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Rescuing Infertility of *Pick1* Knockout Mice by Generating Testis-specific Transgenic Mice via Testicular Infection

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PICK1 (protein interacting with C-kinase 1) is a peripheral membrane protein with high expression in brain, testis, pancreas and other neuroendocrine tissues. Male *Pick1* knockout mice are completely infertile, with a phenotype resembling the human disease globozoospermia. Since PICK1 is expressed in both testis and neuroendocrine tissues, infertility of *Pick1* knockout mice may be due to either impaired neuroendocrine function or abnormal spermatogenesis. To distinguish these two possibilities, we restored PICK1's expression in the testis by seminiferous tubule microinjection of PICK1-containing lentivirus. By examining the testis-specific *Pick1* transgenic mice, we found that PICK1's expression in testis rescued the spermatogenic abnormalities and male infertility in *Pick1* knockout mice. Our results indicate that the infertility is caused by the lack of PICK1 in the testis rather than in other organs. In addition, we found that seminiferous tubule microinjection of lentivirus has a strong preference to produce testis-specific transgenic mice.

Infertility is still a worldwide medical problem nowadays, despite of the development of assisted reproductive technologies. According to World Health Organization approximately 60–80 million couples worldwide suffer from infertility[1], and around 50% of the cases result from male factors[2]. A variety of causes lead to the male infertility, ranging from systematic disorders to urogenital abnormalities and spermatogenesis[3]. The defective spermatogenesis, such as abnormal sperm count, morphology and function, accounts for nearly half of male infertility cases[4–6]. The regulation of spermatogenesis is comprised of the endocrine regulation from the hypothalamic-pituitary-gonadal axis, the autocrine, paracrine and juxtacrine regulation within testis and the environmental factors[3,7]. Normal male reproductive function is mainly regulated by the hypothalamic-pituitary-gonadal axis. Disturbance of hormone secretion from these neuroendocrine tissues leads to impaired spermatogenesis[8,9]. Besides, insulin and leptin have also been shown to regulate the male productive function by promoting sperm motility, acrosome reaction and nitric oxide production[10,11], and also by acting on hypothalamic pro-opiomelanocortin (POMC) neurons[12].

PICK1 (protein interacting with C-kinase 1) is a peripheral membrane protein that is highly expressed in brain, pancreas and testis[13]. The most studied function of PICK1 is its role in regulating the intracellular trafficking of its binding partners, such as AMPA receptors in the brain[14]. PICK1 is also important for the trafficking of proacrosomal granules and its deficiency in mice leads to male infertility with a globozoospermia-like phenotype, including fragmented acrosomes, abnormal sperm morphology and defective sperm function[15]. More recently, PICK1 was found to control dense core vesicle (DCV) trafficking in pancreatic beta cells and pituitary neurons[16,17]. *Pick1* knockout mice have reduced insulin secretion from pancreatic beta cells, and diminished GH (growth hormone) release from pituitary, resulting in impaired glucose tolerance and growth retardation[16,17].

Since PICK1 functions not only in testis but also in other neuroendocrine tissues, it raises the possibility that the infertility of *Pick1* knockout mice could be a consequence of neuroendocrine defects, rather than a local defect in the testis. In order to distinguish these two possibilities, we tried to re-express PICK1 specifically in the testis of *Pick1* knockout mice and test whether their infertility could be rescued. In our testing of the seminiferous tubule microinjection of lentivirus method to produce transgenic animals, we found this method had a strong tendency to generate testis-specific transgenic male mice. Therefore, we attempted to employ testicular microinjection of lentivirus to establish testis-specific expression of PICK1. Our results indicate that the infertility of *Pick1* knockout mice could be successfully rescued by two different strains of testis-specific expression of PICK1 in transgenic mice, demonstrating that the defects of spermatogenesis are the primary defect of PICK1 deficiency in testis rather than that of the upstream of the hypothalamic-pituitary-gonadal axis or any other systems.

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Results

In vivo infection by seminiferous tubule microinjection of lentivirus. In order to re-express PICK1 protein specifically in the testes of *Pick1* knockout mice, we used the method of seminiferous tubule microinjection of lentivirus due to its convenience of handling and high efficiency of *in vivo* infection[18–20]. Before expressing PICK1, we first tested the method by expressing green fluorescent protein (GFP). Concentrated GFP-containing lentiviruses with titers around 1×10^8 IU/ml were prepared and applied to microinjection via the efferent duct of wild-type mice (Fig. 1A, B).

