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Establishment, characterization, and application of pAcr–SP–NTP–EGFP transgenic mice in visualizing the oviduct-migrating ability of sperm from Prss37-null mice

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Abstract

Transgenic mouse model with fluorescently labeled sperm has extensive application value. It is an auxiliary tool for investigating the mechanism of fertilization, especially for visualizing the oviduct-migrating ability of sperm *in vivo*. Here, we produced transgenic mouse lines whose sperm were tagged with enhanced green fluorescent protein (EGFP) according to the previously described method. Polymerase chain reaction analysis of tail-tip genomic DNA identified 13 founders, of which 5 male founders produced offspring to form transgenic lines. We showed that EGFP was testis-specifically expressed, sharing similar expression pattern with endogenous acrosin. It has luminal side restricted distribution in seminiferous tubules and acrosomal aggregation in mature sperm. In addition, inter-strain hybridization obtained $Prss37^{-/-}EGFP^{tg/+}$ males produced sperm with impaired oviduct-migrating ability as visualized under fluorescence microscope, compared with $Prss37^{+/+}EGFP^{tg/+}$ counterparts. These results indicate that a transgenic mouse model with fluorescently labeled sperm has been successfully established and it is a useful tool for evaluating the oviduct-migrating ability of sperm.

Key words: gene expression, EGFP, transgenic/knockout model, oviduct-migrating ability

Introduction

Oviduct-migrating ability of sperm plays a vital role in natural conception. Although the male mouse ejaculates millions of sperm into the female's uterus, only a small amount of the sperm are able to migrate into the oviduct and finally reach the egg in ampulla, due to the complexity of the female genital tract [1–3]. In mice, the female genital tract is mainly consisted of uterus, colliculus tubarius (CT), uteral tubal junction (UTJ), oviduct isthmus, and oviduct ampulla [4]. In most mammals, the UTJ presents anatomical, physiological, and/or mucous barriers to sperm transport in the female genital tract. So far, ~10 kinds of gene knockout mouse models with migration defects through UTJ have been reported in the published literatures, including *Ace* [5], *Adam2* [6], *Clgn* [7,8], *Adam1a* [9], *Adam3* [10], *Calr3* [11], *Tpst2* [12,13], *Pdilt* [14], *Pmis2* [15], and *Prss37* [16]. Migration defect of sperm from *Prss37*-null males was previously evaluated by observation of sections containing UTJ and by counting sperm flushed

out from oviducts several hours after coitus. However, both methods are indirect and not as convincing as visualizing sperm migration from uterus into oviduct directly.

Biological studies using fluorescent proteins have been growing fast these years. Transgenic models with wide expression of fluorescent proteins driven by strong promoters have been established in a number of laboratories [17–20], including ours (unpublished study). Although multiple organs or tissues can emit fluorescence under excitation light, the fluorescence signal in sperm is very weak, even invisible, due to the removal of residual bodies during spermiogenesis. If the fluorescence label of sperm could be realized, it would be easy to trace the sperm in the female reproductive tract, thus bringing great convenience for the in-depth study of the mechanisms of sperm fertilization. In fact, Nakanishi *et al*. produced such a mouse model more than a decade ago, which accumulate enhanced green fluorescent protein (EGFP) in sperm acrosome [21]. In the present study, we replicated a part of the experiment and obtained a transgenic mouse model with fluorescently labeled sperm by placing EGFP coding sequence after mouse proacrosin promoter (pAcr), proacrosin signal peptide (SP), and acrosin N-terminal peptide (NTP), and used it as a tool to evaluate the oviduct-migrating ability of sperm from *Prss37*-deficient mice, which were formerly demonstrated to be infertile due to a defect in sperm migration from uterus into oviduct, by crossing it with *Prss37*$^{-/-}$ females and then by mating between siblings. The transgenic mouse model presented here has a good prospect in application and is supposed to be a new resource for the scientific community.

Materials and Methods

Construction of the transgenic vector

The preparation of the transgene vector pAcr–SP–NTP–EGFP was modified based on the literature. In brief, the protein-coding region of EGFP, the bovine growth factor gene polyadenylation signal (bGHpA) fragment, and the 2.4 kb mouse genomic region carrying the promoter of proacrosin gene as well as sequences of its SP and NTP were amplified using pEGFP-C1 vector (Clontech, Mountain View, USA), PL452 vector (a gift from Dr. Pentao Liu, Wellcome Trust Sanger Institute, Hinxton, UK), and mouse genomic DNA (gDNA) (prepared in our laboratory) as the templates, respectively. Primers for EGFP were P1, 5′-ATACTCGAGgccaccatggtgagc-3′, where CTCGAG is the *Xho*I site and P2, 5′-ATAGGTACCttacttgtacagctcgtcc-3′, where GGTACC is the *Kpn*I site. Primers for bGHpA fragment were P3, 5′-ATAGGTACCcttcttgacgagttcttctgag-3′, where GGTACC is the *Kpn*I site, and P4, 5′-ATAGGATCCttatattaagggttccgcaagc-3′, where GGATCC is the *Bam*HI site. Primers for pAcr-SP-NTP fragment were P5, 5′-ATAAAGCTTcttacttttagttagg-3′, where AAGCTT is the *Hin*dIII site, and P6, 5′-ATACTCGAGcgtggtgttatccttggca-3′, where CTCGAG is the *Xho*I site. First, the polymerase chain reaction (PCR) products of EGFP and bGHpA were digested with *Xho*I–*Kpn*I (Takara, Dalian, China) and *Kpn*I–*Bam*HI, respectively, and introduced into the *Xho*I–*Bam*HI sites of pBR322–MK–MCS to produce pBR322–EGFP–bGHpA. Then, the resulting plasmid was cut by *Hin*dIII and *Xho*I, and inserted by pAcr–SP–NTP fragment digested with the same enzymes, obtaining the transgenic vector pBR322–pAcr–SP–NTP–EGFP–bGHpA, which was evaluated by restriction endonucleases and sequencing.

