Fertilization: a sperm's journey to and interaction with the oocyte

Masahito Ikawa,[1] Naokazu Inoue,[1] Adam M. Benham,[1,2] and Masaru Okabe[1]

[1]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. [2]School of Biological and Biomedical Sciences, Durham University, United Kingdom.

Mammalian fertilization comprises sperm migration through the female reproductive tract, biochemical and morphological changes to sperm, and sperm-egg interaction in the oviduct. Recent gene knockout approaches in mice have revealed that many factors previously considered important for fertilization are largely dispensable, or if they are essential, they have an unexpected function. These results indicate that what has been observed in in vitro fertilization (IVF) differs significantly from what occurs during "physiological" fertilization. This Review focuses on the advantages of studying fertilization using gene-manipulated animals and highlights an emerging molecular mechanism of mammalian fertilization.

Introduction

In the early 1950s, Min Chueh Chang and Colin Russell Austin independently found that mammalian sperm must spend some time in the female reproductive tract before they acquire the ability to fertilize eggs (1, 2). The phenomenon underlying the acquisition of fertilization capability is called sperm capacitation (3), and the discovery of Chang and Austin made it possible to perform mammalian fertilization in vitro by mixing capacitated sperm with ovulated eggs, a procedure used by in vitro fertilization (IVF) clinics today (4) (Figure 1). This simplified in vitro experimental system (5) also allowed researchers to intensively study the mechanism of fertilization. Indeed, using this system together with biochemical approaches, such as competitive binding of antibodies and ligands that interact with sperm, eggs, and their surroundings, various factors were reported to be important for mammalian fertilization.

The development of gene-knockout technology (6, 7) has allowed researchers to test in vivo the findings from IVF studies. Numerous factors considered to be key molecules have been examined for their importance during fertilization (Tables 1 and 2). Surprisingly, these experiments revealed that many of the sperm factors thought to be important for fertilization, including the acrosomal protease acrosin (Acr) (8), the zona pellucida (ZP) binding protein β-1,4-galactosyltransferase 1 (B4galt1) (9, 10), and the egg fusion protein fertilin (a heterodimer that consists of two subunits, a disintegrin and metallopeptidase domain 1b [Adam1b] and Adam2) (11), were not essential. It can be argued that in vivo, compensatory mechanisms supplant the function of the gene targeted in a knockout mouse. However, these factors were identified as important for fertilization in studies using antibodies that blocked IVF by targeting a single epitope of these factors, and no compensatory mechanisms were detected. Although the likelihood is low that compensatory mechanisms are revealed in gene-knockout mice, it can still be argued that in knockout animals upregulation of unknown, functionally related molecules could take place. Despite these arguments, the view that genes essential for mammalian fertilization do exist has been confirmed by the discovery of proteins whose gene disruptions prevent fertility both in vitro and in vivo (e.g., cation channel, sperm associated 1 [Catsper1], ref. 12, and izumo sperm-egg fusion 1 [Izumo1], ref. 13). An additional benefit of gene-knockout studies is that they reveal factors that would never have emerged as essential for fertilization from IVF experiments (e.g., angiotensin-converting enzyme [Ace], ref. 14; calmegin [Clgn], ref. 15; and Cd9, refs. 16–18). In this Review, we discuss the current understanding of the mechanism of mammalian fertilization, which has been defined mainly through in vivo analysis of various gene-manipulated animals.

Sperm migration in the female reproductive tract

Capacitation is the process by which sperm become competent to fertilize an egg (Figure 1). Capacitation takes place after ejaculation, in the female reproductive tract, and is required only by mammalian sperm. Cholesterol is particularly abundant in seminal plasma (19) and has an inhibitory effect on sperm capacitation. During capacitation, cholesterol and other sterols are removed from the sperm surface, and non-covalently attached glycoproteins acquired in the epididymis are released from the sperm surface (20). Together, these modifications create a more fluid membrane environment, making the sperm competent for subsequent fertilization cues.

There are many papers demonstrating the importance of protein phosphorylation, particularly tyrosine phosphorylation, and calcium ion influx for capacitation. These signaling events may also be important for the release of putative "decapacitation factor(s)" from sperm (21). In the IVF clinic, sperm are prepared by the "swim up" method and/or by Percoll density gradient centrifugation to ensure that the sperm are free from seminal plasma, which is believed to contain decapacitation factor(s). Since individual sperm change their biochemical status after collection and incubation and there is no good method to separate them, it is inevitable that in the laboratory, mixed populations of millions of gametes are analyzed at different stages of capacitation and/or the acrosome reaction. It is important to be aware that the global biochemical status of many unsuccessful sperm may not necessarily reflect the molecular events that occur in the one successful sperm that navigates the oviduct and penetrates the ZP, the transparent glycoprotein extracellular matrix that surrounds the egg (Figure 1).

Mammalian sperm have to travel a long distance through the female reproductive tract to the oviduct, in which fertilization takes place. To accomplish the journey to the egg, sperm are

