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Equatorin is not essential for acrosome biogenesis but is required for the acrosome reaction

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ABSTRACT

The acrosome is a specialized organelle that covers the anterior part of the sperm nucleus and plays an essential role in mammalian fertilization. However, the regulatory mechanisms controlling acrosome biogenesis and acrosome exocytosis during fertilization are largely unknown. *Equatorin* (*Eqtn*) is a membrane protein that is specifically localized to the acrosomal membrane. In the present study, the physiological functions of *Eqtn* were investigated using a gene knockout mouse model. We found that $Eqtn^{-/-}$ males were subfertile. Only approximately 50% of plugged females were pregnant after mating with $Eqtn^{-/-}$ males, whereas more than 90% of plugged females were pregnant after mating with control males. Sperm and acrosomes from $Eqtn^{-/-}$ mice presented normal motility and morphology. However, the fertilization and induced acrosome exocytosis rates of *Eqtn*-deficient sperm were dramatically reduced. Further studies revealed that the *Eqtn* protein might interact with Syntaxin1a and SNAP25, but loss of *Eqtn* did not affect the protein levels of these genes. Therefore, our study demonstrates that *Eqtn* is not essential for acrosome biogenesis but is required for the acrosome reaction. *Eqtn* is involved in the fusion of the outer acrosomal membrane and the sperm plasma membrane during the acrosome reaction, most likely via an interaction with the SNARE complex.

1. Introduction

The acrosome is a specialized organelle containing many hydrolytic enzymes that help sperm penetrate the zona pellucida (ZP) of oocytes. During the interaction between sperm and oocyte, the fertilizing spermatozoon must undergo a series of terminal morphological changes called the acrosome reaction (AR). In this process, the outer acrosomal membrane is disrupted, and the stored hydrolytic enzymes are released. The AR process is required for physiological fertilization, and an abnormal AR results in male reproductive defects [1]. Several genes have been reported to be involved in sperm acrosome exocytosis, including members of the SNARE family [2–4]; aSNAP [5]; NSF [6,7]; SNARE binding proteins, such as complexin [8,9]; and synaptotagmin calcium-binding proteins [7].

The *Equatorin* gene (also called *MN9* or *Afaf*) encodes a membrane protein that is specifically localized to the acrosomal membrane [10–12]. Previous in vitro studies showed that pretreatment of sperm with an anti-*Eqtn* antibody significantly reduced the in vitro fertilization (IVF) rate [13,14]. However, the exact in vivo physiological function of this gene is still unknown. To explore the physiological functions of *Eqtn* using an in vivo system, we obtained an *Eqtn* knockout mouse strain (Jackson Laboratory). We show that *Eqtn* is not involved in acrosome biogenesis but is required for the acrosome reaction and that the *Eqtn* protein might interact with SNAP25 and Syntaxin, which are core components of the SNARE complex.

2. Materials and methods

2.1. Animals

The *Eqtn* knockout mouse strain was purchased from Jackson Laboratory (Stock No. 007733) and maintained on a C57BL/6

Abbreviations: ZP, zona pellucida; AR, acrosome reaction; IVF, in vitro fertilization; HTF, human tubal fluid.

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background. *Eqtn*$^{-/-}$ mice were obtained by intercrossing *Eqtn*$^{+/-}$ males and females. All animal work was approved by the Committee on Animal Care at the Institute of Zoology, Chinese Academy of Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Zoology (Permit No. EET-015-03-2010). The mice were genotyped by PCR on DNA isolated from tail biopsies, and the presence of the *Eqtn* null allele was determined according to the recommendations of Jackson Laboratory.

2.2. Antibodies

The rabbit polyclonal anti-Eqtn antibody was produced by Yinchuan Li, as previously described [10]. The rabbit anti-Syntaxin1a antibody (ab118359) was purchased from Abcam, and the rabbit anti-SNAP25 antibody (3132-1) was purchased from Epitomics. The mouse anti-sp56 antibody (55101) was purchased from QED Biologicals, and the mouse monoclonal anti-β-tubulin was purchased from Sigma.

2.3. Tissue collection and histological analysis

The testes of *Eqtn*-deficient and control mice were dissected immediately after euthanasia, fixed in 4% paraformaldehyde for up to 24 h, stored in 70% ethanol, and embedded in paraffin. Sections were cut to a thickness of 5 μm and mounted on glass slides. After deparaffinization, the sections were stained with H&E for histological analysis.

