Title: ADAM5 is required for sperm-zona pellucida binding and sperm oviduct migration

Short title: ADAM5 is essential for male fertility in mice

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Abstract

ADAM5 is a testis-specific transmembrane protein whose role in male fertility remains poorly understood. In this study, we report that *Adam5* knockout (KO) male mice are sterile, despite exhibiting normal testicular morphology, sperm structure, and motility. *Adam5* KO sperm failed to transit the uterotubal junction (UTJ) and displayed severe defects in zona pellucida binding, phenotypes that resemble those observed in *Adam2* and *Adam3* knockout mice. Western blot analysis revealed a significant reduction in the levels of ADAM2 and ADAM3 in *Adam5* KO sperm, supporting the previous finding that ADAM5 interacts with these proteins to form a complex. Additionally, *Adam5* KO sperm exhibited reduced binding to extracellular matrix (ECM) components, including Laminin I and Fibronectin. These findings suggest that ADAM5 plays a crucial role in sperm ECM interaction, a process likely critical for successful UTJ transit. While ADAM5 is a pseudogene in humans, our results provide valuable insights into the function of ADAM family proteins in mammalian reproduction.

Introduction

Among mammalian organs, the testis exhibits a uniquely complex and dynamic transcriptome [1], in which more than 1200 genes in the mouse show enriched expression [2]. This extensive gene expression activity reflects the elaborate molecular orchestration required for spermatogenesis and sperm function. However, the biological functions of most of these testis-enriched genes remain largely unexplored. Given the current lack of a culture system capable of supporting the complete differentiation of functional spermatozoa in vitro [3], gene knockout approaches in mice continue to serve as a principal method for examining gene function within the testis. Notably, because many of these genes are not expressed in other tissues, their disruption does not typically compromise overall viability, enabling a direct evaluation of their contribution to sperm development and fertilizing capacity.

The ADAM (A Disintegrin and Metallopeptidase) protein family is characterized by a conserved structure consisting of a prodomain, metallopeptidase domain, disintegrin domain, cysteine-rich domain, EGF-like domain, and transmembrane domain [4-6]. The ADAM family comprises more than 30 members, which are collectively characterized by their involvement in cell adhesion [7] and sperm function required for fertilization. Among the 1,262 testis-enriched genes we identified by *in silico* analysis [2], 20 genes encode members of the ADAM family. Of these, male infertility or significant subfertility has been reported in knockout models for Adam1a, *Adam2, Adam3*, *Adam6a*, and *Adam6b*. In contrast, the roles of *Adam5*, *Adam26b*, *Adam34*, and *Adam34l* remain to be elucidated.

Previous studies have identified Fertilin (A heterodimer of ADAM1 and ADAM2) and Cyritestin (ADAM3) as candidates for mediating sperm-egg fusion. In mice, two distinct heterodimeric complexes of Fertilin —ADAM1A/ADAM2 and ADAM1B/ADAM2—are expressed in the testis. Genetic ablation of *Adam2* results in the loss of both heterodimers. Although sperm from *Adam2* knockout (KO) mice retain the ability to fuse with oocytes, they exhibit impaired zona pellucida binding and fail to

penetrate the uterotubal junction (UTJ) [8–11]. Similarly, *Adam1a* KO spermatozoa show normal fusion capability but are defective in zona pellucida binding and UTJ migration [12]. In contrast, Adam1b KO males exhibit no observable abnormalities and maintain normal fertility [13]. Notably, both *Adam1a* and *Adam2* KO sperm lack ADAM3 on their surface, suggesting that ADAM1A and ADAM2 are required for ADAM3 maturation in the ER. *Adam3*-deficient spermatozoa also retain the ability to fuse with oocytes but exhibit impaired binding to the zona pellucida, a phenotype shared by the other mutants; thus, these cases can be collectively explained by the loss of ADAM3.

