Correction

DEVELOPMENTAL BIOLOGY

Correction for "Acrosin is essential for sperm penetration through the zona pellucida in hamsters," by Michiko Hirose, Arata Honda, Helena Fulka, Miwa Tamura-Nakano, Shogo Matoba, Toshiko Tomishima, Keiji Mochida, Ayumi Hasegawa, Kiyoshi Nagashima, Kimiko Inoue, Masato Ohtsuka, Tadashi Baba, Ryuzo Yanagimachi, and Atsuo Ogura, which was first published January 21, 2020; 10.1073/pnas.1917595117 (*Proc. Natl. Acad. Sci. U.S.A.* **117**, 2513–2518).

The authors note that on page 2517, left column, third full paragraph, line 25, "(500 V/cm, 50-ms duration, 5-ms intervals)" should instead appear as "(500 V/cm, 5-ms duration, 50-ms intervals)."

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Acrosin is essential for sperm penetration through the zona pellucida in hamsters

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During natural fertilization, mammalian spermatozoa must pass through the zona pellucida before reaching the plasma membrane of the oocyte. It is assumed that this step involves partial lysis of the zona by sperm acrosomal enzymes, but there has been no unequivocal evidence to support this view. Here we present evidence that acrosin, an acrosomal serine protease, plays an essential role in sperm penetration of the zona. We generated acrosin-knockout (KO) hamsters, using an in vivo transfection CRISPR/Cas9 system. Homozygous mutant males were completely sterile. Acrosin-KO spermatozoa ascended the female genital tract and reached ovulated oocytes in the oviduct ampulla, but never fertilized them. In vitro fertilization (IVF) experiments revealed that mutant spermatozoa attached to the zona, but failed to penetrate it. When the zona pellucida was removed before IVF, all oocytes were fertilized. This indicates that in hamsters, acrosin plays an indispensable role in allowing fertilizing spermatozoa to penetrate the zona. This study also suggests that the KO hamster system would be a useful model for identifying new gene functions or analyzing human and animal disorders because of its technical facility and reproducibility.

acrosin | fertilization | hamster

Mammalian spermatozoa deposited in the vagina or uterus ascend the female genital tract and penetrate the outer layers of the oocyte (cumulus oophorus and zona pellucida) before reaching its plasma membrane. It is generally assumed that spermatozoa penetrate the zona by mechanical force, aided by the acrosomal enzymes that are bound to the inner acrosomal membrane (1). A biophysical analysis suggested that the calculated force generated by the sperm alone is not sufficient to penetrate the zona mechanically (2). Of the many acrosomal enzymes, acrosin has been thought to be a major player in this process, because of its strong hydrolyzing activity and widespread distribution in mammals (3, 4). Indeed, it was reported that anti-acrosin antibodies significantly decreased the incidence of in vivo fertilization in rabbits (5), and inhibition of acrosin by soybean trypsin inhibitor prevented human spermatozoa from penetrating the zona (6). However, in contrast to these findings, acrosin-deficient mouse spermatozoa could pass through the zona, although dispersion of the cumulus oophorus was delayed to some extent (7). It is possible that mouse spermatozoa are exceptional, in that they do not rely on acrosomal enzymes to penetrate the zona, because sperm acrosin activity is weaker in mice compared with in other mammalian species such as rats and hamsters (8). The recent advent of gene-editing technology has enabled the generation of knockout (KO) rats (9), and although Isotani et al. (10) successfully disrupted the acrosin gene in rats, acrosin-KO rat spermatozoa were able to penetrate the zona and fertilize oocytes. Thus, there has been no conclusive evidence for the involvement of acrosomal enzymes in mammalian sperm penetration through the zona.

The golden hamster (*Mesocricetus auratus*) is a small rodent that has been extensively used in biomedical research in fields including oncology, immunology, metabolic disease, cardiovascular disease, infectious disease, physiology, and behavioral and reproductive biology (11). Unlike laboratory mice and rats, which belong to the *Muridae* family of rodents, hamsters belong to the *Cricetidae* family. Hamsters have many advantages as a laboratory species, including small body size (between mice and rats), short gestation period (16 d), large litter size (5 to 10 pups), and a very stable 4-d estrous cycle (12). Indeed, the golden hamster is the species in which in vitro fertilization (IVF) using epididymal spermatozoa was first reported (13). The large acrosome of hamster spermatozoa enables researchers to observe the acrosomal reaction in live spermatozoa under a phase-contrast microscope (14, 15). However, hamster embryos are highly vulnerable to in vitro conditions,

Significance

Mammalian oocytes are surrounded by the zona pellucida, a glycoprotein coat that protects the oocyte and embryo from mechanical damage during their preimplantation development within the oviduct. Fertilizing spermatozoa must penetrate the zona, but we do not know the exact mechanisms underlying this process. Sperm proteases were thought to work as zona lysins, but gene-knockout studies in mice did not support this assumption. In this study, we generated hamsters without acrosin, the major acrosomal protease, to examine its role in both in vivo and in vitro fertilization. Surprisingly, mutant male hamsters were completely infertile because their spermatozoa were unable to penetrate the zona. We thus demonstrated that, at least in hamsters, acrosin is essential for sperm penetration through the zona.