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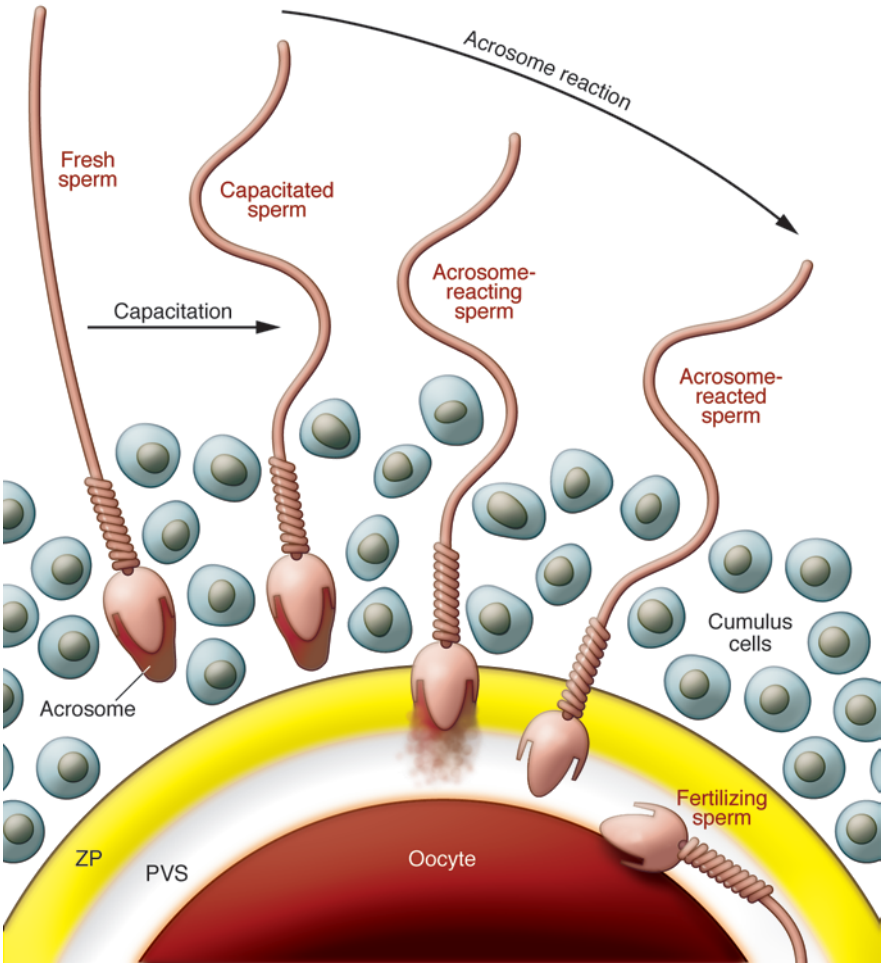

Figure 1
Mechanism of sperm-egg interaction. Over the nucleus of each mammalian sperm is a membranous sac known as the acrosome, which is filled with many kinds of hydrolytic enzymes. In the female reproductive tract or in an IVF medium, sperm undergo capacitation, which permits the acrosome reaction. Near the eggs, probably stimulated by the cumulus cells and the ZP, sperm release their acrosomal contents by exocytosis and penetrate the ZP. Only acrosome-reacted sperm fuse with eggs, but their competency for fusion does not last long. Cumulus cells are packed together by hyaluronic acid at ovulation and become diffuse during fertilization. PVS, perivitelline space.

equipped to overcome various obstacles that lie ahead, such as navigating the uterotubal junction (UTJ) and penetrating the egg extracellular matrices. For example, the cysteine-rich cationic polypeptide defensin β 126 (DEFB126) has been suggested to coat sperm, and its overall negative charge facilitates sperm attachment to the oviduct epithelium, promoting penetration of the cervical mucus (22). While the function of DEFB126, and other related epididymis-specific defensins, remains to be fully tested in knockout animals, it seems that proteins secreted from male accessory glands regulate sperm migration.

The oviduct is comprised of three major segments: the UTJ, the isthmus, and the ampulla (Figure 2). These structures seem to play distinct roles in ensuring successful fertilization. The UTJ connects the uterus and oviduct by intersecting the distal part of the oviduct. Several lines of evidence obtained from knockout mice suggest that sperm migration through the oviduct is biologically regulated. The sperm from male mice lacking one of the molecules Clgn, Ace, Adam1a, Adam2, or Adam3 are motile but not able to pass through the UTJ (23–27). For Ace, it is the testicular isoform of the protein, which is transcribed from testis-specific promoters in intron 12, that is required for fertility. Adam1a, Adam2, and Adam3 are all glycoproteins with EGF-like and peptidase M12B domains. Clgn is a testis-specific homolog of the ER-resident lectin-like chaperone calnexin. Proteomic analysis of sperm from the mutant mouse lines revealed that the sperm surface protein Adam3 is commonly absent or dislocated into detergent-rich membrane domains in all five lines of mutant mice (28). Initially, *Adam3*$^{-/-}$ mice were reported to have no defect in sperm migration (29), but using fluorescent protein–tagged transgenic sperm and live imaging technology (Figure 2), a defect in the oviductal migration of *Adam3*$^{-/-}$ sperm was revealed (27). Adam3 is now considered to be the most important factor in sperm migration in the mouse. The precise mechanism of how Adam3 facilitates the passage of sperm through the UTJ is unknown. The relationship between the five molecules that cause the disappearance of Adam3 from sperm will be discussed later in the section on sperm-ZP binding.

After passing through the UTJ, sperm are held on the surface of mucosal folds in the isthmus and remain there until the time of ovulation draws near. In cows, binder of sperm protein (BSP) on the sperm surface and annexin family proteins on the oviduct epithelial surface have been suggested to play important roles in sperm storage in the isthmus (30, 31). BSPs are fibronectin type II domain–containing proteins released from the male seminal vesicles (a pair of simple tubular glands that release their contents into the vas deferens), and include PDC109 (BSPA1/A2), BSPA3, and BSP30K. Recently, BSP homologs were found in the human (BSPH1) and mouse (32). It has also been suggested that the reducing redox environment of the oviduct could promote the release of sperm by facilitating the reduction of cell surface thiols on sperm cell proteins important for sperm-oviduct binding (33).

Table 1
Genes related to fertility that have been knocked out in mice (A to O)

