Possible Function of the ADAM1a/ADAM2 Fertilin Complex in the Appearance of ADAM3 on the Sperm Surface*

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Running Title: Role of Mouse ADAM1a
(SUMMARY)

In mouse, two different isoforms of ADAM1 (fertilin α), ADAM1a and ADAM1b, are produced in the testis. ADAM1a is localized within the endoplasmic reticulum of testicular germ cells, whereas epididymal sperm contain only ADAM1b on the plasma membrane. In this study, we show that the loss of ADAM1a results in the male infertility because of the severely impaired ability of sperm to migrate from the uterus into the oviduct through the uterotubal junction. However, epididymal sperm of ADAM1a-deficient mice were capable of fertilizing cumulus-intact, zona pellucida-intact eggs \textit{in vitro}, despite the delayed dispersal of cumulus cells and the reduced adhesion/binding to the zona pellucida. Among testis (sperm)-specific proteins examined, only the level of ADAM3 (cyritestin) was strongly reduced in ADAM1a-deficient mouse sperm. Moreover, the appearance of ADAM3 on the sperm surface was dependent on the formation of a fertilin protein complex between ADAM1a and ADAM2 (fertilin β) in testicular germ cells, although no direct interaction between the fertilin complex and ADAM3 was found. These results suggest that ADAM1a/ADAM2 fertilin may be implicated in the selective transport of specific sperm proteins including ADAM3 from the endoplasmic reticulum of testicular germ cells onto the cell surface. These proteins then can participate in sperm migration into the oviduct, the dispersal of cumulus cells, and sperm binding to the zona pellucida.
A family of ADAM (a disintegrin and metalloprotease) transmembranous proteins belonging to the zinc protease superfamily is implicated in various biological processes such as fertilization, neurogenesis, myogenesis, cancer, and inflammation (1-3). ADAMs have a unique organization containing an N-terminal signal peptide domain followed by pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic tail domains, each of which has been believed to fulfill distinct function(s). The metalloprotease domain exhibits a “sheddase” activity toward the ectodomain of membranous precursor proteins (4), whereas cell-to-cell adhesion is mediated by the disintegrin domain (5-7). Although many members of the ADAM family are exclusively or predominantly expressed in the testis, their roles in spermatogenesis and fertilization have not yet been completely elucidated.

Mammalian fertilization is a successive, multi-step process of events including the adhesion and binding of sperm to the zona pellucida (ZP), an extracellular glycoprotein matrix surrounding the egg, and the membrane fusion between sperm and egg (8, 9). Male mice lacking calmegin (10, 11), a testis-specific isoform (tACE) of angiotensin-converting enzyme (12, 13), and ADAM2 (14) are all sterile owing to the sperm defects in binding to the ZP and in migrating from the uterus into oviduct. Sperm from ADAM3-deficient mice are also defective in ZP binding, but can ascend into the oviduct normally (15, 16). An intriguing finding is that the disruption of the functional ADAM2 gene results in the loss or severe reduction of ADAM1 and ADAM3 in testicular germ cells (TGC) and epididymal sperm (14, 16). Thus, it is reasonable to postulate that ADAM1, ADAM2, and ADAM3 interact with one another to make sperm fertilization competent.
Fertilin is a heterodimeric protein complex present on the sperm surface consisting of ADAM1 and ADAM2 (17-19). In mouse, two different genes encoding ADAM1a and ADAM1b corresponding to guinea pig ADAM1 are both localized on the chromosome 5 (20). ADAM1a is present within the endoplasmic reticulum (ER) of TGC, whereas epididymal sperm contain only ADAM1b on the cell surface (21). Moreover, while either ADAM is capable of forming the fertilin protein complex with ADAM2 in the ER of TGC, only the ADAM1b/ADAM2 fertilin complex is present on the sperm surface (21). These data imply that ADAM1a and ADAM1b have critical, distinct roles in spermatogenesis and fertilization, respectively.