A group of injected mice were examined by both western blotting and cryosection analyses for the *in vivo* infection efficiency about 1
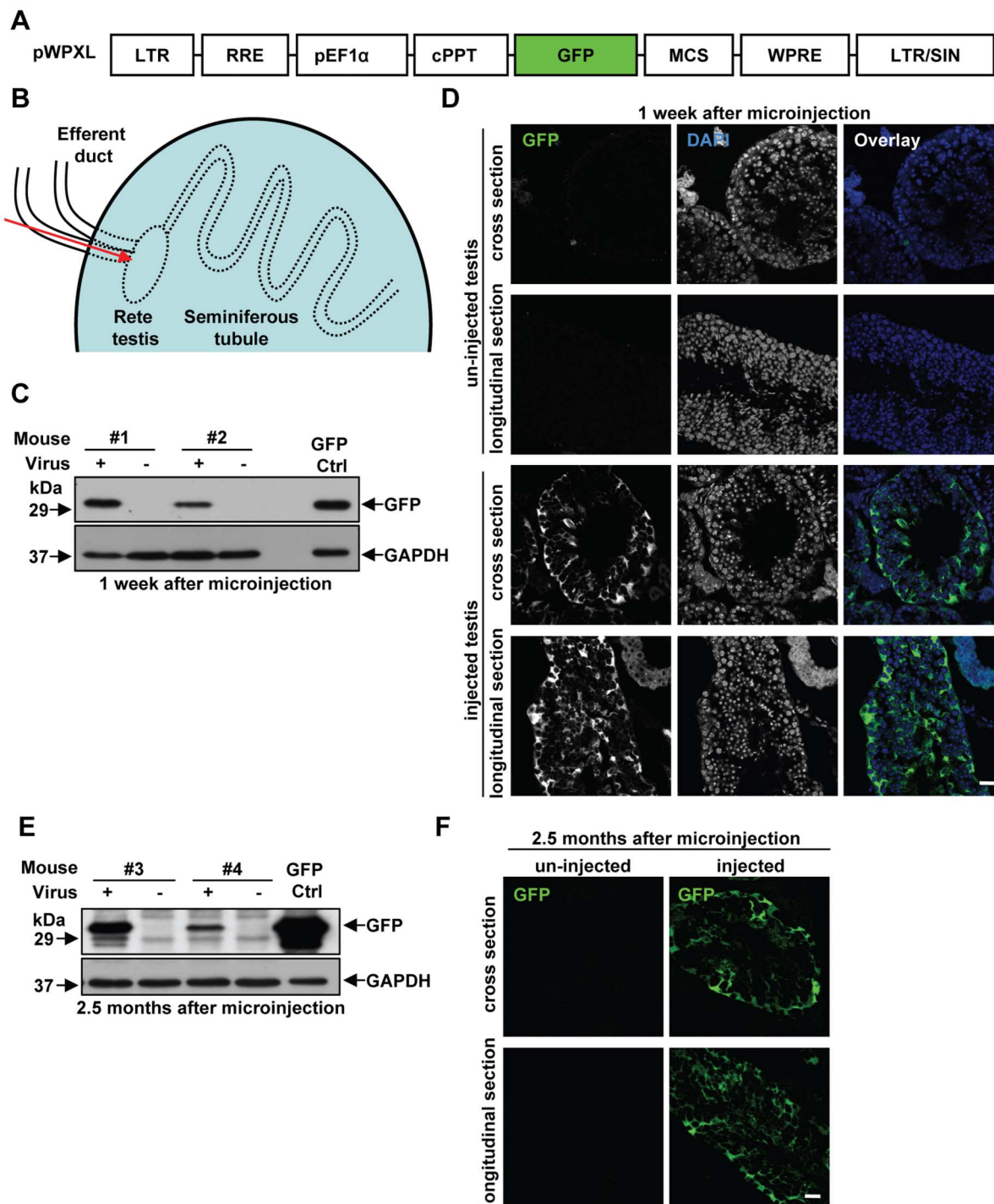

Figure 1 | *In vivo* infection by seminiferous tubule microinjection of lentivirus. (A) Schematic diagram of the pWPXL lentiviral vector. LTR: long terminal repeat. RRE, ref-responsive element. cPPT, central polypurine tract. pEF1α, human elongation factor alpha promoter; GFP, green fluorescent protein. WPRE, woodchuck hepatitis virus posttranscriptional control element. SIN, self-inactivating. (B) Schematic diagram of intratubular microinjection through the efferent duct. (C) Western blotting analysis of injected testes, 1 week after microinjection. GAPDH served as the loading control. Cell lysate from pWPXL-GFP lentivirus infected 293T cells was used as positive control. Full-length blots are presented in Supplementary Fig. S1. (D) Cryosection analysis of the injected testis and un-injected control. Nucleus was labeled by DAPI. Cryosections were cut at 10 μm in thickness. Scale bar, 20 μm. (E) Western blotting analysis of injected testes, 2.5 months after microinjection. GAPDH served as the loading control. Full-length blots are presented in Supplementary Fig. S1. (F) Cryosection analysis of the injected testis, 2.5 months after microinjection. Scale bar, 20 μm.

Table 1 | Transgenic mice from seminiferous tubule microinjection of lentivirus

Mouse	Lentivirus	Titer (IU/ml)	Injected mice			F0
			Total	Fertile (%)	With Tg F0(%)[a]	Tg/total (%)
WT	pWPXL-GFP	1.94×10^8	24	21 (87.5%)	3 (14.3%)	8/336 (2.4%)
	IRES2-based	1.97×10^8	22	19 (86.4%)	1 (5.3%)	4/497 (0.8%)
	Dual-promoter	1.81×10^8	7	6 (85.7%)	1 (16.7%)	4/127 (3.2%)
KO	IRES2-based	1.97×10^8	37	0	-	-
	Dual-promoter	1.81×10^8	15	0	-	-

[a]Tg stands for transgenic mice, and Tg F0 stands for the transgenic founders.

week after microinjection. Expression of GFP was detected in the injected testes but not the un-injected contralateral ones (Fig. 1C, D), suggesting that a lentiviral titer around 1×10^8 IU/ml is appropriate for intratubular infection. Furthermore, GFP signals were observed throughout all the layers of the seminiferous tubule including the basal compartment where the SSCs (spermatogonial stem cells) were located (Fig. 1D)[21]. Widespread GFP expression remained detectable about 2.5 months after microinjection (Fig. 1E, F). Since the duration of an entire spermatogenic cycle in mouse is about 35 days[21], the persistent expression of GFP protein over 70 days indicates that the *Gfp* gene has been integrated into the genome of SSCs and could be passed to their daughter cells during spermatogenesis.

Intratubular infection by lentivirus has a high preference to generate testis-specific transgenic mice. To further investigate the efficiency of this intratubular infection method, a group of 24 mice injected with pWPXL-GFP lentivirus were mated with wild-type females for generating transgenic pups (Table 1). Among them, 3 of the 21 fertile injected mice (14.3%) successfully produced transgenic pups, and 8 transgenic founders were obtained from the total 336 pups. The transgenic efficiency in our method is around 2.4%, which is similar to the previous report[18]. The expression of *Gfp* transgene in transgenic progenies was examined by both western blotting and GFP fluorescent signal analyses. Intriguingly, two different expression patterns were observed in the generated transgenic mice. Among the seven F1 transgenic males examined, six of them (85.7%) were found to have GFP specifically expressed in their testes. Only one (14.3%) F1 mouse was found to have GFP ubiquitously expressed in all the tissues tested, including brain, liver, pancreas and testis (Fig. 2A). For the first pattern, we examined more tissues including the female reproductive organs but still could not detect GFP signals (Fig. 2B). These results indicate that the intratubular infection method we used has a strong preference to generate testis-specific transgenic mice.