2.4. Immunohistochemical analysis

Immunohistochemical analysis was performed using a Vectastain ABC (avidin–biotin-peroxidase) kit (Vector Laboratories, Burlingame, CA, USA) as recommended by the manufacturer. Deparaffinized sections were incubated with 10% goat serum in phosphate-buffered saline (PBS) for 30 min at room temperature and then incubated for 1 h at room temperature with the primary antibody. After being washed three times with PBS, the sections were incubated with a biotinylated secondary antibody (Santa Cruz Biotechnology) for 45 min at room temperature. After incubation with the avidin–biotin-peroxidase complex for 45 min, the sections were washed with PBS. The color was developed using 3,3′-diaminobenzidine substrate, and the sections were dehydrated and mounted with Permount and examined using a Nikon microscope and an Olympus CCD for image capture.

2.5. Immunofluorescence analysis

Mature sperm were collected from the cauda epididymis and washed in PBS. The sperm were spotted onto slides, dried at room temperature, and fixed with methanol at 20 °C for 30 s. The *Eqtn* polyclonal antibody was used at 1:200, and the sp56 polyclonal antibody was used at 1:500. The appropriate FITC- or TRITC- conjugated secondary antibodies were used, and the slides were counterstained with Hoechst 33342 (Sigma) and examined using a laser-scanning microscope (Zeiss).

2.6. Western blot analysis

Western blot analysis was performed as described previously [15]. Protein concentrations were estimated using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). The proteins were electrophoresed under reducing conditions in 12% SDS–PAGE gels and transferred to nitrocellulose membranes. The blots were then incubated with the primary antibody overnight at 4 °C, followed by a 1 h incubation at room temperature with an HRP-labeled secondary antibody (1:20,000; Li-COR Bioscience). Specific signals were detected using an Odyssey Infrared Imaging System and software (Version 3.0).

2.7. Transmission electron microscopy

Testes collected from control and *Eqtn*$^{-/-}$ males were fixed overnight with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and then washed in phosphate buffer, postfixed with 1.0% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in EPON/Araldite resin. Thin sections were cut using an Ultracut ultramicrotome (Leica) and stained with uranyl acetate and lead citrate.

2.8. In vivo and in vitro fertilization analysis

To evaluate the in vivo fertilization rate, control and *Eqtn*$^{-/-}$ males were mated with wild-type ICR females, and the fertilization rate was estimated using the percentage of 2-cell embryos at day 1.5 postcoitus. To evaluate the in vitro fertilization rate, mature sperm were collected from the cauda epididymis and capacitated for 2 h in HTF medium at 37 °C in a humidified incubator with 5% CO_2, 95% air. Highly motile sperm were collected from the upper portion of the medium. Female mice were superovulated, and stage MII oocytes were collected from the oviducts. The oocytes were incubated with the capacitated sperm at a final concentration of 2×10^6 sperm/ml in HTF medium for 6 h and then transferred to KSOM medium [16]. The IVF rate was measured based on the proportion of 2-cell embryos at 24 h after insemination. The data were analyzed using chi-square tests.

2.9. Acrosome reaction (AR) analysis

The A23187-induced acrosome reaction was analyzed as previously reported [17]. Briefly, mature sperm were collected from the cauda epididymis and capacitated for 2 h in HTF medium at 37 °C in a humidified incubator with 5% CO_2 and 95% air. Highly motile sperm were then collected from the upper portion of the medium, and the calcium ionophore A23187 (Sigma) was added (final concentration: 10 μM) to induce the acrosome reaction. 15 min later, the sperm were spotted on a glass microscope slide, dried at room temperature and fixed with methanol at 20 °C for 30 s. Intact acrosomes were stained with FITC-PNA, and the sperm nuclei were labeled with Hoechst 33342 [18]. Sperm that had undergone the acrosome reaction could not be labeled with FITC-PNA. More than 200 sperm were examined for all experimental conditions.