Gene	Expected roles	Fecundity	Fertility defects observed in knockout mice	Refs.
Ace	Blood pressure control	Male, infertile	Severely reduced ZP binding and defect of UTJ migration	24
Acr	ZP binding, ZP penetration	Male, delayed fertility	Delayed acrosome reaction	8, 116
Adam1a	Spermatogenesis	Male, infertile	Severely reduced ZP binding and defect in UTJ migration	25
Adam1b	Sperm-egg fusion	Male and female, fertile	None	11
Adam2	Sperm-egg fusion	Male, infertile	Severely reduced ZP binding and defect in UTJ migration	26
Adam3	Sperm-egg interaction	Male, infertile	Severely reduced ZP binding and defect of UTJ migration	27, 29
Ambp	Covalent binding to hyaluronan	Female, subfertile	Reduced ovulation and fertilization rates accompanied by absent ZP	51
B4galt1	ZP binding	Male, fertile	Reduced acrosome reaction in response to Zp3	9
Bmp15	Cumulus expansion and ovulation	Female, subfertile	Defects in ovulation and the integrity of the COC	117
C1galt1	Synthesis of core-1–derived O-glycans	Male and female, fertile	None	96
Catsper1	Sperm capacitation	Male, infertile	Severely decreased sperm motility and lack of calcium ion influx	40
Cd9	Participation in migration and adhesion in many cell types	Female, infertile	Severely reduced sperm-egg fusion	16–18
Cd46	Sperm-egg interaction	Male, accelerated fertility	Increased acrosome reaction	118
Cd81	Participation in migration and adhesion in many cell types	Female, severely reduced fertility	Severely reduced sperm-egg fusion	119
Clgn	Spermatogenesis	Male, infertile	Severely reduced ZP binding and defect in UTJ migration	15
Cplx1	Binding to the SNARE complex and regulation of neuronal exocytosis	Male and female, fertile	Reduced acrosome reaction in soluble ZP	79
Crisp1	Sperm-egg interaction	Male and female, fertile	Reduced sperm-egg fusion	120
Defb22[A]	Microbicidal and cytotoxic peptides	Not examined	Not examined	22
Dkkl1	Placental development	Male and female, fertile	Severely reduced ZP binding in vitro	121
Gdf9	Cumulus expansion and ovulation	Female, subfertile	Defects in ovulation and the integrity of the COC	122
Hyal5	Penetration through the cumulus layer	Male and female, fertile	None	61
Inpp5b	Hydrolyzation of lipid substrates	Male, subfertile	Severely reduced sperm motility and defect in Adam2 processing	123
Itga6	Sperm-egg interaction	Not examined	Normal sperm-egg fusion	113
Itgb1	Sperm-egg interaction	Not examined	Normal sperm-egg fusion	113
Izumo1	Sperm-egg fusion	Male, infertile	Failed sperm-egg fusion	13
Mfge8	ZP binding	Male and female, fertile	Reduced ZP binding in vitro	124
Mgat1	Synthesis of complex and hybrid N-glycans	Male and female, fertile	Thinner ZP, but eggs fertilized	95
Napa	Membrane fusion and exocytosis	Male, infertile	Reduced acrosome reaction	77

[A]Defb22 (defensin β 22) is a mouse homolog of DEFB126. *Bmp15*, bone morphogenetic protein 15; *Crisp1*, cysteine-rich secretory protein 1; *Dkkl1*, dickkopf-like 1; *Gdf9*, growth differentiation factor 9; *Inpp5b*, inositol polyphosphate-5-phosphatase B; *Itga6*, integrin α_6; *Itgb1*, integrin β_1; *Mfge8*, milk fat globule-EGF factor 8 protein.

During sperm storage, the isthmic epithelium creates a microenvironment that delays capacitation and stabilizes sperm for a period of approximately 24 hours, at least in humans (34, 35). When ovulation draws near, unknown female factors trigger the sperm to leave the reservoir and move up to the ampulla. Release of sperm from the isthmic epithelium is reported to depend mainly on sperm changes that are associated with capacitation. For example, bull sperm are reported to shed their BSPs and lose their oviduct epithelium–binding ability (36). Hyperactivated sperm movement and asymmetrical beating of the sperm flagellum occurs after capacitation and is thought to assist sperm escaping from the oviduct epithelium (37). The importance of hyperactivated sperm movement in release from the isthmic epithelium is supported by a report that *Catsper1*$^{-/-}$ sperm are unable to detach from oviduct epithelium (38). A functional CATSPER channel has been reported to control calcium entry, which stimulates the hyperactivated state (12, 39, 40). Of clinical interest, human male infertility caused by mutations in the gene encoding CATSPER1 was reported recently (41).

The gradual release of sperm from the isthmus helps to reduce the number of sperm available at the point of fertilization and avoids polyspermy (fertilization of an egg by more than one sperm), which is fatal for embryonic development (42). Surgical removal of the isthmus in the pig leads to increased numbers of sperm entering the ampulla and polyspermy in about one-third of eggs (43), suggesting that the isthmic portion of the female reproductive tract regulates the number of fertilization-competent sperm that

Table 2
Genes related to fertility that have been knocked out in mice (P to Z)

Gene	Expected roles	Fecundity	Fertility defects observed in knockout mice	Refs.
Pcsk4	Family of calcium-dependent serine proteinase	Male, subfertile	Reduced capacitation, acrosome reaction, and egg binding in vitro	125
Pgap1	GPI inositoldeacylase that removes palmitate from inositol	Male, infertile	Severely reduced ZP binding and defect in UTJ migration	103
Piga	Enzyme involved in GPI anchor biosynthesis	Female, infertile	Failed sperm-egg fusion	114
Pkdrej	Acrosome reaction	Male and female, fertile	Reduced acrosome reaction in soluble ZP	126
Plcd4	Hydrolyzes phosphatidylinositol-4,5-bisphosphate	Male, subfertile	Severely reduced acrosome reaction in soluble ZP	71
Press21	Serine protease family	Male and female, fertile	Severely reduced ZP binding and ZP-induced acrosome reaction and sperm-egg fusion in vitro, but rescued by exposure of the sperm to uterine fluids	106
Ptger2	Prostaglandin E2 receptor	Female, severely reduced fertility	Reduced fertilization and defects in cumulus expansion	56, 127
Ptgs2	Regulation of prostaglandin synthesis	Female, infertile	Reduction in ovulation number and severe failure in fertilization	128
Ptx3	Early folliculogenesis factor regulated by Gdf9	Female, subfertile	Defects in the integrity of the COC	54
Smcp	Enzymatical inactivation of structural protein in the mitochondrial capsule	Male, subfertile	Reduced sperm motility, migration into the oviduct and fertilization	129
Spam1	Penetration through the cumulus layer and binding to ZP	Male and female, fertile	Delayed COC dispersion	59
Spag11a	Microbicidal and cytotoxic peptides	Not examined	Reduced sperm motility in knockdown rat	130
Tnfip6	Early folliculogenesis factor regulated by Gdf9	Female, infertile	Failed COC expansion	52
Tssk6	Spermiogenesis and sperm function	Male, infertile	Abnormal sperm morphology and defects in sperm-egg fusion through the aberrant regulation of Izumo1 localization	110
Zan	ZP binding	Not examined	Not examined	131
Zp1	ZP formation	Female, reduced fertility	Abnormal ZP and defects in fertilization	86
Zp2	ZP formation and sperm binding	Female, severely reduced fertility	Fragile oocytes with defects in developmental competence	87
Zp3	ZP formation, sperm binding, and acrosome reaction	Female, severely reduced fertility	Lack of ZP and fragile oocytes	88
Zp3r	ZP binding	Not examined	Not examined	101
Zpbp	ZP binding	Male, infertile	Asthenoglobozoospermia	132

Pcsk4, proprotein convertase subtilisin/kexin type 4; *Piga*, phosphatidylinositol glycan anchor biosynthesis, class A; *Pkdrej*, polycystic kidney disease (polycystin) and REJ (sperm receptor for egg jelly, sea urchin homolog)-like; *Prss21*, protease, serine, 21; *Ptgs2*, prostaglandin-endoperoxide synthase 2; *Smcp*, sperm mitochondria-associated cysteine-rich protein; *Spag11a*, sperm-associated antigen 11a; *Zp3r*, Zp3 receptor (also known as Sp56); *Zpbp*, ZP-binding protein.

reach the egg in vivo. This control step is bypassed by IVF, which usually requires thousands of sperm to occur successfully.

