose is known to be essential for successful capacitation. It not only serves as an energy source allowing spermatozoa to swim, but also functions to enable spermatozoa to fertilize eggs (Goodson et al., 2012; Okabe et al., 1986). Cholesterol, bicarbonate, intracellular Ca^{2+} and many other factors are also involved in capacitation (Bailey, 2010; Evans, 2012; Florman and Ducibella, 2006; Yanagimachi, 1994); however, the precise molecular mechanisms underlying their action are yet to be clarified.

Once capacitated, spermatozoa demonstrate a vigorous swimming pattern called hyperactivation, a movement considered to give spermatozoa the strong thrusting power that allows them to penetrate the ZP. It is characterized by asymmetrical flagellar beating consisting of pro- and anti-hook bends accompanied by an increase in cytoplasmic Ca^{2+}. The sperm-specific CatSper ion channels have been suggested to control the intracellular Ca^{2+} concentration and, thereby, the swimming behavior of spermatozoa (Chang and Suarez, 2011). CatSper1 to 4 are essential for fertilization in mouse (Qi et al., 2007). Furthermore, the CatSper channel seems to be activated by progesterone in human spermatozoa (Brenker et al., 2012; Lishko et al., 2011), which is interesting from the sperm-egg interaction viewpoint because cumulus cells are known to produce progesterone. However, progesterone is not reported to activate mouse spermatozoa.

After capacitation, the final step in sperm development prior to fertilization is the acrosome reaction (Fig. 1B). The acrosome, which is a subcellular organelle found at the apical tip of the sperm head, is filled with a variety of lytic enzymes and ZP-binding proteins. Through poorly understood mechanisms, which may involve SNARE proteins (De Blas et al., 2005), the plasma membrane and outer acrosomal membrane fuse and acrosomal contents are exocytosed into the environment (Yanagimachi, 1994). This process, referred to as the acrosome reaction, renders spermatozoa able to penetrate the ZP.

Factors regulating fertilization

Fertilization is a complicated multi-step event, involving maturation and development of the spermatozoa and eggs, followed by sperm migration into the oviduct, and ending with sperm-egg interaction and then fusion. As such, various factors that play a role in each of these events are emerging.

The classical models of fertilization were mainly focused on sperm-zona binding and the zona-induced acrosome reaction, based on *in vitro* experiments, and many factors were postulated to be required for fertilization. With time, however, the genes encoding these factors were cloned and their roles were investigated by gene disruption experiments in mice (Box 2). Surprisingly, almost all of them showed no or a very minor phenotype in fertilization (Ikawa et al., 2010); although they may play a role, the knockout of these individual factors did not affect fertilization. In addition, various

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genes essential for successful fertilization began emerging from such gene disruption experiments. Most of these gene-disrupted mouse lines shared common phenotypes, showing: (1) no migration into the oviduct *in vivo* with aberrant zona-binding ability *in vitro*; or (2) no fusing ability (Table 1, Class I and II). Interestingly, all the spermatozoa from class I mouse lines lacked ADAM3 (Shamsadin et al., 1999) without exception, suggesting that ADAM3 plays a key role in fertilization in the mouse.

Below, I highlight the various factors that have been implicated in the fertilization process, placing an emphasis on those for which an essential role has been identified based on gene disruption approaches.

Factors regulating sperm migration

All of the 11 Class I lines of gene-disrupted males shown in Table 1 exhibited the same defect of sperm migration into oviduct, a defect which would not have been identified by *in vitro* studies. In mice, the uterus and oviduct meet in a structure called the uterotubal junction (UTJ), which significantly reduces the number of spermatozoa reaching eggs. The flow in the UTJ is bidirectional. After coitus, spermatozoa must move up to reach the eggs and, after gamete fusion, embryos resulting from fertilized eggs must move down the UTJ for implantation into the uterus. How the UTJ regulates this bidirectional flow is not known. It is clear, however, that sperm migration is not regulated by simple opening and closing of the UTJ entrance in mouse. A testis-specific molecular chaperone, calmegin, is one of the essential genes required for spermatozoa to migrate into oviduct (Ikawa et al., 1997). Using chimeric mice that ejaculate both wild-type and GFP-tagged, calmegin-disrupted spermatozoa, it was observed that only wild-type spermatozoa migrated into the oviduct, whereas the equally motile calmegin-disrupted spermatozoa remained in the uterus (Nakanishi et al., 2004). These observations indicated that entry into the oviduct is regulated by a recognition system between an individual spermatozoon and the UTJ, but the molecular mechanism underpinning this system remains to be determined.

