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Mechanisms of sperm-egg interactions emerging from gene-manipulated animals

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ABSTRACT Sperm-egg interactions have been studied for many years using biochemical approaches such as the employment of antibodies and ligands that interact with sperm or with eggs and their vestments. As a result, various factors that participate in fertilization have emerged. However, when animals were genetically manipulated to examine the roles of those factors, most of them were found, to our surprise, to be "not essential". Of course, all biological systems contain redundancies and compensatory mechanisms, but at least some factors were found to be "essential" after gene disruption. As a whole, the explanations of sperm-egg interactions require significant modification from the gene manipulation point of view. In this review, information about sperm-egg interactions obtained from genetically manipulated animals is mainly revisited in order to propose a new vision.

KEY WORDS: *fertilization, knockout mouse, sperm-zona interaction, sperm-egg fusion*

Introduction

About a billion years ago, living organisms devised sex (Margulis and Sagan, 1986) to facilitate a genetic shuffling for better survival and quick evolution. Since then enormous numbers of recognition and fusion of males and females have been successfully accomplished and the process has evolved immensely. In mammals, males produce innumerable tiny sperm while females produce much larger eggs with a thin glycoprotein layer: the zona pellucida. How do sperm and egg recognize each other, make contact, and achieve fusion? Various factors have been reported with convincing evidence. However, the recently-introduced, homologous gene disruption technique revealed many of the genes were dispensable because the animals devoid of those genes showed substantial fertilizing ability. One can argue that the disappearance of a factor could be compensated by adjusting the amount of equally functioning factors in gene disruption experiments. However, sperm are like "rockets" with limited resources available once launched. Sperm DNA are tightly packed with protamine and the transcriptions are shut down. Even if the sperm can carry out some *de novo* protein synthesis during capacitation using stored mRNA and mitochondrial ribosomes (Gur and Breitbart, 2006), it is difficult to imagine that the sperm rearrange their function by replacing one factor with others. One can also argue that, based on the importance of fertilization phenomena, various steps are made with backup systems. However, recent gene disruption experiments indicated that there are "essential" factors in fertilization, because in various cases, the lack of a factor leads to the complete loss of fertilizing ability of gametes. Thus the quest for more "essential" factors and analysis of the relationship among those factors represent the most promising path to elucidate the mechanism of fertilization in molecular bases. In the present review, we introduce essential factors in fertilization and clarify their relationships.

Eggs

Eggs are released into the peritoneal or bursal cavity and then are picked up by the cilia of the infundibulum. Mammalian eggs are almost invariably covered by numerous cumulus cells and an extensive extracellular matrix, which is recognized by specific receptors on the tips of the cilia. Pickup of this complex by the oviduct has been recorded in beautiful video pictures available online by Talbot *et al*. using hamsters (Talbot *et al.*, 1999) (Fig. 1).

After being picked up from the infundibulum, the eggs move to the ampulla of the oviduct and wait to be fertilized by sperm. It is not clear why or how they stay in this area, but they do, enveloped in the cumulus mass until fertilization occurs. Eggs have fertilizing ability depending on the nature of cumulus cells and zona pellucida (Yanagimachi, 1994), but surprisingly, fus-

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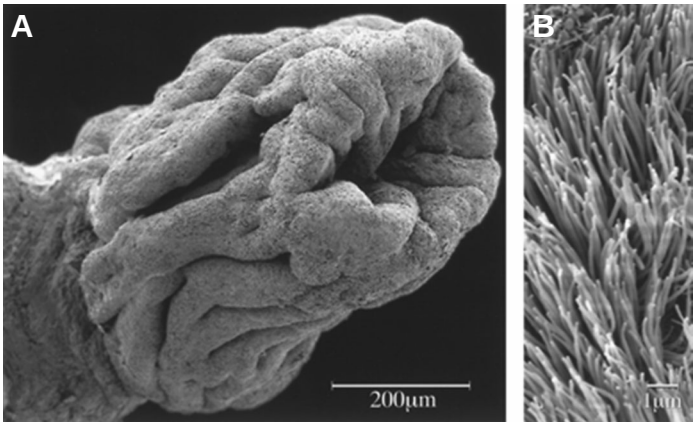

Fig. 1. Hamster infundibulum and magnified view of cilia on its surface *(Talbot* et al.*, 1999). An online video showing the movement of ovulated eggs into the oviduct is available at: http://www.molbiolcell.org/content/vol10/issue1/images/video/mk0190776002b.mov*

ing ability with sperm is formed even as - the immature eggs - develop without cumulus cells inside mouse testis (Isotani *et al.*, 2005).

