Expression of TEX101, regulated by ACE, is essential for the production of fertile mouse spermatozoa

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Formation of spermatozoa of normal shape, number, and motility is insufficient for the male siring of pups. The spermatozoa must be accompanied by sound fertilizing ability. We found that males with disrupted testis-expressed gene 101 (*Tex101*) produce normal-looking but fertilization-incompetent spermatozoa, which were accompanied by a deficiency of a disintegrin and metallopeptidase domain 3 (ADAM3) on sperm plasma membrane. It was also found that the existence of TEX101 on spermatozoa was regulated by angiotensin-converting enzyme (ACE). The removal of GPI-anchored protein TEX101 by ACE was essential to produce fertile spermatozoa, and the function of ACE was not depending on its well-known peptidase activity. The finding of TEX101 as a unique specific substrate for ACE may provide a potential target for the production of an awaited contraceptive medicine for men.

fertility | cyritestin | germ-cell | GPIase | knockout

Angiotensin-converting enzyme (ACE) regulates blood pressure by its ability to cleave angiotensin I and bradykinin (1). Disruption of Ace gene causes low blood pressure, but simultaneously leads to male infertility (2, 3). ACE was reported to have a glycosylphosphatidylinositol (GPI)-anchored protein-releasing activity (other than its peptidase activity) using enhanced green fluorescent protein (EGFP)-tagged GPI and placental alkaline phosphatase (PLAP) as model substrates, and this distinctive enzymatic activity was postulated to be associated with male fertility (4). However, the putative GPI-anchored proteins that could serve as targets for ACE and be essential in fertilization remain unidentified. Among candidate GPI-anchored proteins found in spermatozoa, sperm adhesion molecule 1 (SPAM1) and protease, serine, 21 (PRSS21) were once considered to have important roles in fertilization, but KO mouse lines for the genes encoding these proteins showed normal fertility in natural conditions (5, 6).

TEX101 (encoded by testis-expressed gene 101, *Tex101*) is known as a germ cell-specific, GPI-anchored protein (7, 8) that appears on the plasma membranes of prospermatogonia in the fetal testis, but mostly disappears from epididymal spermatozoa (9, 10). However, other groups reported that TEX101 was found in lipid raft fractions of epididymal sperm membranes (11) and that an anti-TEX101 (NYD-SP8) antibody inhibited in vitro fertilization (IVF) in the mouse (12). To resolve these discrepancies and dissect the role of TEX101 as one of the GPI-anchored proteins of germ cells, we produced a *Tex101*-disrupted mouse line and investigated the effects on male fertility. As a result, we demonstrated that TEX101 was not found in mature spermatozoa, but was nevertheless required as an essential factor during the formation of fertile spermatozoa in spermiogenesis.

Results

Generation of *Tex101*$^{-/-}$ Mice. The existence of TEX101 on epididymal spermatozoa was controversial (9, 12), but by Western blotting, it was shown that TEX101 was a testicular germ-cell–specific protein (Fig. S1 *A* and *B*). We generated *Tex101* gene-disrupted mice using a targeting vector substituting exons 2 and 3 with a neomycin-resistant gene cassette in reverse orientation relative to the *Tex101* transcriptional unit (Fig. S1*C*). The correct targeting event in embryonic stem (ES) cells and germ-line transmission was confirmed by PCR analysis (Fig. S1*D*). Mating between heterozygous F1 mice yielded the expected Mendelian ratios of offspring: 18 wild-type (WT, $^{+/+}$), 32 heterozygous ($^{+/-}$), and 16 homozygous ($^{-/-}$) mutants. No overt developmental abnormalities were observed in *Tex101*$^{-/-}$ mice. The TEX101 protein disappeared in the *Tex101*$^{-/-}$ testis (Fig. S1*E*), but the testicular weights of *Tex101*$^{-/-}$ male mice (110.2 ± 19.1 mg; n = 12) were similar to those of *Tex101*$^{+/+}$ (105.5 ± 7.3 mg; n = 8) and *Tex101*$^{+/-}$ (103.5 ± 5.1 mg; n = 4) animals (Fig. S1*F*). Disruption of TEX101 caused no deleterious effect on testicular histology (Fig. S1*G*). Spermatozoa produced by the mutants appeared morphologically normal (Fig. S1*H*).