To elucidate the role(s) of ADAM1a in TGC, we produced male mice carrying a disruptive mutation in the ADAM1a gene by homologous recombination. The ADAM1a-deficient male mice are infertile because sperm have a severely impaired ability to migrate from the uterus into the oviduct through the uterotubal junction (UTJ). ADAM3, but not other testis (sperm)-specific proteins, is strongly reduced on ADAM1a-deficient mouse sperm. Comparative experiments using TGC and epididymal sperm of ADAM1a-, ADAM2-, and ADAM3-deficient mice suggest that the ADAM1a/ADAM2 fertilin complex may be implicated in the transport of sperm proteins, including ADAM3, from the ER of TGC onto the cell surface.
EXPERIMENTAL PROCEDURES

Generation of Mutant Mice Lacking ADAM1a — A mouse genomic clone, mFAG10 (20), encoding ADAM1a was used for construction of a targeting vector containing an expression cassette of the neomycin-resistance gene (neo) flanked by a 1.5-kbp genomic region of the ADAM1a gene (Fig. 1A). The MC1 promoter-driven herpes simplex virus thymidine kinase gene (tk) was also included in the targeting vector for negative selection. Following electroporation of the targeting vector, which had been linearized by digestion with KpnI, into mouse D3 embryonic stem (ES) cells, homologous recombinants were selected by using G418 and gancyclovir, as described previously (22). Seven ES cell clones carrying the targeted mutation were identified from 450 clones resistant to G418 and gancyclovir, and injected into C57BL/6 mouse blastocysts. Chimeric male mice were crossed to ICR female mice (Japan SLC Inc., Shizuoka) to establish heterozygous mutant lines. Homozygous mice were obtained by mating of heterozygous males and females. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in University of Tsukuba.

Blot Hybridization — Genomic DNA was prepared from the mouse tail, digested by EcoRI, separated by agarose gel electrophoresis, and transferred onto Hybond-N+ nylon membranes (Amersham Biosciences), as described previously (23). Total cellular RNA was prepared from testicular tissues using ISOGEN (Nippon Gene, Toyama, Japan) (24). The RNA samples were glyoxylated, separated by agarose gel electrophoresis, and transferred onto the nylon membranes. The blots were probed by 32P-labeled DNA fragments and analyzed by a BAS2000 Bio-Image Analyzer (Fuji Photo Film, Tokyo) (24).

Preparation of Protein Extracts — TGC and cauda epididymal sperm were obtained typically from 3-month-old mice (25, 26). Cells were suspended in a lysis buffer
consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 10% protease inhibitor cocktail (Sigma-Aldrich), kept on ice for 20 min, and centrifuged at 10,000 x g for 10 min at 4°C. The supernatant solution was subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), and then Western blot analysis, as described below. Protein concentration was determined using a Coomassie protein assay reagent kit (Pierce).

**Antibodies** — Affinity-purified rabbit polyclonal antibodies against ADAM1a, ADAM1b, sp32, and PH-20 were prepared as described previously (21, 27, 28). Anti-ADAM3 antiserum was produced in female New Zealand White rabbits by intradermal injection of recombinant protein containing the cysteine-rich and epidermal growth factor-like domains of ADAM3 between residues 487 and 670. The anti-ADAM3 antibody was affinity-purified as described (21, 27). Mouse monoclonal antibodies against ADAM2 (9D2.2) and ADAM3 (7C1.2) were purchased from Chemicon. Horseradish peroxidase-conjugated goat antibodies against mouse IgG (H + L) and rabbit IgG (H + L) were purchased from Jackson Immunoresearch Laboratories. Anti-ADAM2 (11), anti-calmegin (10, 11), and anti-angiotensin-converting enzyme (29-31) antibodies were kind gifts of Dr. M. Okabe.

**Western Blot Analysis** — Proteins were denatured by boiling for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol (2ME), separated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore). After blocking with 1% skim milk, the blots were incubated with primary antibodies for 2 h, and then with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were detected by an ECL Western blotting detection kit (Amersham Biosciences). When complex formation between two ADAMs was examined, protein samples were kept in 0.3% SDS at room temperature for 10 min, separated by SDS-PAGE under non-reducing
conditions, and transferred onto Immobilon-P membranes. To enhance the immunoreactivity, proteins on the membranes were denatured for 30 min at 50°C in 50 mM Tris-HCl, pH 6.8, containing 2% SDS and 0.75% 2ME after transfer. After rinsing thoroughly with water to remove SDS and 2ME, immunoreactive proteins were detected as described above.