Several lines of evidence support that ADAM3 is a key protein required for zona pellucida binding and penetration through the UTJ [14, 15]. The absence of *Adam3* disrupts both of these essential processes, leading to infertility [16]. Furthermore, *Adam2* KO and *Adam3* KO sperm exhibit reduced levels of ADAM4, ADAM5, and ADAM6 proteins, suggesting the formation of ADAM2/ADAM3/ADAM4, ADAM2/ADAM3/ADAM5, and ADAM2/ADAM3/ADAM6 complexes [17]. ADAM6 KO results in the loss of ADAM3 from the spermatozoa and causes defects in zona pellucida binding, UTJ penetration, and extracellular matrix (ECM) binding [18]. These findings highlight the critical importance of ADAM protein complexes, particularly those involving ADAM3, in male fertility. While ADAM5 has been identified as a component of these complexes, its specific role remains unclear.

In the present study, *Adam5* knockout mice were generated using the CRISPR/Cas9 system to investigate its function in male fertility. Our results revealed that ADAM5 is crucial for sperm binding to the extracellular matrix (ECM), zona pellucida, and UTJ penetration.

Materials and Methods

Animal

This study was reported following the ARRIVE guidelines 2.0 and conducted following the principles outlined in the Guide for the Care and Use of Laboratory Animals. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan (#Biken-AP-H30-01). Mice used in this study were obtained from Japan SLC, Inc. (Shizuoka, Japan) and maintained under specific pathogen-free conditions. They were housed at 23 °C with 50% relative humidity, a 12-hour light/dark cycle, and had free access to water and commercial food pellets. Genetically modified mice generated in this study will be made available through the RIKEN BioResource Research Center (Ibaraki, Japan) or the Center for Animal Resources and Development (CARD) at Kumamoto University, Japan.

In Silico Analysis

The Mammalian Reproductive Genetics Database (19; https://orit.research.bcm.edu/MRGDv2) was utilized to examine *Adam5* mRNA expression. Additionally, previously published single-cell RNA sequencing data (20; https://bis.zju.edu.cn/MCA/) were analyzed to investigate *Adam5* mRNA expression in testicular germ cells. The AlphaFold database (21; https://alphafold.ebi.ac.uk/) was employed for the structural prediction of the ADAM5 protein.

Generation of *Adam5* Knockout Mice

Adam5 knockout mice were created using the CRISPR/Cas9 system. Guide RNA design and off-target analysis were conducted with the software tools CRISPRdirect (https://crispr.dbcls.jp/) and CRISPOR (https://crispor.tefor.net/). Fertilized eggs were collected from the oviducts of superovulated B6D2F1 (BDF1) females mated with BDF1 males. Ribonucleoprotein (RNP) complexes, consisting of synthesized CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), and CAS9 protein, were delivered into the fertilized eggs using a NEPA21 super electroporator (Nepa Gene Co., Ltd,

Chiba, Japan). The treated embryos were cultured in potassium simplex optimization medium with amino acids (KSOMaa) until reaching the two-cell stage, after which they were transferred into the oviducts of 0.5-day pseudopregnant ICR females. The identity of the resulting pups was verified by PCR and Sanger sequencing.

Guide RNA and primer sequences are following: crRNA1: 5'-AAATGTGTACGACATGTCTT-3', crRNA2: 5'-GTCATCCCGGATTTGCGCAT-3', KO-Fw: 5'-TCCATTGGAAGATGTTCTTG-3', KO-Rev: 5'-AGAAGGTCACTTGGGAGCAA-3', WT-Fw: 5'-CTGTACGTGGCCATTCAAGA-3', WT-Rev: 5'-AGAAGGTCACTTGGGAGCAA-3'

In vivo male fertility test

Eight-week-old males, either carrying the *Adam5* wild-type or mutated allele, were individually housed with three 8-week-old B6D2F1 females for two months. Mating plugs were checked daily, and the number of offspring produced was recorded. Each experimental group included at least three males for statistical analysis.

