Asian Journal of Andrology (2015) 17, 646–652
© 2015 AJA, SIMM & SJTU. All rights reserved 1008-682X
www.asiaandro.com/www.ajandrology.com

Open Access

INVITED RESEARCH HIGHLIGHT

Sperm Biology

Mechanisms of fertilization elucidated by gene-manipulated animals

Masaru Okabe

Asian Journal of Andrology (2015) **17**, 646–652; doi: 10.4103/1008-682X.153299; published online: 7 April 2015

Capacitation and the acrosome reaction are key phenomena in mammalian fertilization. These phenomena were found more than 60 years ago. However, fundamental questions regarding the nature of capacitation and the timing of the acrosome reaction remain unsolved. Factors were postulated over time, but as their roles were not verified by gene-disruption experiments, widely accepted notions concerning the mechanism of fertilization are facing modifications. Today, although *in vitro* fertilization systems remain our central research tool, the importance of *in vivo* observations must be revisited. Here, primarily focusing on our own research, I summarize how *in vivo* observations using gene-manipulated animals have elucidated new concepts in the mechanisms of fertilization.

Studies of the mechanisms of fertilization date back to Aristotle (384-322 BCE), who thought that the woman provided fertile ground for the male seed to grow. By the 17th century, however, it was recognized that females produce eggs. Leeuwenhoek's microscope provided the next insight, making it possible to visualize the spermatozoa in semen. Using this microscopic observation, Hartsoeker (one of the first spermatologists) claimed that he could observe a small person residing in the head of spermatozoa. Then in 1876, Hertwig found that the nuclei of the sperm and egg fuse during fertilization in sea urchin.[1] In the 1950s, mammalian spermatozoa were found to undergo a physiological change called capacitation[2,3] and a subsequent morphological change known as the acrosome reaction.[4] Thus, when we look back the history, the comprehension of the mechanisms of fertilization sometimes went in the wrong direction, but gradually nearing the true figure by modifying or abandoning old notions. In this process, the evolution of experimental tools such as light microscopy, antibodies, electron microscopy, etc., played important roles. Today, powerful investigative aids such as transgenic animals and/or gene-disrupted KO animals have become available. We can create an animal deficient in a given gene of interest or one with a "designer gene." For example, the latter includes spermatozoa with a green fluorescent protein (GFP) in their acrosome to report acrosomal integrity. These gene-manipulated animals give us deeper insight into the mechanisms of fertilization. In the present article, I describe the new findings, most of which have depended on the use of gene-manipulated animals.

Center for Genetic Analysis for Biological Responses, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan.
Correspondence: Prof. M Okabe (okabe@biken.osaka-u.ac.jp)
This article was presented at the 12th International Symposium on Spermatology, August 10-14, 2014, Newcastle, Australia.

THE *IN VITRO* FERTILIZATION SYSTEM

After the discovery of capacitation[2,3] and the acrosome reaction,[4] it took more than 15 years until Yanagimachi and Chang reported *in vitro* fertilization (IVF) in hamsters,[5] and for mice, it required another 15 years until an efficient fertilization system became available.[6] A few years later, human IVF was successfully achieved, and the first test tube baby was born, which led Robert Edwards receiving a Nobel Prize in 2010. IVF was supplemented by another discovery that fertilization could be achieved by injecting sperm directly into the egg cytoplasm by a pipette (Intra-Cytoplasmic Sperm Injection).[7,8] These findings boosted assisted fertilization for infertile couples, and today, a significant number of IVF babies are born worldwide.

Although IVF showed great clinical success, it had weaknesses as a probe to study the mechanisms of fertilization. One reason may be that a suitable medium for mouse fertilization did not emerge until 20 years after the discovery of capacitation. Even fertile spermatozoa failed to fertilize eggs unless they were incubated in a proper medium. Moreover, there is no consensus as to which currently-used media is the best during IVF. For example, once we learned that frozen C57BL/6 sperm were prone to lose their fertilizing ability in IVF, Takeo *et al.* developed a medium for these spermatozoa allowing them to penetrate eggs by the addition of methyl-beta-cyclodextrin.[9] This indicates that IVF results are significantly affected by the constitution of the medium. It also implies that the addition of various factors in the IVF medium may affect the results of IVF.

THE EMERGENCE OF A NEW TECHNIQUE – KNOCKOUT MICE

After the discovery and establishment of pluripotent embryonic stem cells (ES cells) from the inner cell mass of a blastocyst,[10] Capecchi[11] and Smithies[12] independently demonstrated that a gene of interest could be disrupted by homologous recombination using ES cells. Their finding became a powerful tool in analyzing the role of genes in living mice.

Before describing the results of gene-disruption experiments, I would like to mention the drawbacks of this technique.

Existence of cumulatively functioning genes

If no phenotype is seen after gene disruption, one may conclude that the gene of interest is not essential to the phenomenon one is studying. However, when some genes are paired with others and cumulatively form an essential gene set, a single gene disruption may not result in an apparent phenotype. G1 cyclins in yeast are an example of this. These proteins (CLNI, CLN2 and CldV3) are encoded by three individual genes and are expressed in the G1 phase of the cell cycles, but cells mutant for any two of the three genes are

phenotypically wild type and G1 arrest could be observed only in the triple mutant yeasts.[13]