2.10. Co-immunoprecipitation (Co-IP) assay

Co-IP was performed as previously described [19]. Whole testis extracts were prepared with lysis buffer (10 mM Tris pH 7.4, 1.0% Triton X-100, 0.5% NP-40, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF) supplemented with protease inhibitors. After centrifugation, the supernatant was incubated with anti-SNAP25 and anti-Syntaxin1a antibodies overnight at 4 °C. Protein A/G beads (60 μl) were then added to each sample, and the mixtures were incubated at 4 °C for 1 h. The beads were washed three times with lysis buffer, boiled in sample buffer containing 0.2 M dithiothreitol, and analyzed by Western blotting as described above.

2.11. Statistical analysis

Experiments were repeated at least three times. The data were evaluated for significant differences using Student's *t*-test and the chi-square test. A p-value < 0.05 was considered significant.

3. Results

3.1. *Eqtn*$^{-/-}$ males are subfertile

Eqtn is a vesicle membrane protein. Our previous studies demonstrated that it is specifically expressed in the acrosomal membrane of sperm [10]. To study the physiological function of this protein, we purchased the conventional *Eqtn* knockout mouse strain *Eqtn*$^{+/-}$ from the Jackson Laboratory (Stock No. 007733). This strain was originally generated by Dr. David Garbers at U.T. Southwestern Medical Center. The *Eqtn* gene contains 8 exons, and exons 4–7 play an important role in the function of the protein. These exons were replaced with a neomycin selection cassette to generate the *Eqtn* knockout mouse strain (Fig. 1A). The genotypes of the mice were determined by PCR (Fig. 1B). The 480-bp band represents the wild-type allele, and the 582-bp band represents the null allele. *Eqtn*$^{-/-}$ mice were obtained by intercrossing *Eqtn*$^{+/-}$ male and female mice, and the Mendelian ratio was grossly normal (data not shown). The expression of the *Eqtn* protein in *Eqtn*$^{-/-}$ mice was then examined by Western blotting (Fig. 1C), immunohistochemical analysis (Fig. 1D and E), and immunofluorescence (Fig. 1F and G). These assays showed that the *Eqtn* protein was completely absent in *Eqtn*$^{-/-}$ mice.

To evaluate male fertility, we crossed control and *Eqtn*$^{-/-}$ males with wild-type females. As shown in Table 1, approximately 96% of the plugged females that were crossed with control males were pregnant, with an average litter size of 12.28. In contrast, only approximately 59% of the plugged females that were crossed with *Eqtn*$^{-/-}$ males were pregnant, with an average litter size of 5.77. This difference between control and *Eqtn*$^{-/-}$ males was significant. However, the morphology of testes from *Eqtn*$^{-/-}$ males was similar to the morphology of testes from control mice, with a 50% lower total sperm number in *Eqtn*$^{-/-}$ mice. These *Eqtn*$^{-/-}$ sperm exhibited normal motility when analyzed using CASA (data not shown).

3.2. The fertilization rate of *Eqtn*$^{-/-}$ males is significantly reduced

To explore the mechanisms that cause the reproductive defects of *Eqtn*$^{-/-}$ males, the in vivo fertilization rate was examined. Approximately 90% of the oocytes were fertilized and had developed to the 2-cell stage in the control group (Fig. 2A and C). However, in the *Eqtn*$^{-/-}$ group, most oocytes were at the one-cell stage, and less than 30% were fertilized and had developed to the two-cell stage (Fig. 2B and C). To examine whether the reduced fertilization rate was due to the decreased sperm number of *Eqtn*-deficient males, an in vitro fertilization (IVF) experiment was performed. More than 60% of the oocytes were fertilized when mixed with control sperm. In contrast, when mixed with the *Eqtn*-deficient sperm, less than 20% oocytes were fertilized (data not shown). As the difference between control and *Eqtn*$^{-/-}$ males was significant, we conclude that the fertilization rate of *Eqtn*$^{-/-}$ males was significantly reduced.