Generation of transgenic mice

A 3.5 kb fragment containing the transgene was excised by double digestion with *Hin*dIII and *Bam*HI, and purified by agarose gel electrophoresis followed by universal DNA purification kit (Tiangen Biotech, Beijing, China) and used in the subsequent pronuclear microinjection (**Fig. 1A**). Fertilized eggs were isolated from superovulated C57BL/6J females (5–6 weeks old) mated with sexually mature C57BL/6J males, and incubated in M16 medium (prepared in our laboratory according to the formula of M16 medium (Sigma, St Louis, USA; M1285). After pronuclear microinjection, eggs were transferred into the oviduct of 0.5-day-postcoitum pseudopregnant mice. In total, 280 DNA-injected eggs were transplanted into 14 recipients, 11 of which became pregnant and delivered 42 newborns. The incorporation of the transgene was first examined by PCR analysis using DNA extracted from the tail with primers P1 and P2, and was further confirmed with primers P4 and P5, which amplified the full-length transgene. Primer sequences were described earlier. After founders were established, they were mated with C57BL/6J mice to generate progeny, and genotyping of offspring was characterized by PCR using primers P1 and P2. All mice were housed in a temperature-controlled facility in rooms maintained at a 12L:12D cycle, with free access to a diet of regular chow and water. All research protocols involving animal experiments were approved by Institutional Animal Care and Use Committee of Shanghai Research Center for Model Organisms (Shanghai, China).

mRNA expression analysis

The expression of the transgene and the endogenous *Acr* was assayed by real-time PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). Total RNA (1 μg) was reverse transcribed by PrimeScript RT reagent kit with gDNA eraser (Takara). Real-time PCR was performed with a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using the double stranded DNA-specific fluorophore SYBR Green (Takara). Primers were 5′-TACCCAGGCATTGCTGACAGG-3′ (forward) and 5′-ACTTGCGGTGCACGATGGA-3′ (reverse) for *Actb*, 5′-GCGACGTAAACGGCCACAAGT-3′ (forward) and 5′-TGCTGCTTCATGTGGTCGGGGTA-3′ (reverse) for *EGFP*, and 5′-TTGACCTCGACCTGTGTAACTC-3′ (forward) and 5′-TCCAGTCCAGGTAGTCCCAG-3′ (reverse) for *Acr*. Resolution of the product of interest from nonspecific product amplification was achieved by melting curve analysis. Expression levels of *EGFP* and *Acr* were normalized to *Actb* content.

Western blot analysis

Testes from transgenic and wild-type (wt) mice were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts (60 μg) of protein lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting with antibodies to EGFP (1:1000 dilution; Sigma) and Gapdh (1:2000 dilution; Kangcheng Biotechnology, Shanghai, China). The secondary antibodies conjugated with IRDye 800CW (Li-Cor, Lincoln, USA) were used to visualize the specific protein bands by using an infrared imager (the Odyssey; Li-Cor).

Observation of green fluorescence in testis and epididymis

Testes together with epididymides were removed from transgenic and wt mice, and were subject to examination under a Nikon's stereomicroscope SMZ1500 (Nikon, Tokyo, Japan) using excitation light.