Sperm

Sperm and eggs move in different directions. Sperm need to ascend the female reproductive tract to find eggs residing in the oviduct, while the eggs descend the oviduct and enter the uterus after fertilization. Usually one egg in humans and about ten in mice await sperm in the oviduct. Compared to the numbers of eggs, an overwhelming number of sperm are ejaculated into the female reproductive tract. However, only a small number of sperm reach the fertilization site. The uterus and oviduct are connected at the uterotubal junction (UTJ). The outer portion of the oviduct hangs into the uterus and forms a colliculus in mice, pigs and cows. The tract is very narrow at the UTJ and sperm are not able to migrate freely into the oviduct through the UTJ, decreasing the number of sperm participating in fertilization. It is not known if only selected sperm can penetrate the UTJ, but various factors are known to be essential for sperm to pass through the UTJ (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004). When we made chimeric mice that ejaculated a mixture of wild-type sperm and motile but not fertile sperm from calmegin-disrupted mice, only the former sperm in the same ejaculates could migrate to the oviduct. This result indicates a possibility of selection at the UTJ (Nakanishi *et al.*, 2004).

Sperm chemotaxis toward eggs before fertilization has been demonstrated clearly in ascidians (Yoshida *et al.*, 2002). In humans, olfactory receptors (ORs) on sperm might function in finding eggs. The hOR17-4 was demonstrated to function in human sperm chemotaxis (Spehr *et al.*, 2003). Mouse sperm might also locate eggs by sensing a chemoattractant (Fukuda *et al.*, 2004). The disruption of an ion channel expressed in VNO neurons are reported to cause females to behave like males (Kimchi *et al.*, 2007). The contribution of ORs in fertilization must be proven by gene disruption experiments.

Sperm are produced in the testis, transferred into the epididymis and remain stored in the cauda epididymis. Once ejaculated, they must be activated by stimuli from the female environment and start to swim vigorously. Only capacitated and acrosome-reacted sperm are competent to fertilize eggs, like matches being struck and activated before acquiring capability to cause fire (Fig. 2)

The nature of the capacitation process is not well understood, but there are many papers indicating the importance of protein phosphorylation and calcium ion influx upon release of "decapacitation factor(s)" from sperm (De Jonge, 2005). It should be noted that the acrosome reaction is a change that happens only in capacitated spermatozoa and gradually increases to 30–40% during 1–2h of incubation *in vitro* in mouse sperm population. This means sperm are not homogeneous but are individually different from each other. However, most reports treat sperm as a mixed mass to evaluate sperm status because there is not a convenient method to separate sperm at different stages of capacitation and/or acrosome reaction. We must be aware that this homogeneity problem exists in the experiments which analyze sperm as a combined mass.

Various methods are proposed to observe acrosome reaction (Cross and Meizel, 1989, Larson and Miller, 1999, Saling and Storey, 1979). Our strategy to observe the acrosomal status under microscope is to use transgenic mouse lines with green fluorescent protein (GFP) in their acrosome. We produced transgenic mice whose sperm have GFP in their acrosome; the green fluorescence is clearly seen with no previous treatment of sperm. After the acrosome reaction, GFP disappears within three seconds (Nakanishi *et al.*, 1999). Sperm from these mice could easily be analyzed using a flow cytometer, and real-time analysis of acrosome reaction is possible (Nakanishi *et al.*, 1999). Although the GFP disperses from acrosome rapidly, other acrosomal components such as MN7 antigen and MC41 antigen remain on sperm for at least 15 min. Thus, it was shown that the acrosome reaction is not a simple all-or-none phenomenon, but one with intermediate stages. Sperm–egg interactions must be investigated taking into account factors such as an intermediate stage of acrosome-reacted sperm (Kim and Gerton, 2003).

The acrosin-GFP mice are available to the public through RIKEN BRC or CARD, Kumamoto University under the registered name, B6;C3 Tg(acro3-EGFP)01Osb or C57BL/6-Tg(CAG/Acr-EGFP)C3-N01-FJ002Osb (http://www.brc.riken.jp/lab/animal/en).

Sperm-egg interactions: an original view

PH-20 has been indicated to have a role in sperm binding with the zona pellucida, based on the finding that two out of the three monoclonal antibodies raised against PH-20 inhibited sperm–zona binding (Primakoff *et al.*, 1988). In 1993, a group studying snake venom found a significant homology of hyaluronidase to PH-20 (Gmachl and Kreil, 1993). These structural data seemed to support the long-held view that hyaluronidase plays a role in fertilization. In fact, in macaque monkeys, zona penetration was completely blocked by anti-PH-20 IgG when present during sperm–oocyte interaction (Yudin *et al.*, 1999). However, when PH-20 gene-disrupted mice were produced and examined, the mice showed a reduced ability to disperse cumulus cells but were found to be fertile (Baba *et al.*, 2002). Baba's group found a remaining hyaluronidase activity in PH-20 disrupted mouse sperm, and they discovered an additional sperm specific hyaluronidase

gene Hyal5 very close to PH-20 in the genome (Kim *et al.*, 2005). The role of hyaluronidase in sperm-egg interaction will be concluded when we see the binding ability impaired sperm from Hyal5 knockout or PH-20 and Hyal5 double knockout mice.