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which has hindered the generation of gene-modified hamsters (16). To circumvent this obstacle, we employed a recently developed in vivo gene-editing system (improved genome-editing via oviductal nucleic acids delivery system; *i*-GONAD) (17) to generate gene-KO hamsters. This enabled us to bypass all of the in vitro embryo-handling steps, thus making generation of KO hamsters technically easier and highly reproducible. The present study aimed to determine whether acrosin is essential for sperm penetration through the zona by investigating how acrosin-KO hamster spermatozoa behaved both in vivo and in vitro.

Results

Generation of Acrosin-Deficient Hamsters. We designed six single-guide RNAs (sgRNAs) that targeted the sequences of either the 5′ or 3′ side of the catalytic domain of the hamster acrosin gene (*SI Appendix*, Fig. S1 and Table S1). We injected the six sgRNAs, together with Cas9 protein, into the oviducts of four females

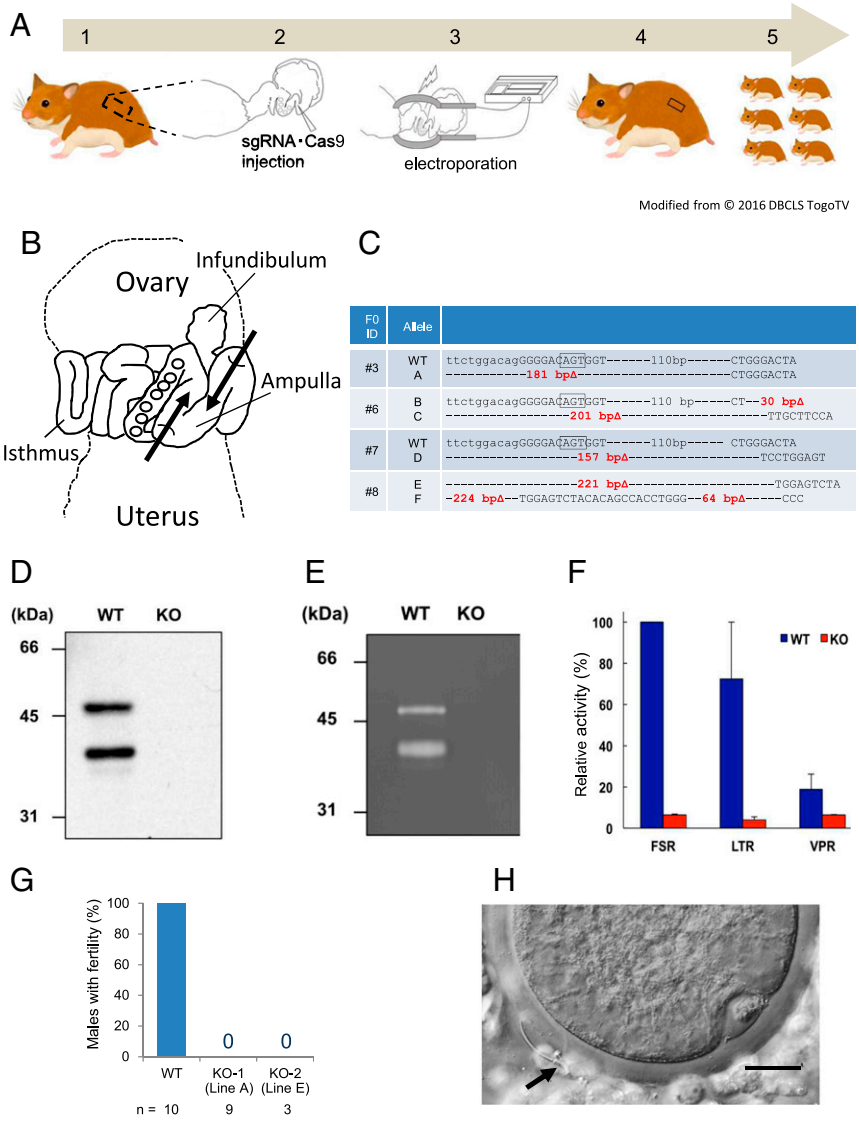

Fig. 1. Generation of acrosin-KO hamsters and biochemical analyses of spermatozoa. (*A*) Generation of KO hamsters by the GONAD method. After the solution containing sgRNAs and Cas9 protein was injected into the exposed oviducts containing zygotes (steps 1 and 2), electric pulses for in vivo transfection into zygotes were applied to the oviducts using a forceps-like electrode (step 3). After surgery, the females were allowed to deliver young (steps 4 and 5). (*B*) The position of sgRNAs and Cas9 injection. These were injected from the *Upper* segment of the ampulla (one of two arrows) toward the *Lower* segments where oocytes reside. (*C*) The mutant alleles found in four founders. Six mutant alleles were identified. For more information on the WT sequence, see *SI Appendix*, Fig. S1. (*D*) Western blot of sperm acid extracts from WT and acrosin-KO hamsters using affinity-purified antibody against the N-terminal 20 amino acids of mouse proacrosin. Acrosin-KO hamster spermatozoa completely lacked the 47- and 40-kDa forms of (pro)acrosin. (*E*) Gelatin zymography of sperm acid extracts. After SDS/PAGE in the presence of 0.1% gelatin, the gels were washed with 2.5% Triton X-100, incubated at pH 8.0 at 37 °C, and then stained with Coomassie brilliant blue. Gelatin-hydrolyzing proteins were detected as transparent bands against a blue background. Note that KO spermatozoa are completely devoid of gelatin-hydrolyzing proteins, whereas 47- and 40-kDa (pro)acrosins in WT spermatozoa