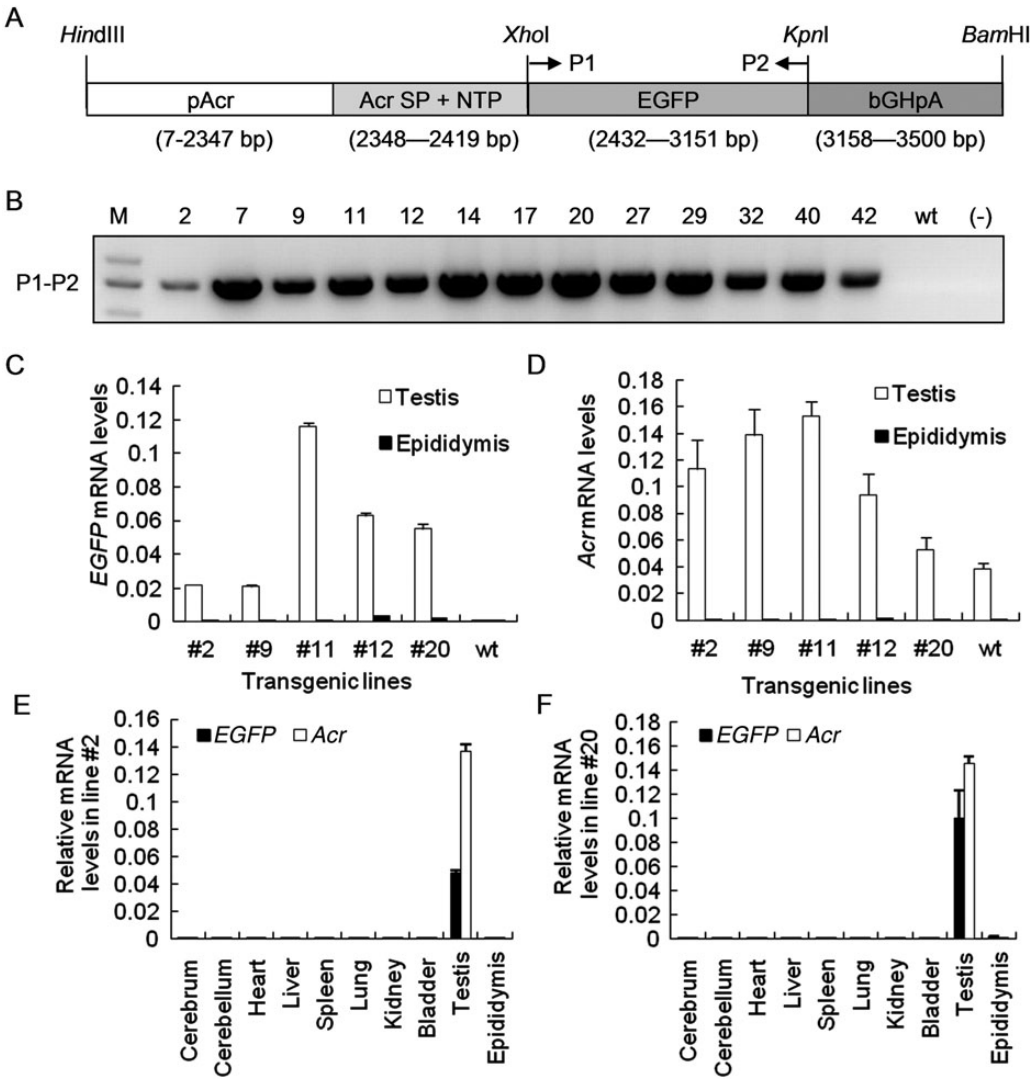

Figure 1. Construction of pAcr–SP–NTP–EGFP transgenic mice (A) Schematic diagram of the transgene construct used for microinjection. The transgenic fragments and their sizes including pAcr, SP, NTP, EGFP, bGHpA, and restriction enzymes are shown. Arrows indicate the PCR primers used for genotyping. (B) Identification of the transgenic founder mice by PCR with primers P1 and P2. M, DNA markers. (C and D) Real-time PCR analyses for *EGFP* and endogenous *Acr* mRNA levels in the testis and epididymis from line #2, #9, #11, #12, #20, and wt control. (E and F) Expression profiles of transgenic *EGFP* and endogenous *Acr* gene in line #2 (E) and #20 (F) by real-time PCR. Bar chart shows mean ± SEM values from three replicates relative to *Actb* mRNA levels.

Immunofluorescence assay

Testes were fixed in Bouin's buffer (combined 75 ml of saturated picric acid, 25 ml of formalin, and 5 ml of glacial acetic acid), fully impregnated in 20% sucrose, frozen in Tissue-Tek OCT compound (Sakura, Torrance, USA) and sectioned at a thickness of 8 μm. Sperm smear slides were prepared by spotting sperm isolated from cauda epididymis onto glass slides. Sperm were air dried and fixed in 4% paraformaldehyde and stored at −20°C. For immunofluorescence, sections or sperm smear slides were washed and incubated in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 10 min. Then, they were blocked in 5% normal goat serum in PBS at room temperature for 1 h and incubated overnight at 4°C with diluted anti-EGFP rabbit antiserum (1:200 dilution; Sigma). After being washed with PBS containing 0.1% Tween-20, slides were incubated for 1 h at room temperature with Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (1:200 dilution; Invitrogen) supplemented with Alexa Fluor 647 conjugated lectin peanut agglutinin (PNA; 1:100 dilution; Molecular Probes, Eugene, USA). After additional washes, sections were counterstained with 4′,6-diamino-2-phenylindole and mounted with fluorescence mounting medium (Dako, Glostrup, Denmark). Images were captured with a laser-scanning confocal microscope (FluoView FV10i, Olympus, Tokyo, Japan).

Examination green fluorescence of sperm ejaculates

Sexually mature males were mated with superovulated wt females at a male-to-female sex ratio of 1:2, and the formation of vaginal plugs was checked every hour. Uteri of plugged females were excised and were subject to transillumination under Nikon's stereomicroscope SMZ1500 (Nikon). Bright field and fluorescent images were taken with equipped color digital camera. Sperm were also collected from the uteri of plugged females, spotted onto glass slides and examined under a Nikon Eclipse 90i fluorescence microscope (Nikon).