Effects on neighboring genes

When myogenic regulatory factor 4 (*Mrf4*), a basic helix-loop-helix *Mrf* family member, was disrupted, Braun and Arnold declared that the mice die at birth,[14] Zhang *et al*. indicated that the mice survive,[15] and Patapoutian *et al*. reported that the mice occasionally die.[16] Afterward, it was found that insertion of a neo gene was detrimental to the neighboring *Myf5* gene and that *Mrf4* disruption was not the cause of the neonatal death.[17] A similar case was reported in the disruption of the *prion* gene, which is responsible for bovine spongiform encephalopathy. Some groups reported the disruption caused an ataxia phenotype, whereas others claimed they found no phenotype. The difference was that when some of the targeting vectors were used, it caused an exon skip and connected the prion gene to the neighboring *doppel* gene to express an aberrant fusion protein ectopically.[18]

Involvement of microRNAs

MicroRNAs (miRNAs) often reside in the intron area of certain genes, and it is known that the disruption of miRNA (s) sometimes causes a severe phenotype in the mouse.[19] Therefore, when we design the targeting vector, we must be careful not to eliminate miRNA (s) unintentionally from the modified area.[20]

Subtle effects

When we observe the phenotype of KO mice, the experimental time frame is limited. Although the gene disruption may not show a significant phenotype, the mice might have a subtle disadvantage. To discover a 5% fitness reduction, the corresponding sample size should be over 2000 and if it were 1%, it might require 600,000.[21] In other words, it is difficult to clarify the subtle effect (s) of gene disruption in normal experimentation. However, these subtle differences could ultimately affect the life of a species from an evolutionary point of view.

In this article, I neglected to describe most genes showing subtle differences and classified them as "nonessential" for the sake of simplicity in describing the fundamental mechanisms of fertilization.

VERIFICATION OF VARIOUS FACTORS IN KO MOUSE LINES

After IVF had become available in mice, various fertilization-related factors were identified using the IVF systems. These factors were subjected to gene KO experiments, and their respective roles were verified *in vivo*. The first gene examined in the KO mouse system in the field of fertilization research was *acrosin*, a sperm acrosomal enzyme. *Acrosin* was widely thought to play an important role in sperm penetration of the zona pellucida. Thus, *acrosin*-null spermatozoa were believed to become fertilization incapable. However, to everyone's surprise, *acrosin* KO mice were fertile, although a slight delay was observed in zona penetration.[22]

Another example was "*fertilin*," which attracted the attention of many researchers.[23] *Fertilin* is a heterodimer consisting of two subunits: *Adam1b* and *Adam2*. Initially, *fertilin* was disrupted by eliminating *Adam2*, and the *fertilin*-disrupted male mice showed an infertile phenotype.[24] *Fertilin* was thought to be a fusion protein, but strangely, the phenotype was loss of zona binding ability of the spermatozoa. As also shown in this example, gene function *in vivo* does not necessarily correspond to expectations. Later, when *fertilin* was disrupted by eliminating *Adam1b* instead of *Adam2*, the *fertilin*-null males showed normal fertility.[25] As mentioned above, when a KO mouse showed two different phenotypes, the wild-type phenotype was normally the true phenotype and any others were caused by disruption of an unrelated factor (s). In this particular case, it was learned that *Adam2* was essentially required in testis (not in spermatozoa) to make fertile spermatozoa by forming a heterodimer with *Adam1a*.[26] Other factors, demonstrated not to be essential using KO mice, are summarized in **Table 1**.

ESSENTIAL FACTORS FOUND BY KO MOUSE LINES

Although various genes predicted to be important for IVF experiments were shown to be dispensable *in vivo*, others were serendipitously found as essential factors for fertilization. The first case was the calmegin KO. Calmegin is a testis-specific molecular chaperone, which is expressed mainly in pachytene stage spermatocytes and disappears from spermatozoa upon spermiation. We expected a phenotype in spermatogenesis, but no abnormality was found in calmegin KO mice. However, we discovered that the males were infertile despite having normal spermatozoa in terms of number and motility.[27] Further investigation revealed that the spermatozoa lost their zona-binding ability. We made two more testis-specific molecular chaperone KO mouse lines, *calsperin* KO and *Pdilt* KO. Lacking these genes, the spermatozoa again became incapable of binding to zona.[28,29] If these genes were only expressed during spermatogenesis, how then was sperm-zona binding affected? As of now, we are aware of

Table 1: Most gene KO mice showed no, subtle or unexpected phenotypes

Genes	*Predicted roles*	*Apparent infertility*	*Number of pups/litter (before vs after gene disruption)*	*References*
Acr (*acrosin*)	Zona penetration	-	10.0 versus 12.5	Baba *et al.*[22]
4 galt1 (*GalTase*)	Sperm-zona binding	-	Fertile *in vivo*, 7.2 versus 6.2	Lu and Shur[56] Asano *et al.*[57]
Spam1 (*Ph-20, hyaluronidase*)	Sperm-zona binding	-	13.8 versus 12.2	Baba *et al.*[58]
Cd46	Sperm-egg fusion	-	9.0 versus 8.9	Inoue *et al.*[59]
Sed1	Sperm-zona binding	-	9.3 versus 3.3, fertile *in vivo*	Ensslin and Shur,[60] Hanayama *et al.*[61]
Adam1a/b (*fertilin*)	Sperm-egg fusion	-	9.9 versus 9.3	Kim *et al.*[25]
Zpbp1	Sperm-zona binding	Infertile*	9.1 versus 0.0	Lin *et al.*[62]
Zpbp2	Unknown	**	9.1 versus 6.9	
Crisp1	Sperm-egg fusion	-	7.3 versus 6.5	Da Ros *et al.*[63]
Pkdrej	Sperm-zona binding	-	8.8 versus 7.1	Sutton *et al.* 2008[64]
Zan (*zonadhesin*)	Sperm-zona binding	-	5.5 versus 6.5	Tardif *et al.*[65]
Zp3r (*Sp56*)	Sperm-zona binding	-	8.6 versus 9.4	Muro *et al.*[66]

**Zpbp1* KO unexpectedly resulted in globozoospermia. ***Zpbp2* KO resulted in spermatozoa with slightly deformed shape but zona binding was normal.
KO: knockout

at least 13 genes involved in the formation of sperm zona-binding ability, and in all 13 cases, the spermatozoa lack *Adam3* (or have aberrant *Adam3*). Since the *Adam3*-disrupted male mice are infertile[30] without affecting other gene products, *Adam3* could be an ultimately essential factor in all of the gene-disrupted mouse lines as shown in **Table 2**. Interestingly, these gene KO mouse lines shared common phenotypes, with (i) no migration into the oviduct and (ii) aberrant zona-binding ability *in vitro*.