Histological analysis

Testes were dissected, fixed in Bouin's solution (Polysciences, Warrington, PA, USA), and embedded in paraffin wax. Subsequently, 5-μm-thick sections were cut from paraffin blocks using a Microm HM325 microtome (Microm, Walldorf, DE, Germany). The sections were sequentially rehydrated with xylene and ethanol, followed by a 15-min incubation in a 1% periodic acid solution. After washing under running water for 15 min, the sections were treated with Schiff's reagent (FUJIFILM Wako, Osaka, JP) for 30 min and then stained with Mayer's hematoxylin solution for 3 min after an additional 15-min wash. Following these processes, the stained samples were observed using SLIDEVIEW VS200 (Olympus, Tokyo, JP).

In Vitro Fertilization and Zona Pellucida (ZP) Binding Test

In vitro fertilization (IVF) was carried out as described previously (22). Cauda epididymal spermatozoa were dispersed in Toyoda, Yokoyama, and Hoshi (TYH)

medium (23) under paraffin oil (26117-45, Nacalai Tesque Inc., Kyoto, Japan) and incubated for 2 hours at 37 °C in 5% CO_2 to achieve capacitation. Eggs collected from the oviducts of superovulated females were placed in TYH drops. Cumulus cells were removed by treating oocytes with 330 μg/mL hyaluronidase (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) for 5 min. For ZP removal, eggs were treated with 1 mg/mL collagenase (C1639, Merck KGaA, Darmstadt, Germany) for 5 min. Capacitated spermatozoa were then added to a drop containing cumulus-intact eggs at a final concentration of 2×10^5 spermatozoa/mL. Pronuclear formation was assessed 8 hours post-insemination. For the ZP binding test, capacitated spermatozoa were added to a drop containing cumulus-free eggs at a final concentration of 2×10^5 spermatozoa/mL. The eggs were incubated for 30 min, after which the number of spermatozoa bound to the ZP was counted.

UTJ penetration assay

The UTJ penetration assay was conducted as previously described (22). B6D2F1 female mice underwent superovulation via intraperitoneal injection of 5 U equine chorionic gonadotropin (eCG), followed by 5 U human chorionic gonadotropin (hCG) 48 hours later. Twelve hours after the hCG injection, the females were housed with test males and vaginal plug formation was monitored every 30 min. Upon detection of a plug, the males were separated from the females. Approximately 2 hours post-plug formation, the oviducts, along with the adjoining uterine tissue, were collected. These tissues were mounted as whole specimens on slides, covered with coverslips, and examined under fluorescence microscopy (BZ-X810; Keyence Corporation, Osaka, Japan) to evaluate the presence of spermatozoa carrying the mitochondrial DsRed2 marker.

Computer-assisted sperm analysis (CASA)

Sperm velocity was evaluated as described previously (22). Cauda epididymal spermatozoa were dispersed in 100 μL drops of TYH medium. Sperm motility parameters were assessed using the CEROS II sperm analysis system (software version 1.4; Hamilton Thorne Inc., Beverly, MA, USA) at 10 min and 2 hours after incubation at 37 °C under 5% CO_2. For each male, more than 200 spermatozoa were analyzed.

Western blot

Immunoblotting procedures closely followed those described previously (22). Testis tissue and spermatozoa from the cauda epididymis were collected and immersed in lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl) supplemented with a protease inhibitor cocktail (Cat. No. 25955, Nacalai Tesque Inc.) and left to incubate overnight at 4 °C. Subsequently, the lysate was centrifuged at 10 000 × g for 15 min at 4 °C. The resulting supernatants were used for SDS-PAGE for immunoblotting.

ECM binding assay

The ECM binding assay was conducted using the CytoSelect™ 48-well Cell Adhesion Assay ECM Array (CBA-070; Cell Biolabs Inc., San Diego, CA, USA). Spermatozoa were suspended in TYH medium, and 1×10^5 spermatozoa were added to each well. After 30 min of incubation, the wells were washed three times with PBS, followed by staining with Hoechst 33342 (Thermo Fisher Scientific Inc., Waltham, MA, USA) to count the number of adhered spermatozoa.