There are more factors suggested to be involved in sperm–zona interactions. Beta 1,4-galactosyltransferase (GalTase) is reported to function not as an enzyme, but as a sperm-egg-binding factor. Various reports exist supporting this notion. However, when a GalTase-disrupted mouse line was produced by Shur's group, unexpectedly it was found that sperm lacking GalTase could fertilize eggs and the males were not sterile although there were some minor defects in sperm-egg interactions (Asano *et al.*, 1997, Lu and Shur, 1997). This could be interpreted as suggesting that the role of GalTase was compensated by other factors and another candidate SED1 was reported as a second zona-binding factor (Ensslin and Shur, 2003). When SED1 gene was disrupted, it was found that the mice were not sterile. Such an unexpected outcome by disrupting genes for sperm-egg interaction dates back to 1994. The first gene disruption aimed at studying sperm-egg interaction was the gene for acrosin. Despite hundreds of papers supporting the importance of sperm acrosin in fertilization, acrosin-null sperm can still fertilize eggs, albeit with a slight delay compared with wild type (Adham *et al.*, 1997, Baba *et al.*, 1994). Baba's group found that protease activity persists in the sperm of acrosin gene-disrupted mouse. Altogether they found five more testis-specific proteases, from TESP1 to TESP5 (Honda *et al.*, 2002), but the roles of these enzymes in fertilization await further investigation.

There are further examples that the disruption of "important" factors results in an unexpectedly mild phenotype or shows an unpredicted phenotype in fertilization. Fertilin was originally clarified as an antigen recognized by the anti-guinea pig sperm monoclonal antibody PH-30. As the PH-30 antibody inhibited sperm from fusing with eggs, the PH-30 gene was cloned and analyzed. The antigen was found to be a heterodimer and one of the monomer genes contains a fusogenic domain similar to viral fusogen. Moreover, the other half of the heterodimer was found to contain a disintegrin domain, which binds to integrin. Thus the antigen that reacts to PH-30 was speculated to be a fusion protein and named "fertilin," which consists of fertilin alpha and beta (ADAM1/2) heterodimer(Blobel *et al.*, 1992). After this report, various types of circumstantial evidence were reported to support the role of fertilin. However, when Adam2, which forms a fertilin heterodimer, disrupted mice were produced, they found that Adam2-null sperm was shown to have fusing ability with eggs but unexpectedly, the sperm failed to bind to zona pellucida (Cho *et al.*, 1998). Later, it was found that Adam1 consisted of two independent genes: Adam1a and Adam1b (Nishimura *et al.*, 2002). Both of the ADAM1 family proteins could make a heterodimer with ADAM2, but "fertilin" on sperm surface was found to be exclusively an ADAM1b/ADAM2 type. Baba's group disrupted the ADAM1b gene but the mice were fertile without fertilin on sperm (Kim *et al.*, 2006).(This will be discussed below.) Similar stories continue in disruptions of CD46 (Inoue *et al.*, 2003), ZPBP1 (Lin *et al.*, 2007) and PKD-REJ (our unpublished data) and in some more factors (private communications).

If the fertilization mechanism consists of–redundantly prepared factors, it is not possible to analyze the role of factors with a single gene disruption experiment. If this was the case, the above mentioned factors may represent the redundant factors. If there is a factor which is not redundant and not able to be compensated, the disruption of a concerned factor must produce sterile mice. Such cases are described below.

Sperm-egg interactions: an emerging view

The first case of normally swimming sperm with normal shape and numbers failing to fertilize eggs was our report on the calmegin gene-disrupted mouse (Ikawa *et al.*, 1997). Calmegin$^{-/-}$ males are almost sterile with a lack of sperm zona-binding ability. When sperm from such males were added to cumulus-free eggs and observed under the microscope, we noted that the sperm had lost their zona-binding ability and were bouncing off the zona pellucida. However the calmegin is not directly involved