Assays of Sperm Migration into the Oviduct — Migration of sperm from the uterus into oviduct was examined as described previously (11). Briefly, female B6C3F1 mice were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (PMSG, Teikoku Zoki Co., Tokyo) followed by human chorionic gonadotropin (HCG, Teikoku Zoki Co.) 48 h later. The female mice were caged with male mice 12 h after the HCG injection, and the formation of vaginal plug was observed every 30 min. The oviducts were excised with a connective part of the uterus approximately 2 h after the copulation, fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS, and frozen in OCT compound (Sakura Finetechnical Co., Tokyo). Sections were prepared, stained with hematoxylin, and then observed under an Olympus BX50 microscope.

Assays of the Dispersal of Cumulus Cells by Sperm — Eggs, tightly packed with cumulus cells, were collected from the oviductal ampulla of superovulated ICR mice 15 to 16 h after HCG injection, and placed in a 0.2-ml drop of TYH medium (32) covered with mineral oil. Fresh cauda epididymal sperm from 3-month-old mice were capacitated by incubation for 2 h in a 0.2-ml drop of TYH medium at 37°C under 5% CO₂ in air. An aliquot of the capacitated sperm suspension (1.5 x 10⁵ sperm/ml) was mixed with the eggs in TYH medium. The eggs and sperm were incubated for 1-6 h at 37°C under 5% CO₂ in air. The status of the cumulus mass was microscopically assessed at time intervals according to the criterion described previously (27).
Assays of Sperm-ZP Binding — Superovulated eggs were treated with bovine testicular hyaluronidase (3 units/ml, Sigma-Aldrich) for 15 min to remove cumulus cells, washed, and placed in a 0.2-ml drop of TYH medium covered with mineral oil. An aliquot of capacitated sperm suspension (1.5 x 10^5 sperm/ml) was added to the drop, and the mixture was incubated for 30 min at 37°C under 5% CO_2 in air. The number of sperm bound to the egg ZP was counted using an Olympus BX50 microscope equipped with HC-2500 digital camera (Fuji Photo Film, Tokyo) (26).

Assays of In Vitro Fertilization (IVF) — Cumulus-intact, ZP-intact eggs were inseminated by capacitated sperm (1.5 x 10^5 sperm/ml) in a 0.2-ml drop of TYH medium. After incubation for 6 h at 37°C under 5% CO_2 in air, the eggs were treated with bovine hyaluronidase (3 units/ml) for 15 min to remove cumulus cells, and washed. The female and male pronuclei in the eggs were stained with 4’-6-diamidino-2-phenylindole (DAPI, 10 µg/ml) for 30 min, and then viewed under an Olympus BX50 fluoromicroscope, as described (14, 16).

Assays of Sperm-Egg Fusion — ZP-intact eggs were incubated with α-chymotrypsin (100 µg/ml, Sigma-Aldrich) for 3 to 5 min, and washed. The ZP-free eggs were treated with DAPI, and then inseminated with capacitated sperm (1.5 x 10^5 sperm/ml). After incubation for 1 h at 37°C under 5% CO_2 in air, the eggs were observed under the above fluoromicroscope, as described (14, 16).