The zona-binding ability of spermatozoa

The sperm-zona interaction is regulated almost in an all or none manner, as illustrated in Fig. 2. *In vitro*, many spermatozoa can bind (or attach) to the ZP of unfertilized eggs but they cannot bind to two-cell-stage eggs, suggesting that sperm-zona binding might be a controlled event. The loss of zona-binding ability, which was commonly observed in Class I knockout mouse lines, could easily be assessed by mixing spermatozoa with cumulus-free eggs, and initially no one doubted that the infertility was caused by the loss of zona-binding ability. However, this assumption was not correct as initially thought.

Mice deficient for the testis-specific molecular chaperone PDILT produce spermatozoa that, based on *in vitro* experiments, exhibit impaired zona-binding ability (Tokuhiro et al., 2012). After crossing with a transgenic fluorescently tagged sperm line (Hasuwa et al., 2010), the failure of sperm migration into the oviduct was indicated by live imaging. However, when the spermatozoa were deposited directly into the ampulla by bypassing the UTJ, the eggs could be fertilized (Fig. 2). Likewise, spermatozoa from infertile *Adam1a*$^{-/-}$ mice, which also show impaired zona-binding ability *in vitro*, could fertilize eggs that were covered by cumulus cells (Nishimura et al., 2004). It is not known what the true sperm-zona interaction entails but these studies suggest that the so-called ‘zona-binding ability’ as measured by binding to cumulus-free eggs *in vitro* appears to be dispensable for fertilization *in vivo* (Fig. 2).

Box 2. Potential limitations of knockout approaches

There are two main drawbacks to gene-targeting approaches. The first occurs when the knockout mice show a minor or no phenotype. As a matter of fact, not all genes are functionally indispensable. If the function of a gene is minor, the knockout mice may not show a strong phenotype, therefore this gene would not be considered a good candidate for knockout-based experimentation. Furthermore, the issue of redundancy can also complicate interpretation of gene-targeting experiments, as the knockout of one factor could be compensated for by other similar factors. It is also known that some genes require the disruption of other gene(s) as a prerequisite to show a phenotype (Rudnicki et al., 1993); some gene products may interact together and knockout of both/all interacting factors might be needed to observe a phenotype. The factors listed in Table 2, which showed almost no phenotype after knockout, might fall into the above category. A second potential drawback relates to the interpretation of the phenotype. For example, fertilin (an ADAM1b/ADAM2 heterodimer) has been depicted as a sperm-egg fusion protein in many textbooks (Blobel et al., 1992) and this was confirmed by the disruption of *Adam2* (Cho et al., 1998), resulting in infertile males. However, when fertilin knockout mice were produced by eliminating *Adam1b* (the second subunit of the fertilin heterodimer) the males were fertile (Nishimura et al., 2004). Infertility in the *Adam2*-deficient/fertilin knockout mice was subsequently attributed to the loss of the testicular ADAM1a/ADAM2 protein dimer, which led to the absence of ADAM3 (a protein that does play an essential role in fertilization) from the spermatozoa.

Thus, the interpretation of a knockout phenotype is not always simple. Moreover, the effect of gene disruption might originate from an unexpected route. This includes the unintentional elimination of microRNAs along with the gene of interest (Osokine et al., 2008). In other cases, the gene manipulation may affect other genes located near the intended target and confound the interpretation of phenotypes from alleles designed to be simple null mutations (Olson et al., 1996). Therefore, caution is required when interpreting knockout results.