Infertility of *Tex101*$^{-/-}$ Male Mice. Adult *Tex101*$^{-/-}$ females and males were mated with WT males and females, respectively, for 2 mo. Whereas the *Tex101*$^{-/-}$ female mice were fertile, the *Tex101*$^{-/-}$ male mice were infertile, despite showing normal mating behavior with successful ejaculation and vaginal plug formation (Fig. 1*A*). Motility of the *Tex101*$^{-/-}$ mouse spermatozoa was assessed by the CEROS computer-assisted sperm analysis system (Hamilton Thorne, Inc.). When we applied the classification of five categories of motility patterns using CASAnova software (13), we found a tendency toward a greater percentage of hyperactivated spermatozoa in the *Tex101*$^{-/-}$ mice than in WT mice (Fig. S2*A*). The ratio of acrosome-reacted to acrosome-intact spermatozoa in *Tex101*$^{-/-}$ mice was similar to that in the WT mice spermatozoa during the first 90 min of incubation, but a statistically significant increase was observed after 180 min of incubation (Fig. S2*B*).

To exclude the possibility that the phenotype was caused by a side effect of genetic and/or epigenetic modifications in the *Tex101* locus, we performed a rescue experiment by generating transgenic mouse lines expressing *Tex101* on a *Tex101*$^{-/-}$ background (Fig. 1*B*). Expression of the transgene was examined by Western blotting in these mice. With a small amount of transgenically produced TEX101, a disintegrin and metallopeptidase domain 3 (ADAM3) reappeared on spermatozoa and concomitantly the infertile phenotype was rescued (litter size of 7.2 ± 2.3, number of litters = 18) (Fig. 1*C* and Fig. S3).

Detailed Analysis of Fertilizing Ability of Spermatozoa from *Tex101*$^{-/-}$ Mice. Cumulus-free eggs were prepared and mixed with spermatozoa from the *Tex101*$^{-/-}$ mice. We found that disruption of *Tex101* resulted in the loss of zona-binding ability by spermatozoa

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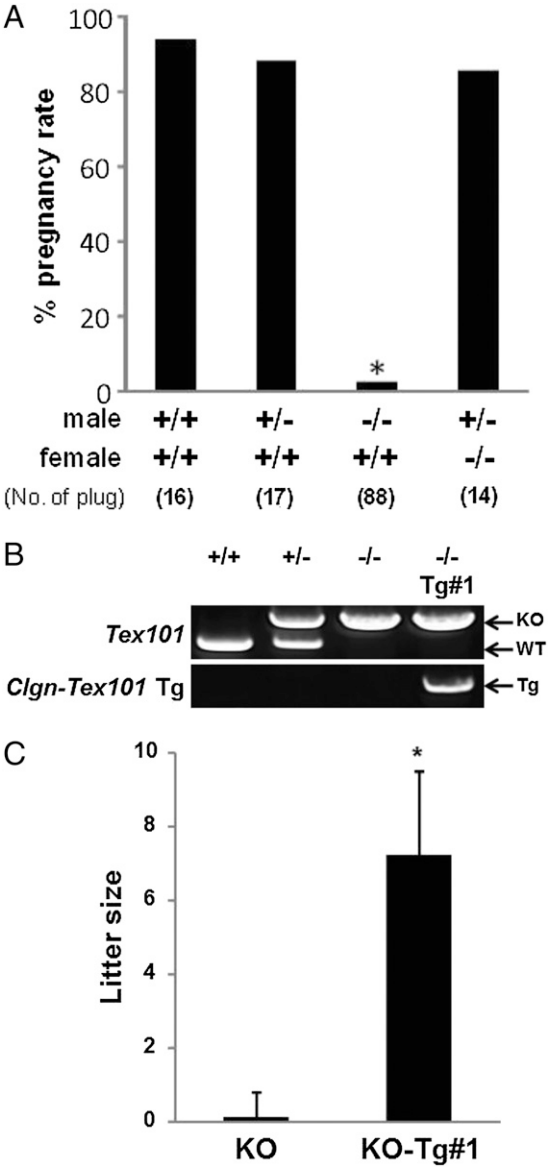