AN INCONVENIENT TRUTH

Although the data in **Table 2** indicated *Adam3* on spermatozoa as a key protein in the fertilization process, *Adam3* is surprisingly a pseudogene in humans. Therefore, to place *Adam3* in the center of the general fertilization scheme may not be appropriate. Do humans have a completely different mechanism of fertilization from mice? Considering the fact that most of the genes in **Table 2** are conserved in human, we could assume the general schema is similar in humans and mice. Our current hypothesis is that we are still missing the ultimate factors contributing to sperm-zona binding. In this context, *Ly6k* is very interesting as spermatozoa from the *Ly6k* KO mice lost zona-binding ability while *Adam3* remains present on spermatozoa.[31] However, *Ly6k* could not be the ultimate key molecule, as it disappears from mature spermatozoa even in wild-type mice. I think we are coming closer to the ultimate factors, but the process of spermatozoa-egg encounters requires further investigation.

IS "SPERM-ZONA BINDING" DISPENSABLE?

In mice, the uterus and oviduct meet in a structure called the uterotubal junction (UTJ), which significantly reduces the number of spermatozoa reaching the eggs. In order to elucidate the mechanisms of UTJ penetration by spermatozoa, we produced chimeric mice that ejaculate both wild-type spermatozoa and GFP-tagged, calmegin-disrupted spermatozoa, and we mated them with wild-type females. We found that only wild-type spermatozoa migrated into the oviduct, while the equally motile calmegin-disrupted spermatozoa remained in the uterus.[32] This indicated that some unknown recognition mechanisms function in the UTJ region. Although spermatozoa from the gene-disrupted mouse lines in **Table 2** fail to migrate into the oviduct, we do not know the reason why the zona-binding ability is always associated with UTJ penetrating ability. What would happen if spermatozoa were directly injected into the oviduct, bypassing the UTJ? We tried this experiment using *Pdilt*,[29] *Tex101*[33] and *Ly6k*[31] KO mouse spermatozoa. To our surprise, the spermatozoa of these three KO mouse lines fertilized the eggs. In other words, spermatozoa could fertilize eggs in the oviduct without the so-called "zona-binding ability." A similar case was reported in *Adam1a* −/− mice; the sperm from *Adam1a* −/− mice could fertilize eggs *in vitro* when they were covered with cumulus layers.[26]

SHOULD THE "ZONA-INDUCED ACROSOME REACTION" BE RENOUNCED?

Many reports indicated that the acrosome reaction was induced upon contact with the zona pellucida, and many researchers considered that spermatozoa undergoing the acrosome reaction before zona contact had no fertilizing ability.[34] In this context, zona-binding proteins were assumed to initiate the signaling cascade leading to the acrosome reaction.[35] We made a transgenic mouse line that expressed GFP in the acrosome. This allowed us to observe the moment of the acrosome reaction. Spermatozoa on the zona pellucida were observed, but zona-binding spermatozoa did not acrosome react under a live imaging system.[36,37] In addition, a recent study by Jin *et al*. indicated that most of the fertilizing spermatozoa were acrosome-reacted before reaching the zona pellucida.[38] The experiments using gene-manipulated animals renounce the "zona-induced acrosome reaction" theory, at least in the mouse.

What about acrosomal exocytosis? If the acrosomal enzymes were released before spermatozoa approach the zona pellucida, it would be difficult for released enzymes to facilitate zona penetration. This question was also investigated using gene-manipulated animals. We previously generated *Izumo1*[39] and *Cd9* KO mouse lines.[40–42] Spermatozoa from the *Izumo1* KO line and eggs from the *Cd9* KO line were not able to fuse with wild-type gametes of the opposite sex. Therefore, we could observe many spermatozoa from *Izumo1* KO males in wild-type eggs or wild-type spermatozoa inside the perivitelline space of *Cd9* KO eggs. We recovered both of these acrosome-reacted and zona-penetrated spermatozoa from the perivitelline space by cracking the zona with a piezo-driven micropipette. The spermatozoa swam out from the perivitelline space and were added to freshly recovered cumulus covered eggs. We found that these spermatozoa could penetrate egg investments (cumulus layers and zona pellucida) a second time and, in the case of wild-type spermatozoa recovered from