exhibit the enzyme activity. (*F*) Acrosin activity in sperm acid extracts. Proteolytic activity of acrosin-KO sperm was measured using Boc-Phe-Ser-Arg-MCA (FSR), Boc-Leu-Thr-Arg-MCA (LTR), and Boc-Val-Pro-Arg-MCA (VPR) as substrates. Data are expressed as the mean ± SEM; $n = 3$. (*G*) Fertility test of acrosin-KO males. No homozygous acrosin-KO males from either of two lines carrying different mutant alleles produced offspring after mating with WT females. All WT males were confirmed to be fertile. (*H*) A cumulus–oocyte complex retrieved from the oviduct of a female that had been mated with an acrosin-KO male. A spermatozoon had reached the zona of an oocyte (arrow) but had not penetrated it. (Scale bar, 20 μm.)

on day 0.5 (the day after mating) and immediately applied electric pulses to the oviducts, using a forceps-like electrode (Fig. 1 *A* and *B*). On day 15.5, the females gave birth to a total of 15 pups, eight of which were weaned. Of these, five (two females and three males) carried mutant alleles, as demonstrated by genomic PCR using ear tissue (*SI Appendix*, Fig. S2). Genomic sequencing analysis identified six types of mutant alleles (alleles A to F) from four founders (F0 #3, #6, #7, and #8; Fig. 1*C*). We could not identify the sequence of each mutant allele in F0 #2 because of extensive mosaicism at the target region (*SI Appendix*, Fig. S2). We mated these four founders with wild-type (WT) hamsters and intercrossed the resultant F1 heterozygous KO hamsters to generate F2 homozygous KO hamsters. Because homozygous KO hamsters carrying allele A originating from F0 #3 (female) were the first obtained, they were used for establishment of the acrosin-KO line (line A) and subjected to a series of phenotypic analyses as described here.

Biochemical Analyses of Spermatozoa. To confirm that KO spermatozoa were devoid of acrosin, we analyzed them by Western blot, using as a probe polyclonal antibody raised against the N-terminal 20-mer oligopeptide of mouse proacrosin (8). As expected, two forms of hamster (pro)acrosin with approximate sizes of 47 and 40 kDa were found only in WT spermatozoa (Fig. 1*D*). These two proteins displayed gelatin-hydrolyzing activity (Fig. 1*E*). When the serine protease activities of sperm acid extracts were measured using three *t*-butyloxycarbonyl (Boc)-dipeptidyl-Arg-4-methylcoumaryl-7-amide (MCA) as substrate, KO spermatozoa exhibited a negligible level of enzyme activity (Fig. 1*F*). Thus, these data demonstrate the absence of acrosin in KO spermatozoa.