Two strategies for expressing exogenous PICK1 in the testes of *Pick1* knockout mice. After demonstrating the intratubular microinjection method as an efficient approach for *in vivo* infection, two strategies were employed to locally re-express PICK1 in the testes of *Pick1* knockout mice. In the first strategy, male *Pick1* knockout mice were directly injected with lentiviruses, with the hope that expression of exogenous PICK1 will rescue their infertility and thus produce progenies that all contain *Pick1* transgene. In the second strategy, wild-type male mice were first injected with PICK1 virus and then mated to *Pick1* knockout mice to test the rescuing effect. In addition, two different lentiviral vectors, an IRES-based and a dual-promoter-based were constructed for delivering the exogenous PICK1 together with a GFP reporter (Fig. 3A). The IRES-based vector was generated by inserting an IRES2 element between PICK1 and GFP to permit their independent expression from a single bicistronic mRNA[22]. The dual-promoter vector was constructed in a way that the expression of PICK1 and GFP was controlled by the pEF1α and pCMV promoters, respectively. Synchronous expression of PICK1 and GFP from these vectors was first confirmed in 293T cells (Fig. 3B), and then the lentiviruses produced from these vectors were used for injection.

For the first strategy, a total of 52 *Pick1* knockout male pups were microinjected and mated with wild-type female mice once they reached adulthood. PICK1 was found to be successfully expressed in the testes of the injected mice (Fig. 3C). However, despite the large number and intensive mating, these injected *Pick1* knockout mice have not produced any progeny (Table 1). For the second strategy, 22 and 7 wild-type pups were injected with the IRES-based and dual-promoter-based dual-gene lentiviruses, respectively (Table 1). Two strains of transgenic mice containing both *Pick1* and *Gfp* (*Pick1&Gfp*), one from the IRES-based and one from the dual-promoter-based, were obtained about 3–3.5 months after microinjection (Table 1). The overall transgenic efficiency is about 1.3%. As shown in Fig. 3D and 3E, expression of GFP was detected in the F1 transgenic mice and in both strains; we again found that GFP was
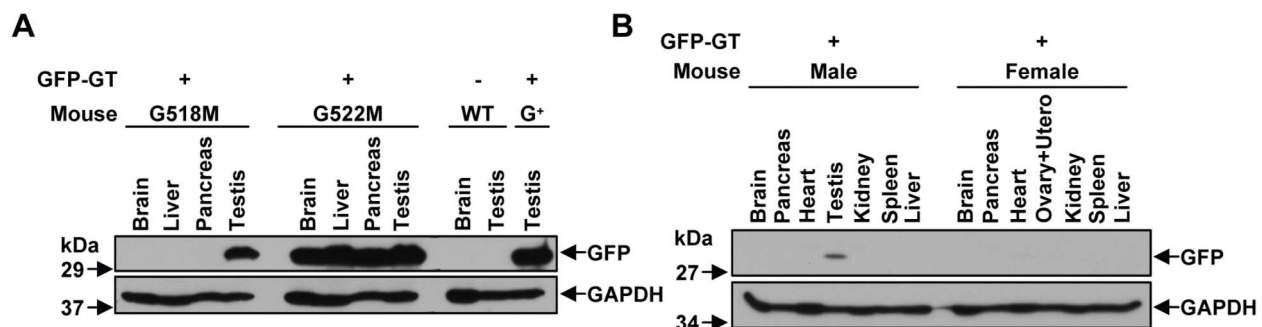