Many studies have also postulated the involvement of carbohydrates in sperm-zona binding. It must be noted, however, that many of these postulations were based on *in vitro* studies, and an essential role for such carbohydrate residues *in vivo* has not yet been confirmed. The enzymatic removal of terminal Gal (Bleil and Wassarman, 1988) or GlcNAc (Shur and Hall, 1982) residues from eggs, for example, abolished the ability of ZP3 to inhibit sperm binding. However, gene-disrupted mice lacking these residues on their zonae were fertile (Asano et al., 1997; Thall et al., 1995), suggesting that these residues are not essential per se for fertilization. Fucose in Lewis X- and A-containing glycans was also indicated to play a role in sperm-zona binding (Kerr et al., 2004), but mice lacking the corresponding fucosyltransferase (Fut9) were fertile (Kudo et al., 2004). A role for mannose present on glycans has also been proposed (Cornwall et al., 1991) but has been negated by N-glycanase treatment (Florman and Wassarman, 1985). *In vitro* experiments have also ascribed sperm binding activity to O-linked oligosaccharide side chains attached to Ser332 and Ser334 of ZP3 (Chen et al., 1998). However, transgenic mice harboring mutations in Ser332 and 334 were fertile (Liu et al., 1995). Moreover, glycosylation was not observed on these residues on native ZP proteins (Boja et al., 2003). Furthermore, disruption of the ZP3-binding protein SP56 (ZP3R – Mouse Genome Informatics) on spermatozoa (Bookbinder et al., 1995) resulted in almost no phenotype in fertilization (Muro et al., 2012). Finally, enzymes that make core glycans have also been disrupted, and eggs lacking complex and hybrid N-glycans as well as core-1-derived O-glycans were fertilized normally (Shi et al., 2004; Williams et al., 2007). The hypothesis that carbohydrates play an important role in sperm-egg interaction thus requires further support.

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Table 1. Factors that are essential for fertilization as indicated by gene-disruption experiments

Class I. Impaired sperm migration into oviduct and aberrant zona binding

Gene	Localization of protein	ADAM3 on spermatozoa	Zona-binding ability	References
Clgn (calmegin)	ER membrane	Disappeared	Impaired	(Ikawa et al., 1997)
Calr3 (calsperin)	ER lumen	Disappeared	Impaired	(Ikawa et al., 2011)
Pdilt	ER membrane	Disappeared	Impaired	(Tokuhiro et al., 2012)
Pmis-2	Sperm surface	Disappeared	Impaired	(Yamaguchi et al., 2012)
Adam1a	Sperm surface	Disappeared	Impaired	(Nishimura et al., 2004)
Adam2	Sperm surface	Disappeared	Impaired	(Cho et al., 1998)
Adam3	Sperm surface	Disappeared	Impaired	(Shamsadin et al., 1999; Yamaguchi et al., 2009)
Ace (angiotensin converting enzyme)	Sperm surface	Aberrantly localized	Impaired	(Hagaman et al., 1998; Yamaguchi et al., 2006)
Tpst2	Acrosomal cap→equatorial segment	Disappeared	Impaired	(Marcello et al., 2011)
RNase10	Epididymis	Disappeared	Impaired	(Krutskikh et al., 2012)
Tex101	Spermatid	Disappeared	Impaired	(Fujihara et al., 2013)
Prss37	Spermatid/sperm	Disappeared	Impaired	(Shen et al., 2013)

ER, endoplasmic reticulum

Class II. Sperm-egg fusion incompetent

Gene	Localization of protein	Phenotype	Average number of pups/litter (wild type versus knockout)	References
Cd9	Egg membrane	Fusion incompetent	8.3 versus <0.10	(Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000)
Izumo1	Acrosomal membrane→plasma membrane	Fusion incompetent	8 versus 0	(Inoue et al., 2005)

A recent view that has been verified by gene-manipulated animals is that the zona loses its affinity for spermatozoa when ovastacin (also known as astacin-like metalloendopeptidase), an enzyme contained in egg cortical granules (Burkart et al., 2012), cleaves ZP2 after fertilization (Bleil et al., 1981; Gahlay et al., 2010). In addition, it has been suggested that liver-originated circulating glycoprotein fetuin-B is essentially required to inhibit premature cleavage of ZP2 by inhibiting ovastacin (Dietzel et al., 2013).

Together, these studies have shown that various sperm-zona interactions can occur but that many of these may not be absolutely required for successful fertilization. We also must remember that the 'sperm-zona binding' described above may not reflect the real sperm-egg interaction required for fertilization *in vivo*, as shown in Fig. 2.

Anatomical or behavioral differences between species guarantee that a male and a female from two different species do not normally mate. Nevertheless, taxon-specific zona penetration has been demonstrated (Rankin et al., 2003). However, if spermatozoa do not require a zona-binding ability for fertilization, how can they distinguish the ZP of the same species? One can anticipate that there is an as yet unclarified affinity between the cumulus-covered eggs and spermatozoa. We can further speculate that the same molecule responsible for zona binding is utilized in sperm-UTJ recognition, which could explain why the defects in zona-binding and UTJ penetration take place inseparably (Table 1).