Homozygous Acrosin-KO Males Are Completely Sterile. Heterozygous acrosin-KO males and females showed normal reproductive performance, as confirmed by their efficient production of offspring. We next examined the fertility of homozygous acrosin-KO males. Nine F2 to F4 males homozygous for acrosin mutations were mated with three WT females each for 2 wk. Ten WT males served as controls. None of the females mated with KO males became pregnant, but all the females mated with WT males became pregnant (Fig. 1*G*). We obtained similar results with another KO line (line E carrying the mutant allele E derived from F0 #8; Fig. 1*G*). These results indicated that acrosin-KO males were completely sterile. Then we examined the behavior of KO spermatozoa within the female genital tract after natural mating. We found spermatozoa within the matrix of the cumulus oophorus (Movie S1) and on the surface of the zona pellucida (Fig. 1*H*) after females were mated with acrosin-KO males. However, there were no spermatozoa within the perivitelline space. Thus, acrosin-KO spermatozoa ascended the uteri and the oviducts normally after mating, but did not penetrate the oocyte zona pellucida.

Acrosin-KO Spermatozoa Have Normal Motility and Can Undergo the Acrosome Reaction. Failure of the acrosome reaction and/or poor motility of spermatozoa are common causes of male infertility. We therefore examined the behavior of the acrosin-KO spermatozoa in vitro. First, we analyzed sperm motility by computer-assisted sperm analysis and found that acrosin-KO spermatozoa were indistinguishable from WT spermatozoa for all parameters examined (Fig. 2*A* and *SI Appendix*, Table S2). We then examined the ability of the spermatozoa to undergo the acrosomal reaction by incubating them in acrosome reaction-inducing medium containing high concentrations of Ca^{2+} (3.4 mM) and BSA (15 mg/mL). Only live, motile spermatozoa were counted because dead spermatozoa often lose their acrosomes. In both WT and KO groups, the acrosome reaction started in a small population of spermatozoa (0% to 2%) at 3 h and was complete in the majority (69% to 77%) by 5 h (Fig. 3*A*). This implied that acrosin plays no essential role in the initiation of the acrosome

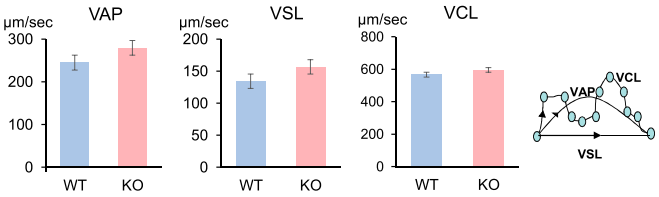

Fig. 2. Sperm motility of acrosin-KO spermatozoa. Three motility parameters of spermatozoa, VAP (average path velocity), VSL (straight-line velocity), and VCL (curvilinear velocity), were not affected by acrosin KO. For other parameters, see *SI Appendix*, Table S1.

reaction. The acrosome status of live hamster spermatozoa was easily determined under a phase-contrast microscope (Fig. 3*B*). We found that acrosin-KO spermatozoa had a protrusion in the acrosome cap region, which disappeared during the acrosome reaction (Fig. 3*B*). Transmission electron microscopic examination revealed that this protrusion was caused by a partial swelling of the acrosome, not by the formation of an additional space beneath the plasma membrane (Fig. 3*C*). Nonetheless, it did not seem to disturb the acrosome reaction because acrosome-reacted KO spermatozoa were indistinguishable from acrosome-reacted WT spermatozoa at both light microscopic (Fig. 3*B*) and electron microscopic levels (Fig. 3*D*).

Acrosin Is Essential for Sperm Penetration through the Zona. We then analyzed the fertilizing ability of acrosin-KO spermatozoa in vitro. First, cumulus-intact oocytes were inseminated with spermatozoa that had been preincubated for 2 h. About 5 h after insemination, most oocytes (about 90%) were fertilized by WT spermatozoa, whereas none were fertilized by acrosin-KO spermatozoa (Fig. 4 *A*–*C*). Acrosin-KO spermatozoa penetrated the cumulus cell layer and bound tightly to the zona pellucida in the same way as WT spermatozoa (Movies S2 and S3), but were never found within the perivitelline space. Next, to determine whether acrosin-KO spermatozoa could fuse with the oolemma, we removed the zona before insemination with KO spermatozoa. We found that all the oocytes ($n = 23$) were fertilized by KO spermatozoa, with multiple male pronuclei (Fig. 4 *A* and *D*). This means that acrosin is essential for sperm penetration through the zona, but not for the acrosome reaction or sperm fusion with the oolemma.

Discussion

Before gene-KO technology became available, it was expected that acrosin-deficient animals would be infertile, because many acrosin inhibitors prevented fertilization in vitro (6, 18, 19). Surprisingly, acrosin-KO mouse spermatozoa were fertile both in vivo and in vitro (7). Furthermore, mice lacking two acrosomal enzymes, acrosin and PRSS21, were also fertile (20). Acrosin-KO rats also showed no distinct phenotype, although they produced smaller litter sizes (10). Although these results implied that a zona lysin was unlikely to be involved in zona penetration by sperm, there was substantial evidence to support the presence of sperm-borne zona lysins: the presence of eroded holes on the zona surface near the attached spermatozoa (21) and the inability of acrosome-intact spermatozoa to penetrate the zona (22). Thus, the involvement of acrosomal enzymes in sperm zona penetration has not been completely excluded. In this study, we demonstrated that in the golden hamster, acrosin is essential for sperm penetration through the zona. Thus, the currently prevailing concept that acrosin is nonessential for fertilization in mammalian species must be reconsidered.

