Cell. Mol. Life Sci. 64 (2007) 1945 – 1958
1420-682X/07/151945-14
DOI 10.1007/s00018-007-7037-7

Cellular and Molecular Life Sciences

Review

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

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Received 26 January 2007; received after revision 7 March 2007; accepted 17 April 2007
Online First 11 June 2007

Abstract. Untangling the molecular nature of sperm-egg interactions is fundamental if we are to understand fertilization. These phenomena have been studied for many years using biochemical approaches such as antibodies and ligands that interact with sperm or with eggs and their vestments. However, when homologous genetic recombination techniques were applied, most of the phenotypic factors of the gene-manipulated animals believed "essential" for fertilization were found to be dispensable. Of course, all biological systems contain redundancies and compensatory mechanisms, but as a whole the old model of fertilization clearly requires significant modification. In this review, we use the results of gene manipulation experiments in animals to propose the basis for a new vision.

Keywords. Fertilization, sperm-egg interaction, gene manipulation, zona pellucida.

Introduction

Living creatures developed the basic structures for ears before they had any way to sense sound and have crafted eyes as sensors for light at least 40 times using a common genetic toolkit [1]. They even invented lenses without any knowledge of physics and succeeded in projecting images of the environment on retinas. They developed means of sensing chemicals in the environment that we recognize today as the ability to smell and taste. About a billion years ago, the ancestors of today's eukaryotic organisms also devised sex [2]: a genetic shuffling and exchange mechanism that functions still as one of evolution's major Generators of Diversity. Originally, there was little disparity in size between the different gamete types, and some organisms such as fungi and protists had – and still have – multiple genders, so the terms "male" and "female" are meaningless for them. One problem the gametes had to overcome was that of finding each other. Not surprisingly, they used chemical sensing, and we now realize that gamete detection mechanisms are still closely related to those used in smell and taste. With the evolution of multicellularity, a division of labor arose. Chordates developed an alternating haploid/diploid life cycle, with the diploid somatic phase dominant and the haploid phase limited to the much smaller gametes. The gametes themselves diverged in form and function, with the male sperm becoming a tiny motile genetic dispersal machine and the female egg remaining as a largely passive recipient carrying the resources needed to fuel early embryonic development [3]. In fact, the terms "male" and "female" are defined by the type of gamete an individual soma produces rather than the specific

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mechanisms involved, and this biology drives the somatic phase phenotype. The system has evolved immensely since the first versions evolved. In mammals, the sharing of genomic resources occurs following fertilization, which occurs between a handful of thousands of millions of small and vigorously moving haploid sperm produced by males and a few oocytes produced by females [4].

In other words, even a mighty African bull elephant weighing several tons must engineer a haploid phase of his life cycle in a specially protected part of his body – the seminiferous tubule – and produce a tiny sperm cell weighing in the order of picograms with a 50–60 μm-long flagellum [5]. This needs to fuse with the much larger – but still very small – egg produced by the female, weighing 20–40 ng, about 100 μm in diameter [6] and protected by a thin glycoprotein layer, the zona pellucida. This size bottleneck is predestined by the haploid/diploid life cycle of sexual reproduction set in place a billion years ago. Because sexual reproduction is such a very fundamental and ancient process, the germ cells must abandon all acquired somatic inventions such as ears, eyes and noses and undergo their ancient fusion process at the time of syngamy ("gamete-joining"). We suspect that the events of mammalian fertilization will reflect that evolutionary history and that the new era of comparative genomics will allow us to uncover surprising links between organisms and cell fusion mechanisms at a molecular level.

How much do we know about the mechanism of reproduction in living creatures? How do sperm and egg recognize each other, contact each other and achieve fusion? This topic formed one of the deepest schisms in Western biological thought for around 200 years [7]. "Spermists", animated by Leeuwenhoek's chance discovery of spermatozoa in 1677, believed that the male "seed" was all-important for reproduction, with the female reproductive tract serving as a mere nurturing garden bed. By contrast, "Ovists" believed that the future life was in the egg and that the spermatozoa either stimulated its growth or were irrelevant parasites. Up to then, ideas of reproduction in Western science were largely based on the works of Aristotle and Galen around 2000 years before; many even believed in spontaneous generation from rotting matter. In Chinese (and presumably other Asian) science, thinking was even less precise, as natural philosophy emphasized the balance of body systems and the flow of energy ("Chi") rather than detailed mechanisms [7]. The war was not resolved until the broad cellular details of vertebrate fertilization were established in the 1870 s. Hertwig and Fol showed that the fertilized zygote contains both male and female pronuclei, and Weissmann postulated the separation of the germ cell lineage from that of the soma [7]. Since then, scientists have established the details of fertilization using physiological experiments, microscopy and biochemistry. However, the era of genomics and gene manipulation is driving a new wave of studies. Here we will review the mechanism of fertilization, mainly in the mouse, introducing experimental results obtained from gene-manipulated animals together with topics and new perspectives that challenge the established view of reproductive biology.

Eggs

Until recently, all eggs were thought to be produced in the fetal ovary, and further development was believed impossible [8]. However, an astonishing publication claimed the existence of proliferative germ cells that could sustain oocyte and follicle production in the postnatal mammalian ovary [9]. Moreover, the same authors reported successful identification of bone marrow cells as a potential source of germ cells that could sustain oocyte production in adulthood [10]. Of course, some have pointed out the weakness of evidence in these papers [11]. However, other groups reported that stem cells isolated from the skin of porcine fetuses had an intrinsic ability to differentiate into oocyte-like cells. According to the authors, these cells formed follicle-like aggregates, which extruded large oocyte-like cells expressing oocyte markers such as zona pellucida [12]. Another report claimed the *de novo* formation of primary follicles in adult human ovaries [13]. Eggs are also reported to differentiate during the culture of embryonic stem (ES) cells [14]. Some very surprising ideas have also been postulated on the male side; for example, Nayermia reported the transdifferentiation of bone marrow-derived stem cells into male germ cells *in vitro* [15]. It seems as if eggs and sperm are popping up everywhere at the moment, though we must note that none of these papers show evidence of these egg-like or spermatid-like cells produced *in vitro* resulting in live-born young after fertilization. This chaotic phase will likely continue for some years before we can clarify the potential of stem cells to generate eggs.