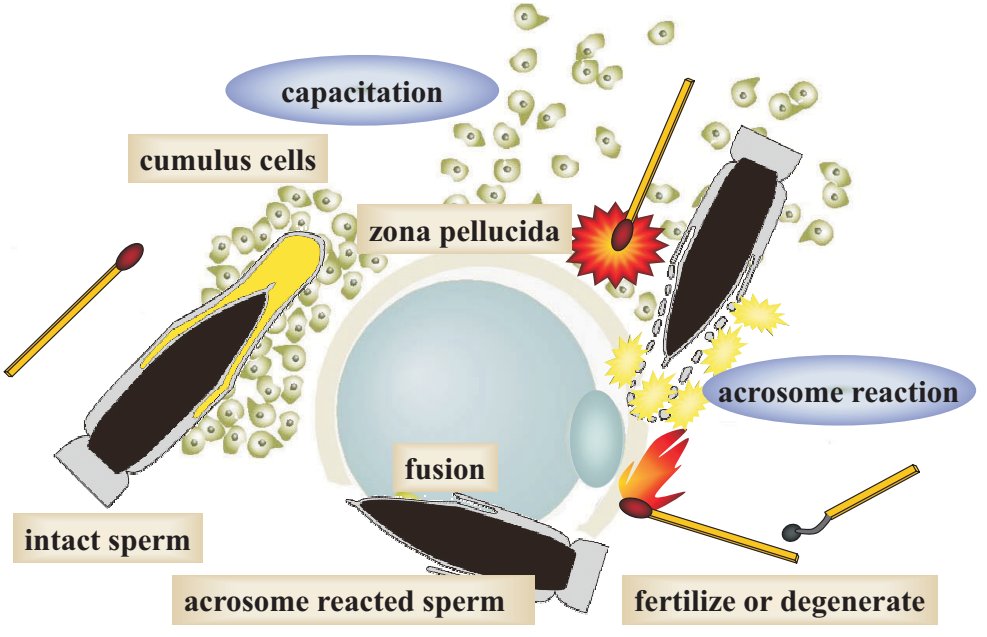

Fig. 2. Mechanism of sperm–egg interaction. *Sperm stored in the epididymis are kept metabolically inert to facilitate prolonged storage (left). Each sperm has a membranous sac over the nucleus called the acrosome which is filled with many kinds of hydrolytic enzymes. After sperm are exposed to the female reproductive environment, they become metabolically active. They undergo capacitation, which permits the acrosome reaction, and start to swim extremely vigorously (hyperactivation). Near the eggs, probably stimulated by the cumulus cells and zona pellucida, they undergo the acrosome reaction to release the acrosomal contents by exocytosis. Only acrosome-reacted sperm are known to fuse with eggs but their competency for fusion does not last long. The integrity of the acrosome in the mouse sperm can be monitored easily by using transgenic mice in which the GFP protein is targeted to the acrosomal contents (Nakanishi* et al.*, 1999).*

in sperm-egg interactions. Calmegin is a testis-specific homologue of the ubiquitously-expressed endoplasmic (ER) molecular chaperone calnexin. During spermatogenesis, sperm shed machineries for protein synthesis, including the ER. Thus, even in wild-type mice, there is no calmegin left on the sperm. Therefore, it is easily speculated that calmegin is acting to fold molecule(s) that are destined to function in sperm-egg binding. After we published the calmegin disruption, two other gene disruptions, Adam2 and angiotensin converting enzyme (ACE), were found to result in male sterility. Peculiarly, these knockout mouse lines and calmegin knockout mice share the phenotype of impaired zona-binding ability and, at the same time, an impaired UTJ penetrating ability. We thought the interaction of calmegin with ADAM2 was conceivable, so we immunoprecipitated calmegin from testicular lysate and examined the interaction of calmegin with ADAM2. Immunoprecipitation followed by western blot analysis revealed that ADAM1a, ADAM1b and ADAM2 formed complexes specifically with calmegin in the ER. The disruption of calmegin was shown to cause impaired heterodimerization of ADAM1a/2 and ADAM1b/2 leading to the complete loss of ADAM2 from mature sperm. Thus the reason why the calmegin and Adam2-disrupted mice share the same phenotypes became clear (Ikawa *et al.*, 2001).

To our surprise, the Adam3-disrupted male mice were also sterile with impaired zona-binding ability (Shamsadin *et al.*, 1999). Why did so many gene disruptions result in a single phenotype? This was explained by a simple reason. The amount of an ADAM family protein in sperm is easily affected by the disappearance of other ADAM family members. Baba's group found the testis specific ADAM1a was essential in fertilization, but they also found that when ADAM1a was eliminated, ADAM3 also disappeared from sperm, (Nishimura *et al.*, 2004). The synchronized disappearance of ADAM family proteins are reported by many researchers (Cho *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004, Stein *et al.*, 2005). A stunning result was reported by Baba's group. When they produced Adam1b (fertilin beta) disrupted mice, they found Adam2 also disappeared from sperm, but the sperm were fertile (Kim *et al.*, 2006). This result clearly indicates that the intensively studied protein "fertilin" is dispensable in fertilization. Combining these data together, ADAM3 emerged to be a putative key molecule in fertilization. The mechanism of infertility caused by ACE disruption remained unclear for many years. However, the relationship between ACE and ADAM3 was demonstrated. When we analyzed the ADAM3 in Ace-disrupted mice, we suggested the membrane micro-domain specific disappearance of ADAM3. We found a significant decrease of ADAM3 on Ace-/- sperm in the Triton X-114 detergent-enriched phase after phase separation, while ADAM2 remain unchanged on sperm (Yamaguchi *et al.*, 2006) (Fig. 3).