In vivo sperm migration analysis

C57BL/6J females (4–5 weeks old) were superovulated by intraperitoneal injection of 5 units of pregnant mare serum gonadotropin, followed by 5 units of human chorionic gonadotropin. After 46–48 h and the C57BL/6J females were mated with *Prss37*$^{-/-}$*EGFP*$^{tg/+}$ males as well as their *Prss37*$^{+/+}$*EGFP*$^{tg/+}$ counterparts. Oviducts, together with the connective part of the uterus, were excised from the mice 2 h after copulation plugs were detected. A ligature was applied to the uterine horn to prevent contraflow of the semen. Oviducts were carefully separated from the uterine horns and straightened out by cutting the mesosalpinx under the stereomicroscope SMZ1500 (Nikon). Images were taken by the equipped digital camera. The migration of sperm through UTJ into oviduct was determined by the presence of the acrosomal EGFP marker.

Results

Generation of transgenic mouse lines

In order to generate a transgenic mouse line, where the fluorescent reporter EGFP was specifically located in the sperm to be a sperm marker for *in vivo* tracing, we constructed a transgenic vector containing a chimeric cDNA encoding EGFP fusion polypeptide with SP and NTP of mouse acrosin prepropeptide under the control of the endogenous proacrosin promoter. After digestion with *Hin*dIII and *Bam*HI, a 3.5 kb fragment (**Fig. 1A**) containing the transgene was excised, purified, and microinjected into the pronuclei of each egg. PCR genotyping of the 42 offspring born from the microinjected eggs identified 13 founders with transgene incorporation (**Fig. 1B**). *EGFP* mRNA expression was primarily examined in testis and epididymis from line #2, #9, #11, #12, and #20. Results showed that *EGFP* mRNA increased tens of thousands of folds in testis when compared with wt control (**Fig. 1C**). On the other hand, *EGFP* was hardly transcribed in epididymis. These transcription features were similar to the endogenous *Acr* gene, as assayed in parallel (**Fig. 1D**). Moreover, the relative expression levels of *EGFP* in mouse tissues from line #2 and #20 were examined in cDNA samples prepared from 10 tissues including cerebrum, cerebellum, heart, liver, spleen, lung, kidney, bladder, testis, and epididymis. The tissue expression of the endogenous *Acr* gene was examined simultaneously. Both genes showed testis-specific expression in both lines (**Fig. 1E,F**). Thus, we generated transgenic mouse lines where the transcription of the transgene mimicked the endogenous *Acr* gene, displaying testis-specific transcription pattern.

Characterization of the transgene expression in mice

To further clarify the transgene expression in mice, relative EGFP protein expression in testis from line #2, #9, #11, #12, and #20 was assayed by western blot analysis (**Fig. 2A**) and fluorescence microscopy (**Fig. 2C**), using samples from wt mice as negative controls. Although each line exhibited extensive expression of the transgene when compared with the wt control, variation of expression levels existed among different lines. Among them, samples from line #20 showed the strongest intensity of green fluorescence compared with those from wt mouse (**Fig. 2B,C**). There were no differences in gross morphology and histology of testis and cauda epididymidis as indicated by hematoxylin and eosin (H&E) staining and light microscopic analysis (**Supplementary Fig. S1**).

Immunofluorescence of frozen testis sections revealed that the transgene was expressed in the luminal side of the seminiferous tubule (**Fig. 3**), corresponding to haploid spermatids during spemiogenesis. PNA staining facilitated the recognition of different spermatogenic

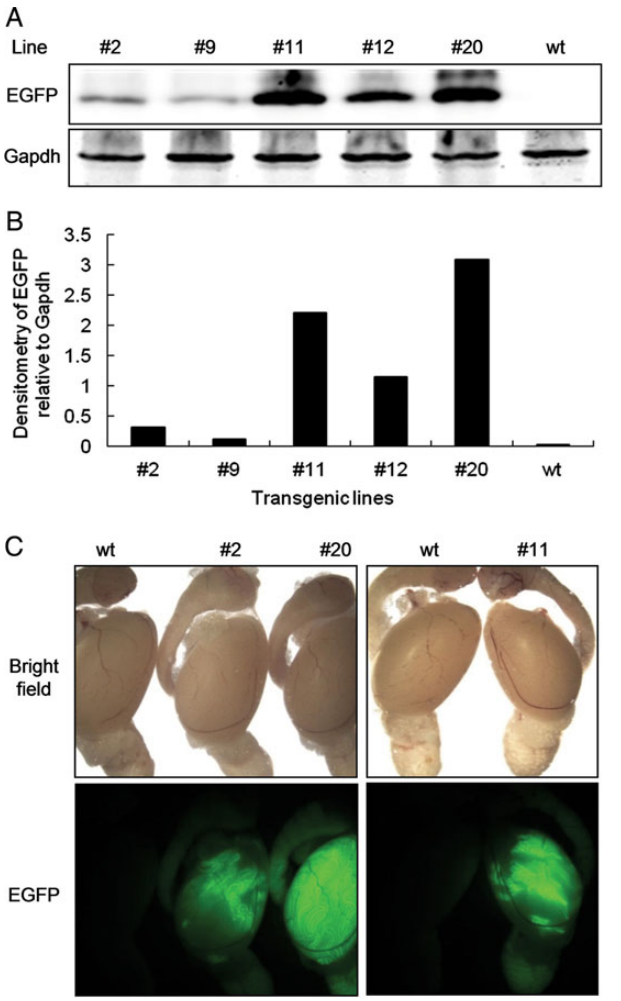

Figure 2. EGFP protein expression in transgenic mouse lines (A) Western blot analysis of EGFP in the testis from the indicated mouse lines. Gapdh is shown as a loading control. (B) Densitometry of EGFP relative to Gapdh in (A) was analyzed. (C) Visualization of EGFP expression in the testis and epididymis from the indicated mouse lines. Representative images are shown.