Fig. 1. Male-specific infertility in *Tex101*$^{-/-}$ mice. (*A*) *Tex101*$^{-/-}$ males copulated normally but failed to induce pregnancy. The total number of plugs observed is shown in parentheses. At least four males were used for each genetic background. Pregnancy rate is the number of pregnant mice/number with vaginal plugs. (*B*) *Tex101* transgenic (Tg) mice were genotyped by PCR. The sequences of primers used were: primer Pr.2, 5′–AGGAGGTGCAGTATACTGGATG–3′; Pr.4, 5′–TTGAGCGGGCCGCTTGCGCACTGG–3′; a 0.6-kb band was amplified by PCR analysis. KO, knockout; WT, wild-type. (*C*) The sterile phenotype of *Tex101*$^{-/-}$ male mice was rescued by the incorporation of a *Tex101* transgene onto their genetic background. Total number of litters, 18. KO, knockout; WT; Tg#1, transgenic #1.

(*Tex101*$^{+/+}$, 87.7 ± 13.3; *Tex101*$^{-/-}$, 1.4 ± 0.6 spermatozoa per egg; Fig. 2*A* and *B* and Movie S1). However, when we performed IVF using cumulus-intact eggs, both *Tex101*$^{+/+}$ and *Tex101*$^{-/-}$ spermatozoa showed fertilizing abilities as effective as that of WT spermatozoa (*Tex101*$^{+/+}$, 90.4 ± 5.8%; *Tex101*$^{-/-}$, 96.1 ± 3.4%; Fig. 2*C*). We then crossed the *Tex101*$^{-/-}$ mice with a transgenic mouse line, in which the acrosome could be visualized by EGFP and mitochondria by DsRed2 (14), and investigated the behavior of spermatozoa in vivo. When we observed the ejaculated spermatozoa through the wall of the female reproductive tract, we found equal amounts of spermatozoa from the uterus of females mated with *Tex101*$^{+/+}$ and *Tex101*$^{-/-}$ males, respectively. However, the latter spermatozoa were shown to have a defect in migrating into the oviduct (Fig. 2*D*).

Disappearance of ADAM3 in *Tex101*$^{-/-}$ Spermatozoa. To reconfirm that the cause of infertility of *Tex101*$^{-/-}$ mice was a failure of sperm migration into oviduct through the uterotubal junction (UTJ), we deposited capacitated spermatozoa (incubated in TYH medium (15) for 90 min) directly into the oviduct and observed if the infertility was rescued (16). When eggs were recovered from the oviducts 24 h later, 60.4 ± 14.0% (n = 111) of the eggs were fertilized by *Tex101*$^{-/-}$ spermatozoa, while 69.6 ± 26.3% (n = 46) were fertilized by WT spermatozoa (Fig. 2*E*). Healthy and fertile offspring were developed from eggs fertilized by *Tex101*$^{-/-}$ spermatozoa when transplanted to pseudopregnant females (Fig. 2 *F* and *G*). This characteristic phenotype was reminiscent of that seen in *Adam3*$^{-/-}$ male mice (15).

We therefore investigated ADAM3 in *Tex101*$^{-/-}$ mice. ADAM3, expressed in haploid spermatids, was found to have disappeared from cauda epididymal spermatozoa of *Tex101*$^{-/-}$ mice (Fig. 3*A* and Fig. S4*B*). We then performed an immunoprecipitation (IP) analysis using testicular germ cell (TGC) extracts. As shown in Fig. 3*B*, ADAM3 was coprecipitated by TEX101 antibody. In wild-type mice, TEX101 was found abundantly in TGCs but mostly disappeared from epididymal spermatozoa (Fig. S1*B*). TEX101 was suggested to influence the fertilizing ability of spermatozoa through interaction with ADAM3 in testis.

ADAM3 on TGCs from *Tex101*$^{-/-}$ mice was degraded when trypsin was added extracellularly (Fig. S4*C*). This indicated that ADAM3 was delivered on sperm surface without the aid of TEX101. This was different from the cases of calreticulin 3 (*Calr3*) and protein disulfide isomerase-like, testis expressed (*Pdilt*) disruption in which ADAM3 failed to reach the surface of TGCs (16, 17). Therefore, the interaction of TEX101 with ADAM3 was assumed to take place on sperm surface rather than on ER membrane as in the case of CALR3 and PDILT disruptions.