In general, gene-KO mice often show no obvious changes in phenotype, probably reflecting the redundancy of the particular gene function or the features of genes specific to mice. Gene KO

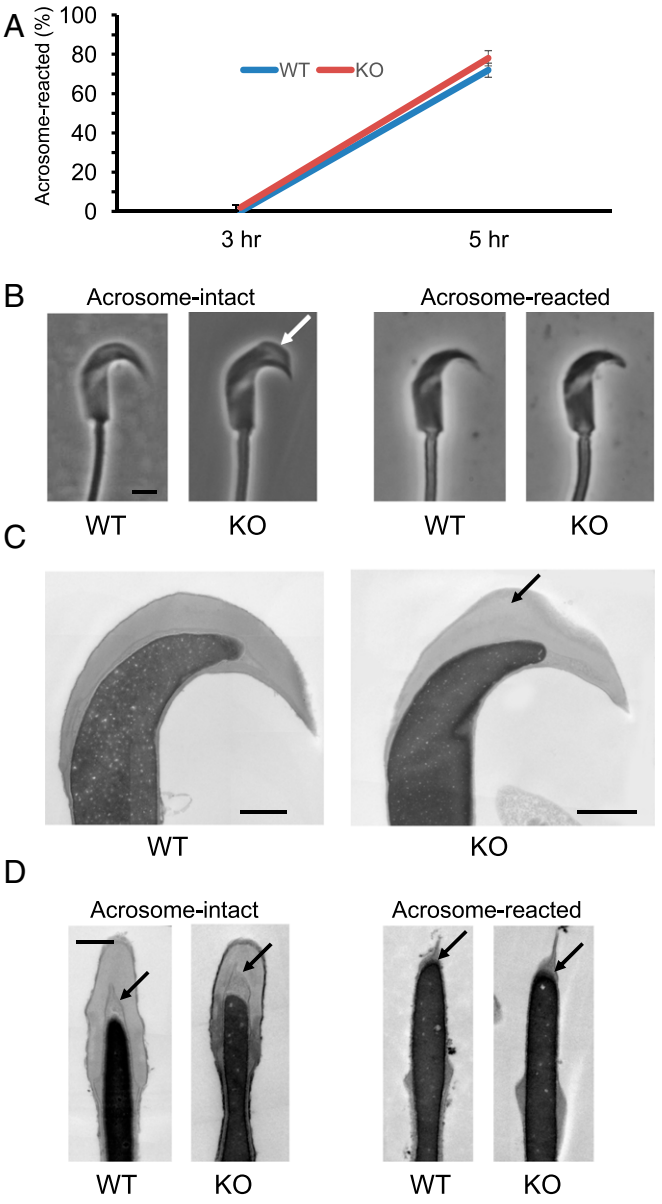

Fig. 3. The acrosomal status of normal (WT) and acrosin-KO spermatozoa. (*A*) The time course of the appearance of acrosome-reacted spermatozoa. Only live, motile spermatozoa were counted (51 to 161 sperm per observation). In both WT and KO sperm, the acrosome reaction started 3 h after the start of incubation and was completed in the majority of spermatozoa (69% to 77%) by 5 h. Results of two replicate experiments expressed as mean ± SEM $P < 0.05$ between the two groups at each point. (*B*) Phase-contrast micrographs of spermatozoa. Acrosin-KO spermatozoa had a protrusion in the acrosome before the acrosomal reaction (arrow). Bar, 2 μm. (*C*) Longitudinal sections of the head of acrosome-intact spermatozoa observed by transmission electron microscopy. The protrusion on the head of the spermatozoa corresponded to a partial enlargement of the acrosome (arrow). Bar, 1 μm. (*D*) Sagittal section of spermatozoa before and after acrosome reaction. Both WT and acrosin-KO spermatozoa exposed the inner acrosomal membrane (arrows) after the acrosome reaction. (Scale bar, 500 nm.)