Combining these facts together, the most important factor that may participate in sperm-zona binding is tentatively ADAM3 (Yamaguchi *et al.*, 2006). However, multiple isoforms of Adam3 transcripts observed in the human were non-functional owing to the presence of deletions and in-frame termination codons (Frayne *et al.*, 1999). Therefore, ADAM3 cannot be an ultimate universal zona-binding factor among mammals.

In terms of other candidate factors for zona binding, sp56 was identified as having the characteristics expected of the sperm protein responsible for recognition of egg zona pellucida. The complementary DNA encoding sp56 was isolated and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors (Bookbinder *et al.*, 1995). Zonadhesin is a multiple-domain transmembrane protein believed to function as a sperm–zona pellucida binding protein(Hardy and Garbers, 1995, Jansen *et al.*, 2001, Lea *et al.*, 2001, Wassarman, 1992). We must examine the fertilizing ability of sperm that lack these factors to reach a definite conclusion.

Moreover, it should be noted that all of the calmegin, ADAM1a-, ADAM2- and ACE-disrupted mouse sperm share the phenotype of inability, not only to bind to zona, but also to migrate into the oviduct. (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004) The puzzle is why the two different inabilities of sperm–zona binding and oviduct migration run in parallel in these gene disruption experiments. We believe this phenomenon could be a good clue in helping to understand the molecular mechanisms of fertilization.

Membrane fusion

The lipid membrane has fluidity both horizontally and transversely and here are various mechanisms to maintain the lipid constitutions of both the outer and inner sides of the membrane. When the cell needs to divide, the membrane must be separated into two sections. Topologically, to achieve this, there must be a membrane break in the lipid bilayer at some point of the cytokinesis. If the egg membrane is broken artificially, as when we make a hole in the egg membrane to do intracytoplasmic sperm injection (ICSI), the opening normally seals back immediately. However, the ability for restoration is not consistent. It differs depending on the stage of the eggs. It also differs depending on the species. For example, mouse eggs are much more fragile than human eggs. We are not aware of the causes, but the adjustment and formation of a characteristic nature of the membrane must occur through a combination of various mechanisms such as the membrane undercoat and the constitution of lipids to form membrane. Sperm membrane is also known to have a complicated structure that contains cholesterol-dense "rafts" which are modified extensively during sperm capacitation (De Jonge, 2005), and evenly observed membrane by electron microscope is actually divided in some areas when examined by anti-sperm antibody.

Various important membrane fusion events exist in several tissues. For example, skeletal muscle cells are formed after myoblast cell fusion. Trophoblast cells fuse with each other to form syncytiotrophoblasts, which are necessary for implantation. Osteoclasts resorb the bone at multiple sites, occasionally containing more than 100 nuclei, and are formed by cell fusion(Yagi *et al.*, 2005). Mitochondrial membranes are known to fuse with neighboring mitochondrial membranes using mitofusin1 and mitofusion2 (Chen and Chan, 2005). One of the most precisely investigated fusion processes is exocytosis. SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) and some kinds of viruses appear to mimic the fusion mechanism using SNAREs for invasion. (Chan *et al.*, 1997, Lu *et al.*, 1995, Weissenhorn *et al.*, 1997). The structures of viral fusion proteins suggest that the fusion machineries employ a fundamentally similar mechanism to coalesce lipid bilayers. Fertilization is the phenomenon that involves membrane fusion between the plasma membrane of an unfertilized egg and the freshly-rear-

ranged sperm membrane soon after the acrosome reaction. Both gametes have to be conditioned properly to accomplish membrane fusion, but the factors involved in fusion were not known for many years.

Sperm–egg fusion in gene disrupted mice

Gene disruption experiments not only destroy the predicted importance of various factors, but also introduce new factors in the mechanism of fertilization. Because all who produce gene knockout mouse lines need to breed them to keep the strain alive, if there is any defect in the fertilization process, it will inevitably draw the attention of researchers in any field. For example, CD9 was disrupted aiming to determine the effects on immune functions. However, the CD9-deficient mice looked healthy and lived normally, but surprisingly, the females lacking CD9 were sterile, while the male mice remain normal (Kaji *et al.*, 2000, Le Naour *et al.*, 2000, Miyado *et al.*, 2000). We analyzed the cause of the sterility using *in vitro* fertilization (IVF) and found that the eggs had no fusing ability with sperm (Miyado *et al.*, 2000). Since fusion did not take place, the cortical granules were not released to block the polyspermy (Barros and Yanagimachi, 1971, Yanagimachi, 1994) which allowed multiple sperm to penetrate into the perivitelline space as shown in Fig. 4A. Thus, the very first discovery of an essential factor in sperm–egg fusion was found serendipitously.