The expression of various chaperones (CALMEGIN, CALR3, and PDILT), which were reported to function transferring ADAM3 onto sperm plasma membrane, is apparently not affected by the disruption of *Tex101* (Fig. S4*A*). The independency of TEX101 from these chaperones was also demonstrated by the unaffected presence of TEX101 in *Adam3*, *Calmegin*, *Calr3*, and *Pdilt* gene-disrupted mouse lines (Fig. S4*D*).

Disappearance of TEX101 from Spermatozoa Mediated by ACE. To investigate the mechanism of disappearance of ADAM3 from *Tex101*$^{-/-}$ spermatozoa, we examined the integrity of TEX101 in Ace$^{-/-}$ testis in which the localization of ADAM3 was reported to be impaired (15). GPI-anchored protein TEX101 disappeared from spermatozoa in wild-type mice (Fig. S1*B*). However, when Ace gene was disrupted, TEX101 remained on the spermatozoa (Fig. 4*A* and Fig. S5*A*).The association of TEX101 with ADAM3 was evident in Ace$^{-/-}$ TGCs (Fig. S5*B*), but aberrantly remaining TEX101 in testicular spermatozoa (and TEX101 in Ace$^{-/-}$ epididymal spermatozoa) no longer associated with ADAM3 examined by immune-precipitation experiments (Fig. S5 *C* and *D*). The dissociation was also indicated by the different distribution of TEX101 remaining in the detergent-enriched phase of sperm extract, while ADAM3 was found in the detergent-depleted phase (Fig. S5*E*).

The effect of ACE on TEX101 was also examined using 293T cells in vitro. TEX101 became undetectable when the cells were cotransfected with somatic type ACE (ACE-s), while sperm acrosome associated 4 (SPACA4) (18), a testis-specific, GPI-anchored protein used as a control, remained unaffected. We also noted the disappearance of TEX101 by testicular ACE (ACE-t). Since the membrane type ACE-t was not detectable after transfection, we used the soluble type. Both the E414D ACE-t and (H413K, H417K) ACE-t mutants were reported to lose peptidase activity (4, 19).