Table 2: KO mice with impaired zona binding ability

Gene	Localization	Adam3 on spermatozoa	Zona binding ability	Migration into oviduct	References
Clgn (*calmegin*)	ER membrane	Disappeared	Impaired	Impaired	Ikawa *et al.*[27]
Adam2	Sperm surface	Disappeared	Impaired	Impaired	Cho *et al.*[24]
Ace (*angiotensin converting enzyme*)	Sperm surface	Aberrantly localized	Impaired	Impaired	Hagaman *et al.*[67] Yamaguchi *et al.*[68]
Adam3	Sperm surface	Disappeared	Impaired	Impaired	Shamsadin *et al.* 1999[30] Yamaguchi *et al.*[70]
Adam1a	Sperm surface	Disappeared	Impaired	Impaired	Nishimura *et al.*[26]
Calr3 (*calsperin*)	ER lumen	Disappeared	Impaired	Impaired	Ikawa *et al.*[28]
Tpst2	Acrosomal cap_equatorial segment	Disappeared	Impaired	Impaired	Marcello *et al.*[71]
Pdilt	ER membrane	Disappeared	Impaired	Impaired	Tokuhiro *et al.*[29]
Pmis-2	Sperm surface	Disappeared	Impaired	Impaired	Yamaguchi *et al.*[72]
RNase10	Epididymis	Disappeared	Impaired	Impaired	Krutskikh *et al.*[73]
Tex101	Spermatid	Disappeared	Impaired	Impaired	Fujihara *et al.*[33]
Prss37	Spermatid/spermatozoa	Disappeared	Impaired	Impaired	Shen *et al.*[69]
Ly6k	Testicular germ cells	Intact	Impaired	Impaired	Fujihara *et al.*[31]

KO: knockout; ER: endoplasmic reticulum

Cd9 KO eggs, fuse with the eggs.[43] Thus, the timing of the acrosome reaction before zona binding seemed to be considerably flexible. This re-penetration experiment indicated that if enzymes are released from the sperm during the acrosome reaction, all enzymes are dispensable for the sperm penetration of the zona pellucida. If enzymes were involved in zona penetration, they might not be the kind released from the acrosome; rather, they remained on the spermatozoa even after the acrosome reaction. In the mouse, it was reported that the acrosomal matrix proteins remain associated with the sperm for prolonged periods of time following the induction of acrosomal exocytosis.[44,45] If acrosomal enzymes (s) were involved, they should have remained on the sperm surface even after zona penetration, sperm recovery, and during the repeated penetration of the fresh egg investments.

In any case, the timing of the acrosome reaction is flexible, as indicated long ago in the rabbits.[46] These findings also indicated that the significant "sperm-zona binding" must occur between acrosome-reacted spermatozoa and the zona pellucida, while most of the classical "sperm-zona binding" assays were observing binding between acrosome-intact spermatozoa and the zona pellucida[47] (**Figure 1**).

FACTORS ESSENTIAL FOR SPERM-EGG FUSION

The first fusion-related factor, *Cd9*, was discovered serendipitously. A tetraspanin protein coding *Cd9* was initially disrupted by researchers in other fields to examine its role in immunology. However, the *Cd9*-disrupted females were infertile, due to the eggs requiring *Cd9* for sound fusion ability with spermatozoa.[40–42] On the sperm side, we had a monoclonal antibody *OBF13*, which inhibited sperm-egg fusion.[48] This was one of the fertilization inhibitory antibodies as shown in **Table 1**. While most of the factors in **Table 1** are shown to be nonessential as a result of KO experiments, the role of the *OBF13* antigen remained unexamined by KO experiments for a long time. This was due to *OBF13* being an IgM class antibody; therefore, there were technical difficulties in identifying the antigen. Once western blot sensitivity improved, we could finally identify the antigen and succeeded in cloning the gene. From its sequence, it was found to be a member of the immunoglobulin superfamily with a single Ig-like domain. We named this gene *Izumo1* based on a Japanese shrine dedicated to marriage. As mentioned in an earlier section, the *Izumo1*-disrupted spermatozoa could

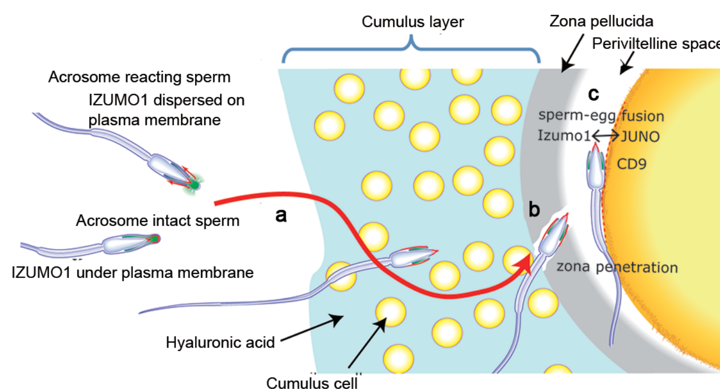

Figure 1: The mechanisms of fertilization, elucidated by gene-manipulated animals. (**a**) Spermatozoa that present *Adam3* (or some unknown factor(s)) can migrate into the oviduct and reach the vicinity of the eggs. Acrosome reaction is induced before spermatozoa reach the zona pellucida and the fusion-related sperm protein *Izumo1* on the outer acrosomal membrane migrates out to sperm surface (indicated by red color). (**b**) Spermatozoa bind to zona pellucida when mixed with cumulus-free oocytes.[74] However, this binding (mostly observed between the acrosome-intact spermatozoa and zona pellucida) was dispensable. The spermatozoa that lost the so-called "zona-binding" ability remained able to fertilize eggs *in vivo* once the oviduct migration step was bypassed.[29,31,33] Moreover, the timing of the acrosome reaction is flexible, as acrosome-reacted spermatozoa recovered from the perivitelline space could penetrate the zona pellucida a second time and fertilize eggs.[43] The mechanism of sperm penetration of zona pellucida is largely unknown. (**c**) Only acrosome-reacted spermatozoa can fuse with eggs. Spermatozoa without *Izumo1* never fused with eggs.[39] *Cd9* on the egg played an important role in fertilization,[40–42] but *Cd9*-disrupted females were not completely infertile. In addition, no direct interaction between *Cd9* and *Izumo1* was observed. This led us to predict a real counterpart for *Izumo1*. Using the newly established AVEXIS assay, JUNO was recently found to be a counterpart for *Izumo1* on the egg.[51] Modified from review.[75]