3.3. *Eqtn* is not essential for acrosome biogenesis

The *Eqtn* protein is localized in the acrosome and expressed from the round spermatid to mature sperm stages, suggesting that *Eqtn* is most likely involved in acrosome biogenesis. We performed a single-sperm immunostaining experiment to test whether the reduction of the fertilization rate of *Eqtn*-deficient sperm was due to a defect in acrosome development. The *Eqtn* protein was absent in sperm from *Eqtn*$^{-/-}$ males (Fig. 1G). However, when the acrosomes in sperm from control (Fig. 1H, white arrow) and *Eqtn*-deficient males (Fig. 1I, white arrow) were labeled with the acrosome-specific protein sp56 [20], no differences between groups were noted. The ultrastructure of the acrosome at different developmental stages was examined using transmission electron microscopy (TEM). Compared with control sperm (Fig. 3A and C), no obvious defects in the acrosome were observed in *Eqtn*-deficient sperm at the cap (Fig. 3B) or mature (Fig. 3D) stage. These results indicate that *Eqtn* is not essential for acrosome biogenesis, and that the loss of this gene does not affect acrosome formation.

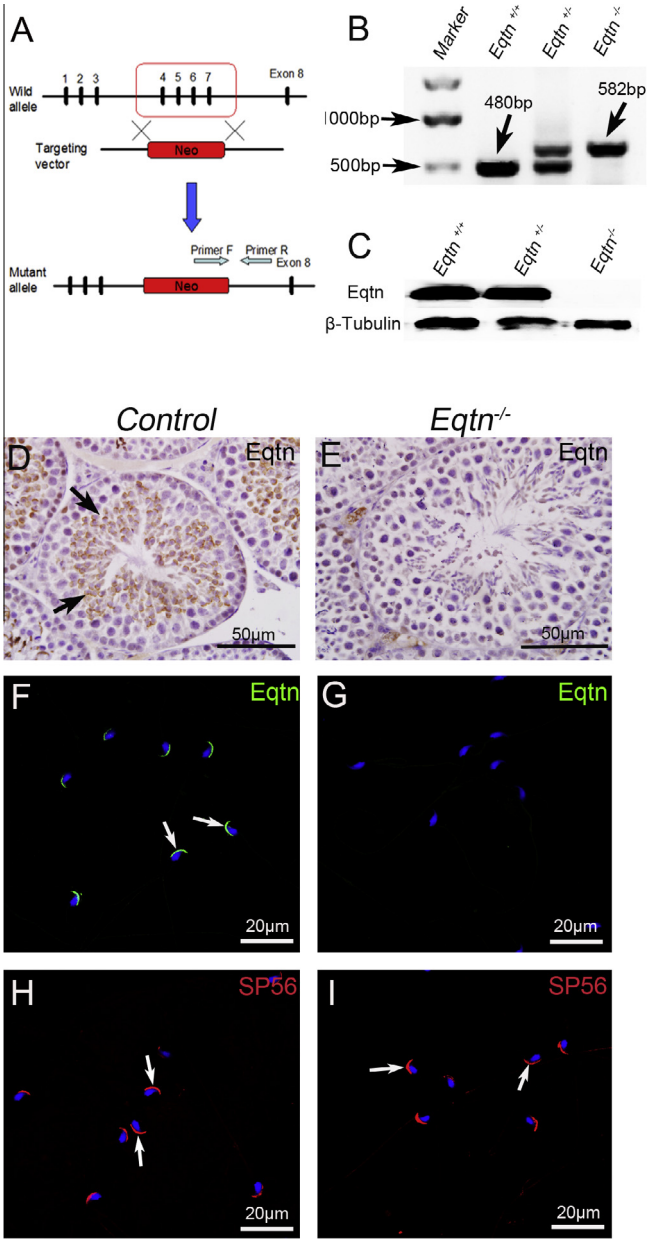

Fig. 1. Generation of the *Eqtn* knockout mouse model. (A) Schematic diagram of the targeting construct. In the targeted allele, exons 4–7 were replaced a neomycin selection cassette. (B) Genotyping by PCR. (C) Western blotting showed that the Eqtn protein was completely absent from *Eqtn*$^{-/-}$ testes. By immunohistochemistry, the Eqtn protein could be detected in the sperm of control testes (D, arrows) but was completely absent from *Eqtn*$^{-/-}$ testes (E). By immunofluorescence, the anti-Eqtn antibody labeled the acrosome of control sperm (F, green, white arrows) but had no detectable signal in *Eqtn*$^{-/-}$ sperm (G). An acrosome-specific protein, sp56, was detected in the acrosomes of control (H, white arrows) and *Eqtn*$^{-/-}$ sperm (I, white arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Eqtn protein is important for the acrosome reaction

To test whether the *Eqtn* protein is involved in the acrosome reaction, we used the A23187 induced acrosome reaction assay.