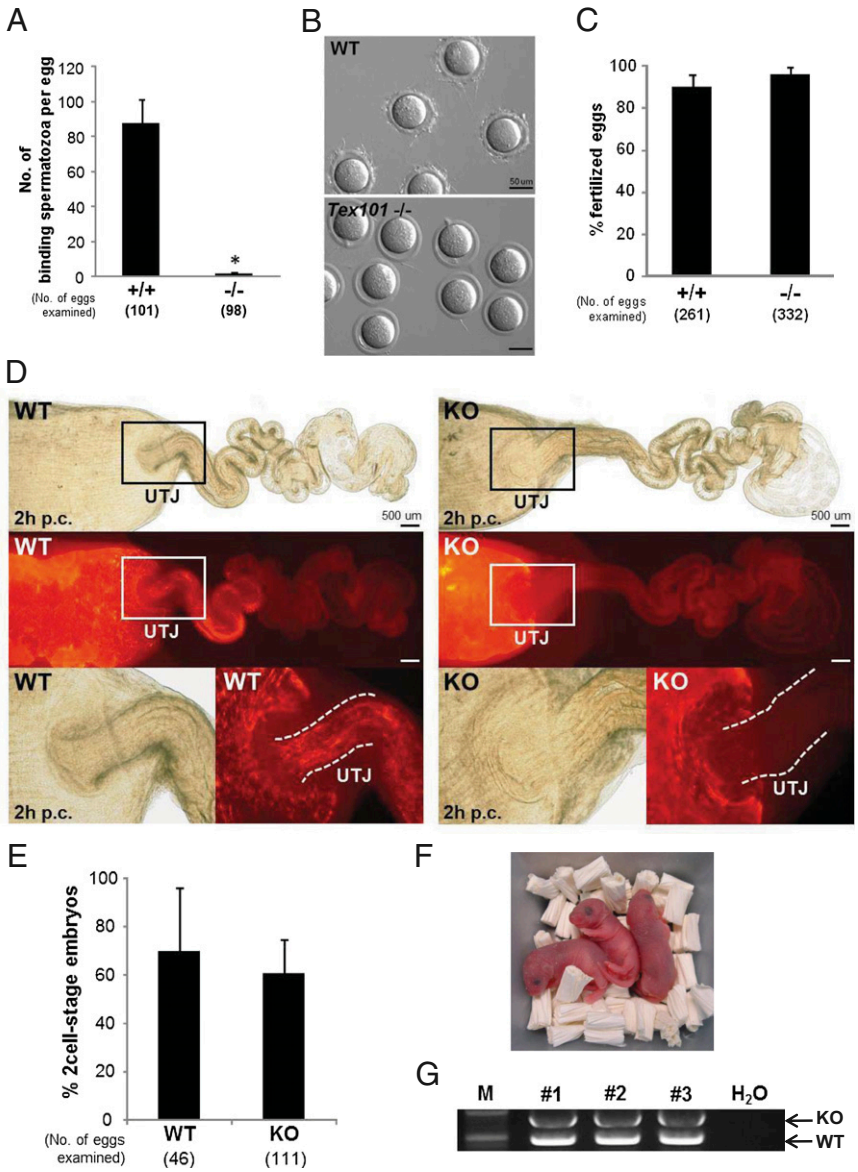

Fig. 2. Fertilizing ability of *Tex101*$^{-/-}$ spermatozoa. (*A*) The number of spermatozoa from *Tex101*$^{-/-}$ males adhering to the zona pellucida was decreased significantly despite frequent collisions. Mean ± SD ($n = 3$, $P < 0.001$). (*B*) Micrograph of zona-adhering spermatozoa using cumulus-free eggs (Movie S1). (Scale bar, = 50 μm.) (*C*) Spermatozoa from *Tex101*$^{-/-}$ males could fertilize cumulus-intact eggs. Mean ± SD ($n = 3$). (*D*) Uteri and oviducts from females mated with *Tex101*$^{+/+}$ and *Tex101*$^{-/-}$ males carrying fluorescent protein-tagged spermatozoa (*Materials and Methods*) indicated the failure of *Tex101*$^{-/-}$ spermatozoa to pass through the UTJ. Photos were taken 2 h after coitus. (*E*) Spermatozoa from *Tex101*$^{-/-}$ male mice showed normal fertilizing ability when capacitated spermatozoa were directly deposited in the oviducts of superovulated females with a fine glass needle under a dissecting microscope. Eggs were collected 24–27 h after the sperm deposition and two-cell stage embryos were judged as fertilized. KO, knockout; WT, wild-type. (*F*) Newborn pups obtained by fertilization using spermatozoa from "infertile" *Tex101*$^{-/-}$ male mice. (*G*) All of the pups derived from *Tex101*$^{-/-}$ spermatozoa and WT eggs had the *Tex101*-disrupted allele as expected. The primers used were Pr. 1, 2, and 3 (the same as in Fig. S1*D*). H_2O, water control; M, molecular weight markers.

Despite the loss of peptidase activity, these mutants were also shown to be effective in removing TEX101 from the 293T cells (Fig. 4*B*).

The removal of TEX101 was mediated by ACE in a substrate-specific manner. In wild-type mice, TEX101 became undetectable on spermatozoa, whereas it remained on spermatozoa in Ace$^{-/-}$ mice. This was in contrast with another testis-specific, GPI-anchored protein, SPACA4 (18), which always remained unaffected on spermatozoa in vivo (Fig. 4*A*).

Discussion

The infertile phenotype of *Tex101*$^{-/-}$ mice was caused by the inability of spermatozoa to migrate into the oviduct. When we analyzed the sperm movement parameters by CEROS, we saw no difference in the motility and number of acrosome-reacted spermatozoa between the wild-type and *Tex101*$^{-/-}$ spermatozoa. We saw more hyperactivated spermatozoa in *Tex101*$^{-/-}$ mice, but the difference remained less than 10% of the entire population (Fig. S2), leading us to consider that motility is not the crucial cause of sperm inability to migrate into oviduct.