After leaving the storage reservoir, sperm move into the ampulla and locate the cumulus-cell oocyte complex (COC). The COC is comprised of ovulated eggs covered by the ZP and a multicellular cumulus oophorus. Sperm chemotaxis is implicated in locating the COC. In particular, human sperm have been reported to sense a chemoattractant from both follicular fluid (44) and COCs (45). Progesterone was proven to be the cumulus-derived chemoattractant by the observation that antiprogesterone treatment abrogated the in vitro chemotactic activity of human (46) and rabbit (47) cumulus-cultured medium. Olfactory receptor, family 1, subfamily D, member 2 (OR1D2) was also demonstrated to function in human sperm chemotaxis and induced calcium signaling when sperm were exposed in vitro to the representative chemical attractant bourgeonal (an aromatic aldehyde) (48). The contribution of these factors to in vivo fertilization awaits analysis by gene-knockout approaches.

Sperm-cumulus interaction

Soon after interacting with COCs, sperm penetrate the matrix of the cumulus oophorus, which is rich in proteins and carbohydrates such as hyaluronan, an unsulfated glycosaminoglycan (Figure 1). In marsupials, eggs shed their cumulus layers just before ovulation, and the cumulus-free eggs are fertilized in vivo (49, 50). Mouse eggs freed from cumulus using hyaluronidase can be fertilized in vitro. However, the cumulus layers surrounding the mouse oocyte are beneficial for fertilization, and genetic deletion of a number of genes involved in synthesizing and stabilizing the COC extracellular matrix suppresses fertilization in vivo. Female mice lacking any one of the factors α1 microglobulin/bikunin (Ambp) (51), TNF-α–induced protein 6 (Tnfip6) (52, 53), and pentraxin 3 (Ptx3) (54) have been shown to exhibit reduced female fertility, due to defects in the integrity of the COC. In these female mice, the number of ovulated oocytes in the oviduct was reduced, but the oocytes that reached the oviduct showed impaired fertilizing ability.

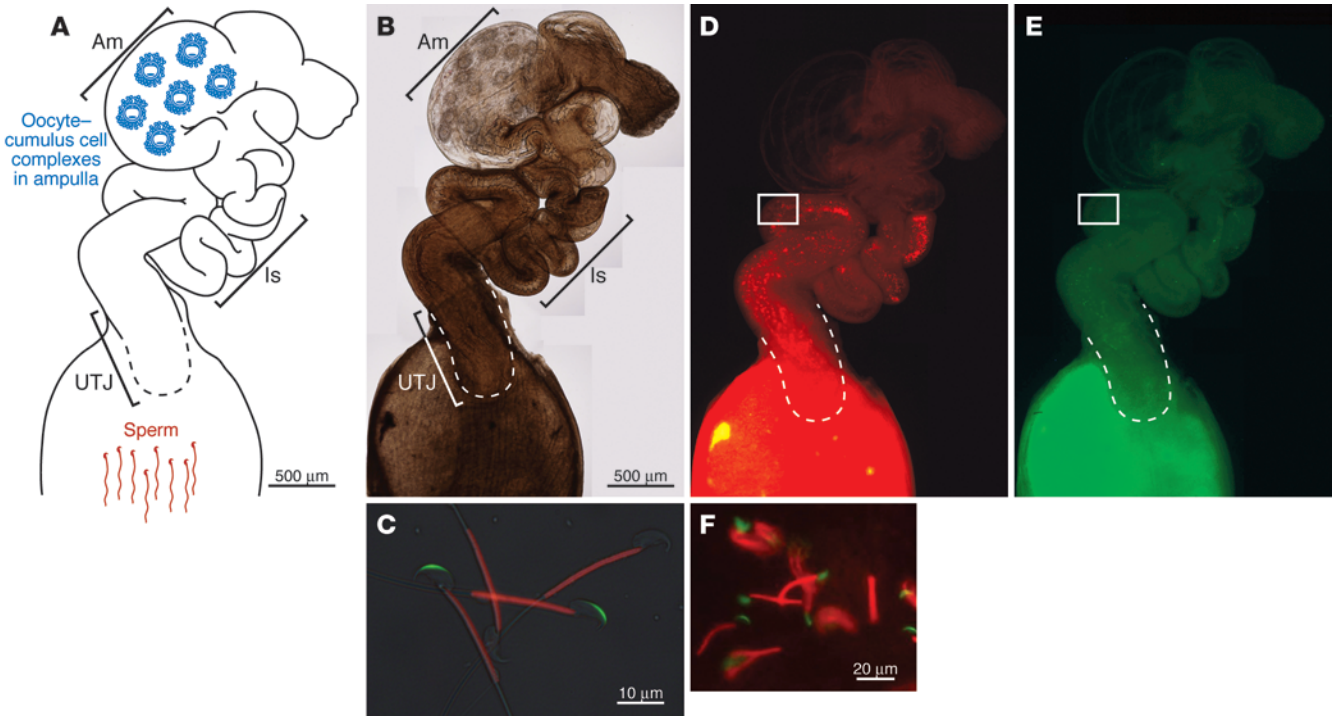

Figure 2
Sperm migration through the female reproductive tract. After sperm are deposited in the female reproductive environment, they become metabolically active and start migrating into the oviduct. (**A** and **B**) Depicted here is one oviduct in a female mouse. The generation of sperm engineered to express fluorescent proteins has facilitated visualization of sperm migration through the female reproductive tract (65, 66). (**C**) Sperm from mice engineered to express acrosome-targeted EGFP (acrEGFP) and mitochondria-targeted red fluorescent protein (mtDsRed2). GFP is released during the acrosome reaction, so that acrosome-reacted sperm are no longer acrEGFP$^+$ but remain mtDsRed2$^+$ (66). The mice are available through public bioresource centers. (**B** and **D**–**F**) The female mouse reproductive tract removed at four hours after coitus. The sperm and their acrosomal status can be monitored through the uterine and oviduct wall. Areas indicated in **D** and **E** are merged in **F**. The images in **B**, **D**, and **E** are a composite of several images. Scale bar: 500 μm (**B**, **D**, and **E**). Am, ampulla; Is, isthmus.

