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—Review—

Factors controlling sperm migration through the oviduct revealed by gene-modified mouse models

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Abstract: Mammalian fertilization is comprised of many steps including sperm survival in the uterus, sperm migration in the female reproductive tract, physiological and morphological changes to the spermatozoa, and sperm-egg interaction in the oviduct. *In vitro* studies have revealed essential factors for these fertilization steps for over half a century. However, the molecular mechanism of fertilization has recently been revised by the emergence of genetically modified animals. Here, we focus on essential factors for sperm fertilizing ability and describe recent advances in our knowledge of the mechanisms of mammalian fertilization, especially of sperm migration from the uterus into the oviduct.
Key words: female reproductive tract, fertilization, male infertility, sperm motility, uterotubal junction

Introduction

The human body is comprised of 60 trillion cells that originate from a fertilized egg produced by the fusion of a spermatozoon with an egg. Mammalian spermatozoa are morphologically differentiated in the testis, but freshly ejaculated spermatozoa are incapable of fertilization. Ejaculated spermatozoa gain their fertilizing ability in the female reproductive tract [2, 18]. The process that renders spermatozoa competent to fertilize an egg is called sperm capacitation [3]. These facts led to the possibility of performing *in vitro* fertilization (IVF) by mixing capacitated spermatozoa with eggs [20, 106]. In the past, factors important for fertilization were discovered via IVF experiments using biochemical approaches. However, recent studies using gene knockout (KO) methods have revealed that several sperm factors identified from the IVF system are not essential for *in vivo* fertilization [37, 76]. KO experiments have also unexpectedly revealed essential factors for fertilization *in vivo*. These essential factors play a role in spermatozoa gaining their fertilizing ability at stages such as spermatogenesis and epididymal transit. In this review, we focus on the molecular mechanism of the sperm fertilizing ability in the female reproductive tract revealed by *in vivo* analysis of genetically modified (GM) mice (Fig. 1).

Sperm Survival in the Uterus

Mammalian spermatozoa must travel a long distance from the uterus to the oviduct, where fertilization takes place. Ejaculated spermatozoa gain their fertilizing ability after remaining in the female reproductive tract for a period of time. This process is called sperm capacitation and is specific to mammalian spermatozoa. Though over 60 years have passed since the discovery of capacitation [3], its molecular mechanism remains to be fully determined [8]. Sperm surface-bound glycoproteins, CD52, CD55, and CD59, transferred from epi-

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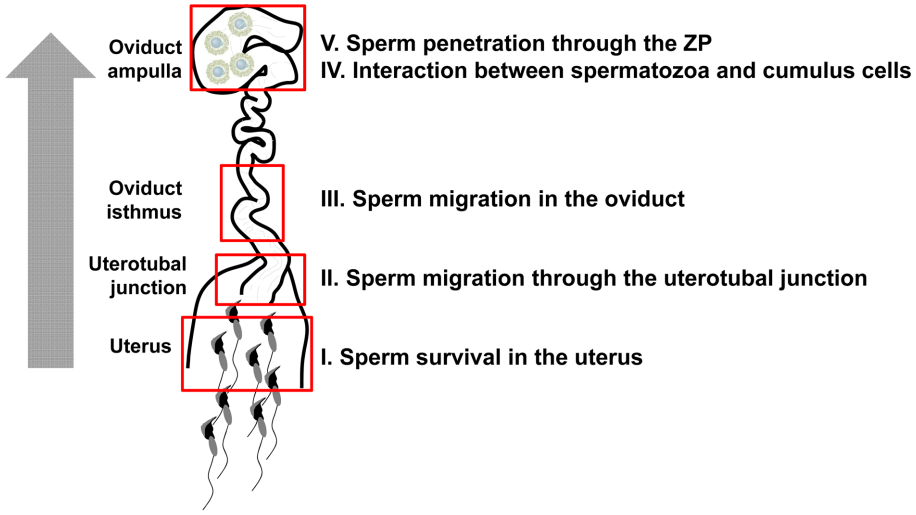

Fig. 1. Overview of the sperm journey into the female reproductive tract. Ejaculated spermatozoa overcome several hurdles in the uterus and the oviduct to fertilize eggs. This review summarizes in five sections the molecular dissection of sperm migration revealed by GM mouse models.

didymal luminal fluids were believed to protect spermatozoa from immunological attacks in the female reproductive tract [51]. Moreover, removal of these glycosylphosphatidylinositol-anchored proteins (GPI-APs) was also thought to induce sperm capacitation [13]. However, KO mouse experiments showed that CD52, CD55, CD59a, and CD59b are not essential for sperm fertilizing ability [34, 83, 91, 103]. Recently, *Cd55b* was identified in mice, but its physiological function has not been clarified.