In terms of molecular weight measured by Western blotting, ADAM3 was processed normally as shown in caput epididymal *Tex101*$^{-/-}$ spermatozoa (Fig. 3*A*). However, it was not resistant to degradation during sperm maturation, and ADAM 3 disappeared in cauda epididymal spermatozoa (Fig. 3*A* and Fig. S4*B*);

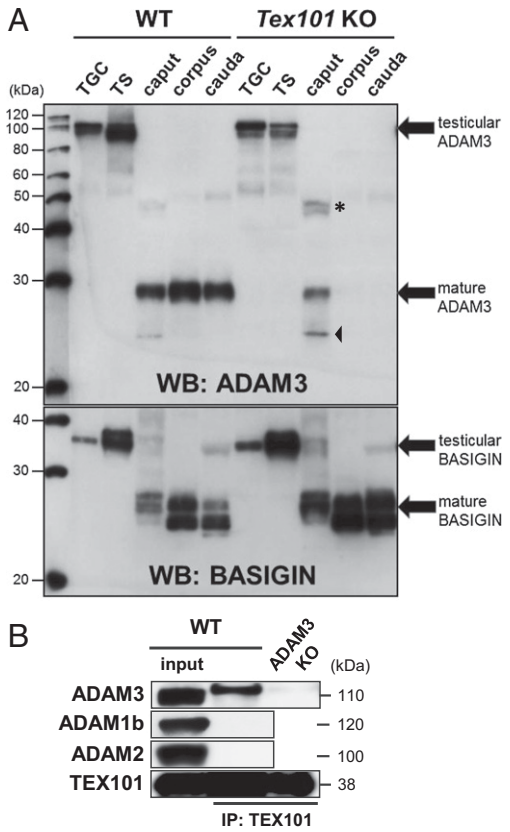

Fig. 3. Failure of ADAM3 to be processed into a mature form caused its disappearance from *Tex101*$^{-/-}$ spermatozoa. (*A*) A small amount of mature ADAM3 was detectable in *Tex101*$^{-/-}$ spermatozoa from the caput epididymis. However, a trace of ADAM3 (arrowhead) was also found in these spermatozoa, indicating an aberrant processing of this protein in the epididymis. The processing of BASIGIN used as a control was not impaired. To compensate for a reduced abundance of spermatozoa, 25 μg of extracts were loaded in the lanes for the caput epididymis, while 5 μg aliquots were used in the others. The asterisk indicates an IgG signal recognized by the secondary antibodies. TGCs, testicular germ cells; TS, testicular spermatozoa. (*B*) TGC extracts (500 μg) from both WT and *Adam3*$^{-/-}$ mice were immunoprecipitated and probed with antibodies against ADAM1b, ADAM2, ADAM3, and TEX101. TGC lysates (20 μg) were loaded as the input control. TEX101 associated with ADAM3, but not with ADAM1b or ADAM2.

moreover, the mice showed a severely subfertile phenotype (Fig. 1*A*). Thus, the role of TEX101 to form fertile spermatozoa seemed to stabilize ADAM3 from protease digestion in epididymis (Fig. S4*C*). As demonstrated in Fig. S1*B*, TEX101 was found almost exclusively in the TGC fraction (containing up to step 8—the spermatid stage—of spermatogenesis) (20) in wild-type mice. Although the existence of remnant TEX101 in cytoplasmic droplets of epididymal spermatozoa from BALB/c mice was indicated (9), such droplets were scarcely seen in the present experiment using spermatozoa from B6D2F1 mice and TEX101 was not detectable in epididymal spermatozoa (Fig. S1*H*).

CALMEGIN (21), CALR3 (16), PDILT (17), and ADAM1a (22) are testis-specific proteins and are not found on spermatozoa, but all of these proteins are required for presenting functionally active ADAM3 on the sperm plasma membrane (23). Since these proteins are considered to function as molecular chaperones, one could speculate that TEX101 is directly functioning as a class of ADAM3-specific molecular chaperones, or indirectly facilitating the folding of ADAM3.