Returning from this digression, at normal ovulation the eggs are released into the peritoneal or bursal cavity and then 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

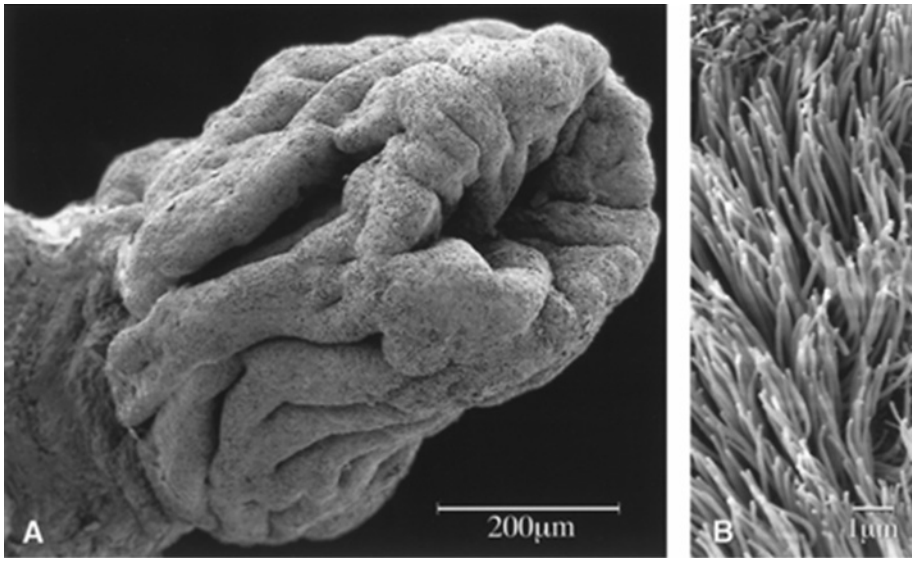

Figure 1. Hamster infundibulum (A) and a magnified view of cilia on its surface (B) [16]. An online video showing the movement of ovulated eggs into the oviduct is available from http://www.molbiolcell.org/content/vol10/issue1/images/video/mk0190776002b.mov.

video pictures available online by Talbot et al. using hamsters (Fig. 1 [16]).

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. The cumulus then disperses and the fertilized zygote resumes its descent down the oviduct towards the uterus where implantation takes place, usually when the embryo reaches the blastocyst stage.

Sperm

Sperm need to ascend the female reproductive tract, whereas the eggs are destined to descend the oviduct and enter the uterus. Naturally, we imagine that the sperm need to use their flagella to swim up to the site where fertilization occurs, but in fact, much distribution throughout the lower tract seems to be passive in response to uterine contractions. As there are millions of sperm, while the eggs usually number one in humans and about ten in the mouse, the fertilizing sperm must swim swiftly and reach the eggs as the "winner" of the race featuring competing sperm. However, this is more than a simple speed race: it is an obstacle course. The uterus and oviduct are connected at the uterotubal junction, where the tract is very narrow and sperm are prevented from migrating freely into the oviduct. The outer portion of the oviduct hangs into the uterus and forms a colliculus in mice, pigs and cows. This is not a wide-open entrance for sperm to migrate into the oviduct. Instead, in some species such as pigs, the junction serves as a mucus-filled reservoir for the fertilizing sperm and restricts the numbers released into the oviduct proper [17]. During their relatively brief life inside the female reproductive tract, sperm must undergo a physiological surface change called capacitation. The nature of this process is not clearly understood, but there are many papers indicating the importance of protein phosphorylation and calcium ion influx upon release of "decapacitation factor" from sperm [18]. The key feature of capacitation is that it frees sperm to undergo the acrosome reaction, which is essential for fertilization and for exposing sperm-egg binding sites [19]. Intriguingly, it now appears that sperm can carry out *de novo* protein synthesis during capacitation, using stored mRNA and mitochondrial ribosomes [20]. Although this needs independent validation, it helps answer the evolutionary puzzle of why sperm actually need midpiece mitochondria: many species (such as human and mouse) can function well using glycolysis, so why bother forming a midpiece when the mitochondria are destined for suicide following syngamy [21]?

How do sperm, which have neither eyes nor ears, find eggs and finish the race? In general, externally fertilizing organisms use a wide variety of chemoattractant and other strategies to ensure syngamy. For example, in ascidians, it is reported that a sperm-activating and -attracting factor (SAAF) is released from eggs and that SAAF is the sulfated steroid 3,4,7,26-tetrahydroxycholestane-3,26-disulfate [22, 23]. Not surprisingly, these ancient strategies have been adapted and modified for internal fertilization and viviparity. In humans, we have long known that olfactory receptors (ORs) reside in spermatozoa. Among these human testicular ORs, hOR17–4 func-

tions in human sperm chemotaxis and is speculated to be a critical component of the fertilization process [24]. Human and mouse sperm may also locate eggs by the aid of a chemoattractant [25], but the intrinsic factors released from the egg to attract sperm are not yet known. There is also evidence of thermotaxis, as a temperature gradient arises in the oviduct around the time of fertilization, and sperm appear to be able to respond to this at a longer range than chemotaxis [26]. Spermatozoa are produced in the testis, transferred into the epididymis and remain stored like canned sardines in the cauda until required. Once ejaculated, they become activated by stimuli from the female environment, like matches being struck (Fig. 2). Excitation continues in the tract as sperm capacitate and various biological indexes change during this event. One reason that the study of capacitation it difficult is the lack of homogeneity of the sperm population used for most experiments *in vitro*. In most species, the number of sperm ejaculated is immense compared with the number of eggs ovulated. However, because fertilization *in vivo* typically occurs between sperm and egg on a one-to-one basis [27], the chance of being a fertilizing sperm (approximately one in 10^8 in human) is far less than of being a lottery winner (approximately one in 10^6). In this circumstance, how can we measure the physiological conditions of the fertilizing sperm accurately? Capacitation itself seems to involve selection of an elite population of sperm as they approach the egg, which of course means that the study of a heterogeneous population of living and dead sperm in a semen sample in the laboratory may have little relevance [28]. Moreover, the acrosome reaction is not an instantaneous event but rather a progressive change to the fully reacted stage [29]. Sperm are known to respond unevenly to environmental conditions; for example, the acrosome reaction is a change that happens only in 30–40% of the sperm population during 1–2 h of incubation *in vitro* in mouse [30]. Nevertheless, most reports treat sperm as a mixed mass to evaluate sperm status. In a way this is inevitable, caused by a limitation in the sensitivity of each measurement and the lack of a convenient method to separate sperm in different stages in capacitation and/or acrosome reaction.