The sperm protein zonadhesin (ZAN) is reported to contribute to species-specific zona binding (Tardif et al., 2010), but this has not yet been proven by rescue of $Zan^{-/-}$ by other species' *Zan*. Furthermore, when mouse ZP proteins were replaced with human homologs by septuple gene manipulation (mZP1 to mZP3 triple gene disruption and hZP1 to hZP4 quadruple transgenic insertion), it was shown that human spermatozoa could bind to and penetrate the humanized ZP (Baibakov et al., 2012). These data may indicate taxon-specific zona penetration. However, Gahlay et al. also mentioned that mouse spermatozoa could also penetrate the humanized ZP, which, in a way, indicated that taxon non-specific zona penetration is possible for mouse spermatozoa. It should also be noted that field vole spermatozoa have been shown to penetrate mouse and hamster zonae pellucidae without the need for an acrosome reaction (Wakayama et al., 1996).

Table 2. Sperm factors involved in sperm-egg interaction indicated by biochemical means but negated by knockout experiments

Gene	Localization of protein	Predicted role	Number of pups/litter (wild type versus knockout)	References
Acr (acrosin)	Acrosome	Zona penetration	10.0 versus 12..5	(Baba et al., 1994)
β4galt1 (GalTase)	Acrosome/sperm surface	Sperm-zona binding	Fertile *in vivo*	(Lu and Shur, 1997)
Sed1 (*Mfge8*)	Sperm surface	Sperm-zona binding	9.3 versus 3.3 Fertile *in vivo*	(Ensslin and Shur, 2003) (Hanayama et al., 2004)
Cd46	Inner acrosomal membrane→plasma membrane	Sperm-egg fusion	9.0 versus 8.9	(Inoue et al., 2003)
Crisp1	Sperm surface	Sperm-egg fusion	7.3 versus 6.5	(Da Ros et al., 2008)
Zan (zonadhesin)	Sperm surface	Sperm-zona binding	7.4 versus 6.0	(Tardif et al., 2010)
Pkdrej	Sperm surface	Sperm-zona binding	8.8 versus 7.1	(Sutton et al., 2008)
Spam1 (*Ph-20*)	Acrosome	Sperm-zona binding	13.8 versus 12.2	(Baba et al., 2002)

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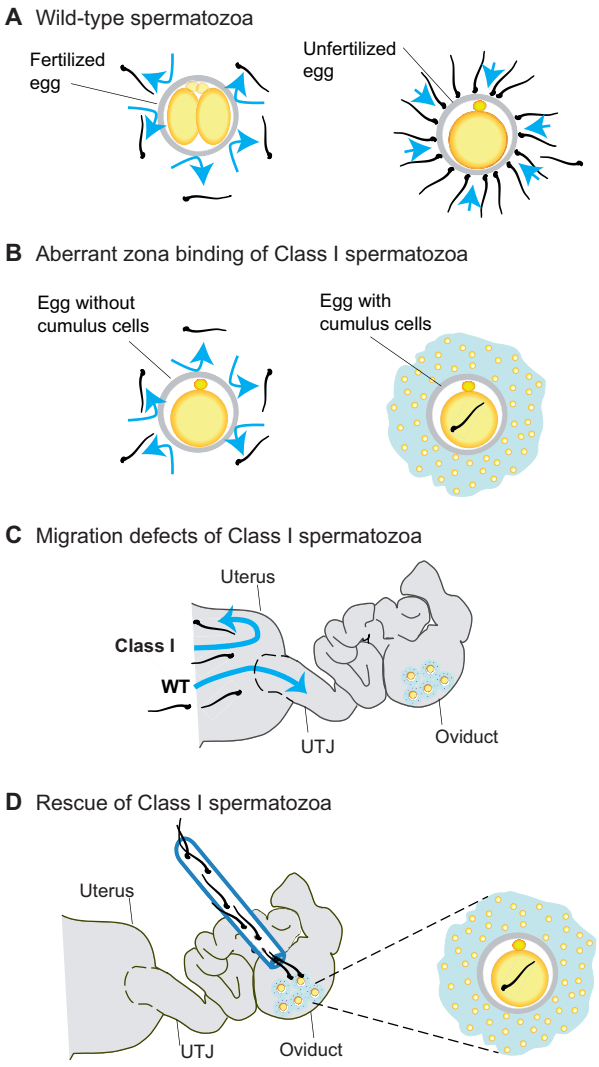