stages. It was used to visualize spermatid acrosomes since it has a specific affinity for them [22]. The transgene was not expressed in spermatids at step 7 of spermiogenesis (**Supplementary Fig. S2**), identified by the presence of PNA lectin-stained round spermatid acrosomes that cover over one quarter of the surface of the nucleus and were randomly oriented within the epithelium [23]. Such an expression pattern was consistent with that of endogenous acrosin prepropeptide, which was first found in step 9 spermatids and was finally located in the acrosome of step 16 spermatids and spermatozoa [24].

The localization of the transgene in the acrosome of mature sperm isolated from cauda epididymis was further elucidated by immunofluorescence of sperm smear slides followed by confocal microscopy. EGFP co-localized with PNA, which bound to the outer acrosomal membrane [25] (**Fig. 4**).

We then tested whether the fluorescence emitted by the EGFP-tagged sperm could be easily detected under fluorescence microscope after they were ejaculated in the female reproductive tract. Sexually mature EGFP positive males from line #2 and #20 were mated with superovulated wt females. Once the vaginal plugs were detected, the

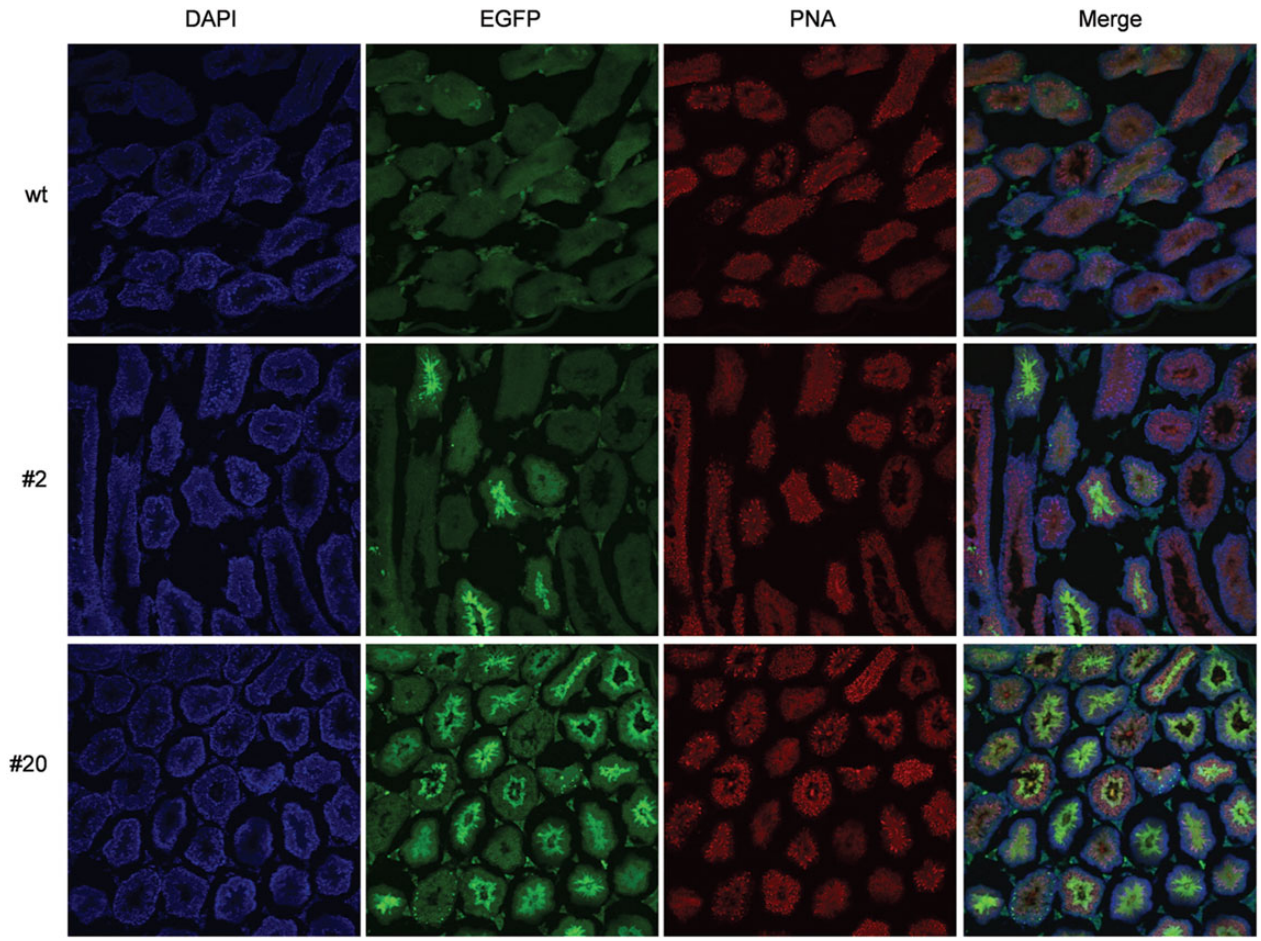

Figure 3. Expression of EGFP in testis is restricted in the luminal side of the seminiferous tubule as illustrated by immunofluorescence on testis frozen sections DAPI, 4′,6-diamino-2-phenylindole; PNA, peanut agglutinin.

