Mechanisms of fertilization elucidated by gene-manipulated animals

Masaru Okabe

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 capacitance 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.

THE IN VITRO FERTILIZATION SYSTEM

After the discovery and establishment of the fertilization system,5,6 the acrosome reaction,7 it took more than 15 years until Yanagimachi and Chang reported in vitro fertilization (IVF) in hamsters,8 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 Injaction).9,23 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.20 developed a medium for these spermatozoa allowing them to penetrate eggs by the addition of methyl-beta-cyclodextrin.24 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 Capecchi11 and Smithies12 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 (CLN1, 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

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)

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phenotypically wild type and G1 arrest could be observed only in the triple mutant yeasts.\textsuperscript{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.\textsuperscript{14} Zhang \textit{et al.} indicated that the mice survive,\textsuperscript{15} and Patapoutian \textit{et al.} reported that the mice occasionally die.\textsuperscript{16}

Afterward, it was found that insertion of a neo gene was detrimental to the neighboring \textit{Myc5} gene and that Mrf4 disruption was not the cause of the neonatal death.\textsuperscript{17} A similar case was reported in the disruption of the \textit{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 \textit{doppel} gene to express an aberrant fusion protein ectopically.\textsuperscript{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.\textsuperscript{19}

Therefore, when we design the targeting vector, we must be careful not to eliminate miRNA (s) unintentionally from the modified area.\textsuperscript{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.\textsuperscript{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 \textit{in vivo}. The first gene examined in the KO mouse system in the field of fertilization research was \textit{acrosin}, a sperm acrosomal enzyme. Acrosin was widely thought to play an important role in sperm penetration of the zona pellucida.

Thus, \textit{acrosin-null} spermatozoa were believed to become fertilization incapable. However, to everyone's surprise, \textit{acrosin KO} mice were fertile, although a slight delay was observed in zona penetration.\textsuperscript{22}

Another example was "\textit{fertilin}," which attracted the attention of many researchers.\textsuperscript{23} \textit{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.\textsuperscript{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 \textit{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.\textsuperscript{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 testsis (not in spermatozoa) to make fertile spermatozoa by forming a heterodimer with Adam1a.\textsuperscript{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 \textit{in vivo}, others were serendipitously found as essential factors for fertilization. The first case was the calmodin KO. Calmodin 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 calmodin KO mice. However, we discovered that the males were infertile despite having normal spermatozoa in terms of number and motility.\textsuperscript{27} Further investigation revealed that the spermatozoa lost their zona-binding ability.

We made two more testsis-specific molecular chaperone KO mouse lines, \textit{calsperrin} KO and \textit{Pdil} KO. Lacking these genes, the spermatozoa again became incapable of binding to zona.\textsuperscript{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 \textit{et al.} \textsuperscript{22}       |
| 4 galt1 (GalTase)       | Sperm-zona binding | -                  | Fertile \textit{in vivo}, 7.2 versus 6.2 | Lu and Shur \textsuperscript{26}                |
| Spam1 (Ph-20, hyaluronidase) | Sperm-zona binding | -                  | 13.8 versus 12.2                      | Asano \textit{et al.} \textsuperscript{27}      |
| Cad46                   | Sperm-egg fusion | -                  | 9.0 versus 8.9                        | Baba \textit{et al.} \textsuperscript{28}       |
| Sed1                    | Sperm-zona binding | -                  | 9.3 versus 3.3, \textit{fertile in vivo} | Inoue \textit{et al.} \textsuperscript{29}      |
| Adam1a/b (fertilin)     | Sperm-egg fusion | -                  | 9.9 versus 9.3                        | Ensilin and Shur \textsuperscript{30}           |
| Zpbb1                   | Sperm-zona binding | Infertile*         | 9.1 versus 0.0                        | Hanayama \textit{et al.}\textsuperscript{31}    |
| Zpbb2                   | Unknown          | **                 | 9.1 versus 6.9                        | Kim \textit{et al.} \textsuperscript{32}        |
| Crisp1                  | Sperm-egg fusion | -                  | 7.3 versus 6.5                        | Lin \textit{et al.} \textsuperscript{33}        |
| Pkdrej                  | Sperm-zona binding | -                  | 8.8 versus 7.1                        | Da Ros \textit{et al.} \textsuperscript{34}     |
| Zan (zonadhesin)        | Sperm-zona binding | -                  | 5.5 versus 6.5                        | Sutton \textit{et al.}\textsuperscript{35}     |
| Zp3r (Sp56)             | Sperm-zona binding | -                  | 8.6 versus 9.4                        | Tardif \textit{et al.}\textsuperscript{36}      |

\*Zpbb1 KO unexpectedly resulted in globozoospermia. **Zpbb2 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 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 humans, 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. 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. 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, "Tex101" and Ly6k 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.

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. In this context, zona-binding proteins were assumed to initiate the signaling cascade leading to the acrosome reaction. 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. In addition, a recent study by Lin et al. indicated that most of the fertilizing spermatozoa were acrosome-reacted before reaching the zona pellucida. 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 and Cd9 KO mouse lines. Sperrmatoozoa from the Izumo1 KO line and eggs from the Cd9 KO line were unable 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 |
|---------------|-------------------------------------|-----------------------|----------------------|-------------------------|------------|
| Cldn (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                | Haganam et al.27 |
| Adam3         | Sperm surface                       | Disappeared           | Impaired             | Impaired                | Yamaguchi et al.28 |
| Adam1a        | Sperm surface                       | Disappeared           | Impaired             | Impaired                | Shamsadlin et al.1999 |
| Calr3 (calserpin) | ER lumen                           | Disappeared           | Impaired             | Impaired                | Yamaguchi et al.29 |
| Tpst2         | Acrosomal cap_equatorial segment    | Disappeared           | Impaired             | Impaired                | Nishimura et al.26 |
| Pdilt         | ER membrane                        | Disappeared           | Impaired             | Impaired                | Ikawa et al.28 |
| Pmis-2        | Sperm surface                       | Disappeared           | Impaired             | Impaired                | Marcello et al.31 |
| RNase10       | Epididymis                          | Disappeared           | Impaired             | Impaired                | Tokuhiro et al.29 |
| Tex101        | Spermatid                          | Disappeared           | Impaired             | Impaired                | Yamaguchi et al.72 |
| Prss37        | Spermatid/spermatozoa               | Disappeared           | Impaired             | Impaired                | Fujihara et al.25 |
| Ly6k          | Testicular germ cells               | Intact                | Impaired             | Impaired                | Shen et al.28 |

KO: knockout; ER: endoplasmic reticulum
Cd9 KO eggs, fuse with the eggs. 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. 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. These findings also indicated that the significant “sperm-zona binding” must occur between acrosome-intact 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 (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. On the sperm side, we had a monoclonal antibody OBF13, which inhibited sperm-egg fusion. This was one of the fertilization inhibition 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 not fuse with the eggs. 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. The mechanism of sperm penetration of zona pellucida is largely unknown. Only acrosome-reacted spermatozoa can fuse with eggs. Spermatozoa without Izumo1 never fused with eggs. Cd9 on the egg played an important role in fertilization 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. Modified from review.

![Figure 1](image)

**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. 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. 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. 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. Cd9 on the egg played an important role in fertilization 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. Modified from review.

![Figure 2](image)

**Figure 2:** Factors involved in sperm-egg fusion. Izumo1, migrated outward from the outer acrosomal membrane to the sperm surface, tender 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. 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 fusion 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 Cd9.⁴⁰ 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.⁴¹ 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.⁴² 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...
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.

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Competing Financial Interests

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

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