Ejaculated spermatozoa mix together with a fluid, called seminal plasma, secreted from a male accessory sexual gland. Capacitated spermatozoa reversibly lose their fertilizing ability when treated with seminal plasma [19]. This finding suggests that seminal plasma contains a decapacitation factor that prevents sperm capacitation [11]. However, this phenomenon has been observed only in *in vitro* experiments that mixed capacitated spermatozoa with seminal plasma or candidate decapacitation factors [58, 61, 74]. Seminal vesicles, which secrete the main component of seminal plasma, contribute to reproduction because removal of seminal vesicles causes a significant reduction in male fertility [78]. Seminal vesicle protein secretion 2 (SVS2) is a major component of the seminal vesicle secretions, and it was found to be a decapacitation factor for mouse spermatozoa *in vitro* [1, 47]. Male *Svs2* KO mice were severely subfertile because of a deficiency in copulatory plug formation and uterus-derived cytotoxicity that damaged the intrauterine spermatozoa [45]. Thus, the authors concluded that SVS2 protects ejaculated spermatozoa from immunological attack in the uterus and is required for spermatozoa to survive in the female reproductive tract. However, the decapacitation functions remain to be determined. Other functions of seminal fluid include influencing the growth and health of offspring [15]. Mammalian seminal plasma proteins may also have a key role in both fertilization and embryo development *in vivo* [64].

Sperm Migration through the Uterotubal Junction

The next obstacle for spermatozoa that survived in the uterus is passage through the uterotubal junction (UTJ) into the oviduct. The UTJ is the connection between the uterus and oviduct and is characterized as the distal portion of the oviduct. Although the structure of the UTJ varies among mammals, the passageway is usually narrow. In mice, the number of spermatozoa that pass through the UTJ is significantly reduced before reaching the oviduct ampulla. It has been proposed that most spermatozoa swim up through the UTJ into the oviduct by self-propulsion. However, KO mice experiments indicate that sperm motility alone is insufficient for sperm migration through the UTJ [37, 77]. There are more than 10 factors found to be essential for UTJ migration. In the following section, we discuss the interactions of these

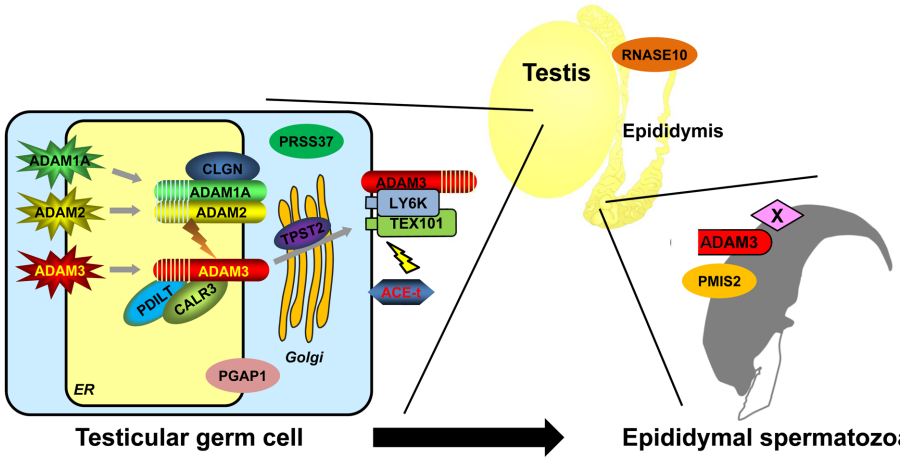

Fig. 2. Essential factors for sperm migration through the uterotubal junction. Currently, fourteen genes are known to be required for sperm migration through the uterotubal junction (Table 1). Testis-specific ADAMs, ER chaperones, and GPI-anchored proteins allow testicular spermatozoa to obtain migrating ability. Epididymal protein RNASE10 works as a sperm maturation factor in the caput epididymis. Sperm protein ADAM3 disappears from spermatozoa in most cases (11/14 genes) and is the leading candidate in interactions with the female reproductive tract in mice. However, ADAM3 remains in *Ly6k* and *Pgap1* KO spermatozoa, although *Ly6k* and *Pgap1* KO mice show impaired sperm migration. Moreover, *Adam3* and *Pmis2* are not conserved in humans. It is possible that an unknown factor X localizes to the sperm membrane and is conserved in mice and humans. An unknown factor X may regulate sperm migrating ability through the uterotubal junction. All of the KO mice with impaired sperm migration have another phenotype, impaired ZP-binding ability *in vitro*. These phenotypes may be correlated with sperm attachment to the epithelial cells of the uterotubal junction.

factors and the current understanding of the mechanism of UTJ migration (Fig. 2).

Testis-specific ADAM proteins

A disintegrin and metalloproteinase (ADAM) family members are membrane-anchored metalloproteinases, and they regulate various events such as cell migration, cell adhesion, and cell interactions [86]. Testicular ADAMs, ADAM1B and ADAM2, heterodimerize to form fertilin. Fertilin is localized to the sperm plasma membrane and has been characterized as a sperm-egg fusion protein [12]. As expected, male *Adam2* KO mice were found to be sterile [21], but the phenotype was not related to sperm-egg fusion [72]. When ADAM1B, a subunit of the fertilin heterodimer, was knocked out, both ADAM1B and ADAM2 disappeared from mature spermatozoa, but the mice were fully fertile [49]. Further investigations indicated that ADAM2 functions to form a dimer with ADAM1A in the endoplasmic reticulum (ER) of spermatogenic cells, leading to the localization of ADAM3 on the sperm surface [73]. Since male *Adam3* KO mice were found to be sterile because of impaired sperm migration through the UTJ [87, 102], ADAM3 is thought to play a pivotal role in sperm migration through the UTJ. More than 10 proteins involved in sperm migration through the UTJ interact with ADAM3, affecting the protein amount and/or the localization of spermatozoa (Table 1).