Figure 2 | Two expression patterns of *Gfp* transgenic mice generated by intratubular microinjection. (A) Western blotting analysis of the expression of GFP in the *Gfp* transgenic mice. G518M and G522M, GFP transgenic male mice; WT, wild-type; G⁺, GFP positive control. GAPDH served as the loading control. In the G522M transgenic mouse GFP was detected in all brain, liver, pancreas and testis, while in the G518M mouse GFP was only expressed in the testis. (B) Western blotting analysis of the tissue distribution of GFP in male and female transgenic mice. GAPDH served as the loading control. Full-length blots are presented in Supplementary Fig. S2.
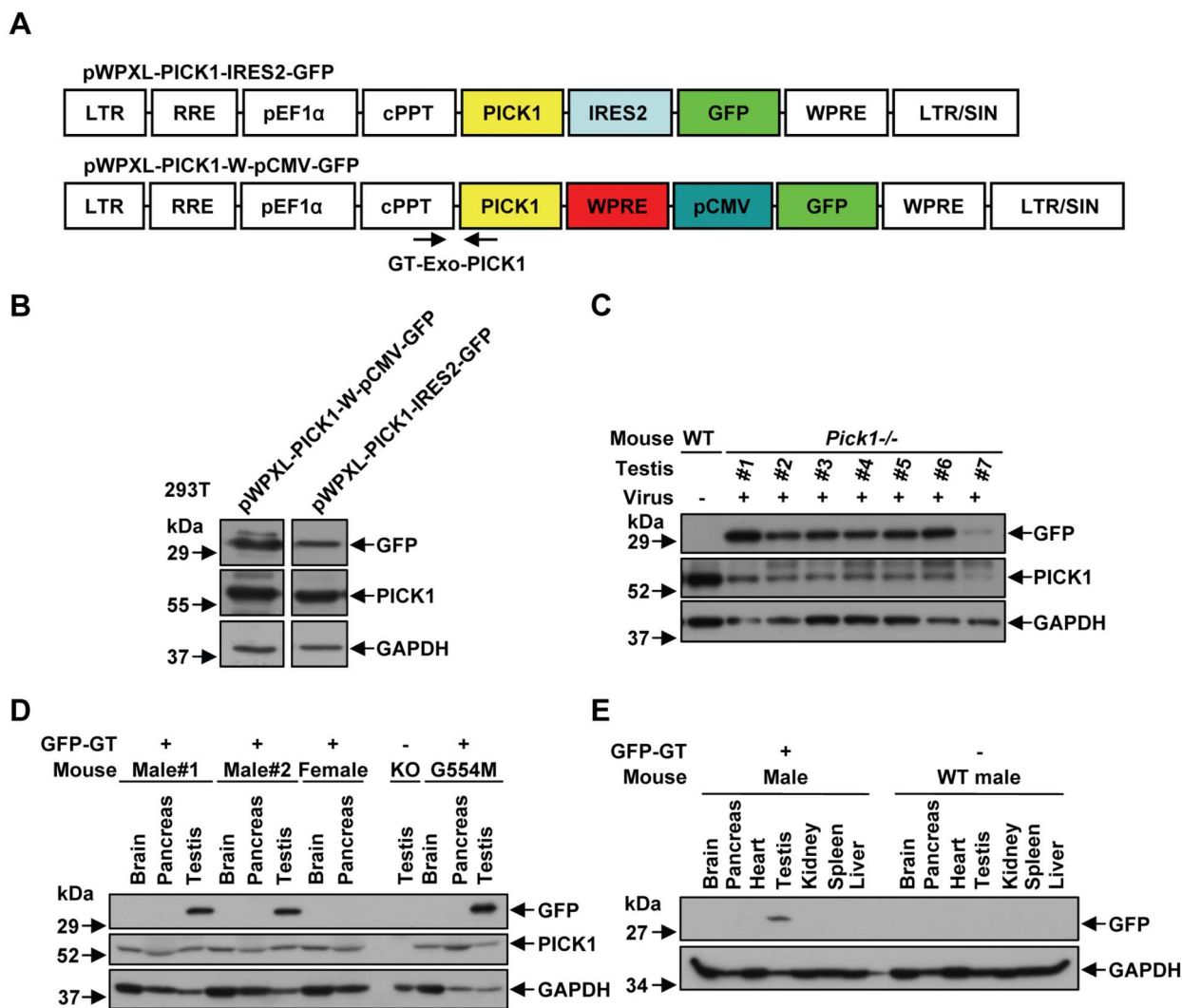
Figure 3 | Generation of testis-specific *Pick1&Gfp* dual-gene transgenic mice. (A) Schematic diagram of the pWPXL-based constructs. IRES, internal ribosome entry site. pCMV, human cytomegalovirus immediate early promoter. Arrows indicate the design of exogenous-PICK1 genotyping (GT-Exo-PICK1) PCR primers. (B) Western blotting analysis of the HEK293T cells transfected with different lentiviral vectors. (C) Western blotting analysis of the testes from the injected *Pick1* knockout mice. The sample from wild-type mouse was used as the positive control for PICK1 and negative control for GFP. (D) Western blotting analysis of the expression of GFP and PICK1 in the *Pick1&Gfp* dual-gene transgenic mice. KO, *Pick1* knockout sample, was used as a negative control for PICK1. G554M, the *Gfp* transgenic male mouse, was used as a positive control for GFP. (E) Western blotting analysis of the tissue distribution of GFP expression in the transgenic mice. Full-length blots are presented in Supplementary Fig. S3 and S4.

specifically expressed in testis. This provided further support to the notion that seminiferous tubule microinjection of lentivirus has a high preference to generate testis-specific transgenic mice.

Rescue of the infertility by testis-specific *Pick1* transgene. To test whether testis-specific *Pick1* transgene can rescue the infertility of *Pick1* knockout mice, we mated the testis-specific *Pick1&Gfp* transgenic male mice with *Pick1* knockout females. The male offsprings with the exogenous *Pick1&Gfp* transgenes and endogenous *Pick1* gene knocked out were selected by genotyping analysis and used for subsequent rescue test (Fig. 4A).

There are some differences between the IRES-based and dual-promoter-based transgenic mice in terms of the levels and patterns of the transgene expression. In the IRES-based transgenic mice, GFP fluorescent signals were detected from almost the entire section of the seminiferous tubules, while in the dual-promoter-based transgenic mice they were much weaker in the basal compartment than in the adluminal compartment (Fig. 4B). In addition, the expression levels of both PICK1 and GFP in the dual-promoter-based transgenic

mice were lower than in the IRES-based transgenic mice (Fig. 4C). Therefore, we focused on the IRES-based transgenic mice for the following rescue studies.