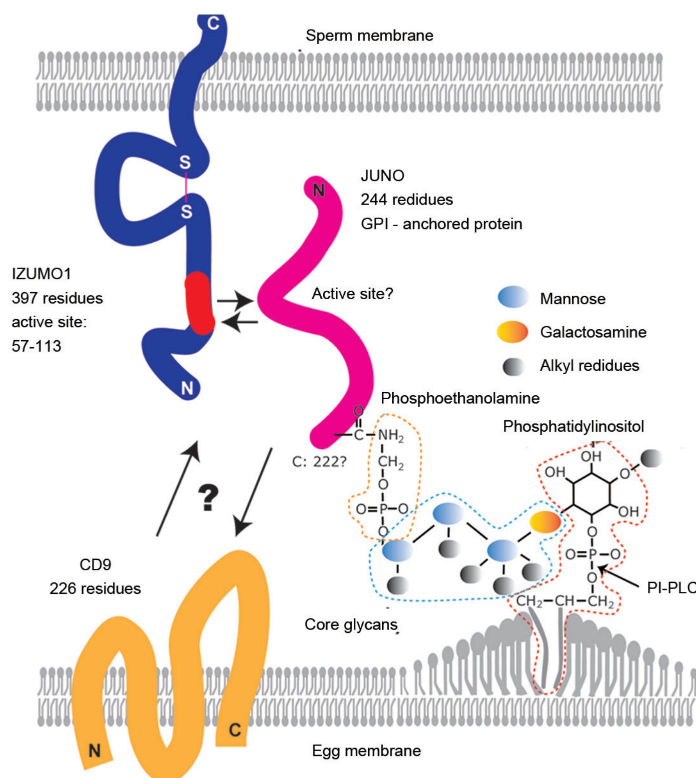

Figure 2: Factors involved in sperm-egg fusion. *Izumo1*, migrated outward from the outer acrosomal membrane to the sperm surface, tending to localize in the equatorial segment of spermatozoa. Various segments of *Izumo1* were examined for their binding ability to eggs and residue 57–113 was indicated to contain an active binding site.[49] Using the AVEXIS assay, JUNO was identified as an *Izumo1* binding protein and its role in fusion was verified by gene-disruption experiments. JUNO is a 244-residue protein but is cleaved at 222 to form a GPI (glycosylphosphatidylinositol)-anchored protein. GPI-anchored proteins are initially formed on the cytosolic side and flipped over to the outer membrane side in the final maturation stage. The next helpful piece of information will be the elucidation of the active site of JUNO. Since *Izumo1* (57–113) bound to *Cd9*-disrupted eggs normally, the elucidation of *Cd9's* role(s) will offer further clarification.

acrosome react and penetrate both cumulus and zona pellucida layers, but were unable to fuse with eggs as we expected.[39]

The fusing ability of *Cd9*-disrupted eggs was severely impaired, but it was not entirely lost, differing from the complete infertility seen in *Izumo1* disruption. In addition, the binding of the putative functional fragment of *Izumo1* in the N-terminus region (*Izumo1*: 57–113) to the egg surface was not altered by disruption of *Cd9*.[49] Thus, *Izumo1* binding to a protein other than *Cd9* was expected on the egg surface. However, as the number of eggs that we can use for the experiment is quite limited, the purification of *Izumo1* binding protein from eggs seemed difficult by conventional means. However, a method called the AVEXIS assay (avidity-based extracellular interaction screen) was invented.[50] Using this method, a soluble, biochemically active, highly avid recombinant mouse *Izumo1* ectodomain was prepared and the reactivity against HEK293 cells transfected with a normalized mouse oocyte cDNA expression library was analyzed and Bianchi *et al*. successfully identified the *Izumo1* binding protein on the egg and named it JUNO after the goddess of marriage.[51] The *Juno*-disrupted female mice were completely infertile. Now that interacting components *Izumo1* and JUNO have been found, rapid progress in the elucidation of the sperm-egg fusion mechanism is expected to follow (**Figure 2**).

LIVE IMAGING OF FERTILIZATION

Observation of fertilization using gene-manipulated animals has given us a new insight. To investigate the role of *Izumo1* in fusion, we made a transgenic mouse line containing the *Izumo1*-mCherry fusion protein and visualized the dynamic movement of *Izumo1* during the fertilization process.[52]

Although *OBF13* was a monoclonal antibody, various staining patterns were obtained in spermatozoa before and after the acrosome reaction.[53] Our long-standing question was how *Izumo1* changed its localization from under the plasma membrane to the sperm surface during the acrosome reaction. Two possibilities were postulated: (i) migration via two steep curves in the equatorial sheath and (ii) Re-adsorption of the antigen after acrosomal vesiculation. However, both hypotheses had their own shortcomings.[54] Moreover, the exact localization of *Izumo1* in live spermatozoa was unclear because it resided under the plasma membrane. First, the red fluorescent protein-tagged *Izumo1*-bearing spermatozoa were observed under a confocal microscope where it was revealed that *Izumo1* was in the acrosomal cap area of both the inner and outer acrosomal membrane. The migration of *Izumo1* upon acrosome reaction was then imaged in live cells. Apparently, *Izumo1* migrated on the sperm surface, not by adsorption of vesicles formed by the acrosome reaction. It was further confirmed that *Izumo1* did not migrate via the acrosomal sheath. This introduced the new hypothesis that *Izumo1* migrated out from the outer acrosomal membrane to the plasma membrane at the beginning of the acrosome reaction when the two membranes fused making tiny holes (**Figure 1a**). *Izumo1* migrated out to the plasma membrane and spread all over the head, but tended to associate in the equatorial segment.[52]

The dynamic movement of *Izumo1* at fusion was also observed using the same transgenic mouse line. *Izumo1* mainly localized to the equatorial segment dispersed in the first step of sperm-egg fusion. However, *Izumo1* on the inner acrosomal membrane did not disperse but was incorporated into the cytoplasm of the egg, together with the inner acrosomal membrane structure. These *Izumo1* movements were recorded in real time.[52] In conjunction with electron microscopic observations reported by many researchers, we realized that the sperm-egg fusion is apparently divided into two different phases as explained in **Figure 3**.