During the past few decades, it has been widely accepted that sperm are activated in the female reproductive tract and undergo the acrosome reaction close to the egg. This normally accompanies a dramatic but short-lived burst of energy termed hyperactivation [19]. Acrosomal enzymes such as hyaluronidase serve to disperse the cumulus matrix and acrosin to penetrate the zona pellucida (Fig. 2). However, studies in hamsters show that the fertilizing sperm enters the egg in less than a minute *in vivo*, long before cumulus dispersion [27]. Even the role of the acrosomal enzymes is far from clear, as it appears that penetration of the zona relies more on mechanical slicing than it does on enzymatic digestion [31]. Textbooks of human physiology still stress the role of enzymes in fertilization, but clearly the widely accepted roles for sperm acrosomal enzymes need major revision.

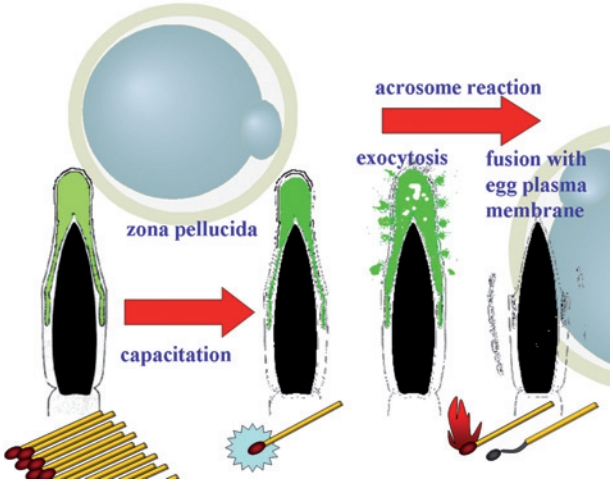

Figure 2. Mechanism of sperm-egg interaction. Sperm stored in the epididymis are kept metabolically inert to facilitate prolonged storage (left). Each sperm has a membranous sac over the nucleus called the acrosome; derived from the Golgi apparatus of the spermatid, this resembles a lysosome and is filled with many kinds of hydrolytic enzymes. After sperm are exposed to the female reproductive environment, they become metabolically active and undergo capacitation, which permits the acrosome reaction, and start to swim extremely vigorously (hyperactivation). Near the eggs, they undergo the acrosome reaction to release the contents by exocytosis. Only acrosome-reacted sperm are known to fuse with eggs, but their competency to fuse does not last long. The integrity of the acrosome in mouse sperm can be monitored easily using transgenic mice in which the GFP protein is targeted to the acrosomal contents [30].

It is important to understand the mechanism of the acrosome reaction in the study of fertilization. In species having a large acrosome, such as the guinea pig, it is easy to investigate acrosomal status using a normal phase contrast microscope [19]. However, in mouse and human spermatozoa, the acrosome is very small, and it is difficult to distinguish acrosome-reacted from acrosome-intact sperm. Various methods are reported to circumvent this problem [32–34]. Our strategy to observe the acrosomal status under a normal microscope is to use transgenic mouse lines with green fluorescent protein (GFP) in their acrosome. To produce such transgenic mice, we added an acrosin signal sequence and part of an N-terminal sequence for GFP, resulting in gene expression under the control of the acrosin promoter [30]. The resulting transgenic mouse lines produce sperm with GFP in their acrosome, and the green fluorescence is clearly

seen with no previous treatment of sperm. After the acrosome reaction, GFP disappears within three seconds. The acrosin-GFP mice with or without CAG-GFP (in which the entire body becomes green) are available to the scientific world through RIKEN BRC or CARD, Kumamoto University, under the registered names B6;C3 Tg(acro3-EGFP)01Osb and C57BL/6-Tg(CAG/Acr-EGFP)C3-N01-FJ002Osb (http://www.brc.riken.jp/lab/animal/en/). Sperm from these mice are easily analyzed using a flow cytometer, and real-time analysis of the acrosome reaction can be performed [30]. Although the GFP disperses from the acrosome extremely rapidly, other acrosomal components such as MN7 and MC41 remain on sperm for at least 15 min. Thus, 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 such an intermediate stage of acrosome-reacted sperm [29].

Sperm-egg interactions: disposing of an old theory?

There are many papers published purporting to explain sperm-egg interactions. For example, beta 1,4-galactosyltransferase (GalTase) is reported to function not as an enzyme but as a sperm-egg-binding factor. Various reports supporting this notion exist. In 1997, a GalTase-disrupted mouse line was produced by Shur's group. Unexpectedly, although there were some minor defects, sperm lacking GalTase could still fertilize eggs, and the males were not sterile [35]. This could be interpreted as suggesting that the role of GalTase was compensated by other factors. Shur's group went on to report another candidate, SED1, as a second zona-binding factor [36]. A SED1 gene-disrupted mouse line was also produced, but again the males were not sterile [37]. The failure to produce sterile males by disruption of factors believed to function in sperm-egg interaction goes back to 1994. The first disruption aimed at studying sperm-egg interaction targeted 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 sperm [38, 39].

Baba's group found that protease activity persists in the sperm of acrosin gene-disrupted mice. In all, they have reported five more testis-specific proteases, numbered from TESP1 to TESP5 [40]. Do all of these enzymes equally participate to compensate for the disruption of acrosin? Alternatively, are there any specific enzymes that play a major role in fertilization? Apart from the proteases, the molecule PH-20 has been indicated to have a role in the sperm's ability to bind to the zona pellucida, based on the finding that two out of the three monoclonal antibodies raised against PH-20 inhibit sperm-zona binding [41]. In 1993, a group studying snake venom found a significant homology of hyaluronidase to PH-20 [42]. 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 (100 μg/mL) when present during sperm-oocyte interaction [43]. However – again paradoxically – when PH-20 gene-disrupted mice were produced and examined, the mice were found to have an almost normal ability to sire pups [44].