CD9 is a member of the "tetraspanin" family of proteins having four transmembrane domains and ability to bind with integrins. Since integrins α6 and β1 were found on the egg membrane, the effect of the addition of synthetic integrin peptides in the IVF system was examined and the inhibition of sperm-egg fusion was reported (Chen *et al.*, 1999). However, again the gene disruption experiments in integrin α6 and β1 revealed that both integrins are not essential for eggs to fuse with sperm (Miller *et al.*, 2000).

Sperm must have completed the acrosome reaction prior to fertilization. This may imply that fusogenic factors are latent in fresh sperm and exposed only after acrosome reaction. We raised monoclonal antibodies against human sperm and screened one which did not react to fresh sperm, but to acrosome- reacted sperm. If the antibody could inhibit sperm–egg fusion, the corresponding antigen could be a good candidate as a fusion factor. Based on this hypothesis, we raised the anti-human sperm monoclonal antibody MH61 to meet

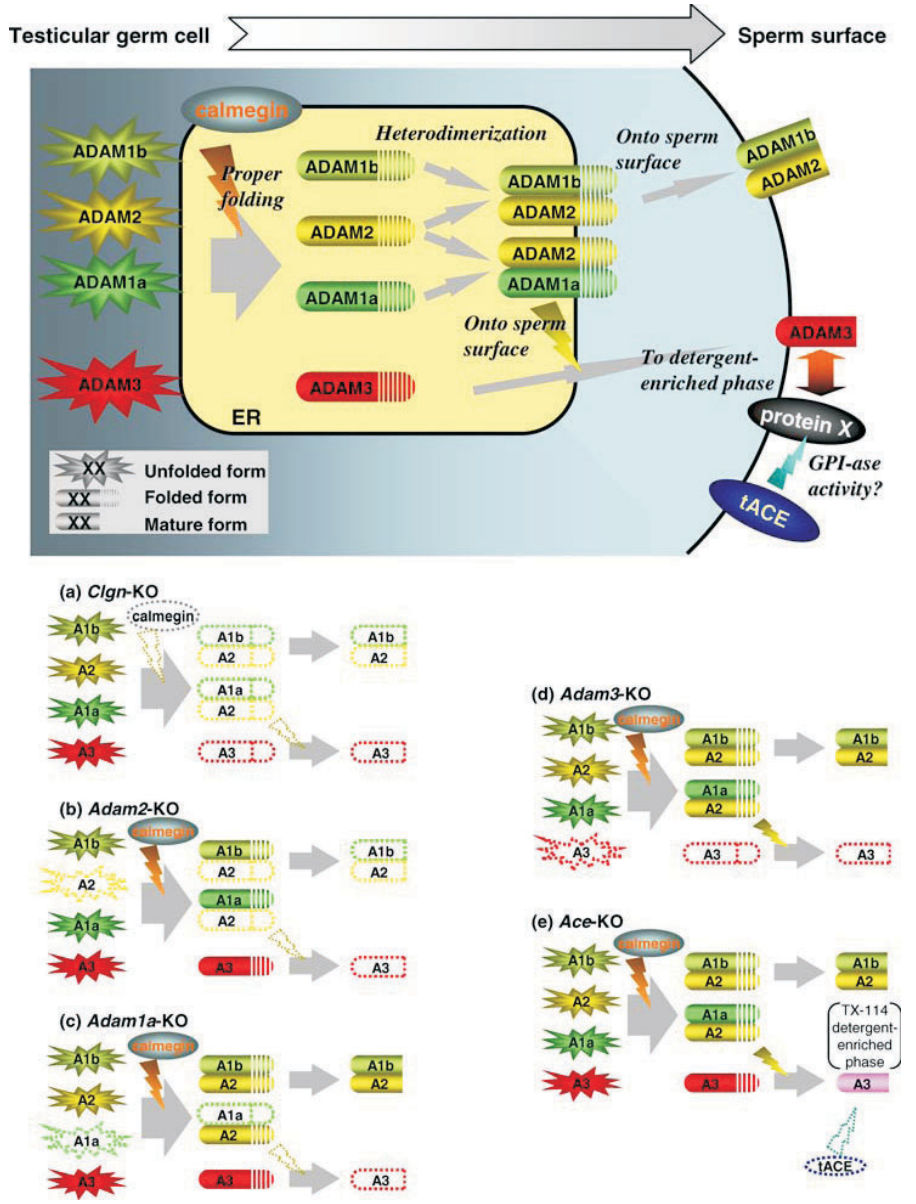

Fig. 3. Schematic model for ADAMs and their roles in sperm function. *The disruption of the genes that encode Adam1a, Adam2, and Adam3 results in impaired sperm-ZP binding. CLGN is required for the folding of ADAM1a, ADAM1b, and ADAM2 and the subsequent dimerization of these proteins. In Clgn-/-* **(a)** *and Adam2-/-* **(b)** *sperm, the disappearance of the ADAM1a/ADAM2 and ADAM1b/ADAM2 heterodimers results in the loss of ADAM1b, ADAM2, and ADAM3 from the sperm. ADAM1a is a testis-specific protein that is not found in sperm (Kim* et al., *2003). When ADAM1a is eliminated* **(c)**, *the ADAM1a/ADAM2 heterodimer disappears from the testis, whereas the expression of ADAM1b/ADAM2 is not affected. However, these sperm lack ADAM3 (Nishimura* et al., *2004). The disruption of ADAM3* **(d)** *is reported to have no significant effect on ADAM1a, ADAM1b or ADAM2 (Nishimura* et al., *2001). These findings suggest that ADAM3 is located downstream of these other ADAM proteins. The disruption of tACE leads to the aberrant localization of ADAM3* **(e)**, *most likely due to a different pathway from the one hypothesized for CLGN/ADAMs. These results indicate the importance of ADAM3 in sperm-ZP interaction and explain why disruption of the individual Ace, Clgn, Adam1a, Adam2, and Adam3 genes produces similar phenotypes.*