It was recently reported that cumulus cells secrete CCL chemokines upon activation of the TLR system by hyaluronan fragments generated by sperm hyaluronidase (55). The study of prostaglandin E receptor subtype EP2–knockout (*Ptger2*-knockout) mice also supports the notion that CCL chemokines facilitate sperm migration to the COC. Ptger2 is expressed in cumulus cells and helps to lower the release of Ccl7. Without *Ptger2*, release of Ccl7 is increased and prevents fertilization by causing the cumulus extracellular matrix to harden (56). Timely interaction between prostaglandin and chemokine signaling in the cumulus may assist monospermic fertilization.

Sperm adhesion molecule 1 (Spam1) was first identified as a sperm receptor for the ZP but was later proven to have hyaluronidase activity and was implicated in sperm passage through the COC (57, 58). The originally described ZP-binding ability is not physiologically essential, because *Spam1*$^{-/-}$ sperm have been shown to bind the ZP. *Spam1*-disrupted mice are fertile, although the mutant sperm show a reduced ability to disperse cumulus cells in vitro (59). Tadashi Baba and colleagues found that *Spam1*$^{-/-}$ sperm retain approximately 40% of their hyaluronidase activity and identified hyaluronoglucosaminidase 5 (Hyal5) as an additional sperm-specific hyaluronidase (60). When they generated *Hyal5*$^{-/-}$ mice, sperm fertility was comparable to that of wild-type mice, both in vitro and in vivo (61), suggesting a more important role for Spam1 than Hyal5 during sperm passage through the cumulus layers. It will be interesting to determine the details of the sperm fertilizing ability in *Spam1*$^{-/-}$*Hyal5*$^{-/-}$ double-knockout mice in the future.

Acrosome reaction

The acrosome is a Golgi-derived exocytotic organelle that covers the tip of the sperm head (Figure 1). Acrosomal exocytosis, the so-called acrosome reaction, happens only in capacitated sperm and is a prerequisite for a sperm to fuse with an egg (Figure 1). Because of its physiological importance, various methods have been proposed to assess acrosomal status (62–64). Taking advantage of mice engineered to express GFP in the acrosome, we have been able to observe, in real-time, the acrosome reaction in live sperm without any pretreatment (Figure 2) (65, 66). It is noteworthy that the acrosome reaction is not a simple all-or-none event but one with intermediate stages. While the soluble GFP disperses from the acrosome within seconds (65), other acrosomal components are only gradually released. For example, the MN7 and MC41 (acrin1 and acrin2) acrosomal antigens remain attached to the sperm head for at least 15 minutes (67). Further investigation of these intermediate stages of fertilization is awaited (68).

The acrosome reaction can be induced in vitro with solubilized ZP (69–71). However, there is a report showing that the intact ZP is not sufficient to induce acrosomal exocytosis (72). Further, according to Ryuzo Yanagimachi, some mouse sperm passing through cumulus layers are already acrosome reacting and have reacted

before reaching the ZP (73). In shrews, the acrosome reaction is induced by cumulus cells but not the ZP (74). Progesterone secreted from human cumulus cells is reported to induce the acrosome reaction (75). Moreover, 20%–40% of capacitated mouse sperm spontaneously undergo the acrosome reaction in an IVF medium, such as TYH (76), without any natural inducer, and those sperm are efficiently able to fertilize in vitro eggs without the cumulus oophorus and ZP. Therefore, a direct sperm-ZP interaction does not seem to be necessary for the acrosome reaction and thus may not be "essential" for fertilization, even in vivo (Figure 1).

The mechanism of the acrosome reaction itself has been well characterized. In brief, transient calcium influx leads to activation of phospholipase C (PLC), and activated PLC generates IP3 and diacylglycerol (DAG) from PIP2. IP3 releases calcium from intracellular stores, and DAG mediates PKC activation and phosphorylation of substrate proteins. These early events promote a subsequent calcium influx via transient receptor potential cation channels (TRPCs), which induces the complete acrosome reaction. Disruption of Plcd4 impairs the in vitro ZP-induced acrosome reaction, while the A23187 ionophore-induced acrosome reaction occurs normally (71).

There is growing evidence that the soluble NSF attachment protein receptor (SNARE) complex regulates the sperm acrosome reaction. Sperm from hydrocephaly with hop gait (Hyh) mice, which have a spontaneous point mutation in the α-SNAP–encoding gene (*Napa*), exhibit severely impaired fertility (77). The problem was traced to a defect in the ZP-induced acrosome reaction. Mice lacking complexin-I (Cplx1), which associates with the SNARE complex and modulates its function (78), also generate sperm with an impaired ability to undergo progesterone-induced acrosome reactions (79). It should be noted that although *Plcd4*$^{-/-}$, Hyh, and *Cplx1*$^{-/-}$ mutant sperm are severely impaired in their ability to undergo ligand-induced acrosome reactions, these sperm are still able to fertilize 56.9%, 46.3%, and 47.3% of eggs, respectively, in vitro. These data support the idea that the ligand-induced acrosome reaction is not essential but assists fertilization.