CONCLUSION

Observation of fertilization using gene-manipulated animals has brought us a new schematic diagram in mammalian fertilization (**Figure 1**). Note that the classical theories of the zona-induced acrosome reaction are not included in the figure. In order to understand the molecular mechanisms of fertilization, we apparently need more information. Reflecting on the progress in fertilization research, the role of gene-manipulated animals seems all the more important. Fortunately, the Crisper/Cas9 system has opened a new (wide) door for gene-disruption experiments.[55] The method is both quick and easy and applicable to mammals, fish, insects, and

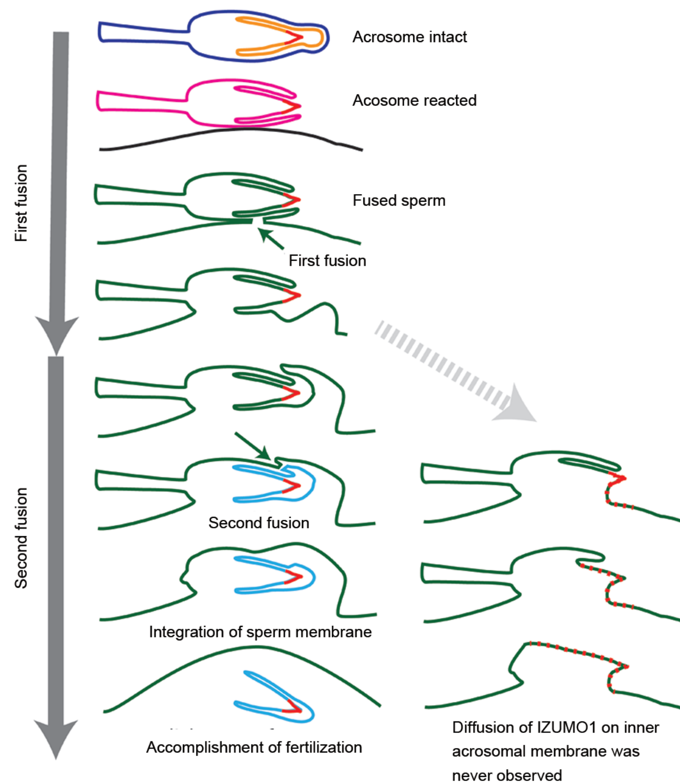

Figure 3: Fertilization requires two independent fusions. Intact spermatozoa have a plasma membrane (blue) and an acrosomal membrane (orange). After the acrosome reaction, these two membranes fuse and form a new sperm membrane (pink). The first fusion takes place between the pink membrane and egg plasma membrane (black). After the first fusion, egg and sperm membrane form a new consecutive membrane (green). If fusion is accomplished in this step, *Izumo1* on the acrosomal cap of the inner acrosomal membrane (indicated by red) should spread on the newly-formed egg surface (green). However, the second fusion (invagination) follows the first fusion that separates the acrosomal cap and acrosomal sheath areas (light blue) from the fused membrane (green). Thus, *Izumo1* on the inner acrosomal membrane is invaginated into the cytoplasm of the eggs. From live imaging, *Izumo1* seems to be required for the first fusion. The nature of the second fusion remains totally unknown.

even to plants. In one sense, gene disruption is easier than antibody production. Use of gene-manipulated animals will soon become as routine as gel-electrophoresis.

ACKNOWLEDGMENTS

I am grateful to Dr. Martin M Matzuk and Ms. Samantha Young for helpful discussion and critical reading of the manuscript. This work was supported in part by grants from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

COMPETING FINANCIAL INTERESTS

The authors would declare no competing interests, or other interests that might be perceived to influence the results and/or discussion reported in this article.