Table 1
Fertility test results.

Genotype	No. of males	No. of pluged females	No. of pregnancy females	Pregnant rate (%)	Litter size
Control	5	25	24	96.0[a]	12.3
Eqtn$^{-/-}$	10	51	31	58.8[b]	5.8

Control and *Eqtn*$^{-/-}$ males were crossed with wild type females, and females with a vaginal plug were counted for the pregnancy test $a > b$, $p < 0.05$.

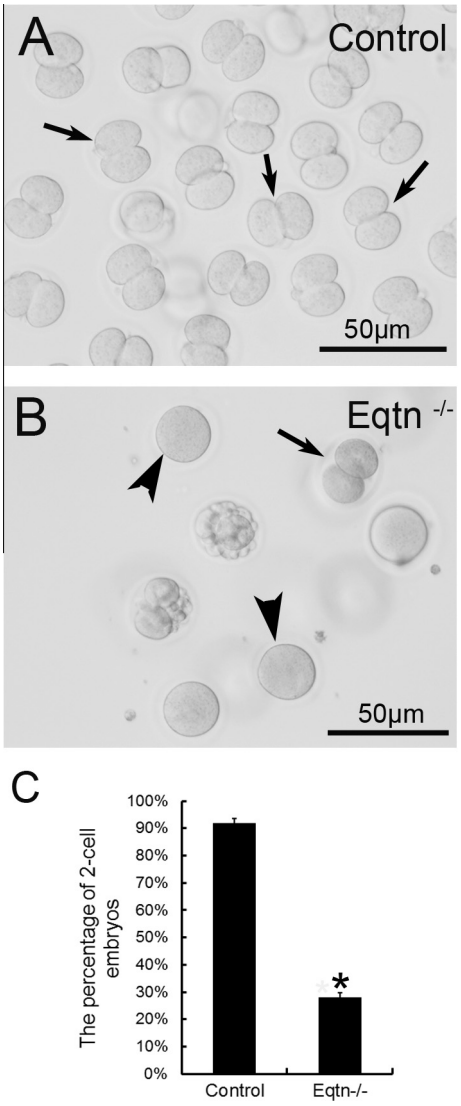

Fig. 2. The in vivo fertilization rate of *Eqtn*$^{-/-}$ males was significantly decreased. Control and *Eqtn*$^{-/-}$ males were crossed with wild-type females, and embryos were collected from the oviducts at 1.5 dpc. Approximately 90% of the collected oocytes were fertilized and had developed to the 2-cell stage in the control group (A, black arrows). However, in the *Eqtn*$^{-/-}$ group, most oocytes were at the one-cell stage (B, black arrow heads), and less than 30% of oocytes were fertilized and had developed to the 2-cell stage (B, black arrows). This difference was significant (C) $^{*}p < 0.01$.

Acrosomal status was evaluated using FITC-PNA (green) staining, intact acrosomes were labeled with FITC-PNA (Fig. 4, white arrows), whereas acrosome-reacted sperm were not labeled with FITC-PNA (Fig. 4, white arrow heads). As shown in Fig. 4A and B, approximately 25% of sperm spontaneously underwent the acrosome reaction in vitro, as evidenced by a loss of PNA staining. Statistical analyses revealed no differences in the rate of the occurrence of a spontaneous acrosome reaction between control and *Eqtn*-deficient sperm (Fig. 4E). However, the rate of A23187-induced AR was dramatically reduced in *Eqtn*-deficient sperm. Upon exposure to A23187, more than 50% of sperm from control mice (Fig. 4C and E) underwent acrosome exocytosis, whereas the AR occurred in less than 20% of sperm from *Eqtn*$^{-/-}$ mice (D, E). The defect in acrosome exocytosis observed in *Eqtn*-deficient sperm was further confirmed using zona pellucida (ZP) protein- and Ca^{2+}-SLO-induced AR models (data not shown). All of these results indicate that the *Eqtn* protein is involved in acrosome exocytosis, consistent with the results of a previous in vitro study [13]. Therefore, we conclude that *Eqtn* is involved in the acrosome reaction.