Mass spectrometry

Protein samples were subjected to mass spectrometry analysis as previously described (24). Proteins were reduced with 10 mM dithiothreitol (DTT), followed by alkylation with 55 mM iodoacetamide, and digested in-gel by treatment with trypsin and purified with C18 tip (GL-Science, Tokyo, Japan). The resultant peptides were subjected to nanocapillary reversed phase LC-MS/MS analysis using a C18 column (25 cm × 75 um, 1.6 μm; IonOpticks, Victoria, Australia) on a nanoLC system (Bruker Daltoniks, Bremen, Germany) connected to a timsTOF Pro mass spectrometer (Bruker Daltoniks) and a modified nano-electrospray ion source (CaptiveSpray; Bruker Daltoniks). The mobile phase consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Linear gradient elution was carried out from 2% to 35% solvent B for 18 min at a flow rate of 400 nL/min. The ion spray voltage was set at 1.6 kV in the positive ion mode. Ions were collected in the trapped ion mobility spectrometry (TIMS) device over 100 ms and MS and MS/MS data were acquired over

an m/z range of 100-1,700. During the collection of MS/MS data, the TIMS cycle was adjusted to 1.1 s and included 1 MS plus 10 parallel accumulation serial fragmentation (PASEF)-MS/MS scans, each containing on average 12 MS/MS spectra (>100 Hz), and nitrogen gas was used as collision gas." Protein identification was carried out using Mascot (version: 2.7.0; Matrix Science, London, UK) regarding Scaffold_4.10.0 (Proteome Software Inc., Portland, OR, USA). Human keratin peptides were excluded from the analysis.

Gene ontology analysis

GO analysis was performed using ShinyGO (25; https://bioinformatics.sdstate.edu/go/), an online tool for functional enrichment analysis.

Statistics

The Shapiro-Wilk test and F-test were performed, followed by a Student's t-test. When normality was not demonstrated, the Wilcoxon rank-sum test was conducted. Statistical significance was defined as $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***.

Results

Generation of *Adam5* Knockout (KO) Mice

ADAM5 is a gene that exhibits testis-specific expression (**Fig. 1A**; Mammalian Reproductive Genetics Database V2: 19). Reanalysis of the public single-cell RNA sequence dataset (**Fig. 1B**; Mouse Cell Atlas: 20) revealed that ADAM5 is expressed as early as the spermatogonia stage. AlphaFold analysis [21] indicated that ADAM5 is a type-I transmembrane protein, containingone transmembrane domain (Fig. 1C). While ADAM5 was predicted to form a heterodimer with ADAM3, ADAM2 was not predicted to form complexes with either ADAM3 or ADAM5.

The mouse *Adam5* gene is located on chromosome 8 at positions 25,217,109-25,314,385 bp on the negative strand. We designed two guide RNAs to delete exons 5 to 16 (**Fig. 1D;** the intron between exons 4 and 5 and within exon 16 were targeted). The 33,528 bp deletion was confirmed by genomic PCR and subsequent Sanger sequencing (**Fig. 1E, 1F)**.

ADAM5 is required for male fertility in mice

To elucidate the role of ADAM5 in male fertility, we housed *Adam5* KO male mice with wild type female mice for 8 weeks. Daily plug observations were conducted, and the number of pups per plug was compared with that of wild-type (WT) male mice. *Adam5* KO mice were highly subfertile and generated significantly fewer pups per plug compared to WT mice (**Fig. 2A**; 9.58±1.67 vs. 0.22±0.83, Wilcoxon rank sum test: P = 2.2 x 10-16). The testis weight/body weight of KO mice was comparable to that of WT mice (3.94±0.49 vs. 3.50±0.19, Student t-test: P = 0.23), and there were no noticeable abnormalities in the appearance of the testes (**Fig. 2B**), or sperm morphology defects observed(**Fig. 2C**). To investigate further, Hematoxylin–Periodic Acid–Schiff (H-PAS) staining was performed on testis sections, revealing no marked abnormalities in