Fig. 2. Journey of spermatozoa to encounter the egg. (A) Wild-type spermatozoa are not able to bind to the zona pellucida (ZP) of fertilized eggs (left) but easily bind to the ZP of unfertilized eggs (right) *in vitro*. (B) By contrast, spermatozoa from the gene-disrupted mice shown in Class I of Table 1 cannot bind to the ZP of the unfertilized egg *in vitro* (left). However, eggs can be fertilized by these spermatozoa if the eggs are covered in cumulus cell layers (right). (C) Class I spermatozoa share the same phenotype: spermatozoa can be observed in the uterus but cannot migrate into the oviduct (top arrow), whereas wild-type (WT) spermatozoa can (bottom arrow). (D) This infertility can be rescued by injecting the gene-disrupted spermatozoa directly into oviduct.

Regulation and importance of the zona-induced acrosome reaction

Various enzymes considered to aid spermatozoa in penetrating the cumulus cell layers and the ZP are released by acrosomal exocytosis (Florman and Ducibella, 2006). The timing of the acrosome reaction was thus believed to be important for spermatozoa as they approach eggs. Therefore, many researchers believed that the acrosome reaction of fertilizing spermatozoa was induced upon contact with the ZP and that spermatozoa that have undergone the acrosome reaction before contact with the ZP have no fertilizing ability (Bleil and Wassarman, 1983). More recently, it was shown that the addition of solubilized ZP can induce the acrosome reaction (Florman and Ducibella, 2006), and a partial ZP3 sequence can accomplish the same effect (Hinsch et al., 2005). In this context, zona-binding proteins were assumed to induce a signaling cascade in spermatozoa (Gong et al., 1995). In fact, various zona-binding proteins were sought and purified from spermatozoa. However, the gene disruption of these factors resulted in no significant phenotype in fertilization [e.g. GalTase (Lu and Shur, 1997), *Sp56* (Muro et al., 2012), zonadhesin (Tardif et al., 2010), Crisp1 (Da Ros et al., 2008), acrosin (Baba et al., 1994)]. Thus, although these factors may play a role in inducing the acrosome reaction, the importance of these factors, and a zona-induced acrosome reaction, during fertilization is unclear.

A zona-independent acrosome reaction

Early studies using transgenic spermatozoa with GFP in their acrosomes were unsuccessful in observing an acrosome reaction on the ZP, and addition of the calcium ionophore A23187 to zona-bound spermatozoa was required to induce an acrosome reaction (Nakanishi et al., 1999). Another group suggested that a penetrating action through the mesh structure rather than by surface binding could induce acrosome exocytosis, because they too could not observe an acrosome reaction on ZP-binding spermatozoa (Baibakov et al., 2007). Recent *in vitro* studies also suggested that spermatozoa did not require contact with the ZP to cause the acrosome reaction. Rather, most of the fertilizing spermatozoa were in fact acrosome-reacted before reaching the ZP *in vitro* (Jin et al., 2011). This, together with the report that spermatozoa can fertilize eggs without zona-binding ability under defined experimental conditions (Nishimura et al., 2004; Tokuhiro et al., 2012), questions the importance of the zona-induced acrosome reaction and whether it is physiologically relevant.

Interestingly, hamster spermatozoa complete the acrosome reaction around the time they pass through the cumulus, or shortly before, or after contacting the surface of the ZP *in vivo* (Cummins and Yanagimachi, 1982). Likewise, guinea pig spermatozoa participating in fertilization appear to undergo the acrosome reaction after reaching the proximal part of the oviduct or when they are very near the eggs (Yanagimachi and Mahi, 1976). In addition, rabbit spermatozoa recovered from the perivitelline space of fertilized eggs can still penetrate and fertilize fresh oocytes (Kuzan et al., 1984). These results suggest that the fertilizing ability of spermatozoa is maintained for a while after the acrosome reaction. Consistent with this notion, a compartmentalized structure within the acrosome (Kim et al., 2001) and asynchronous release of acrosomal proteins during acrosomal exocytosis have been reported (Hardy et al., 1991; Kim and Gerton, 2003; Nakanishi et al., 2001).