3.5. Eqtn protein interacts with Syntaxin1a and SNAP25

The SNARE complex plays an important role in membrane fusion during acrosome exocytosis. Our previous study demonstrated that the *Eqtn* protein might interact with SNAP25, a core component of the SNARE complex, in a transfected cell line [13]. A Co-IP experiment was performed to determine whether the *Eqtn* protein also interacts with other components of the SNARE complex. We found that the *Eqtn* protein could be pulled down by anti-Syntaxin1a and anti-SNAP25 antibodies (Fig. 4F), indicating that the *Eqtn* protein might directly or indirectly interact with the SNARE complex. To further explore the function of *Eqtn* in SNARE complex assembly or integrity maintenance, the expression of Syntaxin1a and SNAP25 was assessed using Western blotting. We found that the protein levels of these two genes were not decreased but rather slightly increased in *Eqtn*-deficient testes (data not shown). These results suggest that the *Eqtn* might interact with the SNARE complex components Syntaxin1a and SNAP25, although loss of *Eqtn* did not affect the expression of the SNARE members.

4. Discussion

The *Eqtn* protein has been reported to be localized to the inner and outer membranes of the sperm acrosome, and previous in vitro studies suggested that this gene might be involved in fertilization [10,11,13,14,21,22]. However, the precise in vivo physiological function of *Eqtn* is still unknown. In this study, we found that *Eqtn*$^{-/-}$ mice were viable and grossly normal, although *Eqtn*$^{-/-}$ males were subfertile. The testicular histology and sperm morphology of *Eqtn*-deficient mice were normal, indicating that *Eqtn* is important for male reproduction but is not involved in testis development.

Given that *Eqtn* is specifically localized to the sperm acrosomal membrane, we investigated sperm and acrosome morphology in the mutants by immunofluorescence and TEM. We found that loss of *Eqtn* did not affect sperm development or acrosome formation. In other words, although the *Eqtn* protein is specifically localized to the acrosomal membrane from the round spermatid to mature sperm stages, it is not essential for acrosome biogenesis.

The in vivo fertilization rate of *Eqtn*$^{-/-}$ males was assessed to explore the underlying mechanism causing the male reproductive defect. The results showed that the proportion of 2-cell embryos was decreased by more than 60% after mating with *Eqtn*$^{-/-}$ males compared with control males, indicating that the subfertility of *Eqtn*$^{-/-}$ male is due to a fertilization defect. In mammalian fertilization, the sperm interact with the ZP and induce the acrosome

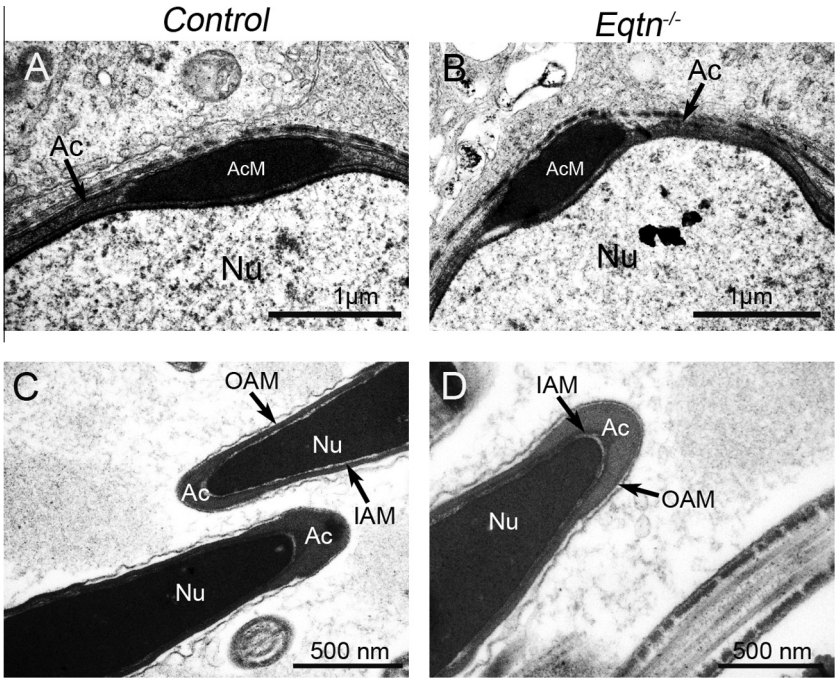

Fig. 3. The ultrastructure of the acrosome in *Eqtn*-deficient sperm was normal. Compared with controls (A, C), the morphology of the acrosome (black arrows) (B, D) was normal in round spermatids (A, B) and mature sperm (C, D) from *Eqtn*$^{-/-}$ mice. Nu, nucleus; Ac, Acrosome; AcM, acrosome matrix; IAM, inner acrosomal membrane; OAM, outer acrosomal membrane.