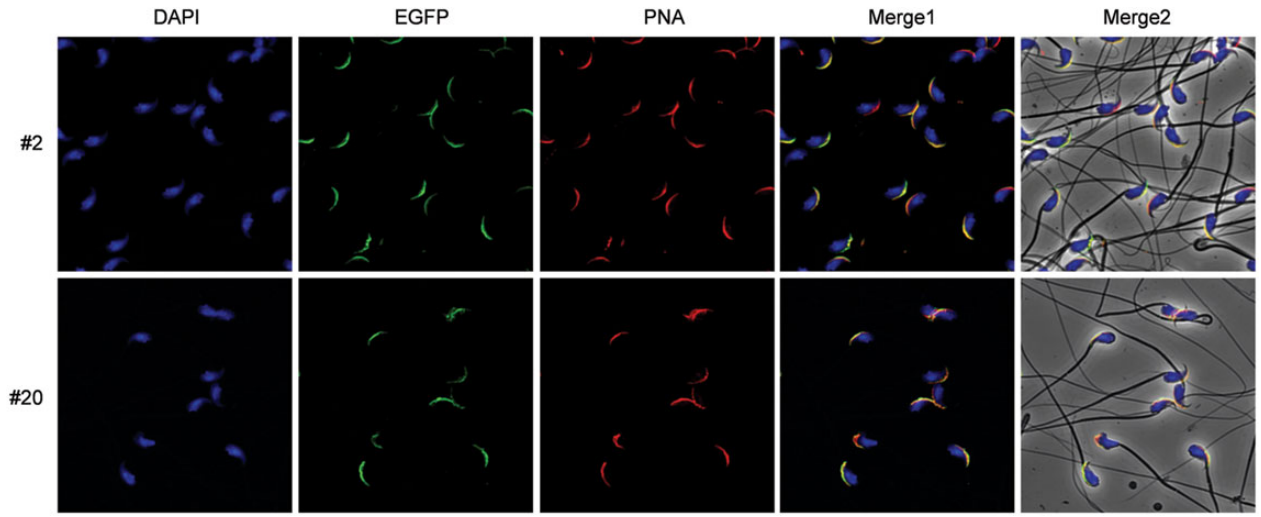

Figure 4. EGFP is maintained in the acrosome region of mature sperm isolated from cauda epididymis, as assayed in line #2 and #20 DAPI, 4′,6-diamino-2-phenylindole; PNA, peanut agglutinin.

uteri of plugged females were excised and subject to observation under Nikon's stereomicroscope SMZ1500. As shown in **Fig. 5**, uterus from plugged female was swollen and exhibited green fluorescence, which was originated from the EGFP-tagged sperm ejaculates, as illustrated under Nikon Eclipse 90i fluorescence microscope with excitation light after they were spotted onto glass slides.