How does the TEX101 relate to ACE? It has been reported that ACE has a GPI-ase activity in spermatozoa using SPAM1 and TESP5 as substrates (4). However, the activity was rather modest and two other groups reported that ACE's GPI-ase activity was irreproducible (24, 25). Moreover, both SPAM1- and TESP5-disrupted mouse lines showed no infertile phenotypes (5, 6). These reports question whether ACE works to produce fertile spermatozoa through its GPI-ase activity (4). However, if we consider that TEX101 (GPI-anchored protein) was the true target for ACE's GPI-ase activity, the removal of TEX101 by ACE could be reasonably explained. If the ACE has a GPI-ase activity, the specificity must be strict, because another testis-specific, GPI-anchored protein, SPACA4 (18), remained unaffected on spermatozoa in vivo (Fig. 4*A*) and in vitro (Fig. 4*B*). This substrate specificity might have hindered other laboratories from reproducing the ACE's GPI-ase activity. Although the disappearance of TEX101 from spermatozoa by ACE seemed to be an essential process in the production of fertile spermatozoa, the possibility remained that TEX101 might have influenced ADAM3 indirectly, or that TEX101 might not be the only protein modified by ACE for sound spermatogenesis (Fig. 4*A* and Fig. S5 *A* and *E*).

In conclusion, by producing a *Tex101*-disrupted mouse line, a molecular mechanism to form fertile spermatozoa in mouse moved one step ahead. This finding also provided the missing link between fertilization-related protein ADAM3 and ACE (Fig. 5). ACE is a peculiar protein having a highly conserved protein sequence among various species including fly (Fig. S6), which seemed to have no angiotensinogen. In this context, ACE's activity to form fertile spermatozoa, which is apparently

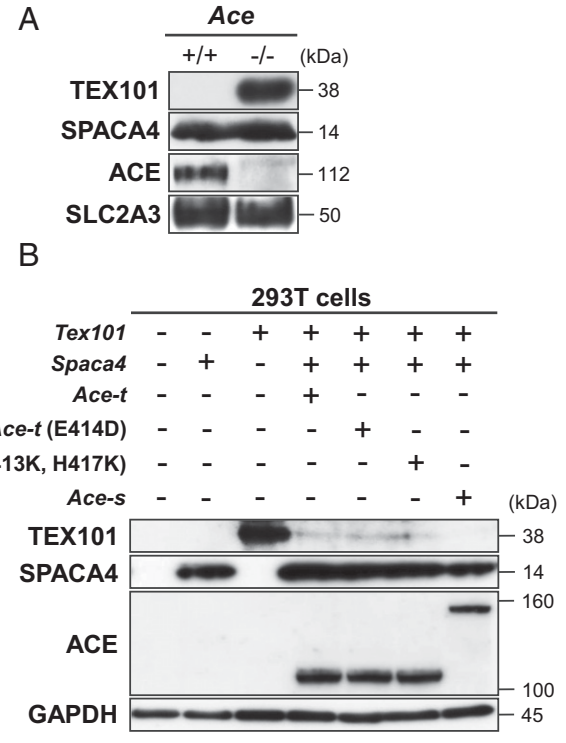

Fig. 4. Shedding of TEX101 did not take place in the Ace$^{-/-}$ testis. (*A*) TEX101 remained on cauda epididymal spermatozoa in Ace$^{-/-}$ mice, while TEX101 became undetectable from WT spermatozoa. Another testis-specific GPI-anchored protein, SPACA4 (18), used as a control, was not removed from the spermatozoa in both WT and Ace$^{-/-}$ mice. A sperm tail protein, SLC2A3, was used as a loading control. (*B*) *Tex101*, Ace, and *Spaca4* were expressed transiently in 293T cells with expression vectors driven by a *CAG* promoter. TEX101 was removed from the 293T cells by the coexpression, while the level of simultaneously expressed SPACA4 remained unchanged. Aliquots of 20 μg of whole cell extracts were loaded in each lane.