There are further examples that the disruption of "important" factors results in an unexpectedly mild effect, or even no effect, on fertilization. The molecule fertilin was originally described as an antigen recognized by the anti-guinea pig sperm monoclonal antibody PH-30. As the PH-30 antibody inhibited sperm fusion with eggs, the PH-30 gene was cloned and analyzed. The antigen was found to be a heterodimer, and one of the monomer genes was found to have a domain similar to virus fusogen. Moreover, the disintegrin domain, which binds to integrin, was discovered in another sequence. The antigen was thus named "fertilin", and the discovery was published in Nature [45]. Various papers have supported the notion that this is a fusion protein. When Myles's group disrupted the gene for Adam2, which forms fertilin as one of its heterodimers, they found that the males were infertile. However, contrary to their expectations, Adam2-null sperm could fuse with the egg surface but could not bind to the zona pellucida [46].

In recent decades, the involvement of many factors in fertilization has been reported based on observations following the addition of antibodies, ligands and inhibitors. Using homologous gene recombination, many of the reported fertilization-related genes were subjected to disruption experiments to test their effect on fertilization. Surprisingly, many of the representative genes thought to be important for fertilization proved inessential or produced unexpected phenotypes when disrupted. These results cast doubt on the credibility of other factors that have not yet been examined for their functions in fertilization under disruption conditions. If we tentatively eliminate those factors not proven *essential* for fertilization, only a few remain. Thus, the theory of the molecular events of fertilization established over decades has been jeopardized.

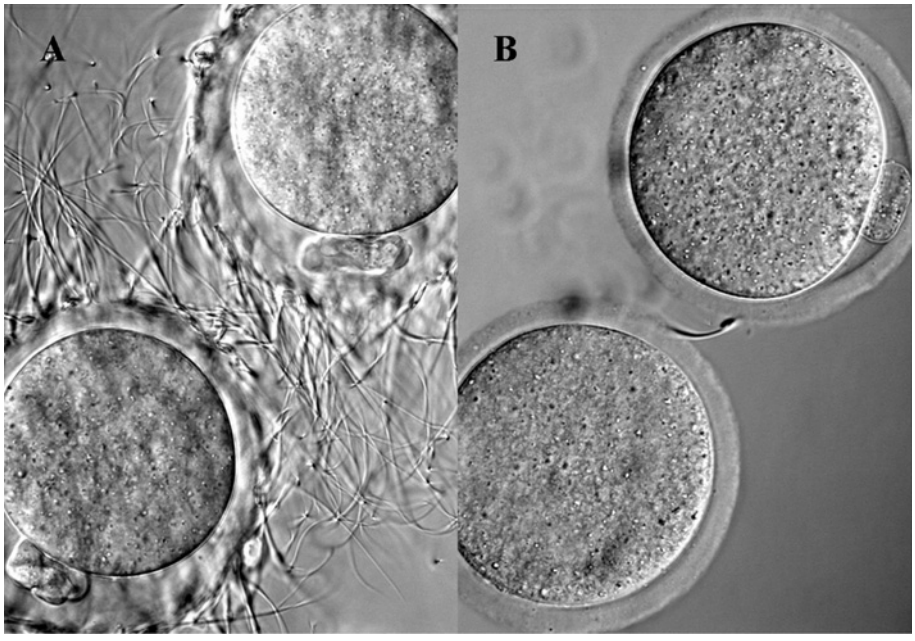

Figure 3. Impaired zona-binding ability of sperm from calmegin-knockout mice [47]. Sperm from calmegin$^{+/+}$ mice adhered successfully to the zona pellucida of eggs (A), but those from calmegin$^{-/-}$ mice failed to attach despite frequent collisions with the zona pellucida (B) (original magnification ×400).

Sperm-egg interaction: an emerging new horizon

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 [47]. Calmegin$^{-/-}$ males are almost sterile; 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 (Fig. 3). It is thus obvious why the calmegin$^{-/-}$ males are sterile, but can we then speculate that calmegin itself functions in sperm–zona interaction? The answer is no, because calmegin is a testis-specific homologue of the ubiquitously expressed endoplasmic (ER) molecular chaperone calnexin. During spermatogenesis, most gene expression is shut down sequentially; during spermiogenesis, sperm shed most of the unnecessary machinery for protein synthesis, including the ER. Thus, even in wild-type mice, there is no calmegin left on the sperm. Therefore, one can speculate that calmegin is acting to fold molecule(s) that are delivered onto the sperm surface during spermatogenesis and that are destined to act later in zona-binding.

After this report of calmegin disruption, reports on the previously mentioned ADAM2-knockout mice were published. Interestingly, both of these gene-disrupted mouse lines share the phenotype of impaired zona-binding ability. Considering calmegin's putative function of folding zona-binding proteins properly and the phenotype of the ADAM2-disrupted mouse, an interaction of calmegin with ADAM2 is conceivable. To study this, we immunoprecipitated calmegin from testicular lysates and examined the interaction of calmegin with ADAM2. Immunoprecipitation followed by western blot analysis revealed that both ADAM1 and ADAM2 formed complexes specifically with calmegin in the ER but did not do so with calnexin. The disruption of calmegin was shown to cause impaired heterodimerization of ADAM1/2 and resulted in the complete absence of ADAM2 from mature sperm. Because ADAM1 was absent from mature sperm when the ADAM2 gene was disrupted [46], ADAM1 was also predicted to be missing from mature calmegin$^{-/-}$ sperm. There is precedence for the disappearance of a membrane protein from the cell surface when the chaperone function of calnexin (a calmegin homologue) is disrupted. For example, in the absence of functioning calnexin, formation of the insulin receptor homodimer is repressed and the receptor is absent from the cell surface [48]. These results indicate not only the importance of calmegin for sperm membrane protein maturation but also reinforce the concept of ER chaperones functioning to form dimerized proteins. Thus, we might be able to explain why mice with two different gene disruptions show the same phenotype in terms of fertilization biology.

However, surprises continued. Reports on other gene disruptions such as those for ADAM1a and ADAM3 also reported the same phenotype of male infertility with impaired zona-binding ability. Expression of the ADAM family in sperm affects the expression of other ADAM family members in turn. According to Nishimura et al., disruption of ADAM1a caused ADAM3 to disappear, leaving the amount of ADAM2 un-

changed. Meanwhile, ADAM3 disruption did not cause a significant effect on the amount of ADAM2 [49]. To date, the most downstream factor – in other words, the closest factor that may participate in sperm-zona binding – is tentatively ADAM3. Unfortunately, a computer search for ADAM3 in the human genome revealed that it is a pseudogene. If ADAM3 is not present in human sperm, the proposal of a scheme including ADAM3 in sperm-zona binding is not applicable to humans. An alternative possibility is that there is a general zona-binding factor and that the disappearance of ADAM3 causes the loss of another factor from sperm; thus, ADAM1a disruption resulted in the loss of ADAM3 from sperm [50].