Immunoprecipitation — Proteins (1 mg) were extracted from TGC in a lysis buffer (0.5 ml) consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 10% protease inhibitor cocktail, as described above, pre-cleared by incubation with 30 µl of Protein A-Sepharose beads (50% slurry, Amersham Biosciences) on ice for 3 h, and centrifuged at 300 x g for 5 min at 4°C. The supernatant solution was mixed with fresh Protein A-Sepharose beads (30 µl), and the mixture was incubated on ice for 3 h with
antibodies. After centrifugation, the pellet was washed 5 times with the lysis buffer, suspended in a Laemmli buffer containing 1% SDS and 1% 2ME, boiled for 5 min, and then analyzed by Western blotting. Anti-sp32 antibody and rabbit pre-immune serum were used as controls.
RESULTS AND DISCUSSION

To investigate the role of ER-resident ADAM1a in spermatogenesis, we produced mutant mice lacking ADAM1a by homologous recombination in ES cells. The targeting construct was designed to replace the protein-coding region containing the pro-, metalloprotease, disintegrin, and cysteine-rich domains in ADAM1a with the neo gene (Fig. 1A). The genotypes of wild-type (Adam1a\(^{+/+}\)), heterozygous (Adam1a\(^{+/-}\)), and homozygous (Adam1a\(^{-/-}\)) mice for null mutation of the ADAM1a gene were identified by Southern blot analysis of genomic DNA (Fig. 1B). Northern blot analysis indicated the absence of ADAM1a mRNA in Adam1a\(^{-/-}\) testis (Fig. 1C). Moreover, protein extracts of Adam1a\(^{-/-}\) TGC completely lacked the precursor form of ADAM1a (Fig. 1D). These data demonstrate the loss of ADAM1a in Adam1a\(^{-/-}\) male mice.

Breeding of Adam1a\(^{+/+}\) male and female mice yielded the expected Mendelian frequency of Adam1a\(^{-/-}\) mice [Adam1a\(^{+/+}\) : Adam1a\(^{+/-}\) : Adam1a\(^{-/-}\) = 67 (20%) : 184 (55%) : 87 (26%)]. Adam1a\(^{-/-}\) mice were normal in health condition, body size, and behavior. Despite normal formation of copulation plugs in mated females, the fertility of Adam1a\(^{-/-}\) males was severely impaired; only a single pregnancy was observed, and only two offspring were born after 24 Adam1a\(^{-/-}\) males were mated with 61 Adam1a\(^{+/+}\) females for more than two months. Therefore, the Adam1a\(^{-/-}\) male mice are essentially sterile. Adam1a\(^{+/+}\) male and Adam1a\(^{-/-}\) female mice exhibited normal fertility, and the average litter sizes were also normal (13.0 ± 1.5 and 10.4 ± 1.5 offspring, \(n = 8\) and 11, respectively).

No significant difference in size and weight of the testis was found between Adam1a\(^{+/+}\) and Adam1a\(^{-/-}\) mice in spite of TGC-specific localization of ADAM1a. Spermatogenic cells in the seminiferous tubules of Adam1a\(^{-/-}\) mice were morphologically normal. Cauda epididymal sperm of Adam1a\(^{-/-}\) mice could not be distinguished from...
those of $Adam1a^{+/+}$ mice by shape, motility, or percentage of spontaneous acrosome reaction (data not shown). In addition, the numbers of sperm in the cauda epididymis (1.4 ± 0.4 x 10^7 sperm, $n = 4$) and uterus after ejaculation (1.1 ± 0.3 x 10^6 sperm, $n = 4$) in $Adam1a^{-/-}$ mice were similar to those (1.2 ± 0.3 x 10^7 and 1.2 ± 0.3 x 10^6 sperm, respectively) in $Adam1a^{+/+}$ mice. Thus, there is no apparent abnormality in spermatogenesis, sperm maturation, and ejaculation in the $Adam1a^{-/-}$ male mice.

We next examined the migration of ejaculated sperm from the uterus into the oviduct through UTJ. As shown in Fig. 2, microscopic analysis of frozen sections of UTJ 2 h after copulation showed the presence of $Adam1a^{+/+}$ sperm in the uterus and colliculus tubarius, a small segment of the oviduct that protrudes into the uterine lumen (33). However, $Adam1a^{-/-}$ sperm were not detectable in the colliculus, and all of them remained in the uterus. The phenotype of $Adam1a^{-/-}$ sperm is consistent with those of sperm lacking calmegin (10), tACE (12), and ADAM2 (14). Thus, the loss of the ER-resident ADAM1a is associated with the ability of sperm to ascend into the oviduct through the UTJ.