ZP

After passing through the cumulus oophorus, sperm encounter the ZP, their last hurdle before meeting the egg (Figure 1). The major components of the ZP are three glycosylated proteins, Zp1, Zp2, and Zp3. There are various reports indicating that Zp3 functions as the primary sperm receptor and can induce the acrosome reaction (80–82). During the passage of sperm through the ZP, Zp2 is thought to function as a secondary receptor for acrosome-reacted sperm. On fertilized eggs, Zp2 is converted to Zp2f by an oocyte secretory enzyme(s) to prevent further sperm binding and fertilizing the egg (83, 84). Zp1 is thought to cross-link Zp2/Zp3 heterodimers and creates the filamentous structure of the ZP (85). Interestingly, knockout studies have revealed that eggs can form the ZP in the absence of either Zp1 or Zp2 and that sperm can fertilize these eggs (86, 87). However, when Zp3 was disrupted, the ZP was not formed (88, 89). Although oocytes lacking a ZP or with only a thin ZP (*Zp3*$^{-/-}$ and *Zp2*$^{-/-}$ oocytes, respectively) were able to be fertilized with wild-type sperm, their developmental ability was compromised (87). These results indicate that the ZP helps to maintain an appropriate interaction between granulose cells and oocytes during oocyte maturation. ZP4 has been identified in some species, including humans, but its species-specific function(s) remains to be determined (90).

The ZP not only functions as a receptor for sperm but also acts as a species-specific barrier (73). Rankin et al. replaced mouse Zp2 and Zp3 with their human homologs and examined the fertilization potential of eggs surrounded with a mouse Zp1/human ZP2/human ZP3 chimeric ZP (91). Mouse sperm, but not human sperm, were able to bind to the chimeric ZP and fertilize the eggs. As Zp3 is thought to be the primary sperm receptor, these data suggest that oligosaccharides attached to the ZP proteins, rather than the peptide sequences themselves, are critical for species-specific sperm binding. This idea is also supported by earlier biochemical studies, showing that enzymatic removal of terminal galactose (Gal) or N-acetylglucosamine (GlcNac) residues from the ZP abolishes its affinity for sperm (92–94). However, it has been reported that disruption of mannoside acetylglucosaminyltransferase 1 (Mgat1), a medial-Golgi enzyme essential for the synthesis of hybrid and complex N-glycans, resulted in oocytes that were efficiently fertilized, even though the ZP was fragile and lacked terminal N-glycan Gal and GlcNac residues (95). The same group also disrupted T-synthase (C1galt1) so that ZP without core 1 and core 2 O-glycans was generated. Since there are no core 3 and core 4 O-glycans in the ZP, these mice can be considered as having O-glycan–deficient ZP. However, oocytes from these animals were still fertilized by sperm (96). Moreover, oocyte-specific disruption of both *Mgat1* and *C1galt1* has been achieved, and ZP with no terminal Gal and GlcNac was shown to be functional (96). The results obtained from gene-knockout studies using oocyte-specific Cre recombinase strongly support the idea that the oligosaccharides on the ZP are far less important for sperm-egg interactions than previously believed. An alternative interpretation could be that there are some unknown ZP-associated glycoproteins (derived from ovary or another source where the glycosyl transferases are still expressed) that are important for sperm-egg interactions.

Sperm proteins required for ZP binding

Various reports exist supporting the notion that B4galt1 on the sperm surface can bind to ZP glycans (97). However, when *B4galt1*-knockout mice were produced, the mutant sperm could fertilize eggs (9, 10). The literature is replete with examples of sperm factors that have been demonstrated to be important for ZP binding, using a biochemical approach, but later proven to be not essential in gene-disruption experiments.

Ironically, the first case of normal-looking sperm with defective ZP-binding capacity was generated unexpectedly (15). When sperm from *Clgn*$^{-/-}$ mice were mixed with cumulus-free eggs, the sperm were unable to bind the ZP and were repelled. However, Clgn is not directly involved in sperm-egg interactions, because Clgn is a testis-specific ER molecular chaperone involved in folding newly synthesized secretory and membrane proteins. Even in wild-type male mice, Clgn is absent from mature sperm. We therefore speculated that Clgn facilitates the maturation of a sperm protein(s) required for ZP binding.

Five knockout mouse strains have now been reported to show defective sperm-ZP binding (*Clgn*-, *Ace*-, *Adam1a*-, *Adam2*-, and *Adam3*-knockout mice) (Figure 3). The disruption of Clgn impairs Adam1a/Adam2 and Adam1b/Adam2 heterodimerization, and because these other ADAM family members regulate the amount of Adam3 on sperm, this leads to the complete loss of Adam2 and Adam3 from the surface of mature sperm (23, 28). When Baba and colleagues produced *Adam1b*-knockout mice, Adam2 was also found to disappear from sperm, but Adam3 remained