Testis-specific ER chaperones

ADAM3 is a cysteine-rich, glycosylated membrane protein that is co-translationally translocated into the ER of spermatids, where numerous molecular chaperones and catalysts promote glycoprotein folding as well as the disposal of misfolded proteins. Membrane-bound calnexin (CANX) and soluble calreticulin (CALR) were originally found as homologous lectin chaperones that mainly mediate nascent glycoprotein folding in somatic cells. Testicular germ cell-specific homologues of CANX and CALR are calmegin (CLGN) and calsperin (CALR3), respectively. CLGN mediates the heterodimerization of ADAM1A/ADAM2 that is required for the maturation of ADAM3 [38]. CALR3 binds directly to ADAM3 and regulates its maturation. Both *Clgn* and *Calr3* KO mice lack ADAM3 in sperm and are sterile [39, 40]. Other chaperones, such as those in the protein disulfide isom-

Table 1. KO mouse lines with impaired sperm-ZP binding *in vitro* and impaired sperm migration from uterus into the oviduct

Gene	Expression pattern	Localization	Phenotype of KO mice			Human ortholog	References
			In vitro sperm-ZP binding ability	Sperm migration ability to oviduct	Localization of ADAM3 on spermatozoa		
Ace-t	Sperm	Sperm surface	Impaired	Impaired	Aberrantly localized	+	Krege JH, *et al.* 1995; Yamaguchi R, *et al.* 2006
Adam1a	Testis	Endoplasmic reticulum	Impaired	Impaired	Disappeared	–	Nishimura H, *et al.* 2004
Adam2	Sperm	Sperm surface	Impaired	Impaired	Disappeared	+	Cho C, *et al.* 1998
Adam3	Sperm	Sperm surface	Impaired	Impaired	Disappeared	–	Shamsadin R, *et al.* 1999; Yamaguchi R, *et al.* 2009
Calr3	Testis	Endoplasmic reticulum	Impaired	Impaired	Disappeared	+	Ikawa M, *et al.* 2011
Clgn	Testis	Endoplasmic reticulum	Impaired	Impaired	Disappeared	+	Ikawa M, *et al.* 1997
Ly6k	Testis	Testicular germ cell	Impaired	Impaired	Localized	+	Fujihara Y, *et al.* 2014
Pdilt	Testis	Endoplasmic reticulum	Impaired	Impaired	Disappeared	+	Tokuhiro K, *et al.* 2012
Pgap1	Ubiquitous	Endoplasmic reticulum	Impaired	Impaired	Localized	+	Ueda Y, *et al.* 2007
Pmis2	Sperm	Sperm surface	Impaired	Impaired	Disappeared	–	Yamaguchi R, *et al.* 2012
Prss37	Testis	Testicular germ cell	Impaired	Impaired	Disappeared	+	Shen C, e*t al.* 2013
Rnase10	Epididymis	Epididymis	Impaired	Impaired	Disappeared	+	Krutskikh A, *et al.* 2012
Tex101	Testis	Testicular germ cell	Impaired	Impaired	Disappeared	+	Fujihara Y, *et al.* 2013
Tpst2	Ubiquitous	Golgi apparatus	Impaired	Impaired	Disappeared	+	Marcello M, *et al.* 2011

erase (PDI) family proteins, have also been implicated in the intra- and intermolecular disulfide bond formation in the ER [79]. Among this protein family, PDIA3 is associated with CANX/CALR and contributes to the quality control cycle of newly synthesized glycoproteins in the ER. Testis-specific PDI-like protein, PDILT, cooperates with CALR3 in testicular germ cells and plays an indispensable role in disulfide bond formation and folding of ADAM3 [95, 97]. Male *Pdilt* KO mice are infertile because of impaired transport of ADAM3 to the sperm surface [95]. Testicular germ cell-specific ER chaperones are essential for the folding and maturation of ADAM3.

Testis-specific GPI-anchored proteins

GPI-APs are anchored to the outer cell membrane by GPI and are critical at various points in mammalian fertilization [26, 52]. The GPI-AP complex, which consists of testis expressed gene 101 (TEX101) and lymphocyte antigen 6 complex locus k (LY6K), is present only in testicular germ cells, and it disappears from epididymal spermatozoa [54, 107]. TEX101 and LY6K are required for sperm migration into the oviduct [28, 29] (Fig. 3). Our study revealed that the transient interaction of the LY6K/TEX101 GPI-AP complex with ADAM3 is a critical step for ADAM3 maturation. Intriguingly, dissociation of the complex from ADAM3 is mediated by the GPI-AP releasing (GPIase) activity of angiotensin-converting enzyme (ACE). ACE is a well-characterized carboxy dipeptidase that regulates blood pressure. *Ace*-deficient mice showed low blood pressure, and the male mice were sterile [30, 53]. *In vitro* analysis demonstrated that TEX101 (but not LY6K) is the specific substrate for not only wild-type ACE but also zinc peptidase-defective ACE. These findings are consistent with the aberrantly remaining TEX101/LY6K protein complex on *Ace* KO mouse spermatozoa [28, 29]. As a result, ADAM3 dislocates from the Triton X-114 detergent-enriched phase to the detergent-depleted phase in *Ace* KO mouse spermatozoa, although ADAM3 localizes to both phases in wild-type mouse spermatozoa [104]. Therefore, ACE-mediated shedding of the GPI-AP complex, TEX101 and LY6K, is required for the correct localization of ADAM3 in epididymal spermatozoa and subsequent sperm fertilizing ability. The release of GPI-APs is one of the key events in activation of the sperm fertilizing ability [26].