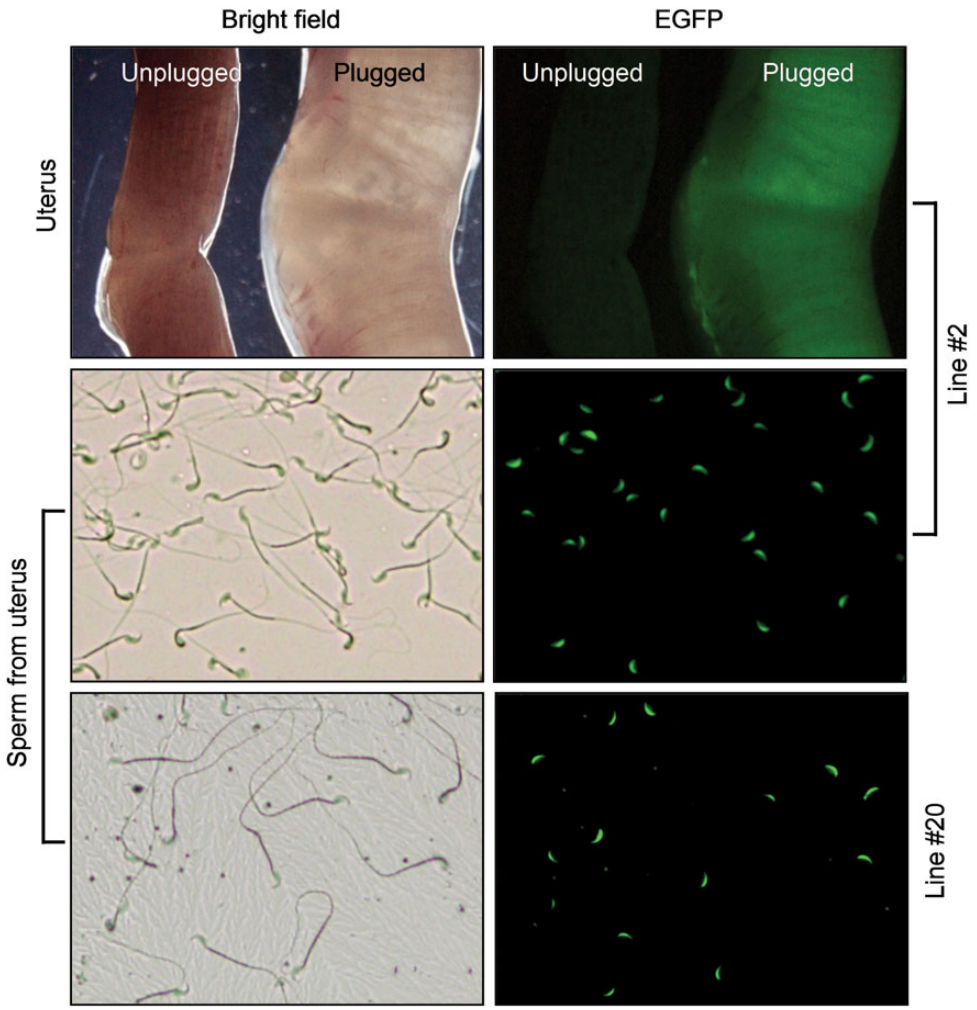

Figure 5. The green fluorescence of sperm ejaculates could be easily observed in the uteri as well as the sperm collected from the uteri of plugged females under fluorescence microscope The top and middle images are about line #2, and the bottom images are about line #20, as indicated on the right of the images.

Evaluation of the oviduct-migrating ability of sperm from Prss37-null mice using EGFP as a sperm marker

In order to evaluate the oviduct-migrating ability of sperm from *Prss37*-null males, we produced a mouse model with $Prss37^{-/-}$ $EGFP^{tg/+}$ genotype by interstrain hybridization, sperm from which were tagged with EGFP in the head. When $Prss37^{-/-}EGFP^{tg/+}$ males were mated with superovulated females, the EGFP-tagged $Prss37^{-/-}$ sperm were confined in the uterus and were hardly able to enter the oviduct (**Fig. 6C,D,F**). On the other hand, when their $Prss37^{+/+}$ $EGFP^{tg/+}$ counterparts were mated with superovulated females, the EGFP-tagged $Prss37^{+/+}$ sperm were easily detected both in the uterus and in the oviduct (**Fig. 6A,B,E**), at 2–4 h after coitus. This evidence thus reinforced the migration defect of sperm from *Prss37*-null mice, as we previously reported [16].

Discussion

For fertilization to occur in mammals, ejaculated sperm must migrate from uterus into oviducts and reach the egg. Sperm migration defect in the female reproductive tract has been reported in many gene knockout mouse models [5–16].

There are several ways to evaluate the oviduct-migrating ability of sperm, including: (i) H&E staining of tissue sections containing UTJ to observe the presence of sperm in the colliculus, the initial part of the oviduct; (ii) flushing out oviductal contents several hours after coitus and counting the number of sperm inside; and (iii) tagging the sperm with fluorescence and tracing their movement. The first two methods were easy to implement in any well-equipped laboratory. However, tagging the sperm with fluorescence required careful design. Nakanishi *et al.* [21] once produced transgenic lines carrying EGFP in the acrosome region of sperm, which allowed them to investigate the oviduct-migrating ability of *Adam3*-disrupted sperm [10]. Furthermore, they established another transgenic mouse called RBGS whose sperm expressed EGFP in the acrosome and RFP (DsRed2) in the middle piece and submitted it to RIKEN BioResource Center and Center for Animal Resources and Development (CARD) [26]. Our group happens to have several infertile mouse models, and the causes of infertility remain unclear. A transgenic model with fluorescently labeled sperm is supposed to facilitate our research programs. In the beginning, we applied to CARD for the supply of RBGS mice. However, only frozen embryos but not live mice were allowed to be supplied to us. Moreover, the import process of mouse embryos is cumbersome, time-consuming, and costly. According to the 'Material transfer