The expression of the exogenous PICK1 was testis-specific (Fig. 4C, D), but lower than the endogenous PICK1 level in wild-type mice ($35.3\% \pm 4.2\%$ of the wild-type level). ICA69 (islet cell autoantigen 69kD), a major binding partner of PICK1, is also expressed in the testis. Its protein level is highly dependent on the presence of PICK1, since it is undetectable in *Pick1* knockout testis[13,16]. Thus, the testis-specific restoration of ICA69 ($45.7\% \pm 8.7\%$ of the wild-type level) in the rescued knockout mice further confirmed that PICK1 was only expressed in the testis (Fig. 4C, D). Male *Pick1* knockout mice have been reported to have round-headed sperm with defective mitochondrial sheaths, malformed acrosomes, abnormal nuclear shapes and smaller testes[15]. To examine whether testis-specific expression of PICK1 could reverse these defects, we examined the sperm morphology, acrosome formation and testes size. In the sperm of rescued mice, the morphology of their heads had been restored from round to hook-shaped (Fig. 5A).
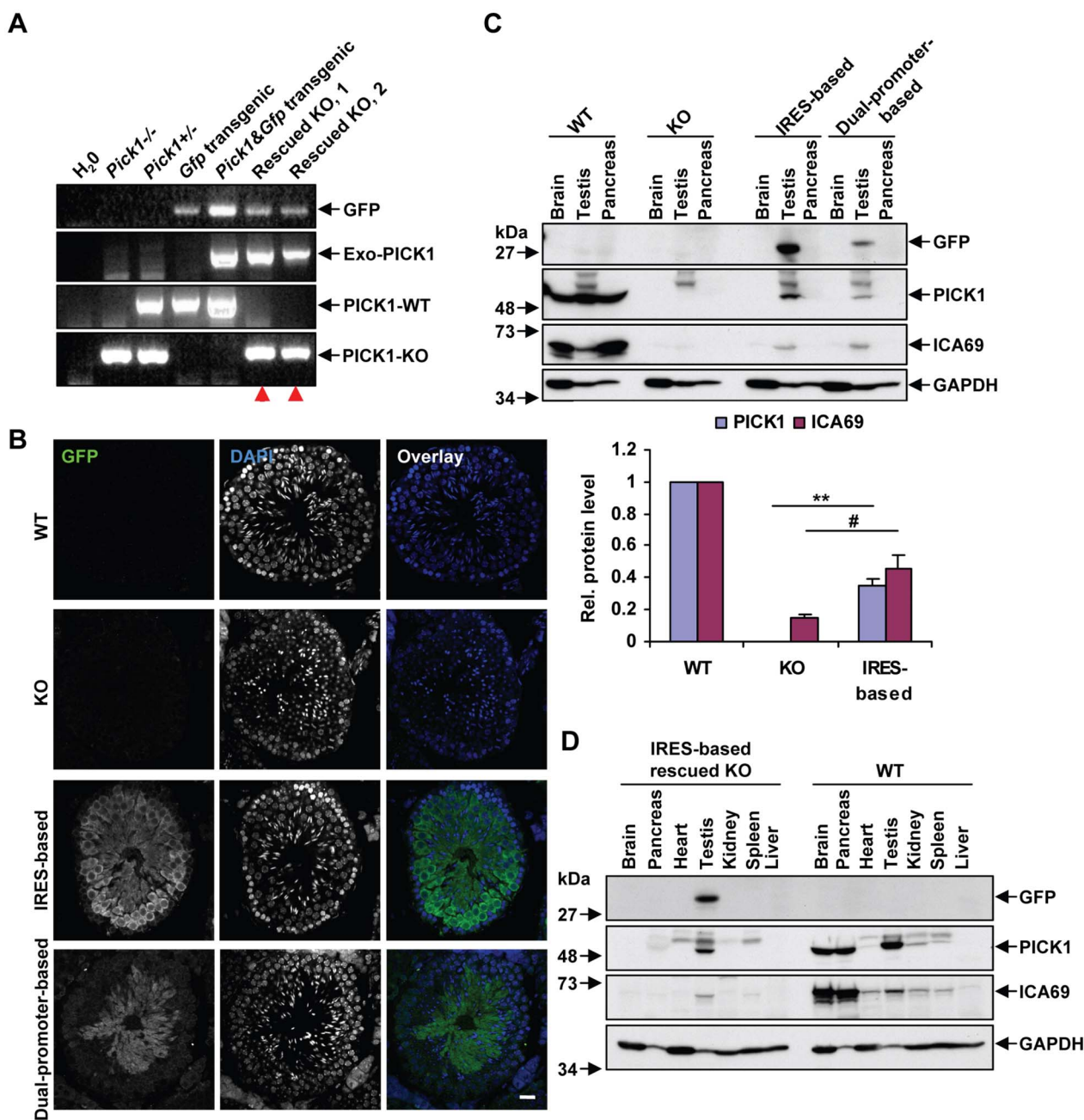
Figure 4 | Re-expression of testis-specific *Pick1&Gfp* transgenes in *Pick1* knockout mice. (A) Representative results of genotyping confirmation of the rescued mice. Black arrows indicate the target bands. Red arrowheads indicate the rescued knockout mice. Exo-PICK1 is short for exogenous PICK1. Full-length gels are presented in Supplementary Fig. S5. (B) Cryosection analysis of the GFP fluorescent signal in the *Pick1* knockout mice with either IRES-based or dual-promoter-based *Pick1&Gfp* transgenes. Nucleus was labeled by DAPI. GFP signal was enhanced by anti-GFP antibody. Scale bar, 20 μm. (C) Western blotting analysis of the expression of GFP, PICK1 and ICA69 in both strains of transgenic mice (with *Pick1* knockout background). Both PICK1 and ICA69 proteins were restored but lower than those of wild-type mice. Quantitative data are presented as mean ± SEM. The protein levels of PICK1 and ICA69 in wild-type samples were considered as 1. In *Pick1* knockout mice, PICK1 level = 0, ICA69 level = 0.15 ± 0.04. In rescued mice, PICK1 level = 0.35 ± 0.04, ICA69 level = 0.46 ± 0.17. Comparison between knockout and rescued mice was conducted using Student's t test (**$p < 0.01$, #$p < 0.05$, n = 4). (D) Western blotting analysis of the tissue distribution of the IRES-based *Pick1&Gfp* transgenes in *Pick1* knockout mice. Full-length blots are presented in Supplementary Fig. S6.

Besides, the protein intensity in their heads, presented by coomassie blue staining, was comparable to the normal level. The defects of mitochondrial sheaths disappeared (Fig. 5A). Moreover, the acrosomes from the sperm of rescued mice were again crescent moon-shaped, compared with the fragmented and mislocalized acrosomes in knockout mice (Fig. 5B). The condensed nuclei of spermatozoa in rescued mice and wild-type mice were the same hook-shaped, while in knockout mice were abnormally round (Fig. 5C). Consistent with the slightly increased size of testes, the weight of the testes from rescued mice was significantly heavier than that from knockout mice (Fig. 5D).