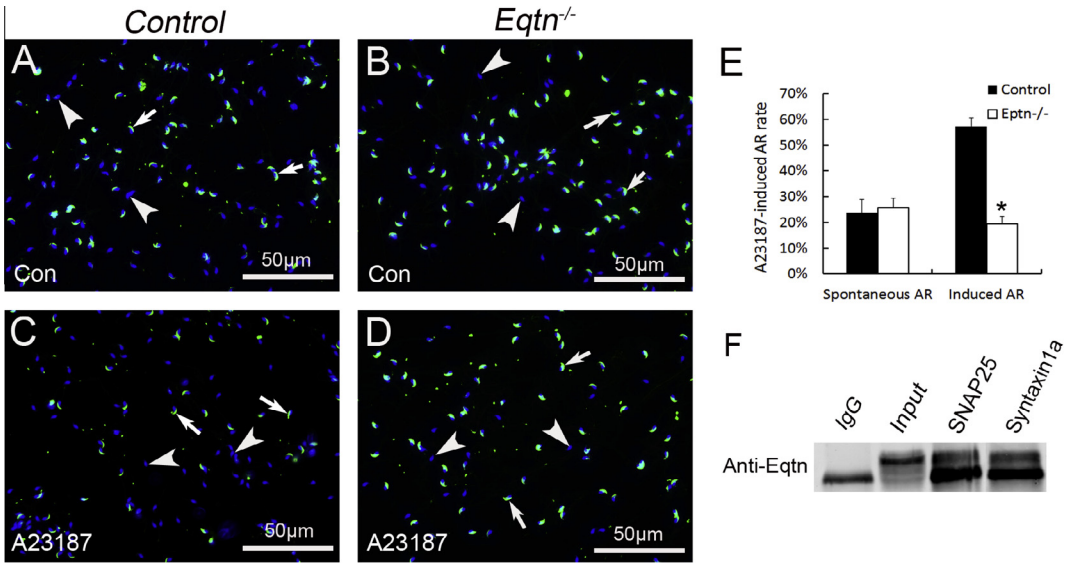

Fig. 4. The acrosome reaction (AR) rate was significantly reduced in sperm from *Eqtn*$^{-/-}$ mice. FITC-PNA (A–D, white arrows, green) was used to label intact acrosomes, and the sperm nuclei were labeled with Hoechst 33342 (blue). The acrosome-reacted sperm had Hoechst-labeled nuclei but lacked PNA staining (A–D, white arrow heads). The proportion of sperm that spontaneously underwent AR was not significantly different between control (A, E) and *Eqtn*$^{-/-}$ samples (B, E). The percentage of sperm that underwent AR after A23187 induction was significantly reduced in *Eqtn*-deficient sperm (D, E) compared with control sperm (C, E). The *Eqtn* protein was pulled down by anti-Syntaxin1a and SNAP25 antibodies, but not by the control IgG (F) $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reaction, which is a critical step for successful fertilization. We found that the proportions of *Eqtn*-deficient sperm that underwent the acrosome reaction following induction by A23187, ZP protein, or Ca^{2+} were all dramatically reduced relative to controls, indicating that the reduced fertilization by sperm from *Eqtn*$^{-/-}$ males is most likely caused by an abnormal acrosome reaction. Recent studies have found that the SNARE complex is involved in the acrosome reaction in mammals [2,5,9,23]. This complex consists of SNAP25, Sytaxin1a, VAMPs, and other components. In the present study, we found that the *Eqtn* protein might either directly or indirectly interact with SNAP25 and Sytaxin1a. However, loss of *Eqtn* did not affect the levels of these two proteins, suggesting that although *Eqtn* might interact with the SNARE complex during the acrosome reaction, it did not affect the expression of this complex members.

Given that the acrosome reaction is a rigorously controlled and complicated process, more experiments are required to explore the exact functions of *Eqtn* in the future.

Declaration of interest

The authors have no conflict of interest to declare.

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