REFERENCES

1 Clift D, Schuh M. Restarting life: fertilization and the transition from meiosis to mitosis. *Nat Rev Mol Cell Biol* 2013; 14: 549–62.
2 Austin CR. Observations on the penetration of the sperm in the mammalian egg. *Aust J Sci Res B* 1951; 4: 581–96.
3 Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 1951; 168: 697–8.
4 Dan J. Studies on the acrosome reaction. I. Reaction to egg water and other stimuli. *Biol Bull* 1952; 103: 54–66.
5 Yanagimachi R, Chang MC. Fertilization of hamster eggs *in vitro*. *Nature* 1963; 200: 281–2.
6 Toyoda Y, Yokoyama M, Hoshi T. Studies on the fertilization of mouse eggs *in vitro*. *Jpn J Anim Reprod* 1971; 16: 147–57.
7 Uehara T, Yanagimachi R. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol Reprod* 1976; 15: 467–70.
8 Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; 340: 17–8.
9 Takeo T, Hoshii T, Kondo Y, Toyodome H, Arima H, *et al*. Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. *Biol Reprod* 2008; 78: 546–51.
10 Robertson E, Bradley A, Kuehn M, Evans M. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* 1986; 323: 445–8.
11 Capecchi MR. Generating mice with targeted mutations. *Nat Med* 2001; 7: 1086–90.
12 Smithies O. Forty years with homologous recombination. *Nat Med* 2001; 7: 1083–6.
13 Reed SI. G1-specific cyclins: in search of an S-phase-promoting factor. *Trends Genet* 1991; 7: 95–9.
14 Braun T, Arnold HH. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *EMBO J* 1995; 14: 1176–86.
15 Zhang W, Behringer RR, Olson EN. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev* 1995; 9: 1388–99.
16 Patapoutian A, Yoon JK, Miner JH, Wang S, Stark K, *et al*. Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* 1995; 121: 3347–58.
17 Olson EN, Arnold HH, Rigby PW, Wold BJ. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* 1996; 85: 1–4.
18 Rossi D, Cozzio A, Flechsig E, Klein MA, Rülicke T, *et al*. Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. *EMBO J* 2001; 20: 694–702.
19 Hasuwa H, Ueda J, Ikawa M, Okabe M. miR-200b and miR-429 function in mouse ovulation and are essential for female fertility. *Science* 2013; 341: 71–3.
20 Osokine I, Hsu R, Loeb GB, McManus MT. Unintentional miRNA ablation is a risk factor in gene knockout studies: a short report. *PLoS Genet* 2008; 4: e34.
21 Brookfield J. Can genes be truly redundant? *Curr Biol* 1992; 2: 553–4.
22 Baba T, Azuma S, Kashiwabara S, Toyoda Y. Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J Biol Chem* 1994; 269: 31845–9.
23 Blobel CP, Wolfsberg TG, Turck CW, Myles DG, Primakoff P, *et al*. A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. *Nature* 1992; 356: 248–52.
24 Cho C, Bunch DO, Faure JE, Goulding EH, Eddy EM, *et al*. Fertilization defects in sperm from mice lacking fertilin beta. *Science* 1998; 281: 1857–9.
25 Kim E, Yamashita M, Nakanishi T, Park KE, Kimura M, *et al*. Mouse sperm lacking ADAM1b/ADAM2 fertilin can fuse with the egg plasma membrane and effect fertilization. *J Biol Chem* 2006; 281: 5634–9.
26 Nishimura H, Kim E, Nakanishi T, Baba T. Possible function of the ADAM1a/ADAM2 Fertilin complex in the appearance of ADAM3 on the sperm surface. *J Biol Chem* 2004; 279: 34957–62.
27 Ikawa M, Wada I, Kominami K, Watanabe D, Toshimori K, *et al*. The putative chaperone calmegin is required for sperm fertility. *Nature* 1997; 387: 607–11.
28 Ikawa M, Tokuhiro K, Yamaguchi R, Benham AM, Tamura T, *et al*. Calsperin is a testis-specific chaperone required for sperm fertility. *J Biol Chem* 2011; 286: 5639–46.
29 Tokuhiro K, Ikawa M, Benham AM, Okabe M. Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male fertility [corrected]. *Proc Natl Acad Sci U S A* 2012; 109: 3850–5.
30 Shamsadin R, Adham IM, Nayernia K, Heinlein UA, Oberwinkler H, *et al*. Male mice deficient for germ-cell cyritestin are infertile. *Biol Reprod* 1999; 61: 1445–51.
31 Fujihara Y, Okabe M, Ikawa M. GPI-anchored protein complex, LY6K/TEX101, is required for sperm migration into the oviduct and male fertility in mice. *Biol Reprod* 2014; 90: 60.
32 Nakanishi T, Isotani A, Yamaguchi R, Ikawa M, Baba T, *et al*. Selective passage through the uterotubal junction of sperm from a mixed population produced by chimeras of calmegin-knockout and wild-type male mice. *Biol Reprod* 2004; 71: 959–65.
33 Fujihara Y, Tokuhiro K, Muro Y, Kondoh G, Araki Y, *et al*. Expression of TEX101, regulated by ACE, is essential for the production of fertile mouse spermatozoa. *Proc Natl Acad Sci U S A* 2013; 110: 8111–6.
34 Bleil JD, Beall CF, Wassarman PM. Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP2. *Dev Biol* 1981; 86: 189–97.
35 Gong X, Dubois DH, Miller DJ, Shur BD. Activation of a G protein complex by aggregation of beta-1,4-galactosyltransferase on the surface of sperm. *Science* 1995; 269: 1718–21.
36 Nakanishi T, Ikawa M, Yamada S, Parvinen M, Baba T, *et al*. Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. *FEBS Lett* 1999; 449: 277–83.
37 Baibakov B, Gauthier L, Talbot P, Rankin TL, Dean J. Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. *Development* 2007; 134: 933–43.
38 Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, *et al*. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proc Natl Acad Sci U S A* 2011; 108: 4892–6.
39 Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* 2005; 434: 234–8.
40 Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, *et al*. The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat Genet* 2000; 24: 279–82.
41 Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C. Severely reduced female fertility in CD9-deficient mice. *Science* 2000; 287: 319–21.
42 Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, *et al*. Requirement of CD9 on the egg plasma membrane for fertilization. *Science* 2000; 287: 321–4.
43 Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R. Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. *Proc Natl Acad Sci U S A* 2011; 108: 20008–11.
44 Hardy DM, Oda MN, Friend DS, Huang TT Jr. A mechanism for differential release of acrosomal enzymes during the acrosome reaction. *Biochem J* 1991; 275 (Pt 3): 759–66.
45 Kim KS, Foster JA, Gerton GL. Differential release of guinea pig sperm acrosomal components during exocytosis. *Biol Reprod* 2001; 64: 148–56.
46 Valdivia M, Sillerico T, De Ioannes A, Barros C. Proteolytic activity of rabbit perivitelline spermatozoa. *Zygote* 1999; 7: 143–9.
47 Gahlay G, Gauthier L, Baibakov B, Epifano O, Dean J. Gamete recognition in mice dependes on the cleavage status of an egg's zona pellucida protein. *Scinece* 2010; 329: 216–9.
48 Okabe M, Yagasaki M, Oda H, Matzno S, Kohama Y, *et al*. Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs. *J Reprod Immunol* 1988; 13: 211–9.
49 Inoue N, Hamada D, Kamikubo H, Hirata K, Kataoka M, *et al*. Molecular dissection of IZUMO1, a sperm protein essential for sperm-egg fusion. *Development* 2013; 140: 3221–9.
50 Crosnier C, Bustamante LY, Bartholdson SJ, Bei AK, Theron M, *et al*. Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum. *Nature* 2011; 480: 534–7.
51 Bianchi E, Doe B, Goulding D, Wright GJ. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* 2014; 508: 483–7.
52 Satouh Y, Inoue N, Ikawa M, Okabe M. Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. *J Cell Sci* 2012; 125 (Pt 21): 4985–90.
53 Okabe M, Adachi T, Takada K, Oda H, Yagasaki M, *et al*. Capacitation-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate *in vitro*. *J Reprod Immunol* 1987; 11: 91–100.
54 Toshimori K. Dynamics of the mammalian sperm membrane modification leading to fertilization: a cytological study. *J Electron Microsc (Tokyo)* 2011; 60 Suppl 1: S31–42.
55 Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, *et al*. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013; 153: 910–8.
56 Lu Q, Shur BD. Sperm from beta 1, 4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. *Development* 1997; 124: 4121–31.