Essential factors regulating sperm migration through the UTJ

To date, more than 10 factors have been reported to be essential for sperm migration through the UTJ (Table 1) and to be involved in ADAM3 maturation; however, there is no direct evidence that ADAM3 functions on the sperm surface during UTJ migration. Considering that ADAM3 is a pseudogene in humans, the contribution of undiscovered novel factors should be taken into account.

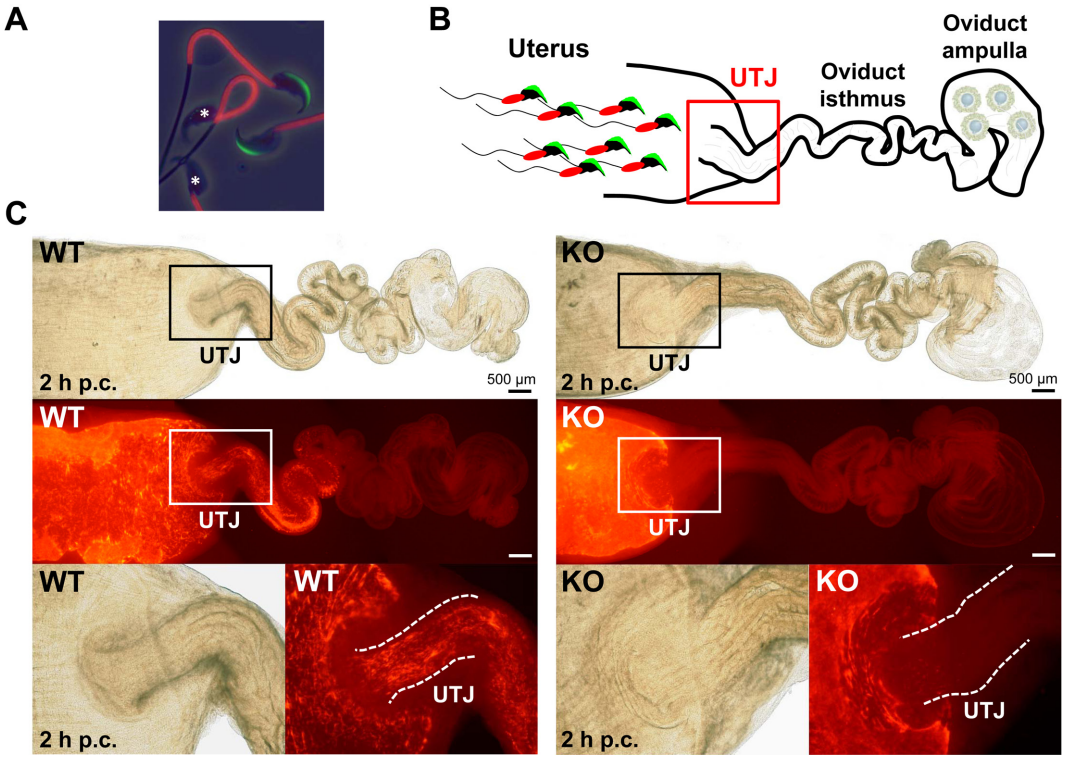

Fig. 3. Observation of ejaculated spermatozoa into the female reproductive tract. (A) Visualization of the acrosome and midpiece of spermatozoa. A transgenic mouse line carrying *Acr-Egfp* and *CAG-Su9/DsRed2* transgenes expressed both a green sperm acrosome and red mitochondria in the sperm midpiece [31]. It is easy to determine if the acrosome reaction occurred in these spermatozoa due to the green acrosome. *Acrosome-reacted spermatozoa. This transgenic mouse line [B6D2-Tg (*CAG/su9-DsRed2*, *Acr3-Egfp*) RBGS002Osb] is available from the RIKEN BioResource Center and the Center for Animal Resources and Development (CARD), Kumamoto University. (B) Scheme of observing sperm migration into the female reproductive tract using fluorescent spermatozoa. (C) Observation and visualization of ejaculated spermatozoa into the female reproductive tract two hours post coitus (p.c.). Observing the red signals, wild-type (WT) spermatozoa passed through the uterotubal junction (UTJ), but *Tex101* KO spermatozoa were unable to migrate from the uterus to the oviduct [29]. Fluorescent spermatozoa could facilitate live imaging of localization and movement *in vitro* and *in vivo*.