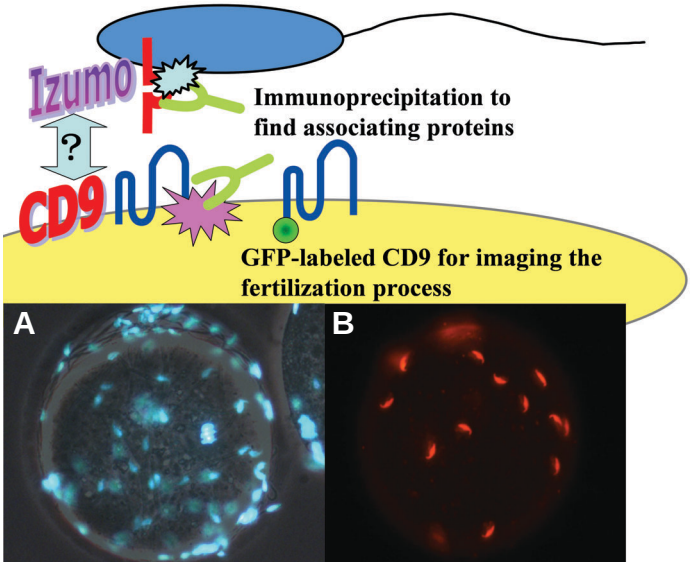

Fig. 4. Accumulation of sperm in the perivitelline space caused by failure of sperm–egg fusion. *Only one factor each on sperm and eggs is available at the moment. As in the case of live imaging acrosome reaction (ftp://ftp.gen-info.osaka-u.ac.jp/ARmovie/), the imaging of the fusion factors at the time of fertilization may be possible by producing fluorescent chimeric proteins. Immunoprecipitation will also clarify the new factors involved in the fusion event.* **(A)** *Sperm accumulated in the perivitelline space of CD9–/– mouse eggs. The sperm could penetrate the zona pellucida but failed to fuse with the egg surface. Many sperm were able to enter because of the lack of egg activation, which normally leads to cortical granule release and the zona block to polyspermy. Sperm nuclei were stained with Hoechst 33342(Miyado* et al.*, 2000).* **(B)** *Similarly, when eggs were inseminated with Izumo–/– sperm, the sperm could penetrate the zona pellucida but failed to fuse with the eggs. This also resulted in the accumulation of many sperm inside the perivitelline space (Inoue* et al.*, 2005). These penetrated sperm had clearly undergone the acrosome reaction, as they were all exposing the acrosome reacted sperm-specific antigen MN9 (Toshimori* et al.*, 1998).*

this criterion (Okabe *et al.*, 1990). The reacting antigen was identified as CD46, whose function is reported to be a complement receptor in human. However, when we cloned the CD46 gene in mouse, CD46 was found to be present only on sperm (Tsujimura *et al.*, 1998). We thought that this suggested the original role of CD46 was to function in sperm–egg interaction. Therefore, we produced CD46 disrupted mice with a strong expectation of causing sterility, but CD46 disrupted mice showed no visible damage to fertilizing ability in males or females (Inoue *et al.*, 2003).