To assess whether the absence of ADAM1a in the ER of TGC affects sperm/egg interaction, IVF assays were carried out using capacitated cauda epididymal sperm. $Adam1a^{-/-}$ sperm showed a significant delay in the dispersal of cumulus cells from the cumulus mass surrounding the eggs (Fig. 3A). The delayed dispersal of cumulus cells may reflect the impaired ability of $Adam1a^{-/-}$ sperm to hydrolyze hyaluronic acid in the extracellular matrix of cumulus cells. However, sperm hyaluronidase PH-20 (27, 34) was normally present on the sperm surface of $Adam1a^{-/-}$ mice, as described below. When cumulus-free, ZP-intact eggs were used, sperm binding to the ZP was severely reduced by the loss of ADAM1a (Fig. 3B). Unexpectedly, $Adam1a^{-/-}$ sperm were capable of fertilizing cumulus-intact, ZP-intact eggs normally (Fig. 3C), despite the delayed dispersal of cumulus cells (Fig. 3A) and the impaired ability to bind the ZP (Fig. 3B). Furthermore,
no significant difference in the fusion of sperm with ZP-free eggs was found between $Adam1a^{++}$ and $Adam1a^{--}$ mice (Fig. 3D). Therefore, we conclude that the infertility of $Adam1a^{--}$ male mice is caused by the inability of sperm to ascend into the oviduct.

ADAM1 and ADAM2 are both synthesized as precursors in TGC, and converted into the mature forms during the transit of testicular sperm into the epididymis and/or once in the epididymis (21, 25, 35). The deficiency of ADAM2 has been reported to result in the reduction and loss of ADAM1 in TGC and epididymal sperm, respectively (14, 16), thus implying the importance of forming the fertilin protein complex between these two ADAMs during spermatogenesis. Calmegin, a homologue of the molecular chaperone calnexin, is present on the ER membrane of TGC, and is required for the heterodimerization between ADAM1 and ADAM2 (11). Because two forms of ADAM1, ADAM1a and ADAM1b, exist in mouse TGC (20, 21), we examined whether the loss of ADAM1a influences the formation of the fertilin protein complexes in TGC, and the appearance of proteins required for fertilization on the sperm membrane.

Western blot analysis indicated that six testis (sperm)-specific proteins, including ADAM3, PH-20, tACE, and calmegin, are normally present in TGC of $Adam1a^{--}$ mice, although only ADAM3 was severely reduced in $Adam1a^{--}$ sperm (Fig. 4A). The levels of ADAM1b, ADAM2, PH-20, and tACE were similar when $Adam1a^{++}$, $Adam1a^{+-}$, and $Adam1a^{--}$ sperm were compared. When the formation of heterodimeric protein complexes between the precursors of ADAM1a or ADAM1b and ADAM2 in TGC was examined, 200-kDa ADAM1a/ADAM2 fertilin was the only complex missing in $Adam1a^{--}$ mice (Fig. 4B). In some experiments, we found that a 110-kDa precursor form of ADAM3 is synthesized in postmeiotic TGC, processed into the 42-kDa mature protein probably via a 50-kDa intermediate form during sperm maturation in the epididymis, and localized on the surface of cauda epididymal sperm (data not shown). Because the
ADAM3 precursor is normally produced in Adam1a<sup>+/−</sup> TGC (Fig. 4A), the reduced level of mature ADAM3 in Adam1a<sup>+/−</sup> sperm is presumably due to the insufficient transport of the ADAM3 precursor from the ER to the cell surface during spermatogenesis. Thus, the formation of the fertilin complex between ADAM1a and ADAM2 in TGC may be involved in the appearance of mature ADAM3 on the sperm surface. Moreover, our results support the possibility that ADAM3 is one of the key molecules directly involved in the binding of sperm to the egg ZP, since ADAM1a-, ADAM2-, and ADAM3-deficient mouse sperm, which all show a defect in binding to the ZP, also all have in common that they lack ADAM3 (14-16).