More recently, acrosome-reacted mouse spermatozoa were shown to penetrate fresh, cumulus-enclosed, zona-intact eggs, and the resultant fertilized eggs developed to term after uterine transplantation (Inoue et al., 2011). These results suggest that the timing of acrosome reaction can be flexible. It also suggests that the crucial sperm-zona interaction occurs between the ZP and acrosome-reacted, rather than acrosome-intact, spermatozoa.

Sperm-egg fusion

Upon fusion, spermatozoa activate the egg thereby inducing calcium oscillations and completion of the second meiotic cell division (Miyazaki and Ito, 2006) PLCζ from spermatozoa is considered to be the responsible activator during this process (Nomikos et al., 2012), but other factors may also be involved (Harada et al., 2011). Activation of the egg leads to exocytosis from peripherally located cortical granules. Ovastacin is an enzyme that accumulates in cortical granules as described earlier, and is reported to result in cleavage of ZP2, which is considered

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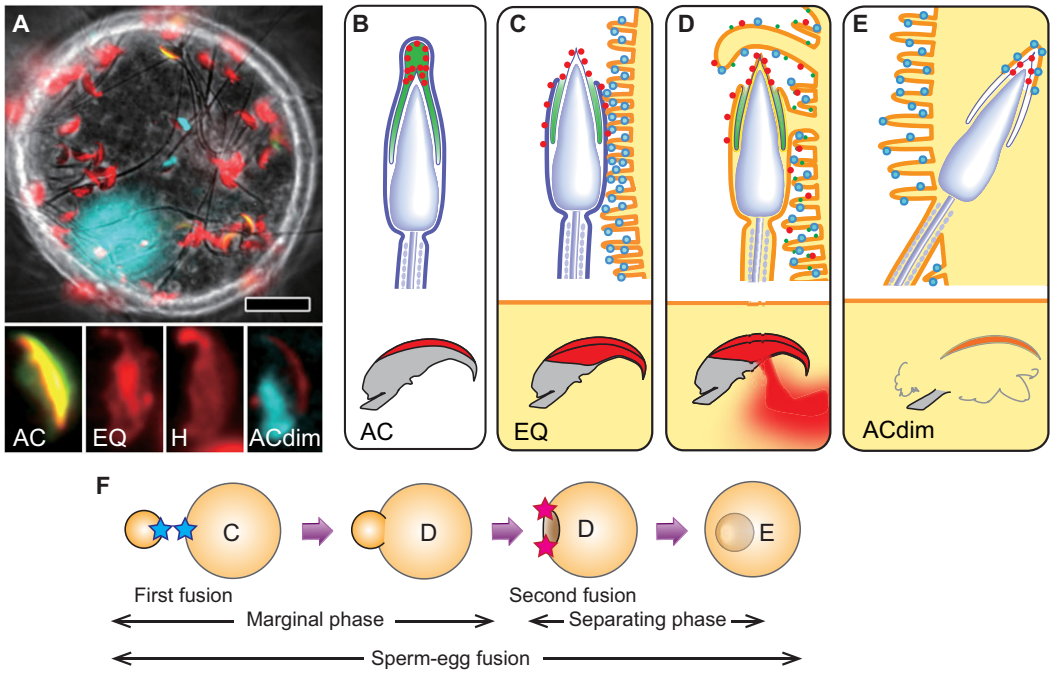

Fig. 3. Two distinct fusion steps in sperm-egg fusion. (A) When zona-free eggs are mixed with spermatozoa expressing mCherry-tagged IZUMO1 (red), various patterns of IZUMO1 localization are observed: acrosomal cap (AC), equatorial (EQ) or head (H), and dim AC (AC_{dim}). Note that the color in the AC appears yellow in A owing to the existence of acrosomal GFP. EQ or H patterns are evident pre-fusion, whereas the AC_{dim} pattern is seen in the fused spermatozoa, indicated by Hoechst staining (blue). (B-F) The dynamic movement of IZUMO1 (red circles and red fill) at the time of sperm-egg fusion, as determined by live imaging (Satouh et al., 2012), is illustrated. CD9 is represented by blue circles. The top panels represent cross sections and the bottom panels show top views of spermatozoa. Intact spermatozoa (B) show an AC pattern. Fusion-competent spermatozoa (C) exhibit an EQ pattern of IZUMO1 and initiate interaction with eggs (yellow). Once fusion starts (D), the IZUMO1 on spermatozoa spreads out from the spermatozoa to the egg plasma membrane. However, IZUMO1 on the inner acrosomal membrane remains on spermatozoa. Acrosome-reacted spermatozoa (E) have a complicatedly invaginated structure, but after fusion the sperm plasma membrane unites with that of the egg and forms a single plane including the tail part of the membrane. However, the inner acrosomal membrane area containing IZUMO1 is detached from the original fusion plane (E) and forms an independent membrane structure, giving rise to the AC_{dim} pattern. (F) The fertilization process can be separated into two phases: (1) a marginal phase (C to D); and (2) a separating phase (D to E). The former can be described as the first fusion event, shown here as a standard cell-cell fusion event using blue stars as fusion focal points. The latter, which can be separated from the first fusion event, has an endocytotic aspect and a different topological characteristic from the first fusion (fusion sites are indicated with red stars).