This idea is also supported by the fact that ADAM3 localized normally in migration-defective *Ly6k* and *Pgap1* KO spermatozoa [28, 96]. Because both LY6K and PGAP1 disappear during epididymal sperm maturation, these molecules do not directly function during UTJ migration. Recently, we identified sperm membrane proteins missing in *Adam3* KO spermatozoa and found that protein missing in infertile sperm 2 (PMIS2) is a novel sperm protein required for UTJ migration [101]. Although many molecules have proven to be essential for sperm migration through the UTJ, the sperm migration mechanism *per se* is still unclear. Interestingly, migration-defective spermatozoa also show impaired binding to the zona pellucida (ZP) [77]. It is also reported that *Adam3* KO spermatozoa are less adhesive than wild-type spermatozoa [87, 102]. To understand these defects, wild-type and *Clgn* KO chimeric mice were produced to test the migration ability of mixed spermatozoa. Although control wild-type spermatozoa could pass through the uterotubal junction, the mixed wild-type spermatozoa could not compensate for the inability of *Clgn* KO spermatozoa to migrate into the oviduct [71]. These data implicate that there is an initial interaction with the UTJ entrance that may be a critical step prior to sperm migration into the oviduct. Further study is needed to resolve the mystery of the factor (s) controlling sperm migration into the oviduct.

Contribution of sperm motility

Sperm motility is also important for the UTJ passage. For example, male mice that lack cation channel, sperm associated 1 (CATSPER1), a component of a Ca^{2+} chan-

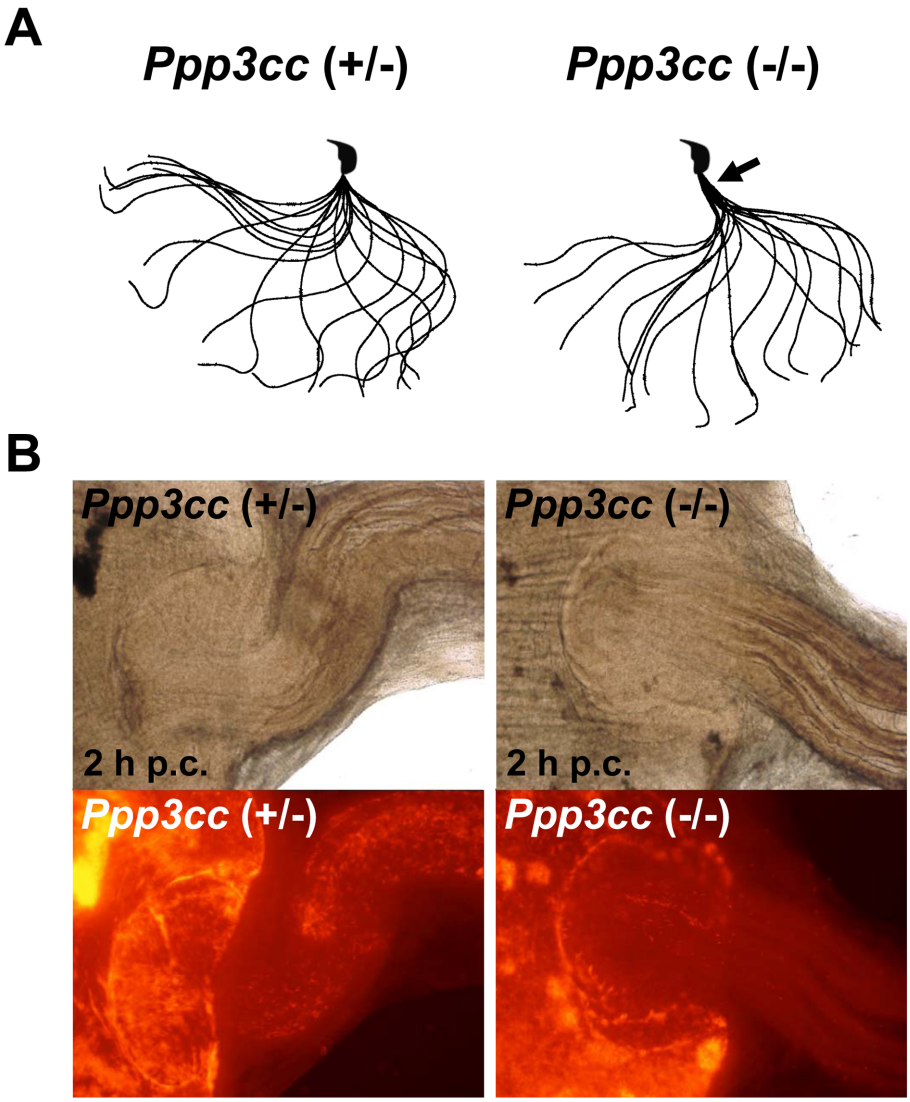

Fig. 4. Waveform and migration of *Ppp3cc* KO spermatozoa. (A) Flagellar movement patterns. Sperm motility was videotaped at 200 frames per second. Single frames throughout one beating cycle are superimposed. The midpiece (black arrow) is rigid in the spermatozoa obtained from *Ppp3cc* KO mice [68]. (B) Visualization of ejaculated spermatozoa in the female reproductive tract two hours post coitus (p.c.). Fewer *Ppp3cc* KO spermatozoa were observed in the oviduct isthmus.