Might there be other sperm factors generally functioning in zona binding? A mouse sperm protein, sp56, that has the characteristics expected of the sperm protein responsible for recognition of egg zona pellucida was identified. The complementary DNA encoding sp56 was isolated, and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors [51]. Zonadhesin is a multiple-domain transmembrane protein believed to function as a sperm-zona pellucida-binding protein [52, 53]. There are reports that sp56 and zonadhesin function in sperm-zona binding [54, 55]. However, in the light of past gene knockout experiments, we must test the fertilizing ability of sperm that lack these factors before reaching a definite conclusion. At least we know that sp56 is present on sperm from the calmegin gene-disrupted mouse [47].

Moreover, it should be noted that the calmegin, ADAM1a-, ADAM2- and ACE-disrupted mouse sperm share the phenotype of an inability not only to bind to zona but also to migrate into the oviduct [46, 50, 56, 57]. The puzzle is why the two different inabilities of sperm-zona binding and oviduct migration run in parallel in these gene disruption experiments. Does this offer a clue to solving the molecular mechanisms of fertilization?

Membrane fusion

Compared with sperm-zona binding, sperm-egg fusion must be more complicated. The cell membrane consists of a double lipid bilayer separating the inside of the cell from the environment. It is not static but has many dynamic features. The cell must transact signals between the outside and inside to adjust its function properly. The lipid membrane has fluidity both horizontally and transversely, and there are various mechanisms to maintain the lipid constitutions of both the outer and inner sides of the membrane. Membranes also contain cholesterol-dense "rafts", and these are modified extensively during sperm capacitation [18]. New membrane needs to be synthesized accompanying cell divisions. Cytokinesis requires the assembly of an actomyosin contractile ring that constricts during cytokinesis [58]. 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; however, this must be repaired immediately. 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 capacity 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. Are there mere differences in lipid constitution or in the membrane restoration mechanism? The adjustment and formation of a characteristic nature of the membrane must be formed by a combination of various mechanisms such as the membrane undercoat and the constitution of lipids forming raft structures.

Fertilization (membrane fusion) takes place only between the plasma membrane of an unfertilized egg in a certain time window and the freshly rearranged sperm membrane soon after the acrosome reaction. Both gametes have to be conditioned properly to accomplish membrane fusion, but the factors involved in fusion are not clarified. Various important membrane fusion events exist in several tissues (Table 1): hepatocytes are multi-nucleated cells that originate after nuclear divisions but without cytokinesis. However, skeletal muscle cells, which are also multi-nucleated, are formed after myoblast cell fusion. Trophoblast cells fuse with each other to form

Table 1. Fusion processes in the body.

Membrane fusion within a cell
Cell division
Exosome [87]
Viral budding
Secretion of neurotransmitters (snare, clathrin)
Sperm acrosome reaction [88]
Phagocytosis (snare, clathrin)
Mitochondrial fusion (mitofusin) [89]
Membrane fusion between the same kind of cells
Muscle cells from myoblasts
Syncytiotrophoblasts from trophoblast
Formation of osteoclast (DC-STAMP) [59]
Membrane fusion between different kinds of cells
Fertilization sperm-egg [66, 75]
Virus infection

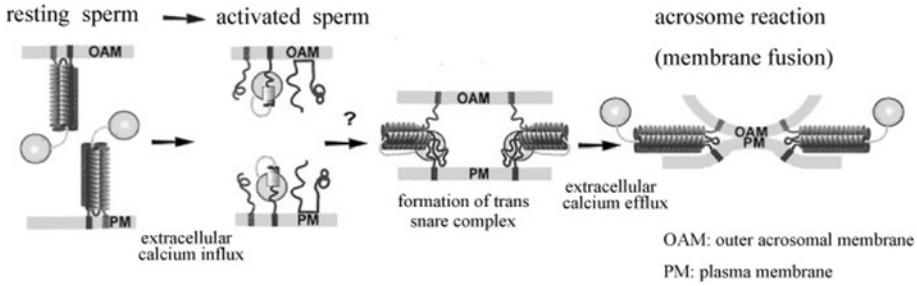

Figure 4. Hypothetical role of SNARE proteins in the acrosome reaction, involving fusion of the outer acrosomal membrane (OAM) and the sperm plasma membrane (PM) [63]. In both the PM and the OAM, Rab3, NSF and aSNAP associate as a heterotrimer. Rab3 is activated after the incorporation of calcium into the acrosome, resulting in dissociation of the trimer. This leads to new trimer formation *in trans.* A local decrease in calcium ion concentration brings the PM and OAM close together to allow fusion.

syncytiotrophoblasts, which are thought to function for further progression of the embryo into the uterine wall by digesting uterine tissue. However, in these three important instances, no cell factors involved in cell fusion have been reported. Osteoclasts, developed from monocytes, can be viewed as specialized macrophages working to resorb bone at multiple sites. They are also multinuclear cells, occasionally containing more than 100 nuclei, and are formed by cell fusion. Recently, a protein named DC-STAMP was reported to function in the fusion process [59], but the role remains indirect [60], and the real fusion mechanism is still unknown.

In another instance of membrane fusion in our body, mitochondrial membranes are known to fuse with neighboring mitochondrial membranes and/or to divide. This fusion is limited to mitochondrial membranes; fusion does not take place with other organelle membranes. In this process, mitofusin1 and mitofusion2 are reported to function in the fusion process [61].

One of the most precisely investigated fusion processes involves exocytosis originating in protein transport from the rough ER. SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) mediate exocytosis from single-cell eukaryotes to neurons. Bilayer fusion is proposed to occur in multiple steps. A tight SNARE pairing force between the two different lipid bilayers causes close apposition of the two layers, and the water molecules are expelled from the interface. Lipids of the two interacting leaflets of the bilayers then fuse between the membranes to form a hemifusion, or half-fusion product. After this hemifusion, rupture of the new bilayer is believed to complete the fusion reaction.