We then analyzed OBF13 monoclonal antibody that was raised against mouse sperm, binds only to acrosome-reacted sperm, and inhibits sperm-egg fusion in the mouse (Okabe *et al.*, 1987). We recently identified the antigen by separation of crude extracts from mouse sperm by two-dimensional gel electrophoresis and subsequent immunoblotting with the monoclonal antibody. The identified spot was analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The antigen gene encodes an immunoglobulin superfamily (IgSF), type I membrane protein with an extracellular immunoglobulin domain that contains one putative glycosylation site. We termed the antigen "Izumo" after a Japanese Shinto shrine dedicated to marriage. We then produced an Izumo-disrupted mouse line. The Izumo-null males showed complete sterility despite normal mating behavior with normal vaginal plug formation. No offspring were fathered by these mice. When the sperm fertilizing ability was examined by *in vitro* fertilization system, many sperm were observed inside the perivitelline space indicating the fertilization was hampered at the sperm-egg fusion stage (Fig. 4B). However, when we performed intracytoplasmic sperm injection (ICSI) using Izumo $^{-/-}$sperm, they could activate the eggs, and the eggs were implanted normally and resulted in normal embryos. Therefore the deficiency of Izumo protein affects only to the sperm-egg fusion stage (Inoue *et al.*, 2005).

Relationship between the gene disruptions and their apparent phenotype

We need to be careful about the "off target" effects of gene disruption. A targeted disruption of the myogenic basic-helix-loop-helix gene Mrf4 is a good example. The phenotypes of three different Mrf4-disrupted mouse lines from three different laboratories with slightly different targeting vectors were very different, ranging from those showing complete viability of homozygotes to those displaying complete lethality. These three similar, but slightly different, targeting vectors had very different effects on expression of the adjacent Myf5 gene, which accounts for much of the phenotypic variation (Olson *et al.*, 1996). Another good example of the potential pitfalls of gene disruption is the case of the disruption of the Prion gene (PrP). Five independent PrP knockout mouse lines have been reported with no phenotype (Bueler *et al.*, 1992, Manson *et al.*, 1994) and with cerebellar symptoms (Moore *et al.*, 1999, Sakaguchi *et al.*, 1996, Silverman *et al.*, 2000). The discrepancy of the observation was associated with inter-gene splicing with neighboring Doppel gene in some of the targeting vectors (Flechsig *et al.*, 2003, Rossi *et al.*, 2001). To examine that the absence of Izumo directly caused the failure to fuse, we made a transgenic Izumo line driven by testis-specific calmegin promoter. The sterile phenotype was rescued with the transgenically-expressed Izumo on mouse sperm. Thus, we are certain that Izumo is the sperm factor shown to be essential for sperm-egg fusion.

Generality of sperm-egg fusion factors

In the sperm-zona binding event, ADAM3 holds the central position in the mouse, but the equivalent gene in human seems to be not producing functional protein (Frayne *et al.*, 1999). We were curious to see if Izumo is species-specific. Therefore, Izumo $^{-/-}$ sperm were mixed with hamster eggs which are able to fuse with sperm from different species. As a result, it was shown that Izumo is essential for mouse sperm to fuse with hamster eggs. Likewise, fusion of human sperm to hamster eggs was inhibited by the addition of anti-human Izumo antibody. This may suggest that Izumo is involved in sperm–egg fusion in humans as well. However, as indicated throughout this review, the addition of antibody in the IVF condition often provides us with different views obtained from various gene-manipulated animals. Therefore, it is too early to conclude that Izumo is functional in humans. It will become clear if men with mutations in their Izumo gene are found to be infertile with a symptom of fusion disability. In any case, the first

unambiguous fusion-related factors on sperm (Izumo) and on eggs (CD9) have been clarified. Again, the mice are available to the public through the Japanese animal distribution systems (indicated earlier). It is now a historic point in time, as we all are standing at the starting line of the elucidation of sperm-egg fusion.

Conclusions

Experiments using gene-manipulated animals are very powerful tools for judging the essentiality of concerned factors in fertilization. Of course, if a certain factor is judged as "not essential," it does not necessarily mean the factor is not functioning *in vivo*. However, the number of genes that are indispensable in fertilization is accumulating and their relationships with fertilization biology are emerging in the field of sperm-zona interaction. Gene disruption experiments are pursued in many research fields and the number of genes disrupted is increasing day by day. Thus, any genes that might affect reproduction will continue to be found even by researchers in different field. The day that we can draw a clear image of the sperm-egg fusion mechanism in molecular biology is definitely nearing.

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