nel localized in the principle piece, were infertile due to impaired sperm motility [84]. Migration of *Catsper1* KO spermatozoa through the UTJ was inefficient as observed by transillumination [33] and fluorescence microscopy [22]. Some *Catsper1* KO spermatozoa were observed in the oviduct isthmus a few hours after coitus but disappeared with time. This suggests that the *Catsper1* KO spermatozoa can pass through the UTJ but that most of the ejaculated spermatozoa in the uterus lose their motility with time before entering the UTJ. Mice lacking protein phosphatase 3 catalytic subunit gamma (PPP3CC), a catalytic subunit of calcineurin localized in the sperm tail, are another example illustrating the importance of sperm motility for UTJ passage [68]. *Ppp3cc* KO spermatozoa showed a rigid midpiece (Fig. 4A), and when the oviduct isthmus was observed two hours after copulation, less KO spermatozoa were observed compared with the control (Fig. 4B). Although *Ppp3cc* KO spermatozoa showed impaired motility, their velocity parameters in a hybrid background were comparable with those in a wild-type C57BL/6 background. Further studies are required to understand the exact role

of sperm motility in sperm migration, but flagellar movement patterns such as midpiece flexibility should also be taken into account for UTJ passage.

Recently, Muro *et al.* observed sperm migration in the female reproductive tract [70] using fluorescent spermatozoa that transgenically expressed green fluorescent protein (GFP) in the acrosome and red fluorescent protein from *Discosoma* sp. (DsRed2) in the midpiece [31]. They observed that the tail of spermatozoa migrating in the intramural UTJ seemed to be motionless. One explanation for this observation is that the midpiece that can be observed by DsRed2 fluorescence is motionless but that the principle piece and endpiece that cannot be observed with fluorescence are motile and play a role in sperm migration. However, analysis of *Ppp3cc* KO mice suggests that midpiece motility may in fact be important for UTJ passage [68]. Spermatozoa may pass through the UTJ with the midpiece "seemingly motionless" because of the viscous environment as suggested by Muro *et al.* [70].

Sperm Migration in the Oviduct

Sperm motility and hyperactivation for sperm migration

Once spermatozoa pass through the UTJ, they need to migrate through the oviduct to the ampulla. Sperm motility may be important for efficient sperm migration in the oviduct, as less *Catsper1* and *Ppp3cc* KO spermatozoa were observed in the oviduct ampulla [22, 68]. A simple explanation for this importance is that spermatozoa swim by self-protrusion in the oviduct. However, there are several studies showing complex interactions between spermatozoa and the oviduct. Chang and Suarez observed that mouse spermatozoa attached to and detached from the epithelium of the oviduct isthmus [17], suggesting that spermatozoa may bind and unbind several times as they migrate through the oviduct. Detachment of spermatozoa may be caused by hyperactivated motility [17] characterized by a high amplitude and asymmetrical beating pattern of the sperm tail. It is interesting to mention that both *Catsper1* and *Ppp3cc* KO spermatozoa cannot exhibit hyperactivated motility [16, 68]. Molecules that mediate the interaction between spermatozoa and the oviduct have not been identified yet using KO mouse models. In bulls, attachment of spermatozoa to the epithelium is mediated by binder of sperm protein 1 (BSP1), which is secreted by seminal vesicles [36]. There are two homologs of BSP in mice, *Bsph1* and *Bsph2* [57]; however, analyses of KO mice that lack these genes have not been performed.

Peristatic movement and sperm migration

Using fluorescent spermatozoa, Muro *et al.* observed that spermatozoa moved back and forth together with peristatic movement in the oviduct isthmus [70], suggesting that oviduct contractions may play a role in sperm migration. Ishikawa *et al.* observed a similar movement of sperm assemblage as well [42]. They showed that this movement was blocked and that fewer spermatozoa were found in the oviduct ampulla when peristatic movement was inhibited by the anticholinergic drug Padrin, suggesting that peristatic movement plays a role in sperm migration in the oviduct. However, a few spermatozoa can still reach the oviduct ampulla even with Padrin administration [42]. Another study that used Nicardipine to block oviduct contractions also showed that the spermatozoa could still reach the first loop of the oviduct isthmus or the oviduct ampulla [17]. These studies indicate that sperm motility may play a larger role in sperm migration in the oviduct isthmus rather than peristatic movement.

Oviductal fluid flow and sperm migration

How spermatozoa orient themselves in the oviduct remains an unanswered question. Miki and Clapham showed that mouse and human spermatozoa tend to swim against the flow (rheotaxis) and suggested that rheotaxis against oviductal flow is a major determinant of sperm guidance in the oviduct [65]. This is supported by an observation that *Catsper1* KO spermatozoa cannot exhibit rheotaxic behavior and cannot migrate through the oviduct efficiently. In addition to rheotaxis, chemotaxis [60, 88] and thermotaxis [7] are also implicated in sperm migration. Because these hypotheses are based on *in vitro* studies, further *in vivo* experiments are necessary to understand how spermatozoa understand direction in the oviduct.