Before acquiring the ability to fuse with eggs, eutherian sperm need to undergo the acrosome reaction. This is a form of calcium-mediated exocytosis resembling mast cell degranulation [19]. It involves point fusions between the plasma membrane and the outer acrosomal membrane over a limited domain of the sperm head but not in a special zone known as the equatorial segment. Significantly, this region is the part of the sperm later involved in fusion with the oolemma [19]. In this context, the involvement of SNARE in the acrosome reaction has been postulated [62, 63] (Fig. 4).

Some kinds of viruses appear to mimic this fusion mechanism, using SNAREs for invasion. The core of certain viral fusion proteins generally consist of continuous polypeptides, within which oppositely oriented (*i.e.* antiparallel) helical hairpin-like structures assemble in a helical bundle, and these have been proposed to link up the two membranes for fusion [64–66]. The resemblance between SNAREs and viral fusion proteins suggests that the two fusion machineries employ a fundamentally similar mechanism to coalesce lipid bilayers (see Fig. 5).

In fact, all contagious pathogens need to be released from the cells to spread themselves to other cells. There must be numerous ways to create fusion between two membranes. Is there a common biological mechanism involved? In an experiment using influenza hemagglutinin peptide (HA) to induce the fusion of giant liposomes under visualized conditions, shrinkage of liposomes is always observed before fusion (Fig. 6). During the shrinkage, some parts of the membrane become highly flexed. Imaging of the shrunken liposomes indicates that liposomes fuse with each other as single-layered structures. These results suggest that a perturbation of lipid bilayers, probably arising from the acute bending in the membranes, is a critical factor in fusion efficiency, even during fertilization [67]. Of course, another possibility is that the shrunken liposome could have produced an unknown structure composed of lipids and amphiphilic peptides, but this is impossible to resolve microscopically.

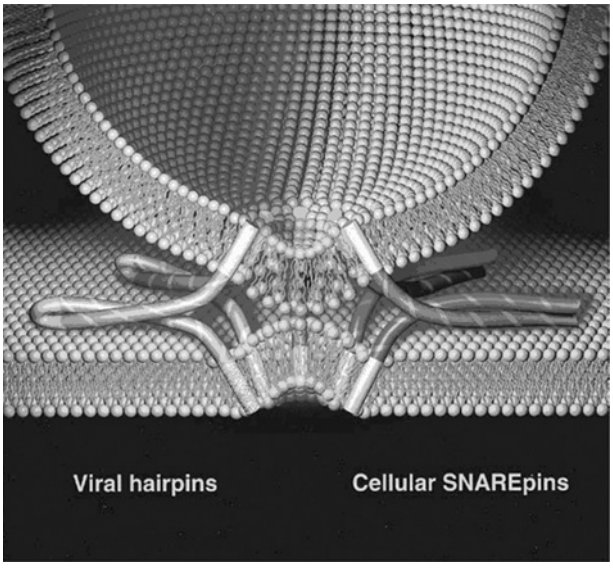

Figure 5. Viral fusion proteins that mimic cellular SNAREs. In cellular fusion events, plasma membrane and vesicle SNAREs bind together to coalesce the lipid bilayers [88]. The left side of the image illustrates viral SNARE-like single-chain proteins; the right side illustrates cellular SNAREpins. During viral fusion with a host cell, one viral hairpin protein spans the viral and cellular membranes and forms SNARE-like structures upon fusion [88]. Membrane folding as shown here might occur in the vicinity of SNAREs.

Sperm-egg fusion in gene-disrupted mice

Gene disruption experiments involving already-known factors have worked to dismantle the old models of fertilization. However, the new techniques will lead to the introduction of new factors to the scheme. 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 be known rapidly. For example, in the case of CD9 knockouts, the authors were aiming to determine the effects on immune functions. The CD9-deficient mice look healthy and live normally, but, surprisingly, if a female lacks CD9, she is sterile, while the fertilizing ability of male mice is normal [68–70]. We analyzed why these female mice are sterile using *in vitro* fertilization (IVF) and found that the ovulated oocytes could not fuse with sperm. This lack of sperm fusion meant that there was no release of cortical granules to induce the zona block to polyspermy [19, 71], and this allowed penetration of further sperm into the perivitelline space of the eggs. The CD9-deficient eggs with multiple perivitelline sperm are shown in Fig. 7A.

Thus, this gene disruption experiment by scientists whose initial interest lay elsewhere resulted serendipitously in the very first finding of an essential factor in sperm-egg fusion. This fusion-related factor on the egg membrane has a so-called "tetraspanin" structure. This family of proteins has four transmembrane