to decrease the affinity to spermatozoa (Burkart et al., 2012). This phenomenon, which prevents polyspermy, is called the zona reaction.

To identify factors involved in sperm-egg fusion, the antigens of monoclonal antibodies that inhibit fusion *in vitro* (Blobel et al., 1992; Okabe et al., 1987; Okabe et al., 1992) were characterized, and this led to the identification of molecules such as CD46 (Taylor et al., 1994), IZUMO1 (Inoue et al., 2005) and fertilin (ADAM1b/ADAM2 heterodimer) (Blobel et al., 1992). To assess the roles of these molecules *in vivo*, researchers turned to gene-disruption experiments in mice. Initially, fertilin gathered attention but turned out not to be required for fertilization *in vivo* (Box 2). A *Cd46*-disrupted mouse line was also produced but, even though the testis is the only place in which CD46 is expressed in mouse, no effects on the fertilizing ability of spermatozoa from these mice was observed (Inoue et al., 2003). It is possible that these proteins may play redundant roles and, when knocked out, their function can be compensated for by other proteins. Alternatively, it is possible that these factors interact with or rely on other factors, and knockout of additional interacting factors is required to observe an effect *in vivo* (see Box 2 for a further discussion of the limitations of such knockout approaches).

Despite their potential limitations, gene disruptions sometimes bring us serendipitous findings. For example, the gene encoding the tetraspanin CD9 was initially disrupted in order to clarify its role in immunology, but it became the first known essential factor for sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). More recently, we disrupted yet another gene, *Izumo1*, that was shown to be essential for fertilization; *Izumo1*$^{-/-}$ mice produce normal-looking spermatozoa but are completely infertile. The *Izumo1*-disrupted spermatozoa are able to penetrate cumulus layers and the ZP normally but they fail to fuse with eggs (Inoue et al., 2005), thus highlighting the importance of IZUMO1 in the sperm-egg fusion event.

Based on such gene-disruption studies, CD9 on the egg and IZUMO1 on spermatozoa are the only two essential factors for fusion described to date. However, interaction between the two factors has not been observed and, structurally, neither factor has a fusogenic domain, which suggests that they might act via additional interacting proteins. However, the recent identification and disruption of an IZUMO1-associating protein (angiotensin converting enzyme 3; ACE3) resulted in fertile males (Inoue et al., 2010), implying that there must be other additional factors that are involved in fusion.

To assess the role of IZUMO1 during fertilization, the localization of IZUMO1 at the time of fusion was investigated using mCherry-tagged IZUMO1. IZUMO1 is initially hidden under the plasma membrane before the acrosome reaction but then moves out to the

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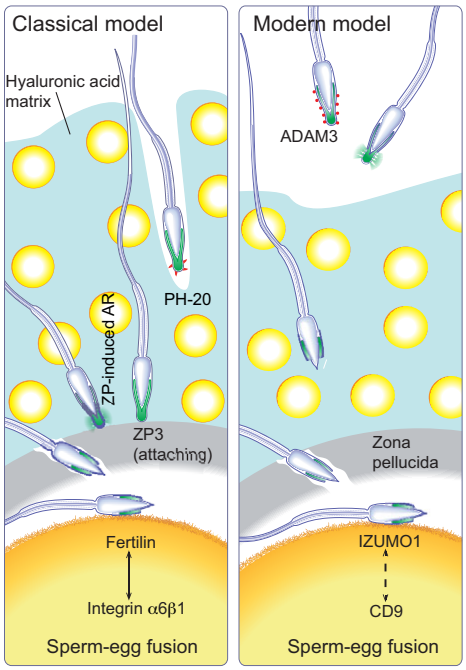