spermatogenesis (**Fig. 2D**). Additionally, sperm motility and progressive motility were unaffected in KO mice (**Fig. 2E**; 10 min motility: P = 0.625, progressive: P = 0.875; 120 min motility: P = 1, progressive: P = 1, Wilcoxon rank sum test, alternative = greater).

Adam5 KO spermatozoa exhibit zona pellucida binding deficiency and UTJ penetration failure.

Given that sterility resulting from the lossof ADAM family members expressed in the testis can likely be attributed to zona pellucida binding deficiency and UTJ penetration failure (*Adam1a*, *Adam2*, *Adam3* [9], *Adam6* [19]), we next explored sperm function in vitro. First, we tested whether *Adam5* KO sperm could bind to the zona pellucida by removing the cumulus cells from unfertilized eggs through hyaluronidase treatment and incubated them with *Adam5* KO sperm for thirty minutes. We found that sperm adhesion to the zona pellucida was almost absent in KO (**Fig. 3A, 3B**; P = 2.2 x 10-16). To test the capacity of sperm to migrate into the oviduct, we introduced a fluorescent marker of the sperm midpiece [Tg(CAG::Su9-DsRed2, Acr::Acr3-EGFP; 26)] into *Adam5* KO mice and observed the spermatozoa post-mating using a red fluorescent signal. In WT, spermatozoa passed through the UTJ two hours after mating, whereas in KO, UTJ penetration was not clearly observed (**Fig. 3C-3E**). Collectively, these results highlighted that ADAM5 is required for UTJ penetration and sperm-zona pellucida binding.

Adam5 KO leads to decreased expression of ADAM2, ADAM3

We next used western blotting to investigate whether the quantity of ADAM family proteins was altered in ADAM5 KO testes and spermatozoa(**Fig. 4**). Since ADAM2, ADAM3, and ADAM5 are predicted to form a complex [17], we used antibodies against ADAM2 and ADAM3. We also used an antibody against ADAM1B as a negative control because ADAM1B is not predicted to be a component of this complex.

As we expected, we found that ADAM1B levels were comparable in both the testis and spermatozoa. In contrast, ADAM2 levels were reduced in both the testis and spermatozoa of the KO. ADAM3 levels were equivalent in the testes between WT and KO, but reduced in the spermatozoa of the KO. These results support the hypothesis that ADAM2, ADAM3, and ADAM5 form a complex. Since antibodies against ADAM5 were not available, we performed mass spectrometry on wild-type and KO sperm protein (**Supplemental data 1**). Peptides derived from ADAM5 were detected in wild-type spermatozoa but were not detected in *Adam5* KO spermatozoa, validating our KO model. ADAM6A and ADAM6B were detected in spermatozoa from *Adam5* KO mice. Among the ADAM family proteins, we found ADAM24 and Gm4787(ADAM4B) were reduced in the KO (**Supplemental Data 2**). In mass spectrometry analysis using *Adam3* knockout spermatozoa, ADAM6A and ADAM6B were not detected, whereas there was no significant difference in ADAM5 levels compared to wild-type (P = 1, two-sided t-test, **Supplemental Data 3**). Since ADAM3 and ADAM5 are suggested to form a complex [17], we compared their expression levels in knockout spermatozoa to assess potential interdependence. The fold change (FC) of ADAM5 in ADAM3 knockout spermatozoa relative to wild-type was 1.01, while the FC of ADAM3 in *Adam5* knockout spermatozoa was 0.52 (Fig. 4C) , highlighting that ADAM5 is required to stabilise ADAM3 levels in sperm but not conversely. Gene ontology (GO) term enrichment analysis revealed a significant overrepresentation in glycosaminoglycan degradation (Fig. 4D) in *Adam5* KO sperm, with a particular enrichment in heparan sulfate degradation (Fig. 4E).