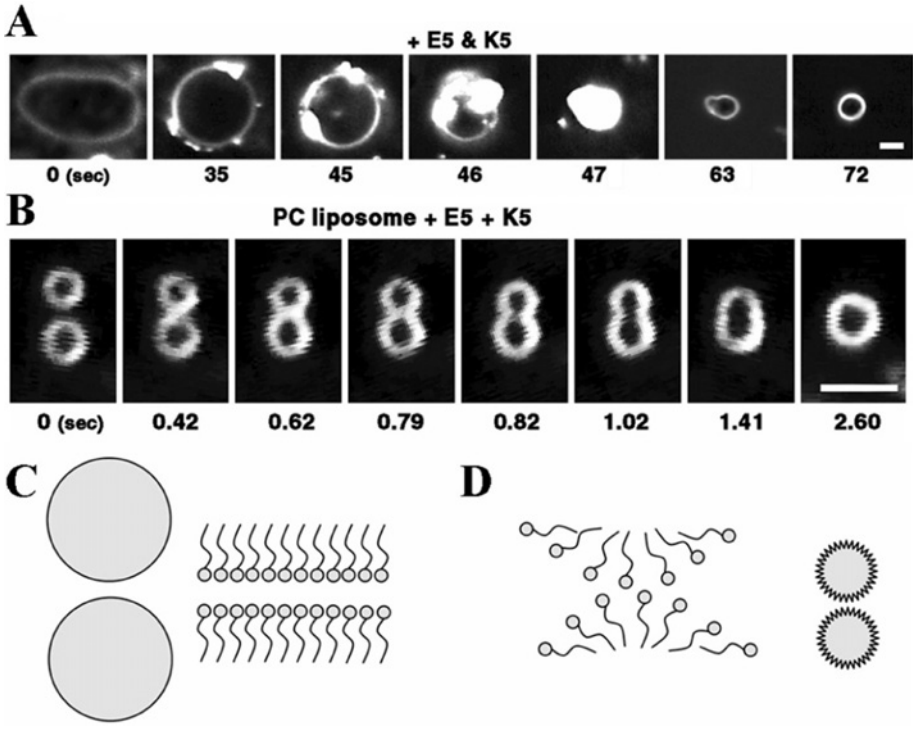

Figure 6. Artificial membrane fusion model using virus fusion protein-embedded liposomes. [67]. Shrinkage of giant liposomes was found to be necessary before liposomes acquired the competency to fuse. (A) A sequential image of dark-field images of a shrinking liposome. Numbers under the images indicate the time in seconds. E5 and K5 denote the recombinant virus fusion proteins in liposomes. (B) Sequential view of virus fusion protein embedded in shrunken liposomes (bars indicate 5 μm.). When any two shrunken liposomes made close contact, 40% of them detached after a short time, 20% of them remained attached and 40% of them fused as shown in this sequence. (C) Lipid bilayer before shrinkage. (D) Lipid bilayer after shrinkage. The liposome becomes smaller because of the folding of the membrane, as shown (bars indicate 5 μm).

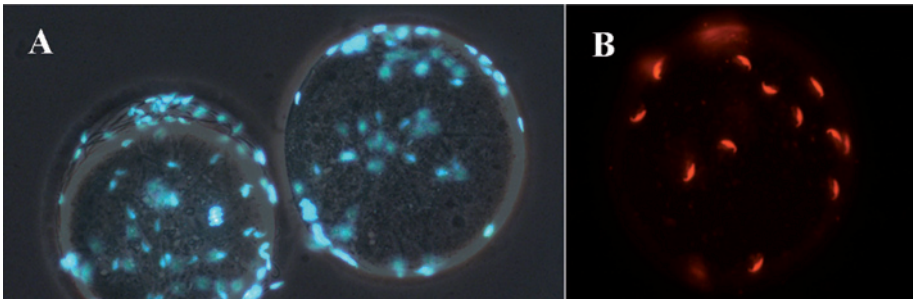

Figure 7. Accumulation of sperm in the perivitelline space caused by failure of sperm-egg fusion [89]. (A) Sperm accumulated in the perivitelline space of $CD9^{-/-}$ mouse eggs. The sperm could penetrate the zona pellucida but failed to fuse with the egg surface. Many sperm were able to enter because 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. (B) Similarly, when eggs were inseminated with $Izumo^{-/-}$ sperm, the sperm could penetrate the zona pellucida but failed to fuse with the eggs, resulting in the accumulation of many sperm inside the perivitelline space. These penetrated sperm had clearly undergone the acrosome reaction, as they were all exposing the acrosome-reacted sperm-specific antigen MN9 [47].

domains and binds with integrins. Soon it was learned that there are integrins α6 and β1 on the egg membrane, and the addition of synthetic peptides of a partial sequence of integrins were reported to inhibit sperm-egg fusion [72]. The methods employed in these experiments were to add ligands in the IVF system, such as when various sperm-zona-binding factors were examined. However, the "integrin-assisted fusion" theory was proven false, because mouse eggs that are deficient in integrin α6 and β1 are still able to fuse with sperm [73].

Sperm have to have completed the acrosome reaction prior to fertilization. This may imply that fusogenic factors are expressed on the sperm membrane only after this stage. If we could raise a monoclonal antibody that did not react to ejaculated sperm but only to acrosome-reacted sperm and if the same antibody could inhibit sperm-egg fusion, the corresponding antigen must be involved in the sperm-egg fusion event. Based on this hypothesis, we raised the anti-human sperm monoclonal antibody MH61 [74]. This antibody inhibited the fusion of human sperm to hamster zona-free eggs. Because the antibody could react only to acrosome-reacted sperm, it was then used to evaluate the fertilizing ability of human sperm in clinics. To clarify the antigen, we performed western blotting of the sperm extract and analyzed the band by its N-terminal protein sequence. A search for the antigen revealed that one of the complement receptors, CD46, reacted with the antibody. At this point, it became clear that at least three different laboratories were investigating the involvement of CD46 in sperm-egg fusion. However, the ortholog of CD46 was not found in the mouse. Why is this complement receptor functioning in sperm-egg interaction? We were interested to note that when Seya's group identified the CD46 gene in mouse, it was also found that this gene was expressed only in the testis (more precisely, only in sperm) [75], whereas the human *CD46* gene is expressed throughout the body. This suggests that although CD46 now functions as a complement-regulating factor in primates, its original role was to function in sperm-egg interaction. With this assumption in mind, we produced a mouse line whose CD46 gene was disrupted by homologous recombination. However, unexpectedly, the disruption of CD46 caused no visible damage to the fertilizing ability of males or females [76], adding another example to the pile of genes classified as "not essential in fertilization". Thus, the inhibition of IVF by the addition of antibodies to certain factors does not necessarily mean that those factors are essential for fertilization.

The role of CD46 in fertilization was only shown using human sperm fusing with hamster eggs. To identify other putative factors involved in sperm-egg fusion, we continued our quest using another monoclonal antibody against mouse sperm, OBF13, which specifically inhibits fusion [77]. The antigen was identified 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), and ten peptides that were 100% identical to a part of the sequence listed in the RIKEN full-length database (NCBI accession number XM_133424) were found. The registered DNA sequence was confirmed by sequencing after reverse transcription polymerase chain reaction (RT-PCR) amplification with total RNA prepared from the testis. A human homolog was found as an unverified gene in the NCBI database (accession number BC034769). This gene encodes an immunoglobulin superfamily (IgSF) type I membrane protein with an extracellular immunoglobulin domain that contains one putative glycosylation site. The antigen was shown to be a testis (sperm)-specific

56.4-kDa antigen by western blotting with a polyclonal antibody raised against recombinant antigen. We termed the antigen "Izumo" after a Japanese Shinto shrine dedicated to marriage. However, as described above, it was not clear if the antigen plays an indispensable role in sperm-egg fusion until we could examine the fertilizing ability of sperm lacking the Izumo protein. Therefore, we produced an Izumo gene-disrupted mouse line and found that the males were sterile despite normal mating behavior with normal vaginal plug formation. No offspring were fathered by these mice, but it was unclear whether the defect was limited to fusion or extended to later developmental stages. To address this question, we performed ICSI to insert Izumo$^{-/-}$ sperm directly into the cytoplasm of wild-type eggs and thereby bypass the fusion step. Eggs could not fuse with Izumo$^{-/-}$ sperm (Fig. 7B) but were successfully fertilized when injected with Izumo$^{-/-}$ sperm; the fertilized eggs implanted normally, and the resulting embryos developed appropriately to term.