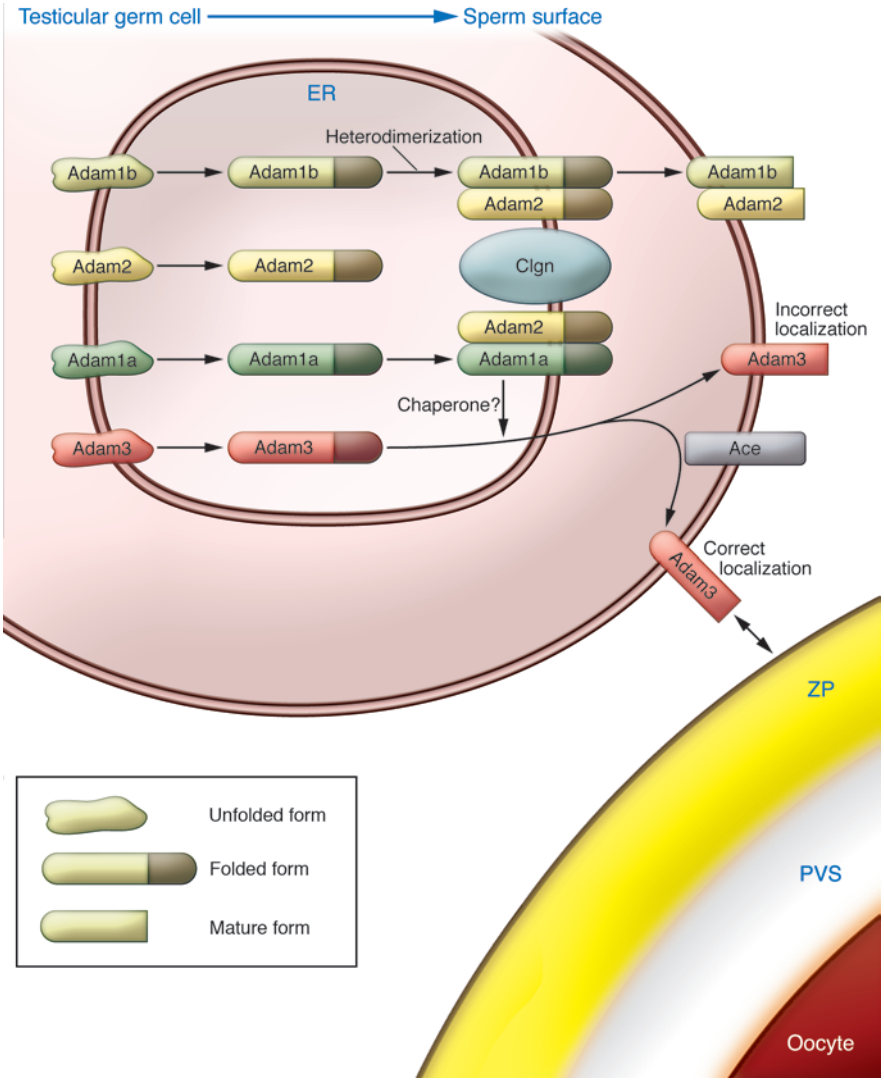

Figure 3
Maturation of ADAMs and their roles in sperm function. Disruption of the genes *Clgn*, *Ace*, *Adam1a*, *Adam2*, and *Adam3* results in impaired sperm-ZP binding and impaired migration through the UTJ. Clgn is required for Adam1a/Adam2 and Adam1b/Adam2 heterodimerization. Lack of Adam1a/Adam2 heterodimerization in *Clgn*$^{-/-}$, *Adam1a*$^{-/-}$, and *Adam2*$^{-/-}$ mice causes Adam3 disappearance from the surface of mature sperm. Disruption of *Ace* leads to aberrant localization of Adam3, as evidenced by reduced amounts of Adam3 protein in the Triton X-114 detergent-enriched phase of sperm membranes (28). The diagram illustrates why disruption of the individual *Ace*, *Clgn*, *Adam1a*, *Adam2*, and *Adam3* genes produces similar phenotypes and indicates the importance of Adam3 in sperm fertilizing ability.

intact and the sperm were fertile (11). When *Adam1a*, which is ER specific and not found in mature sperm, was knocked out, Adam2 remained intact, but the sperm lost surface expression of Adam3 and ZP-binding ability (25). This result clearly indicates that Adam1b/Adam2 (i.e., fertilin) is dispensable for fertilization, at least in mice. This result is surprising, because there was strong evidence that the disintegrin domain of fertilin on sperm interacted with the RGD domain of integrins on oocytes and that the addition of RGD fragments to sperm inhibited fertilization. One could argue that some other factor(s) compensates for the loss of fertilin in gene-disrupted mice during spermatogenesis, but that the same factor(s) is not able to compensate for the loss of fertilin function when inhibitory antibodies or exogenous ligands are added. However, it could be counter-argued that the fertilization inhibiting activity of the antibody and/or ligands was not physiological but rather an artifactual effect. Since the entire mechanism of fertilization is yet to be clarified, the outcome of the debate awaits further investigations.

The Adam3 protein can directly bind to the ZP (60). It appears that Adam3 requires the concerted action of Clgn and Adam1a/Adam2 for its quality control and maturation during spermatogenesis. The mechanism of infertility caused by *Ace* disruption remained unclear for many years, since overall levels of ADAMs in sperm are not influenced by Ace. Recently, however, we noticed a substantial decrease in the amount of Adam3 in membrane microdomains and suggested that erroneous distribution of Adam3 in *Ace*$^{-/-}$ mice might cause the defective ZP-binding phenotype (28) (Figure 3). These observations point toward Adam3 as the most important factor that participates in sperm-ZP binding. However, in humans, other ADAMs, or another protein, are likely to function as an alternative to Adam3, because the human homolog of *Adam3* seems to be a pseudo gene (98, 99). In fact, there are various papers showing that other proteins are involved in human sperm binding to the ZP, including the 56-kDa Zp3 receptor (ZP3R) and zonadhesin (ZAN). Zp3r is a peripheral membrane glycoprotein that has been suggested to be a receptor for Zp3 in the mouse (100, 101), whereas ZAN is a large sperm head protein, with multiple isoforms, that has been implicated in species-specific gamete recognition (102).

In addition to their inability to bind to the ZP, sperm from *Clgn*-, *Ace*-, *Adam1a*-, *Adam2*-, and *Adam3*-knockout mice share another notable phenotype: they are unable to migrate into the oviduct

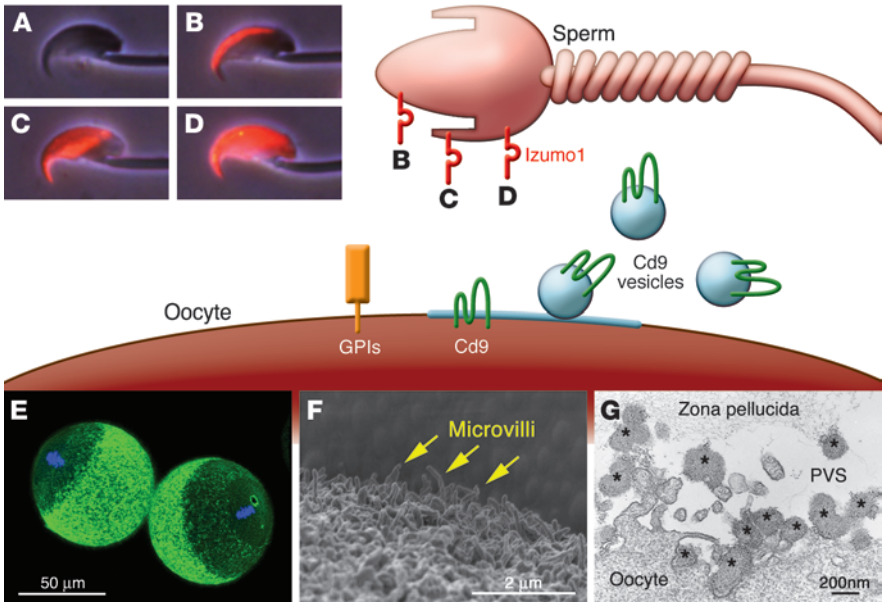

Figure 4
Potential mechanism of sperm-egg fusion. Sperm Izumo1 and egg Cd9 are essential factors for sperm-egg fusion (13, 16). GPI-anchored proteins on the egg surface are also essential (114), but none of the individual proteins have been identified. (**A**) Izumo1 is an acrosomal membrane protein that is not exposed before the acrosome reaction is complete. Acrosome-reacted sperm can be classified into three major groups by their Izumo1 staining pattern: acrosomal cap (**B**), equatorial (**C**), and whole head (**D**). (**E** and **F**) Cd9 is localized on the cilia distributed across the surface of unfertilized eggs, except the cilia at the metaphase plate. (**G**) Cd9-containing vesicles (asterisks) secreted from the oocyte are able to translocate onto the sperm surface and may play a role in sperm-egg fusion. **G** is reproduced with permission from *Proceedings of the National Academy of Sciences of the United States of America* (115).