in rats may have similar results to that in mice because of the phylogenetic closeness of the two species. In contrast, *Cricetidae* rodents (hamsters) diversified from *Muridae* rodents long before *Mus* (mouse) and *Rattus* (rat) emerged (23, 24). Therefore, if some physiological mechanisms underwent specific patterns of evolution in murine rodents, the related KO phenotypes could be different between murine rodents and other animals. Perhaps the mechanisms of fertilization are one such case. Indeed, the acrosome cap of mouse and rat spermatozoa is much smaller than those of many other rodent species (1), and acrosin-bound markers (e.g., enhanced green fluorescent protein) are necessary for clear visualization of their acrosome (25). This small acrosomal cap in mice and rats may be related to the lesser dependence of their spermatozoa on acrosin for fertilization. Interestingly, both acrosin-KO mice and rats showed a delayed sperm penetration of cumulus layers, implying that acrosin in these species functions on cumulus layers, not on the zona pellucida. In contrast, acrosin-KO hamster spermatozoa readily dispersed cumulus cells as WT spermatozoa at least in vitro. In mice, KO of many other fertilization-related factors such as hyaluronidase and fertilin also resulted in no or subtle changes to adult phenotypes (26). It is possible that hamsters and some other animals with large acrosome caps would have serious problems with fertilization when spermatozoa lack these substances.

Important questions relating to sperm acrosin are its intracellular location and its role in fertilization. In cattle and in humans, acrosin is present on the inner acrosome membrane of

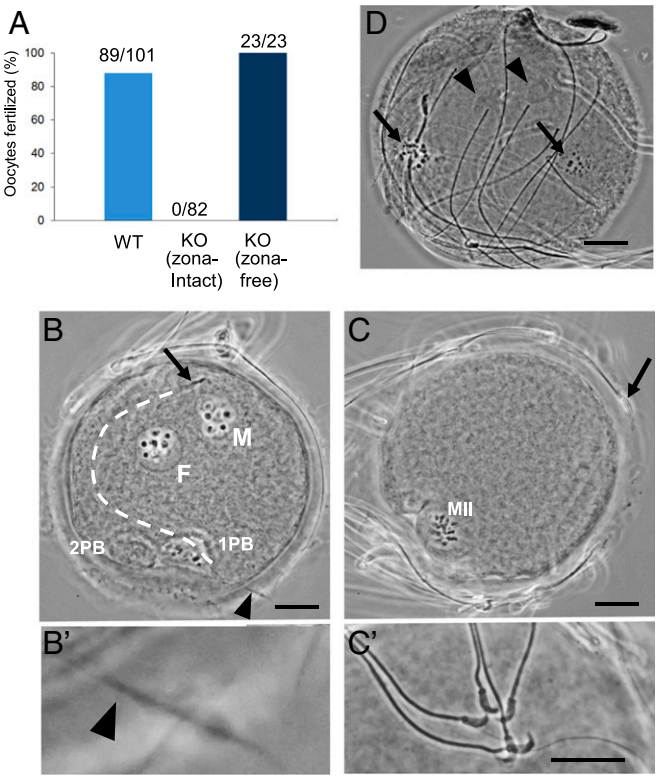

Fig. 4. Fertilizing ability of acrosin-KO spermatozoa assessed by IVF. (*A*) Although ~90% of oocytes were fertilized by WT spermatozoa, no oocytes were fertilized by acrosin-KO spermatozoa. When the zona was removed, all oocytes were fertilized by acrosin-KO spermatozoa. (*B*) An oocyte fertilized by WT spermatozoon. F, female pronucleus; M, male pronucleus; 1PB, first polar body; 2PB, second polar body; arrow, sperm tail in the egg cytoplasm in focus. The rest of the tail was out of focus (dotted line); tip of the sperm tail crossing the zona (arrowhead). (*B′*) High magnification of the tip of the sperm tail seen crossing the zona in *B*. (*C*) An unfertilized oocyte inseminated with acrosin-KO spermatozoa. Spermatozoa attached to the zona but did not penetrate it (arrow). MII, metaphase II chromosomes. (*C′*) Spermatozoa on the zona of the oocyte in *C*. They are all acrosome-reacted. (*D*) A zona-free oocyte inseminated with acrosin-KO spermatozoa. Arrows and arrowheads indicate male pronuclei and decondensing sperm heads, respectively. (Scale bar, 20 μm.)

spermatozoa after the acrosome reaction (27, 28). Membrane-bound acrosin may well serve as a zona lysin, as the sperm head advances through the zona pellucida. Although Yanagimachi and Teichman (29) and Yunes et al. (30) were unable to detect proteolytic activity on the inner acrosome membrane of acrosome-reacted hamster spermatozoa by cytochemical and immunocytochemical methods, the results of the present study have prompted us to reinvestigate this.