This seems to be compelling evidence that Izumo is a central player in sperm-egg fusion. However, we need to be careful about the "off-target" effects of gene disruption. A targeted inactivation of the myogenic basic-helix-loop-helix gene *MRF4* is a good example. The phenotypes of three different MRF4-deficient mouse lines from three different laboratories with similar design of the targeting vector were very different, ranging from complete viability of homozygotes to complete lethality; these three similar but slightly different targeting vectors had different effects on expression of the adjacent *Myf5* gene, which accounts for much of the phenotypic variation [78]. Another good example of the potential pitfalls of gene disruption is the case of the gene for the PRION protein (PrP), which is a glycoprotein expressed constitutively on the neuronal cell surface. A protease-resistant isoform of the prion protein is implicated in the pathogenesis of a number of transmissible spongiform encephalopathies. Five independent PrP-knockout mouse lines have been reported [79, 80], and three of these show cerebellar symptoms and loss of Purkinje cells upon ageing [81–83]. However, it is now accepted that the disruption of PrP causes no apparent phenotype; the discrepancy in the observations was associated with inter-gene splicing with neighboring Doppel in some of the targeting vectors. In this case, the resulting truncated PrP expression in Purkinje cells was shown to cause Purkinje cell death and ataxia [84, 85]. Are these gene knockouts exceptional cases? We are not able to estimate how frequently unpredictable side effects might happen. However, to be cautious, we need to be reassured that the infertile phenotype of the Izumo gene-disrupted mouse is really caused by the absence of Izumo and not by some other, indirect, effect. Some scientists prefer to compare knockout mouse lines from two different ES cell lines, but this is not enough to reveal a side effect brought about by the characteristic nature of the targeting vector. One of the ways to confirm that a phenotype corresponds directly to the targeted gene is to examine if the defect is rescued by introduction of the transgene into the knockout mouse line.

To examine whether the infertility phenotype of Izumo$^{-/-}$ mice was directly derived from the lack of Izumo on sperm, we performed a rescue experiment by crossing Izumo$^{-/-}$ mice with transgenic mouse lines generated to express Izumo using the testis-specific calmegin promoter12. The sterile phenotype was rescued by transgenic expression of Izumo on mouse sperm. Thus, we have come to believe that Izumo is really functioning in sperm-egg fusion. This was the first factor shown to be essential not only by the inhibitory activity of antibodies or ligands but also by using genetically modified animals.

Normally, interaction of gametes is limited within the taxon, and xenogeneic gametes do not meet each other; if they do, they generally fail to interact properly. However, as an exceptional case, hamster eggs are known to be able to fuse with sperm from different species, such as mouse and human, when the zona pellucida is removed. We were curious to see if Izumo$^{-/-}$ sperm could fuse with hamster eggs; the experiment showed that without Izumo, the mouse sperm failed to fuse with hamster eggs. Likewise, fusion of human sperm to hamster eggs was inhibited by the addition of anti-human Izumo antibody, suggesting that Izumo might also have a role in human sperm-egg fusion. However, we have been claiming throughout this review that the inhibition of *in vitro* fertilization by antibodies may not always indicate the importance of the antigen in the fertilization process. Therefore, we prefer to hold back on any conclusion that Izumo is functional in humans until we learn if men with mutations in their Izumo gene are infertile and if it is clear that their sperm cannot fuse with eggs.

In any case, the first unambiguous fusion-related factors on sperm (Izumo) and on eggs (CD9) have been clarified. However, it is not yet known whether sperm Izumo interacts with egg CD9, as occurs with placental IgSF protein PSG17 [86]; neither do we know why the localization of Izumo after the acrosome reaction is not limited to the equatorial segment, where fusion initially takes place. All we can say now is that continued study of this protein's function will undoubtedly lead to a fuller understanding of the cell-cell fusion process in fertilization. The results from gene-disrupted mice in relation to fertilizing ability are summarized in Table 2.

Table 2. Various gene knockout mouse lines and their phenotypes.

origin/protein Sperm	(initially predicted) functions	fertility*	impaired step	reference
acrosin	zona penetration	fertile	–	[38, 39]
GalTase	sperm-zona binding	fertile	–	[35]
calmegin	folding of nascent protein	infertile	zona binding, UTJ transition	[47]
ADAM2#	sperm-egg fusion	infertile	zona binding, UTJ transition	[46]
ACE	regulation of blood pressure	infertile	zona binding, UTJ transition	[56]
ADAM3	sperm-egg fusion	infertile	zona binding	[90]
SedI	sperm-zona binding	fertile	–	[36]
C3	sperm-egg fusion	fertile	–	[91]
PH20	cumulus mass passage	fertile	–	[44]
CD46	sperm-egg fusion	fertile	–	[75]
ADAM1a#	unpredicted	infertile	zona binding, UTJ transition	[50]
Izumo	sperm-egg fusion	infertile	sperm-egg fusion	[70]
ADAM1b	sperm-egg fusion	fertile	–	[92]
Egg				
CD9	unpredicted	infertile	sperm-egg fusion	[66-68]
Integrin alpa6	sperm-egg fusion	fertile	–	[72]
Integrin beta	sperm-egg fusion	fertile	–	[93]

* Fertile lines are defined here as those for which homozygous mice could be used to maintain the lines.
# Fertilin is an ADAM1b and 2 heterodimer.

Conclusions

Experiments using gene-manipulated animals are clearly very powerful for judging whether candidate "important factors" in fertilization are indeed essential. If a certain factor is judged as "not essential" by gene disruption experiments, we cannot necessarily conclude that it does not function *in vivo*. However, at least these studies can show whether such factors affect sperm viability. As the number of genes that are truly indispensable for fertilization are accumulating, their relationships with fertilization biology are emerging [87]. For any study using gene disruption, the first thing scientists need to do is to mate the animals and establish a mutant line. Thus, any genes that might affect reproduction will be discovered immediately. We trust that this review will alert gene biologists to the importance of such serendipitous findings for the elucidation of sperm-egg interactions and for unraveling the molecular mechanisms involved. The more difficult process will be to extrapolate from laboratory models to real life, but we think the future looks bright. We will continue to look to Izumo for inspiration.

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