Fig. 4. Mechanism of fertilization: old and new models. In the classical scheme (left), the spermatozoa have hyaluronidase (PH-20; also known as SPAM1) (pink triangles) on their surface and penetrate the cumulus layer and attach to the zona pellucida (ZP). Many zona-binding proteins on spermatozoa have been postulated (Table 2). ZP3 was reported to be the molecule to which spermatozoa bind (or attach) and had acrosome reaction-inducing ability. The timing of the acrosome reaction was also proposed to be important for zona penetration because it was believed that the acrosomal enzymes (such as acrosin) must be released upon binding to zona. Sperm fertilin was also thought to function during egg fusion, with eggs using integrin α6β1 for its counterpart. However, none of these gene-disrupted mice became infertile (He et al., 2003). In the modern scheme (right), the spermatozoa penetrate the cumulus layer with or without the acrosome reaction. Acrosome-reacted spermatozoa can even penetrate the cumulus and zona for a second time (Inoue et al., 2011). Although the so-called 'zona-binding' ability is not required for spermatozoa to fertilize eggs, it is known that the 'zona-binding' ability is attributable to ADAM3 (pink dots) on the surface of sperm. If ADAM3 became aberrant, spermatozoa could not migrate into the oviduct and hence failed to approach eggs. If ADAM3 became aberrant, spermatozoa could not migrate into the oviduct and hence failed to approach eggs. However, ADAM3 is a pseudogene in human (Grzmil et al., 2001). Therefore, if a general mechanism does exist among mammalian species, we might still not have found the key factor for fertilization. IZUMO1 on spermatozoa and CD9 on eggs were shown to be essential for fusion but the precise mechanism of fusion is yet to be clarified.

plasma membrane from the outer acrosomal membrane during the acrosome reaction in live spermatozoa (Satouh et al., 2012). This suggests that an additional function of the acrosome reaction is to render spermatozoa capable of fusing with the egg by transferring IZUMO1, and possibly other proteins, onto the sperm surface (Fig. 3A). Of note, three IZUMO1 localization patterns after acrosome reaction were observed: (1) an equatorial (EQ) fluorescence pattern; (2) a head (H) fluorescence pattern; or (3) a dim acrosomal cap (AC) fluorescence pattern (AC_{dim}) (Fig. 3C-E). The formation of the AC_{dim} pattern was noteworthy, because this pattern was considered to be produced by the phagocytosis of the inner acrosomal membrane after the first (marginal) fusion event is accomplished, with part of the sperm membrane thereby detaching from the fusion plane (dividing fusion) (Fig. 3D,E). The formation of an AC_{dim} pattern is the product of fusion step 2 (Fig. 3F). As each fusion event has a characteristic nature, each might require individual fusion factors. In any case, we need to identify more factors involved in sperm-egg fusion.

Perspectives

A new view (Fig. 4) on the mechanism of fertilization is emerging. This view includes diminished importance of the so-called 'zona-binding ability' of sperm, a zona-independent acrosome reaction, and the zona-penetrating ability of spermatozoa recovered from the perivitelline space. This updated view is largely based on *in vivo* observations of gene-manipulated animals, whereas classical models were mainly centered on observations of *in vitro* fertilization (IVF). In IVF conditions, eggs must be exposed to 10^5 spermatozoa per ml (comprising a mixture of acrosome-intact, acrosome-reacted, and degrading spermatozoa). However, only a few (presumably acrosome-reacted) spermatozoa approach eggs for fertilization *in vivo*. Furthermore, recent studies have shown that many factors reported to be important for fertilization *in vitro* can be genetically disrupted with little or no perturbation of fertility (although see Box 2 for further discussion of potential reasons for this). These studies emphasize that, to avoid misplaced enthusiasm that could mislead the field, it will be important to validate candidate molecules in gene-manipulated animals in order to focus on those essential for fertility. Fertilization might be one of the most suitable research fields to require the use of gene-manipulated animals to perform *in vivo* studies, as the mysterious behavior of gametes is seemingly difficult to reproduce *in vitro*.

Competing interests

The author declares no competing financial interests.

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