To further explore the functional roles of fertilin complexes in TGC, protein extracts from TGC and epididymal sperm of mice lacking ADAM2 (Adam2<sup>−/−</sup>) or ADAM3 (Adam3<sup>−/−</sup>) were analyzed by Western blotting. Compared to wild-type mice, the level of ADAM1b was reduced in TGC of Adam2<sup>−/−</sup> mice despite the presence of ADAM1a, ADAM3, and PH-20 (Fig. 4C). Neither fertilin complex between ADAM1a and ADAM2 nor between ADAM1b and ADAM2 was found in Adam2<sup>−/−</sup> TGC due to the loss of ADAM2 (Fig. 4D). Also, Adam2<sup>−/−</sup> sperm completely lacked ADAM1b and barely contained ADAM3 (Fig. 4C), as described previously (16). In TGC and epididymal sperm of Adam3<sup>−/−</sup> mice, only ADAM3 was missing, while the levels of three other proteins were similar between wild-type and Adam3<sup>−/−</sup> mice (Fig. 4C). The ADAM1a/ADAM2 and ADAM1b/ADAM2 fertilin complexes were formed in Adam3<sup>−/−</sup> TGC (Fig. 4D). These data suggest that the appearance of mature ADAM3 on the sperm surface may depend upon the presence of the ADAM1a/ADAM2 fertilin complex in TGC. Moreover, the formation of the heterodimeric protein complex between the ADAM1b and ADAM2 precursors in TGC may be essential for the localization of the mature forms of these two ADAMs on the sperm surface.
The severely reduced levels of ADAM3 in Adam1a<sup>−/−</sup> and Adam2<sup>−/−</sup> sperm (16) led us to postulate a direct association of ADAM3 with ADAM1a, ADAM1b, or ADAM2, and with ADAM1a/ADAM2 or ADAM1b/ADAM2 fertilin complex in TGC. To investigate this possibility, we carried out immunoprecipitation analysis of protein extracts from TGC of wild-type and three mutant mice using antibodies against ADAM1a, ADAM1b, ADAM2, and ADAM3 (Fig. 5). In TGC of the wild-type and Adam3<sup>−/−</sup> mice, ADAM2 was immunoprecipitated with antibodies against both ADAM1a and ADAM1b, while the immunoprecipitate with anti-ADAM2 antibody contained ADAM1a and ADAM1b in addition to ADAM2. Only ADAM1b and ADAM2 were immunoprecipitated from Adam1a<sup>−/−</sup> TGC with anti-ADAM1b or anti-ADAM2 antibody. These data are consistent with the presence of ADAM1a/ADAM2 and ADAM1b/ADAM2 fertilin complexes in both wild-type and Adam3<sup>−/−</sup> TGC, and with the absence of only ADAM1a/ADAM2 fertilin and both fertilins in Adam1a<sup>−/−</sup> and Adam2<sup>−/−</sup> TGC, respectively (Figs. 4B and 4D). However, no ADAM3 was immunoprecipitated with antibody against ADAM1a, ADAM1b, or ADAM2 (Fig. 5), indicating that ADAM3 is incapable of forming a protein complex with ADAM1a, ADAM1b, and ADAM2 in TGC.

Although many ADAM members exclusively expressed in the testis have been identified, little is known of their roles in TGC during spermatogenesis. We show here a link between the loss of ER-resident ADAM1a (ADAM1a/ADAM2 fertilin) and the insufficient transport of ADAM3 onto the cell surface. This finding may provide a new viewpoint for the regulation of the localization of fertilization-related sperm proteins by fertilin in TGC. ADAM1a/ADAM2 fertilin may be implicated in the selective transport of specific sperm proteins, including ADAM3, that function in the sperm ascent into the oviduct (Fig. 2) and/or in the sperm penetration through the layer of cumulus cells (Fig. 3A). Our preliminary experiments indicated that ADAM1a and ADAM3 are partially
localized in lipid rafts of TGC (data not shown). The ADAM1a/ADAM2 fertilin complex may organize the membrane microdomain as a platform where the selected sperm proteins such as ADAM3 are present, even though this fertilin complex is incapable of interacting directly with ADAM3 in TGC (Fig. 5). ADAM1a/ADAM2 fertilin may be also associated with an unknown protein(s) mediating the transport of ER proteins. Regardless, the molecular mechanism of protein transport catalyzed by ADAM1a/ADAM2 fertilin in the ER of TGC remains to be further elucidated.