Our study may have broad implications in diverse fields of biology. Our hamster genome-editing system is technically easy and highly reproducible. Although the mouse KO system has contributed immeasurably to our understanding of physiology and pathology in general, it is not always perfect. We expect that KO hamsters could substitute for KO mice in the analysis of gene functions and the generation of new human disease models that have not been achieved in mice.

Materials and Methods

Animals. Golden (Syrian) hamsters purchased from Japan SLC, Inc. were housed under controlled lighting conditions (daily light period, 0700 to 2100) and provided with water and food ad libitum. All animal experiments were approved (T2019-J004) by the Animal Experimentation Committee at the RIKEN Tsukuba Institute and were performed in accordance with the committee's guiding principles.

Generation of KO Hamsters. Mature females were induced to superovulate by i.p. injection of 10 IU equine CG (eCG) at 0900 to 1200 on the day of conspicuous, postestrus vaginal discharge (day 1 of the estrous cycle), followed by mating with fertile males during the night of day 4 until the next morning (day 1 of pregnancy). The sgRNAs were designed using CRISPOR (http://crispor.tefor.net) and produced using a GeneArt Precision gRNA Synthesis Kit (#A29377; Thermo Fisher Scientific). Potential off-target sites in the golden hamster genome (MesAur1.0) were identified using the latest version of the CRIPSR Design Tool website (CRISPRdirect: http://crispr.dbcls.jp/). We confirmed that there was no potential off-target site containing one to two nucleotide mismatches with the 20-nt target sequence of the sgRNAs used. There is one sperm-related gene, RABL2B, in the vicinity of the Acrosin gene. This is known to be a risk factor for the fertilizing ability of spermatozoa in humans (31). We confirmed that the RABL2B expression in the testes was not affected by Acrosin gene deletion, as shown by quantitative RT-PCR using specific primers (*SI Appendix*, Fig. S3 and Table S1). *i*-GONAD was performed as described (17). Briefly, the solution contained six sgRNAs (*SI Appendix*, Table S1) and Cas9 protein (#1081059; IDT). Approximately 2.5 to 3.0 μL of solution was injected from the upper segment of ampulla toward the lower segments using a fine glass micropipette (Fig. 1*B*). After injection, the oviduct was covered with a piece of Kimwipe wetted with PBS and then pinched by a forceps-type electrode (#CUY650P5; NEPA GENE). Electroporation was performed using NEPA21 (NEPA GENE). The electroporation conditions consisted of three sequential poration pulses (500 V/cm, 50-ms duration, 5-ms intervals) followed by three transfer pulses (100 V/cm, 50-ms duration, 50-ms intervals). On day 16 of pregnancy, fetuses were delivered naturally and live pups were examined for CRISPR-Cas9-induced mutations at the target sites.

Identification of Mutations on the Acrosin Gene. Genomic DNA was extracted from small pieces of ear tissue from the pups. Approximately 400-bp genomic fragments containing the target site were amplified by PCR using primers (*SI Appendix*, Table S1) and 0.625 U of Tks Gflex (Takara Bio). PCR was performed under the following conditions: 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 1 cycle of 72 °C for 3 min. After confirmation of an indel mutation at the target site, PCR fragments were subcloned into a pGEM T Vector system (Promega) and sequenced for the determination of each allele.

Western Blot. Freshly excised epididymides were minced in PBS. Sperm were collected by centrifugation at 3,000 rpm for 5 min and extracted on ice for 2 h in 1 mM HCl solution containing 5 mM *p*-aminobenzamidine (15). After centrifugation at 12,000 rpm for 10 min, the supernatant solution was dialyzed against 1 mM HCl to remove *p*-aminobenzamidine and used as sperm acid extracts. Proteins were separated by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) under nonreducing conditions and transferred onto Immobilon-P polyvinylidene difluoride membranes (Merck Millipore). After being blocked with 2% skim milk, the blots were incubated with affinity-purified antibody against the N-terminal 20-mer oligopeptide of mouse proacrosin (8), and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The immunoreactive proteins were detected by an enhanced chemiluminescence Western blotting detection kit (GE Healthcare UK).

Gelatin Zymography. Proteins exhibiting gelatin-hydrolyzing activities in sperm acid extracts were visualized by SDS/PAGE in the presence of 0.1% gelatin under nonreducing conditions, as described previously (8). After electrophoresis, gels were washed twice with 0.1 M Tris·HCl at pH 8.0, containing 2.5% Triton X-100 at room temperature to remove SDS, and then incubated in the same buffer free of Triton X-100 at 37 °C overnight. The gelatin-hydrolyzing proteins were detected by staining the gels with Coomassie Brilliant Blue.