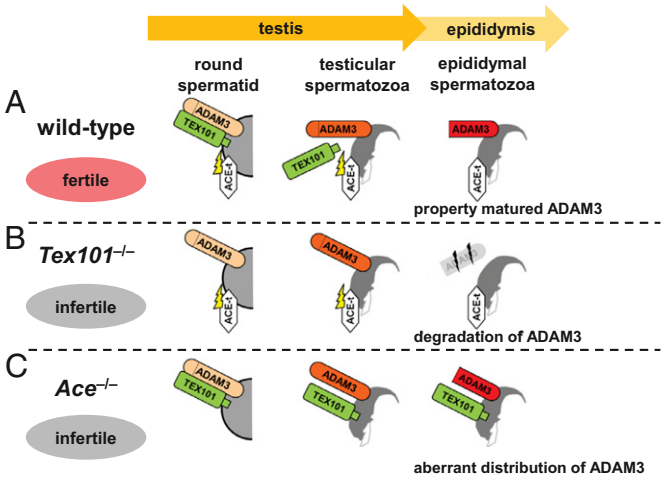

Fig. 5. TEX101, ADAM3, and ACE on spermatozoa during mouse spermatogenesis. (*A*) In round spermatids, TEX101 and an immature form of ADAM3 were both localized on the plasma membrane and could be coprecipitated by antibodies (Fig. 3*B*). This association must have been essential for ADAM3 (expressed from pale orange to dark orange) to be spliced properly to form a mature ADAM3 (expressed in red) on spermatozoa. (*B*) Without TEX101, the males became infertile (Fig. 1*A*), and ADAM3 (gray) on epididymal spermatozoa was totally degraded during epididymal maturation of spermatozoa (Fig. 3*A*). (*C*) The removal of TEX101 was mediated by the presence of ACE (Fig. 4*A*). When TEX101 remained on spermatozoa by ACE disruption, the localization of ADAM3 on epididymal spermatozoa became aberrant, and the males became infertile probably due to the production of nonfunctional ADAM3 (26). The reason for the dissociation of TEX101 with ADAM3 in $Ace^{-/-}$ testicular spermatozoa is unknown. TEX101 might not be the only protein modified by ACE for sound spermatogenesis.

independent from its well-known dipeptidase activity, might be indicating the more versatile function of ACE common in a variety of animals.

Materials and Methods

Animals. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University. The *Tex101*-disrupted mouse line has been submitted to RIKEN BRC (www.brc.riken.jp/inf/en/index) and is available to the scientific community.

Antibodies. The monoclonal antibodies used here were as described: TES101 for TEX101 (8), 1D5 for ACE, KS64-10 for solute carrier family 2, member 3 (SLC2A3), KS64-125 for IZUMO1, KS107-158 for ADAM1b, and KS139-281 for SPACA4 (16, 26). The others were purchased from Chemicon (7C1 for ADAM3 and 9D for ADAM2) and Santa Cruz Biotechnology (sc-9757 for BASIGIN and sc-25778 for GAPDH). Rabbit antisera against CALMEGIN, CALR3, PDILT, and PMIS2 were as described (16, 17, 27). The anti-ADAM5 polyclonal antibody was kindly provided by Dr. Chunghee Cho (Gwangju Institute of Science and Technology, Gwangju, Korea) (28).

Fertility Testing and Sperm Migration Assay in *Tex101*$^{-/-}$ Mice. Sexually mature male mice of the *Tex101*$^{+/+}$, $^{+/-}$, and $^{-/-}$ genotypes were caged with (C57BL/6N × DBA/2) F1 (also known as B6D2F1) female mice (>2 mo old) for 2 mo, and the number of pups in each cage was counted within a week of birth. Copulation was confirmed by checking for vaginal plugs every morning. Sperm migration analysis was performed as described (14, 15).

Sperm-ZP Binding Assay. Sperm-zona pellucida (ZP) binding assay was performed as described previously (26). Briefly, after mixing with spermatozoa, eggs were fixed with 0.25% glutaraldehyde and the bound spermatozoa were observed with an Olympus IX-70 fluorescent microscope after Hoechst 33258 staining.

IVF. IVF using mouse spermatozoa was performed as described previously (17).

Immunoblotting. Immunoblot analysis was performed as described previously (26).

IP. IP was performed as described previously (17).

Sperm Deposition into the Oviduct. Sperm deposition into oviducts was performed as described previously (29). After 24–27 h, eggs were collected by flushing the oviducts and fertilized eggs were implanted into the oviducts of a pseudopregnant female mouse (30).

Transfection of 293T Cells. Transient transfection into 293T cells with plasmid DNA was performed as a standard transfection method using calcium phosphate. Total cell lysates for 44–48 h posttransfection were used for Western blotting.

Statistical Analysis. All values are shown as the mean ± SD of at least three independent experiments. Statistical analyses were performed using Student *t* test inserted in Exel after the data were tested for normality of distribution.

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