Interaction between Spermatozoa and Cumulus Cells

Sperm enzymes involved in sperm passage through cumulus cell layers

Spermatozoa move into the ampulla of the oviduct and encounter the cumulus-cell oocyte complex (COC). The COC consists of ovulated eggs covered by an ex-

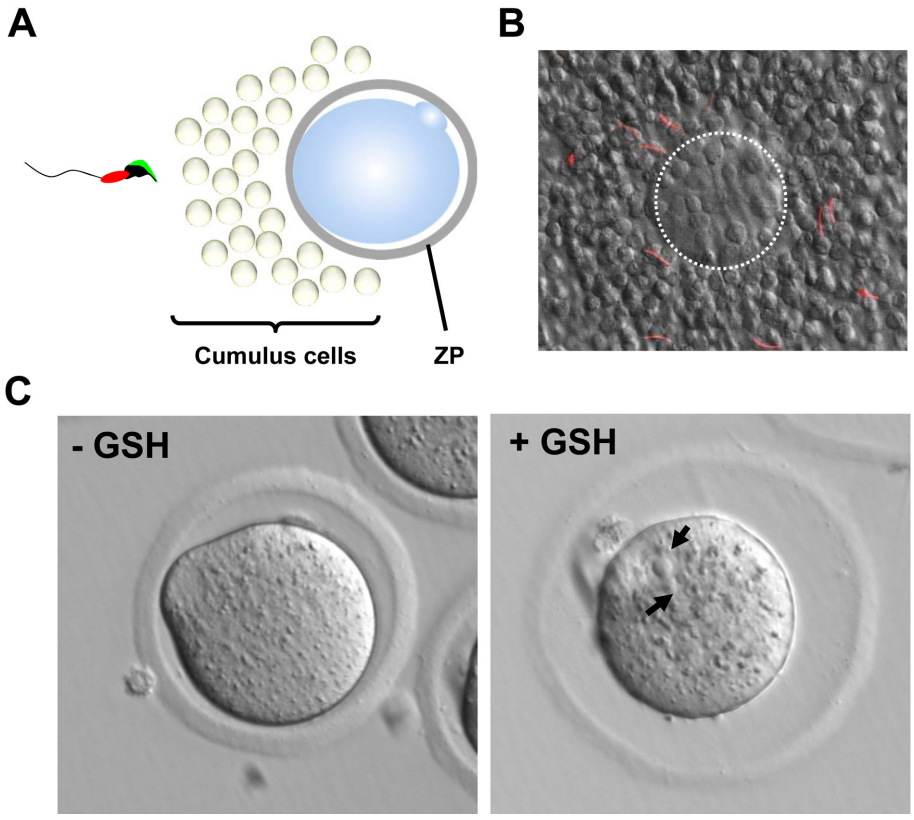

Fig. 5. Sperm passage through the cumulus cell layer and the ZP. (A) The cumulus-cell oocyte complex (COC). Ovulated eggs are covered by a cumulus cell layer and the zona pellucida (ZP). (B) Sperm passage through the cumulus cell layer. The spermatozoa penetrating through the cumulus cell layer were observed using the red fluorescence localized in the midpiece. The egg is highlighted with a white dotted line. (C) Rescue of ZP penetration failure using glutathione (GSH). When GSH is used, the ZP is destabilized and expanded. *Ppp3cc* KO spermatozoa can penetrate the ZP in the presence of GSH and fertilize the egg. Black arrows indicate the pronuclei.

tracellular matrix (ECM), the ZP, and a cumulus cell layer filled with hyaluronic acid (Figs 5A and B). Mouse spermatozoa have at least two hyaluronidases, sperm adhesion molecule 1 (SPAM1) and hyaluronoglucosaminidase 5 (HYAL5) [48]. SPAM1 was first identified as a sperm receptor for the ZP and was later reported to have hyaluronidase activity that enables spermatozoa to pass thorough the COC [59, 80, 81]. While *Spam1* KO mice are fertile, *Spam1* KO spermatozoa show a reduced ability to disperse cumulus cells *in vitro* [4]. *Hyal5* KO mice are fertile both *in vitro* and *in vivo* [50], suggesting functional redundancies in these genes.

Proteinase activity is also implicated in COC penetration as shown below. Acrosin (ACR) and protease, serine 21 (PRSS21), are trypsin-like serine proteases and are localized on the sperm head [6, 35]. Although these sperm proteases were thought to play an essential role in ZP binding and penetration, *Acr* KO spermatozoa were fertile, albeit with a slight delay in ZP penetration *in vitro* [5]. *Prss21* KO mouse lines were also fully fertile *in vivo* [105]. Moreover, *Acr* and *Prss21* double KO mice were subfertile because of impaired sperm penetration through the cumulus matrix and ZP *in vitro* [46]. This indicates that the sperm trypsin-like activity is not essential for *in vivo* fertilization in mice. Therefore, the sperm factor required for penetration through the cumulus matrix *in vivo* remains to be determined.