To date, 9 of the 14 IRES-based (64.3%) and 3 of the 14 dual-promoter-based (21.4%) target male mice (*Pick1* knockout mice with *Pick1&Gfp* transgenes) had successfully sired pups. We also noticed
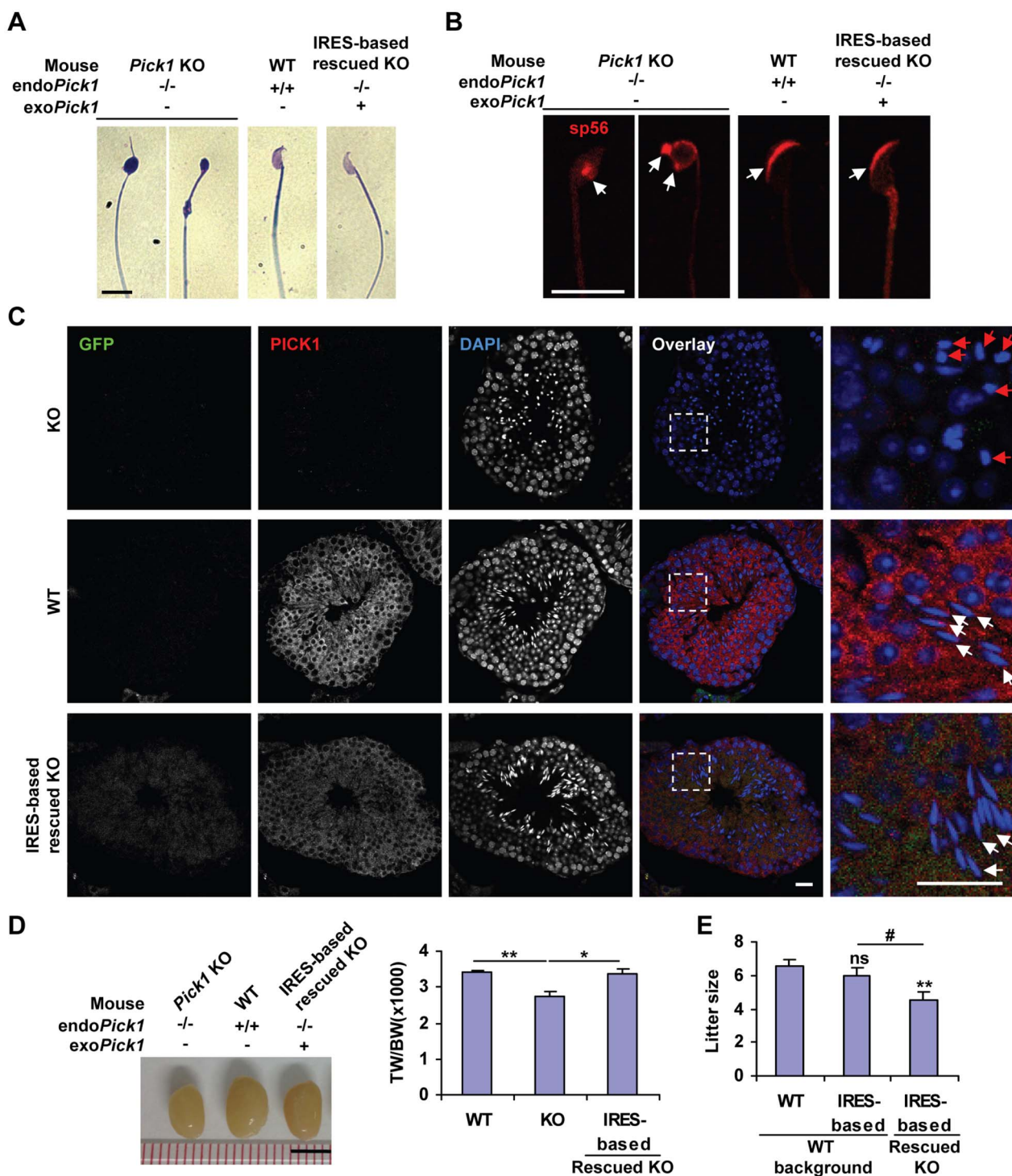
Figure 5 | Rescue of the infertility of *Pick1* knockout male mice by testis-specific *Pick1* transgene. (A) Morphology of the sperm with coomassie blue staining. Scale bar, 10 μm. (B) Immunostaining of acrosome matrix protein sp56 in sperm. White arrows indicate the acrosomes. Scale bar, 10 μm. (C) Cryosection analysis of the spermatogenesis in the rescued testis. Nucleus was labeled by DAPI. Red and white arrows indicate the abnormally round and normal hook-shaped nuclei, respectively. Scale bar, 20 μm. (D) The size of the testes of IRES-based rescued mice increased compared with knockout control. TW/BW, testis weight/body weight. Scale bar, 5 mm. Quantitative data are presented as mean ± SEM. Wild-type = 3.43 ± 0.05, knockout = 2.73 ± 0.13, IRES-based rescued = 3.36 ± 0.14. Student's t test (*$p < 0.05$, **$p < 0.01$, n = 4). (E) The litter size of the IRES-based rescued mice was smaller than that of the wild-type mice or the corresponding transgenic mice. Quantitative data are presented as mean ± SEM. Wild-type mice = 6.5 ± 0.4, IRES-based transgenic mice (WT background) = 6.0 ± 0.4, IRES-based rescued KO mice = 4.5 ± 0.5. Unpaired student's t test (#$p < 0.05$, **$p < 0.01$, n ≥ 19).

that the litter size of these rescued mice was smaller than that of the wild-type mice or the corresponding transgenic mice (Fig. 5E), probably due to the lower expression level of exogenous PICK1. Nevertheless, the successful production of progenies confirmed the functional recovery of sperms in the rescued knockout males. Taken together, these results suggest that the infertility of *Pick1* knockout male mice could be rescued by local re-expression of exogenous PICK1 in the testis.