57 Asano M, Furukawa K, Kido M, Matsumoto S, Umesaki Y, *et al.* Growth retardation and early death of beta-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. *EMBO J* 1997; 16: 1850–7.
58 Baba D, Kashiwabara S, Honda A, Yamagata K, Wu Q, *et al.* Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J Biol Chem* 2002; 277: 30310–4.
59 Inoue N, Ikawa M, Nakanishi T, Matsumoto M, Nomura M, *et al.* Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. *Mol Cell Biol* 2003; 23: 2614–22.
60 Ensslin MA, Shur BD. Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding. *Cell* 2003; 114: 405–17.
61 Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, *et al.* Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 2004; 304: 1147–50.
62 Lin YN, Roy A, Yan W, Burns KH, Matzuk MM. Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. *Mol Cell Biol* 2007; 27: 6794–805.
63 Da Ros VG, Maldera JA, Willis WD, Cohen DJ, Goulding EH, *et al.* Impaired sperm fertilizing ability in mice lacking Cysteine-RIch Secretory Protein 1 (CRISP1). *Dev Biol* 2008; 320: 12–8.
64 Sutton KA, Jungnickel MK, Florman HM. A polycystin-1 controls postcopulatory reproductive selection in mice. Proc Natl Acad Sci U S A. 2008; 105: 8661-6.
65 Tardif S, Wilson MD, Wagner R, Hunt P, Gertsenstein M, *et al.* Zonadhesin is essential for species specificity of sperm adhesion to the egg zona pellucida. *J Biol Chem* 2010; 285: 24863–70.
66 Muro Y, Buffone MG, Okabe M, Gerton GL. Function of the acrosomal matrix: zona pellucida 3 receptor (ZP3R/sp56) is not essential for mouse fertilization. *Biol Reprod* 2012; 86: 1–6.
67 Hagaman JR, Moyer JS, Bachman ES, Sibony M, Magyar PL, *et al.* Angiotensin-converting enzyme and male fertility. *Proc Natl Acad Sci U S A* 1998; 95: 2552–7.
68 Yamaguchi R, Yamagata K, Ikawa M, Moss SB, Okabe M. Aberrant distribution of ADAM3 in sperm from both angiotensin-converting enzyme (Ace)- and calmegin (Clgn)-deficient mice. *Biol Reprod* 2006; 75: 760–6.
69 Shen C, Kuang Y, Liu J, Feng J, Chen X, *et al.* Prss37 is required for male fertility in the mouse. *Biol Reprod* 2013; 88: 123.
70 Yamaguchi R, Muro Y, Isotani A, Tokuhiro K, Takumi K, *et al.* Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. *Biol Reprod* 2009; 81: 142–6.
71 Marcello MR, Jia W, Leary JA, Moore KL, Evans JP. Lack of tyrosylprotein sulfotransferase-2 activity results in altered sperm-egg interactions and loss of ADAM3 and ADAM6 in epididymal sperm. *J Biol Chem* 2011; 286: 13060–70.
72 Yamaguchi R, Fujihara Y, Ikawa M, Okabe M. Mice expressing aberrant sperm-specific protein PMIS2 produce normal-looking but fertilization-incompetent spermatozoa. *Mol Biol Cell* 2012; 23: 2671–9.
73 Krutskikh A, Poliandri A, Cabrera-Sharp V, Dacheux JL, Poutanen M, *et al.* Epididymal protein Rnase10 is required for post-testicular sperm maturation and male fertility. *FASEB J* 2012; 26: 4198–209.
74 Hartmann JF, Gwatkin RB, Hutchison CF. Early contact interactions between mammalian gametes *in vitro*: evidence that the vitellus influences adherence between sperm and zona pellucida. *Proc Natl Acad Sci U S A* 1972; 69: 2767–9.
75 Okabe M. The cell biology of mammalian fertilization. *Development* 2013; 140: 4471–9.