COC factors that modulate sperm functions

Sperm chemotaxis is found in not only marine invertebrates but also mammals [44]. Chemoattractants are present in oviductal fluid and are also secreted from the COC [24]. In humans, the COC secretes sperm chemoattractants after ovulation [90]. Progesterone secreted from

the COC influences several functions including hyperactivation and the acrosome reaction in human spermatozoa [9]. The extranuclear-mediated effects of progesterone stimulate an influx of calcium, tyrosine phosphorylation of proteins, and other signaling molecules [82]. Progesterone-induced calcium influx is mediated by a sperm-specific calcium channel CATSPER in human spermatozoa [60, 88]. It has recently been revealed that human CATSPER activation by progesterone is triggered by the steroid binding to a serine hydrolase, abhydrolase domain containing 2 (ABHD2) [66]. Furthermore, the CATSPER channel complex may serve as a polymodal sensor for multiple chemicals (odorants, 8-Br-cNMPs, or menthol) [14]. However, mouse *Catsper* does not react to progesterone induction *in vitro*, and mouse *Abhd2* is not essential for male fertility [69]. Since the sequence similarity of *Catsper* orthologs is low (less than 50%) [85], the mechanism of sperm calcium entry may differ in each species.

Prostaglandin E2 (PGE2) is a key mediator of ovulation [23]. One of the four subtypes of PGE2 receptor, prostaglandin E receptor 2 (*Ptger2*), is expressed in the cumulus cells. Female *Ptger2* KO mice are severely subfertile due to impaired cumulus expansion in the oviduct [32]. PGE2-PTGER2 signaling facilitates cumulus ECM assembly and sperm passage through cumulus cell layers. The gene expression profile indicates that cumulus cells upregulate a set of immune response- and chemokine-related genes during ovulation [93]. One of the chemokines, chemokine ligand 7 (*Ccl7*), is overexpressed abnormally in *Ptger2* KO cumulus cells, and excessive cumulus ECM assembly interferes with sperm migration through the COC [94]. These results suggest that CCL7 promotes cumulus ECM assembly to protect the oocyte and functions as a chemoattractant for spermatozoa. While it is unclear why this occurs, proper interaction between prostaglandin and chemokine signaling is required for successful fertilization [89]. Further studies have shown that CCL7 facilitates sperm migration towards the COC *in vitro*. Recently, we found that *Adam3* KO spermatozoa are able to fertilize cumulus-intact eggs but not cumulus-free eggs [95]. The supernatant of cumulus cells is able to partially restore *Adam3* KO sperm fertilizing ability. These data also suggest that COC factors can modulate sperm fertilizing ability.

Sperm Penetration through the ZP

During *in vitro* fertilization, numerous spermatozoa bind to the ZP, and it has been long believed that ZP binding ability is critical for sperm fertilizing ability. However, when *Adam3* KO spermatozoa, which cannot bind to the ZP [87], were deposited directly into the oviduct to circumvent sperm migration through the UTJ, the ovulated eggs were fertilized [95]. These data questioned the importance of ZP binding ability.

There are two possible factors that are necessary for sperm penetration through the ZP, namely, proteases and sperm motility. Proteases were thought to be important because the acrosome contains proteases that are released during the acrosome reaction. However, ACR and PRSS21, trypsin-like serine proteases in the sperm head, are not essential for male fertility in mice, as mentioned previously [5, 46, 105]. Further, recent live-imaging studies demonstrated that mouse spermatozoa underwent the acrosome reaction before contact with the ZP [43, 56, 70], and rabbit and mouse spermatozoa that penetrated the ZP once could penetrate the ZP again and fertilize ZP-intact eggs [41, 55]. These results suggest that the proteases that are released from the acrosome are not necessary for ZP penetration. However, careful interpretation is required because there is a possibility that proteases remain attached to the sperm head after the acrosome reaction, and this may contribute to ZP penetration.

There is positive evidence that sperm motility is crucial for ZP penetration. Field vole spermatozoa can penetrate through the ZP of mice and hamsters without the acrosome reaction [98], suggesting that the mechanical force generated by sperm motility is important for ZP penetration. This idea is supported by *Catsper1* or *Ppp3cc* KO mice that exhibit impaired sperm motility and failure to penetrate the ZP [68, 84]. Both *Catsper1* and *Ppp3cc* KO spermatozoa do not exhibit hyperactivated motility as mentioned previously. However, *Ppp3cc* KO spermatozoa could penetrate through the ZP when the eggs were treated with glutathione (GSH), which reduces disulfide bonds and destabilizes the ZP (Fig. 5C) [10, 68, 92]. The fertilized eggs developed to term when they were transplanted into the oviduct of pseudopregnant mice. This method using GSH in IVF could be useful to further identify factors that are involved in ZP penetration.

Conclusion

Use of GM animals is a powerful approach to clearly identify the *in vivo* function (s) of a given gene. In mammalian fertilization research especially, most findings based on biochemical *in vitro* approaches have been revised by the analyses of KO mouse models [37, 77]. However, the conventional KO method is expensive, laborious, and time-consuming to perform. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has emerged as a genome editing tool in mice, rats, and other animal models [99]. This system enables researchers to make GM mice easier and quicker than the conventional KO method [100]. We have also established a method to generate GM mice using a CRISPR/Cas9 expression plasmid [25, 62, 63] and have analyzed reproductive phenotypes of GM mice using this method [27, 67, 75, 108]. Reproductive biology is one of the most suitable research fields that can use GM animals. We therefore believe that mutant animals will soon unravel whole gene functions through gene-disruption experiments.

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