Reduced Binding Ability of *Adam5* KO Spermatozoa to Extracellular Matrix Components

Based on previous reports indicating that *Adam6* KO spermatozoa show reduced binding to extracellular matrix plates [19], we observed the binding ability of *Adam5* and *Adam3* KO spermatozoa to ECM-coated plates. The results showed a significant decrease in the binding ability of *Adam5* and *Adam3* KO spermatozoa to Fibronectin and Laminin I (**Fig. 5**; Fibronectin: WT vs *Adam5*; $P = 3.0 \times 10^{-4}$, WT vs *Adam3*; P = 2.0

x 10^{-4}, Laminin-I: WT vs *Adam3*; P = 0.014, WT vs *Adam5*; P = 0.013 student t-test). These results indicate that ADAM5 is required for sperm ECM binding.

Discussion

In this study, we highlight that *Adam5* exhibits testis-specific expression and is required for male fertility (**Fig. 1**). *Adam5* KO male mice were sterile despite normal spermatogenesis, sperm morphology, and motility (**Fig. 2**). Our study revealed the critical role of ADAM5 in sperm adhesion that is essential for UTJ migration and sperm-zona pellucida binding.

The impaired UTJ transit and reduced zona pellucida binding observed in *Adam5* KO mice (**Fig. 3**) resemble the phenotypes of *Adam2* and *Adam3* KO mice [8]. These proteins are thought to form a complex with ADAM5 [17]. We demonstrated a significant reduction in ADAM2 and ADAM3 protein levels in *Adam5* KO spermatozoa (**Fig. 4**), supporting the formation of a complex involving ADAM2, ADAM3, and ADAM5. The presence of ADAM6 in *Adam5* knockout spermatozoa suggests that ADAM5 does not form a complex with ADAM6. A possible reason for the observed decrease in ADAM2 but not in ADAM3 in the testis is that ADAM2 may form a complex with ADAM5, which subsequently associates with ADAM3.

Additionally, since ADAM5 content was not decreased in *Adam3* KO spermatozoa, butADAM3 content was reduced in *Adam5* KO spermatozoa, it is plausible that ADAM3 functions as a structural platform that stabilizes ADAM5. An examination of proteins lost in *Adam5* KO sperm revealed that peptides derived from ADAM24 and Gm4787 (ADAM4B) were detected in the wild-type but not in the KO. These findings suggest that the loss of ADAM5 could disrupt the stability or expression of these related proteins. While ADAM24 KO mice are fertile [27], the role of ADAM4B remains uncharacterized. Further investigation is needed to understand the precise role of these proteins in the ADAM family complex.

Furthermore, GO analysis of proteins absent in *Adam5* KO spermatozoa indicated a significant enrichment in glycosylation-related pathways, including glycosaminoglycan degradation, and highlighted GUSB as a notable factor. GUSB has been reported to bind to the sperm surface in the cauda epididymis [28], and our data suggest that this binding does not occur in *Adam5* KO mice. Although *Gusb* mutant mice exhibit male

infertility, detailed mechanistic studies have not yet been conducted [29]. Given that *Gusb* mutant mice also cause systemic abnormalities, conditional KO models will be essential to delineate the specific role of GUSB in male reproduction. Finally, *Adam5* KO spermatozoa showed reduced binding to Laminin I and Fibronectin (**Fig. 5**). These results align with findings in *Adam6* KO spermatozoa, where a similar reduction in binding to ECMs was reported [18]. These findings reinforce the hypothesis that ECM components are interaction partners of ADAM protein complexes. While the mechanism underlying sperm transit through the UTJ remains unclear, future studies should explore the relationship between ECM-binding capacity and UTJ transit.