Discussion

In order to validate whether the infertility of male *Pick1* knockout mice is caused by the primary defect in the testis, we utilized two different strategies to rescue PICK1 specifically in testis. One strategy uses direct intratubular microinjection of PICK1-containing lentivirus to *Pick1* knockout mice. The other is to first generate testis-specific *Pick1* transgenic mice by injecting wild-type testis and then backcross the *Pick1* transgene into the *Pick1* knockout mice. Using the second approach, testis-specific expression of PICK1 was achieved and it was sufficient to rescue the defective spermatogenesis and infertility in *Pick1* knockout male mice. This result indicates that the abnormal spermatogenesis and infertility in *Pick1* knockout mice are the consequences of PICK1 deficiency in testis, but not a secondary defect due to lack of PICK1 in neuroendocrine systems.

An interesting observation of this study is the high tendency of producing testis-specific transgenic mice by seminiferous tubule microinjection of lentivirus, despite the transgenes were driven by ubiquitous promoters. Around 85% of the examined F1 *Gfp* transgenic mice and all the examined *Pick1&Gfp* transgenic mice displayed testis-specific expression pattern. As far as we know, this is the first report that the testicular microinjection method could produce tissue-specific transgenic progeny. In previous studies, the intratubular injection of retrovirus or intertubular injection of lentivirus both generated ubiquitously expressed transgenic mice[18,23]. The difference may be due to the use of different viruses and injection approaches. One possible mechanism of the testis-specific expression found in our study is that the HIV (human immunodeficiency virus)-derived lentiviral vectors prefer integrating into the transcriptionally active regions and local hotspots[24–26], which are associated with LEDGF/p75 (lens epithelium-derived growth factor, also called DFS70/p75/PSIP1) and epigenetic marks such as H3K4me (methylated histone 3 on lysine 4)[27,28]. Therefore, while infecting the SSCs *in vivo*, lentivirus may easily integrate into the transcriptional regions specifically active in the testis[29].

We were not able to restore the fertility of *Pick1* knockout mice by injecting lentiviruses directly into their testes. There are several possible reasons, such as the inappropriate expression pattern of the exogenous PICK1, the probable developmental defects of the reproductive system occurred during the embryonic stage and/or the scarcity of infected SSCs. The success in rescuing the infertility by the *Pick1* transgenes delivered by the same lentiviruses through backcrossing suggests the first possibility is unlikely. The other two reasons still could not be ruled out, especially the low infection rate of SSCs. By using the intratubular injection method, only a fraction of the testis could be infected. The overall ratio of transgenic pups generated by this method was around 0.8%–3.2% (Table 1), suggesting that the maximal overall ratio of infected SSCs was only 1.6%–6.4% (if only half of the parental chromosomes were transmitted to the offspring)[18]. Therefore, the future work of increasing viral titer and combination of intratubular and intertubular injection may help tackle this problem.

Methods

Construction of lentiviral vectors. PICK1 cDNA used here was described previously[30]. The GFP-containing pWPXL lentiviral vector (pWPXL-GFP) was from Addgene (plasmid 12257, Didier Trono)[31]. In order to facilitate further modifications, a backbone construct, pWPXL-PICK1, was first generated by replacing GFP with PICK1 via BamHI/EcoRI (New England Biolabs). The IRES (internal ribosome entry site)-based dual-gene lentiviral vector, pWPXL-PICK1-IRES2-GFP, was constructed by subsequently inserting an IRES2 element from pIRES2-GFP (Clontech) and a GFP into the pWPXL-PICK1 backbone, using EcoRI/SpeI and SpeI/SpeI, respectively. The dual-promoter lentiviral vector, pWPXL-PICK1-WPRE-pCMV-GFP, was constructed by subsequently inserting a CMV promoter (human cytomegalovirus immediate early promoter) from pIRES2-GFP, a GFP and a WPRE element (woodchuck hepatitis virus posttranscriptional control element) from pWPXL into the pWPXL-PICK1 backbone, using EcoRI/SpeI, SpeI/SpeI and EcoRI/EcoRI, respectively. The additional WPRE element inserted between the two promoters was to make sure both expression cassettes can work independently[32].

Lentivirus production. Recombinant lentiviruses were produced by transient co-transfection of HEK293T cells and titrated by FACS (fluorescence-activated cell sorting) as previously described[33,34]. Briefly, 24 hrs prior to transfection, about 2–2.5 × 10⁶ cells were placed into a 10 cm tissue culture dish. Then the cells were co-transfected with pWPXL lentiviral vector (10 μg), psPAX2 plasmid (6.5 μg) and pMD2.G plasmid (3.5 μg) (Addgene) by calcium phosphate precipitation method. Medium was changed about 9 hrs later. After transfection for 36–48 hrs, medium collected was spun at 1000 rpm for 5 mins to remove cell debris and filtered through a 0.45 μm filter. Crude medium containing lentiviruses was aliquoted and stored at −80°C or concentrated by centrifuging at 83,000 g for 1.5 h at 4°C (HITACHI, CP 100MX) and resuspended in PBS (phosphate-buffered saline).

To determine the titer of lentivirus by FACS, 24 hrs prior to infection, about 2–3 × 10⁴ cells in 1 ml medium were placed to each well of a 24-well plate. The number of cells was counted again on 1 well (about 6–8 × 10⁴ cells) just before adding the virus. Then viruses with 10-fold serial dilutions were added to the cells. Cells were split and analyzed by FACS 3 days later. Usually, the titer of crude lentivirus was around 1 × 10⁵ IU/ml (infectious units per ml) while the titer of concentrated lentivirus was around 1 × 10⁸–10⁹ IU/ml.