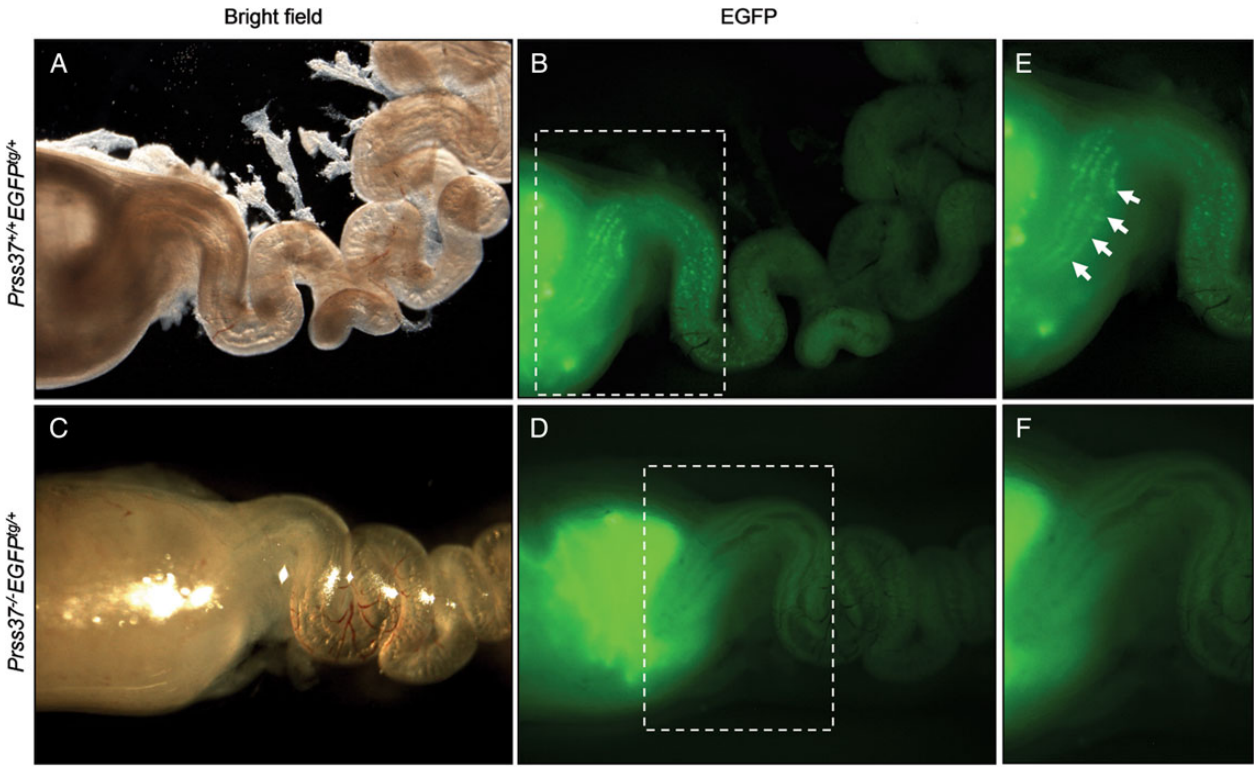

Figure 6. Evaluation of the oviduct-migrating ability of sperm from *Prss37*-null mice (A, B, and E) EGFP-tagged *Prss37*$^{+/+}$ sperm is visualized (white arrows) in the oviduct. (C, D, and F) EGFP-tagged *Prss37*-null sperm shows inability of migrating from uterus into oviduct. Boxed areas in (B) and (D) were magnified in (E) and (F).

agreement', we were only allowed to use the material 5 years upon receipt of it. To make matters worse, no pups were produced by thawing of the frozen embryos due to complicated reasons. In the end, we decided to reproduce such a transgenic model with EGFP-tagged sperm according to the method reported. We do not mean to prove that our model outperforms other models, but to use such a model as a tool to investigate the mechanisms of mammalian fertilization. The transgenic lines presented here well displayed the required features, such as testis-specific expression (**Fig. 1E,F**) and acrosome localization (**Fig. 4**). The new transgenic mice have similar application values as the models reported by other research groups, and are supposed to be a new resource for the scientific community, especially for the researchers in mainland China. The generation strategy of the present pAcr–SP–NTP–EGFP transgenic mice is almost the same as Acr3–EGFP reported by Nakanishi *et al.*, which is better than Acr1–EGFP and Acr2–EGFP as it showed 100% of lines with a green acrosome. The proacrosin signal peptide and its N-terminal peptide are suggested to be essential for EGFP to be localized in the acrosome. Owing to the nature of random gene insertion, expression of the transgene and the subsequent transgenic mouse phenotype may vary from line to line. Even when different lines exhibit common phenotypic characteristics, each of them represents a unique strain of mice. RBGS transgenic mouse, which expresses EGFP in the acrosome and DsRed2 in the mitochondria of sperm, was established to overcome the drawback that acrosome-reacted Acr–EGFP sperm lost the green fluorescence, which interfered with the accurate detection of sperm in the oviduct. However, the sperm migration defect reported in many gene knockout infertile mouse models happens when sperm migrate from uterus into oviduct, i.e. migration through UTJ, where the acrosome reaction of most sperm has not taken place yet. Thus, the Acr–EGFP transgenic mouse lines, including ours, are suitable for evaluating the oviduct-migrating ability of sperm. As an illustration, when the EGFP transgene was introduced into *Prss37*-null mice by interstrain hybridization, sperm ejaculates from *Prss37*$^{-/-}$*EGFP*$^{tg/+}$ males were confined in the uterus and were hardly able to enter the oviduct (**Fig. 6C,D,F**), reinforcing the migration defect of sperm from *Prss37*-null mice, as we previously reported [16].

In summary, we have successfully established transgenic mouse lines with green sperm and they are supposed to be a new resource for the scientific community, especially for the researchers in mainland China. The fluorescence nature of the sperm from these transgenic mouse lines would facilitate their use in the investigation of fertilization mechanisms, especially sperm migration, acrosome reaction, and so on.

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

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