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1 The abbreviations used are: ZP, zona pellucida; tACE, a testis-specific isoform of angiotensin-converting enzyme; TGC, testicular germ cells; ER, endoplasmic reticulum; UTJ, uterotubal junction; neo, neomycin-resistance gene; tk, herpes simplex virus thymidine kinase gene; ES, embryonic stem; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; 2ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; IVF, in vitro fertilization; DAPI, 4'-6-diamidino-2-phenylindole.
FIGURE LEGENDS

FIG. 1. **Production of mice lacking ADAM1a.**  
**A**, targeting strategy of the mouse ADAM1a gene. A part of the protein-coding region (closed box) in the ADAM1a gene was replaced by the neomycin-resistant gene (*neo*, darkly shaded box). The herpes simplex virus thymidine kinase gene (*tk*, lightly shaded box) was included in the targeting construct for negative selection. Open boxes represent the 5’- and 3’-untranslated regions. Restriction enzyme sites indicated are as follows: E, *Eco*RI; G, *Bgl*II; K, *Kpn*I; S, *Sac*I; X, *Xho*I.  
**B**, Southern blot analysis of genomic DNA from wild-type (+/+, *Adam1a*+/+), heterozygous (+/−, *Adam1a*+/−), and homozygous (−/−, *Adam1a*−/−) mice for the targeted mutation of the ADAM1a gene. Genomic DNA was digested with *Eco*RI, separated by agarose gel electrophoresis, and subjected to Southern blot analysis using a 32P-labeled *Sac*I-*Xho*I fragment (S-probe, see Fig. 1A) as a probe. The wild-type and targeted alleles yielded 21- and 11-kbp DNA bands, respectively.  
**C**, Northern blot analysis of total cellular RNA from *Adam1a*+/+ (+/+), *Adam1a*+/− (+/−), and *Adam1a*−/− (−/−) testes. The blots were probed by a 32P-labeled DNA fragment containing the 3’-untranslated region of ADAM1a cDNA.  
**D**, Western blot analysis of protein extracts from testicular germ cells (TGC) of *Adam1a*+/+ (+/+), *Adam1a*+/− (+/−), and *Adam1a*−/− (−/−) mice, using affinity-purified anti-ADAM1a antibody. No immunoreactive protein corresponding to the 100-kDa ADAM1a precursor was found in TGC of *Adam1a*−/− mice.

FIG. 2. **Defect in sperm migration from uterus into oviduct.** Female mice, which had been superovulated by injection of human chorionic gonadotropin, were caged with wild-type (*Adam1a*+/+, *A* and *C*) or ADAM1a-deficient (*Adam1a*−/−, *B* and *D*) males, and the oviducts were excised with a connective part of the uterus 2 h after copulation.
Frozen sections were prepared and stained with hematoxylin. Arrows indicate sperm present in the uterine lumen (U) and colliculus tubarius (CT). The boxed regions in A and B are enlarged in C and D, respectively. Note that Adam1a<sup>−/−</sup> sperm are absent in the colliculus (CT), and all of them remain in the uterus (B and D).