Cell culture and transfection. HEK293T cells culture and transfection were performed as previously described[15,35]. Briefly, HEK293T cells were cultured in humidified atmosphere containing 5% CO_2 in minimal essential medium (MEM, Invitrogen, 11700-077) with 10% fetal bovine serum (FBS, Invitrogen, 10270-106), 1 mM sodium pyruvate (Invitrogen, 3126), 1× Penicillin-Streptomycin-Glutamine (Invitrogen, 10378-016) and passaged every 2 to 3 days when the cell confluence reached 80–90%. Calcium phosphate coprecipitation method was used for transient tranfection and medium was changed about 9 hrs later.

Mice and microinjection procedure. C57BL/6 (B6) mice were purchased from the APCF (Animal and Plant Facility) of HKUST (The Hong Kong University of Science and Technology). *Pick1* knockout mice used in this study were described previously[15,36].

Seminiferous tubule microinjection was performed as previously described[18,19]. Briefly, around 2–10 μl of PBS containing concentrated lentiviral particles was introduced into the seminiferous tubules of immature mice (5–10 days old). The pups were placed on ice to cause hypothermia-induced anesthesia. The pups were returned to their dams after the operation and used for mating with wild-type B6 females to produce transgenic offspring after at least 6 weeks. In some experiments, the injected mice were sacrificed by cervical dislocation method for different analyses.

The Animal Research Panel of the Committee on Research Practice of the Hong Kong University of Science and Technology approved all of the animal experimentation protocols.

Genotyping. Mouse ear tissue was used as a source of DNA for genotyping. A specific punch, Ear Punch Pliers from Harvard Apparatus, was used to produce a small (0.5–2 mm) notch near the edge for mice identification, according to The Jackson Laboratory, U.S.A. & IACUC.

PCR (polymerase chain reaction) was used for determining the genotype of transgenic mice according to a modified protocol from The Jackson Laboratory. GFP genotyping (GT-GFP) and WPRE genotyping (GT-WPRE) were designed to amplify a fragment from GFP and WPRE elements in the pWPXL integrant, respectively. Exogenous PICK1 genotyping (GT-Exo-PICK1) was designed to detect the exogenous *Pick1* gene inside the viral integrant but not the endogenous *Pick1* gene. Genotyping of endogenous *Pick1* was the same as previously described[16]. Primers used: for GFP genotyping were forward 5′ AAGTTCATCTGCACCACCG 3′ and reverse 5′ TCCTTGAAGAAGATGGTGCG 3′, for WPRE genotyping were forward 5′ TCCTGGTTGCTGTCTCTTTATG 3′ and reverse 5′ GCAGAATCCAGGTGG CAAC 3′, for Exo-PICK1 genotyping were forward 5′ TCTTCCATTTCAGGTGTC GTG 3′ and reverse 5′ GCAGGCGTGTTATCAAATACC 3′, for PICK1-WT genotyping were forward 5′ CACTCGCAGCTTGTTCTGATCTG 3′ and reverse 5′ TCACTTGCCAGAGGAGAAAACTG 3′, and for PICK1-KO genotyping were forward 5′ AAAAATAGGCGTATCACGAGGC 3′ and reverse 5′ TCACTTGCCAG AGGAGAAAACTG 3′.

Antibodies. The rabbit anti-GFP polyclonal antibody, rabbit anti-ICA69 polyclonal antibody, guinea pig and rabbit anti-PICK1 polyclonal antibodies and the mouse anti-sp56 antibody (QED Bioscience Inc.) were described previously[15,16]. All the secondary antibodies were also the same as previously described[15]. Briefly, the rhodamine red X-conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories Inc., while the Alexa Fluor 488-conjugated secondary antibody was purchased from Molecular Probes (Invitrogen).

Epididymal sperm extracting and morphology classification. Epididymal sperm analyses were conducted as previously described[15]. Briefly, sperm from cauda epididymis of adult mice were extracted by incubation of cauda epididymis at 37°C under 5% CO_2 for 30 mins. For morphology classification, about 15–20 μl of the sperm extract medium was spread on a pre-coated slide for morphological observation or further staining. Quick coomassie blue staining for morphological observation was performed by incubating the sperm slides with 0.22% coomassie solution for 1 min[37].

Histology. Cryosection experiments were conducted as previously described[13]. Briefly, mouse tissues were dissected out from adult mice, fixed with 4% PFA (paraformaldehyde) and 4% sucrose in PBS for at least 4 hrs at 4°C, and then incubated in gradient sucrose-PBS solution (10% sucrose for 1 hr, 20% sucrose for 1 hr, and 30% sucrose overnight) at 4°C. Cryosections were cut on a Cryostat (Leica Histopathology System, CM1850) at 10 µm thickness.

Immunohistochemistry. Fluorescent immunostaining was performed as previously described[13,16]. Briefly, cryosections were first rinsed with PBS, then permeabilized and blocked with 10% normal donkey serum (NDS) plus 0.2% Triton X-100 in PBS for 1 hr at RT (room temperature). After incubating with primary antibodies at 4°C overnight, rinsing with PBS three times and incubating with secondary antibodies for 1 hr at RT, slides were then incubated with 0.5 µg/ml DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) for 15 mins at RT to stain the nucleus. Finally, the slides were mounted with Vecta Mount solution (Vector Labs) for fluorescent signal analysis.

Imaging and softwares. The images were captured by Nikon Eclipse TE2000 inverted fluorescence microscope, Zeiss LSM510 confocal microscope, or Zeiss LSM710 confocal microscope. Image processing was preformed using MetaMorph (Universal Imaging) and Adobe Photoshop.

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Acknowledgments

Author contributions

X. Li performed majority of the experiments, analyzed the data and wrote the manuscript, Z. Mao established the seminiferous tubule microinjection of lentivirus method and contributed to the writing of the manuscript. M. Wu contributed to the experiments in Fig. 4 and 5. J. Xia designed the study, analyzed the data and wrote the manuscript.

Additional information