(23–27). This suggests that oviduct migration and ZP binding might share a common mechanism. This hypothesis is supported by the phenotype of a sixth mutant mouse, the post-GPI attachment to proteins 1–knockout (*Pgap1*-knockout) mouse. Pgap1 is an ER resident GPI inositol-deacylase that is involved in the maturation of GPI-linked proteins. Few *Pgap1*$^{-/-}$ mice reach sexual maturity. Those male mice that do reach adulthood have sperm that are normal in number and motility but that cannot ascend the oviduct or bind to the ZP (103). If the reason underlying this commonly observed dual defect is clarified, it will help us to understand the molecular mechanisms of fertilization in more detail.

ZP penetration

The enzymatic hypothesis for ZP penetration posits that proteolytic cleavage of ZP proteins by sperm cell–surface proteases clears a path for the incoming sperm (73). The enzyme heralded as the prime candidate for the controlled proteolytic clearance of ZP proteins was Acr. Acr is an acrosomal enzyme with chymotryptic activity that is released during the acrosome reaction. Despite several papers supporting an important role for Acr in ZP binding and penetration, *Acr*$^{-/-}$ sperm can still fertilize eggs, albeit with a slight delay compared with wild-type sperm (8, 104). Baba and colleagues found that protease activity persists in the sperm of *Acr*-knockout mice, suggesting that alternative proteases could be involved in ZP penetration. Five more testis-specific serine proteases have been identified and named Tesp1–Tesp5 (105). Thus far, only mice lacking Tesp5 (also known as Prss21) have been generated and analyzed (106). Sperm from these mice have a diminished ability to bind to the ZP and fuse with eggs, but curiously, the phenotype is rescued by treating the sperm with uterine fluids. This observation further demonstrates the complex interplay between male and female factors during the course of fertilization.

Sperm-egg fusion

After penetration of the ZP, sperm immediately meet and fuse with the egg plasma membrane (Figure 4). Electron microscopic observation has shown that sperm that penetrate the ZP are acrosome reacted (107, 108). The fact that only acrosome-reacted sperm are able to fuse with eggs implies that a sperm fusogen(s) is hidden or latent in fresh sperm and becomes exposed or activated only after the acrosome reaction. Many sperm antigens, such as Mn9, Cd46, and Izumo1, become reactive to antibodies only after the acrosome reaction has been completed. We previously raised a monoclonal antibody, OBF13, that inhibited sperm-egg fusion. The cognate antigen was a good candidate for a fusion factor (109). We identified the antigen as an Ig superfamily, type I membrane protein, with an extracellular Ig domain that contains one putative glycosylation site. The antigen was named "IZUMO" after a Japanese Shinto shrine dedicated to marriage. *Izumo1*$^{-/-}$ male mice are completely sterile, even though the mutant sperm can penetrate the ZP and contact the egg plasma membrane (13). When the fusion step was bypassed by intracytoplasmic sperm injection into unfertilized eggs, *Izumo1*$^{-/-}$ sperm activated eggs, and the fertilized eggs developed to term normally when transferred to the uterus of female mice. Therefore, the Izumo1 protein is essential for sperm-egg fusion (13). During the acrosome reaction, Izumo1 relocates from the anterior head of the sperm to the site(s) in which fusion will take place (Figure 4, A–D). Testis-specific serine kinase 6 (Tssk6) is a male germ cell–specific serine kinase, and sperm from mice lacking Tssk6 have defects in ZP binding and do not redistribute Izumo1 properly (110, 111).

What is essential on the egg surface? When fertilin was believed to be the essential sperm factor, $\alpha_6\beta_1$ integrin on the egg surface was considered a likely candidate binding partner (112). However, gene disruption experiments showed that neither α_6 integrin nor β_1 integrin were essential (113). Instead, a chance discovery led to the identification of the tetraspanin Cd9 as an egg cell–surface protein essential for fertility (16–18) (Figure 4). Cd9 is ubiquitously expressed and was expected to function in various cells. However, the *Cd9*-knockout mouse showed a defect restricted to eggs, in which the protein was found to be essential for sperm-egg fusion. Given that Izumo1 is essential for sperm to bind to eggs and that Cd9 is essential for eggs to bind to sperm, it is tempting to speculate that they interact with each other to form a fusogenic

complex. However, we, and others, have not detected any direct interaction between sperm Izumo1 and egg Cd9. If these proteins do indeed interact, it is likely that they both require associating proteins on the sperm and egg cell surface, and the identity of these putative factors is being intensively investigated.

Conclusions

Experiments using gene-manipulated animals are very powerful tools for judging which factors are essential in fertilization. Of course, if a certain factor is judged as "not essential," it does not necessarily mean the factor does not function at all in vivo. The number of genes that are indispensable for fertilization is growing, and their roles and relationships in sperm-ZP interactions are becoming clearer. Gene-disruption experiments are conducted in many research fields, and the number of genes disrupted is increasing day by day. Thus, genes that affect reproduction will continue to be found, even by researchers in different fields. The analysis of both expected and serendipitous fertility phenotypes is steadily bringing into focus a clear image of sperm-egg interaction mechanisms. We therefore believe that the day that we can portray the sequential events in fertilization is drawing closer

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Address correspondence to: Masaru Okabe, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka, 565-0871, Japan. Phone: 81.6.6879.8338; Fax: 81.6.6879.8376; E-mail: okabe@gen-info.osaka-u.ac.jp.

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