FIG. 3. Characterization of ADAM1a-deficient mouse sperm. Capacitated epididymal sperm of wild-type (Adam1a<sup>+/+</sup>) and ADAM1a-deficient (Adam1a<sup>−/−</sup>) mice were used in all experiments. The numbers in parentheses indicate the numbers of eggs examined. A, delayed dispersal of cumulus cells from the cumulus mass surrounding eggs. Cumulus masses were inseminated by Adam1a<sup>+/+</sup> (open box) or Adam1a<sup>−/−</sup> (shaded box) sperm. After incubation, the eggs free from cumulus cells (pattern d, see Baba et al. 2002) were counted. Total numbers of 177 and 166 cumulus masses were examined for Adam1a<sup>+/+</sup> and Adam1a<sup>−/−</sup> sperm, respectively. Data are expressed as the means ± S.E., where n = 5. B, defect in adhesion/binding to egg zona pellucida. Cumulus-free, zona pellucida (ZP)-intact eggs were incubated with Adam1a<sup>+/+</sup> (+/+ or WT) or Adam1a<sup>−/−</sup> (-/-) sperm for 30 min, and the number of sperm bound to the ZP was counted. Data are expressed as the means ± S.E., where n = 4. C, fertilization with cumulus-intact, ZP-intact eggs in vitro. The unfertilized eggs were incubated with Adam1a<sup>+/+</sup> (+/+ or WT) or Adam1a<sup>−/−</sup> (-/-) sperm for 6 h. The egg possessing female and male pronuclei was defined as “fertilized egg”. Data are expressed as the means ± S.E., where n = 4. D, fusion with ZP-free eggs. The eggs were incubated with Adam1a<sup>+/+</sup> (+/+ or WT) or Adam1a<sup>−/−</sup> (-/-) sperm for 60 min. Data are expressed as the means ± S.E., where n = 4.

FIG. 4. Presence of testis (sperm)-specific proteins in ADAM1a-deficient mice. Proteins from testicular germ cells (TGC) and epididymal sperm of wild-type (+/+ or WT,
Adam1a<sup>+/+</sup>, heterozygous (+/-, Adam1a<sup>+/−</sup>), and homozygous (-/-, Adam1a<sup>−/−</sup>) mice were extracted with 1% Triton X-100, separated by SDS-PAGE under reducing (A, C) or non-reducing conditions (B and D), and subjected to Western blot analysis using antibodies against testis (sperm)-specific proteins indicated. The protein samples from male mice lacking ADAM2 (A2) or ADAM3 (A3) were also examined. A, testis (sperm)-specific proteins in TGC and sperm of Adam1a<sup>−/−</sup> mice. Only Adam1a<sup>−/−</sup> sperm showed a noticeable reduction of ADAM3. Note that ADAM1a and calmegin are both present specifically in the endoplasmic reticulum of TGC. B, formation of fertilin protein complexes in TGC of Adam1a<sup>−/−</sup> mice. An arrow indicates the location of the heterodimeric complex between ADAM1a and ADAM2. C, testis (sperm)-specific proteins in TGC and sperm of mice lacking ADAM2 or ADAM3. The levels of ADAM1b and ADAM3 were severely reduced in TGC and sperm of the ADAM2-deficient mice, respectively. D, fertilin complex formation in TGC of mice lacking ADAM2 or ADAM3. Neither ADAM1a/ADAM2 nor ADAM1b/ADAM2 complex was formed in the ADAM2-deficient mice.

FIG. 5. Immunoprecipitation analysis of protein extracts from testicular germ cells. Proteins from TGC of wild-type (WT), ADAM1a- (A1a), ADAM2- (A2), and ADAM3-deficient (A3) mice were immunoprecipitated (IP) with anti-ADAM1a, ADAM1b, ADAM2, or ADAM3 antibody, and then subjected to Western blot analysis using the above antibodies as probes. Anti-sp32 antibody and rabbit pre-immune serum were used as controls. Note that no immunoreactive signal was detected when the blots were probed by anti-sp32 antibody and pre-immune serum, and the immunoprecipitates from the four protein samples with anti-sp32 antibody contained only sp32.
| IP: ADAM1a | IP: ADAM1b | IP: ADAM2 | IP: ADAM3 |
|------------|------------|------------|------------|
| WTA1a A2 A3 | WTA1a A2 A3 | WTA1a A2 A3 | WTA1a A2 A3 |
| ADAM1a | | | |
| ADAM1b | | | |
| ADAM2 | | | |
| ADAM3 | | | | kDa
| 100 | 120 | 100 | 110 |
Possible function of the ADAM1a/ADAM2 fertilin complex in the appearance of ADAM3 on the sperm surface
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