Measurement of Enzyme Activity. Proteolytic activity of acrosin in sperm acid extracts was measured using Boc-Phe-Ser-Arg-MCA, Boc-Leu-Thr-Arg-MCA, and Boc-Val-Pro-Arg-MCA as substrates (32). The reaction mixture (0.25 mL) consisted of 50 mM Tris·HCl at pH 8.0, 10 mM $CaCl_2$, 40 μM enzyme substrate (Peptide Institute), and sperm acid extracts (1 μg of protein). After incubation at 30 °C for 30 min, the reaction was terminated by addition of 0.1 M acetate buffer at pH 4.3 (0.75 mL). The amount of 7-amino-4-methylcoumarin formed from the substrates was measured fluorometrically with excitation at 380 nm and emission at 460 nm. One unit of the enzyme activity was defined as 1 nmol of 7-amino-4-methylcoumarin formed per minute under these conditions.

IVF. Actively motile spermatozoa for IVF were collected by the swim-up method. Briefly, about 2 μL sperm mass was collected from the cauda epididymis and placed at the bottom of a 15-mL round-bottom polystyrene tube. About 2 mL of modified TALP medium (33), which had been equilibrated in 5% CO_2 at 37 °C, was gently laid on the sperm mass. Approximately 2 to 3 min later, the upper 150 μL of the medium with actively motile spermatozoa was sucked up and transferred to a plastic dish, which was then covered with mineral oil. The spermatozoa were incubated for 3 h in 5% CO_2 at 37 °C before they were used for insemination. To collect mature unfertilized eggs, female hamsters (8 to 16 wk old) were each injected with 7.5 units of eCG, followed by 7.5 units of human CG 48 h later. Cumulus–oocyte complexes were collected from the oviducts 15 h after human CG injection and placed in 150-μL drops of modified TALP medium. They were inseminated with preincubated spermatozoa and kept in 5% CO_2 at 37 °C. The final concentration of spermatozoa in the insemination medium was ∼150 sperm/μL. About 5 to 6 h later, the oocytes were mounted and compressed between a slide and coverslip and fixed with 2.5% glutaraldehyde in cacodylate buffer, and their nuclear status was examined as described previously (34).

Zona-Free Oocyte IVF. To induce the acrosome reaction of spermatozoa, cauda epididymal spermatozoa were preincubated in modified TALP medium with higher concentrations of BSA (15 mg/mL) and Ca^{2+} (3.4 mM) for 5 to 6 h. At the end of this preincubation, about 70% of spermatozoa were acrosome-reacted and actively motile, irrespective of their genotype (Fig. 3*A*). Oocytes collected as described here were freed from both cumulus cells and the zona pellucida by treatment with 0.1% hyaluronidase and acid Tyrode's solution, respectively. After coculture with preincubated spermatozoa for 2.5 h, oocytes were examined for the presence or absence of the male pronucleus as described here.

Transmission Electron Microscopy of Spermatozoa. Spermatozoa were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 30 mM Hepes buffer containing 100 mM NaCl and 2 mM $CaCl_2$ (pH 7.4) for >2 h at room temperature, followed by postfixation in an aldehyde–OsO_4 mixture (1% OsO_4, 1.25% glutaraldehyde, 1% paraformaldehyde, and 0.32% $K_3[Fe\{CN\}_6]$ in 30 mM Hepes buffer [pH 7.4]) for 2 h. Fixed spermatozoa were washed three times with Milli Q water and stained en bloc with 0.5% uranyl citrate for 10 min. Stained spermatozoa were centrifuged and placed in Milli-Q-water-washed citrus pulp to facilitate later handling of spermatozoa. Each aliquot of citrus pulp containing fixed spermatozoa was washed with 50% ethyl alcohol, dehydrated in an ethanol series, and dipped in epoxy resin (Quetol 812, Nisshin EM). Sperm masses dissected out of the pulp were reembedded in the same resin. Each sample was sectioned at 80-nm thickness with an ultramicrotome (EM UC7; Leica). Sections were examined in a transmission electron microscope (JEM-1400; JEOL).

Sperm Motility Analysis. Cauda epididymal spermatozoa were collected and preincubated as described here for 2 h at 37 °C in 5% CO_2. The overall sperm motility, progressive motility, average path velocity, straight-line velocity,

curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, linearity, and straightness were assessed by computer-assisted sperm analysis, using a Hamilton Thorne IVOS computerized semen analyzer (Hamilton Thorne). All the parameters were measured in >200 spermatozoa in at least three different fields.

Statistical Analysis. The results from the sperm motility assay, enzyme reactivity test, and observation of the time course of the acrosome reaction were analyzed by two-way analysis of variance. The percentages were subjected to arcsine transformation before the statistical analysis. *P* values less than 0.05 were considered to indicate significance.

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