A limitation of this study is that *ADAM5* is a pseudogene in humans [30], as is *ADAM3*, which plays a crucial role in zona pellucida binding and spermatozoa migration through the oviduct in mice. However, the observed reductions in ECM binding and oviduct migration provide valuable insights into the functions of other ADAM family genes. Among ADAM family members expressed in human testes (e.g., ADAM2, ADAM18, ADAM20, ADAM21, ADAM29, ADAM30, and ADAM32 [31]), only *Adam2* KO mice have shown reduced litter sizes [8, 32-35], except for *Adam30*, for which no KO study has been conducted. Given that ADAM3, ADAM4, ADAM5, and ADAM6, which form complexes with ADAM2 in mice, are not expressed in humans, ADAM2 may create a different complex in humans and be related to binding to ECM. This hypothesis could be tested using immunoprecipitation and mass spectrometry with ADAM2 in human spermatozoa.

In this study, we demonstrated that *Adam5* KO spermatozoa exhibit a reduced ECM-binding capacity and fail to transit the UTJ, resulting in male sterility. The impaired ECM-binding ability appears critical for successful UTJ transit, highlighting the importance of ADAM5 in mouse sperm function.

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Declarations

The authors declare no conflict of interest.

Data availability

All relevant data supporting the findings of this study are included in the manuscript.

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Figure Legends

Fig. 1. Generation of Adam5 Knockout Mice

A. Expression pattern of Adam5 as registered in a public database. B. Cells expressing Adam5, identified by reanalysis of scRNA-seq data. Red labels indicate log2(TPM+1). C. Predicted 3D structure of ADAM5 generated using AlphaFold. D. Gene structure of Adam5 and knockout strategy. Red arrows indicate guide RNAs and black arrows indicate primers. E. Typing PCR results. Negative control (N.C.) was performed using water. F. Sanger sequencing results show deletions at indicated regions.

Fig. 2. Adam5 Knockout Mice Exhibit Normal Sperm Morphology and Motility but Are Severely Subfertile

A. Comparison of pups per plug between WT and KO mice. B. Visual appearance of testes and normalized testis weights in WT and KO mice. C. Comparison of sperm morphology between WT and KO mice. D. Histological sections of WT and KO testes. E. Sperm motility at 10 min and 120 min after incubation in WT and KO mice.

Fig. 3. Adam5 Knockout Spermatozoa Show Defects in ZP Binding and UTJ Penetration

A. Representative images of eggs after insemination with WT or KO spermatozoa. B. Comparison of the number of ZP-bound spermatozoa between WT and KO mice. C. Picture of RBGS spermatozoon.D. Illustrated diagram of the UTJ penetration test. E. Images after the UTJ penetration test. Yellow arrowheads indicate fluorescence from the spermatozoa midpiece.

Fig. 4. Loss of ADAM5 Induces Reduction of ADAM2 and ADAM3 in Spermatozoa

A. Western blot analysis of testis-derived proteins for ADAM1B, ADAM2, and ADAM3. BASIGIN was used as a control. B. Western blot analysis of sperm-derived proteins for ADAM1B, ADAM2, and ADAM3. C. The fold changes (KO/WT) of

ADAM5 and ADAM3 in Adam3 KO and Adam5 KO spermatozoa, respectively, as determined by mass spectrometry. D. The chart of enriched pathway E. Glycosaminoglycan degradation pathway. Red shows the proteins undetected in Adam5 KO spermatozoa.

Fig. 5. Adam5 and Adam3 KO Spermatozoa Show Reduced Binding Capacity to ECM

A graph showing the number of spermatozoa bound to the ECM-coated plate (y-axis) with different ECM coatings (x-axis). BSA was used as a control.

Supplemental data 1 MS data of Adam5 KO (n=3 mixed)

Supplemental data 2 ADAM family proteins detected in Adam3 or Adam5 KOSupplemental data 3 MS data of Adam3 KO (n=3 mixed)