



Published in final edited form as:

Andrology. 2019 September ; 7(5): 644–653. doi:10.1111/andr.12621.

Nine genes abundantly expressed in the epididymis are not essential for male fecundity in mice

Taichi Noda^{1,#}, Nobuyuki Sakurai^{1,#}, Kaori Nozawa^{2,3}, Sumire Kobayashi⁴, Darius J. Devlin^{2,3,5}, Martin M. Matzuk^{2,3,6,7,8}, Masahito Ikawa^{1,4,9}

¹Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 5650871, JAPAN

²Center for Drug Discovery, Baylor College of Medicine, Houston, TX 77030, USA

³Department of Pathology & Immunology, Baylor College of Medicine, Houston, TX 77030, USA

⁴Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 5650871, JAPAN

⁵Interdepartmental Program in Translational Biology & Molecular Medicine, Baylor College of Medicine, Houston, TX 77030, USA

⁶Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

⁷Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

⁸Department of Pharmacology and Chemical Biology, Baylor College of Medicine, Houston, TX 77030, USA

⁹Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, JAPAN

Abstract

Background—Spermatozoa become competent for fertilization during transit through the epididymis. As spermatozoa from the proximal caudal epididymis can fertilize eggs, proteins from the caput and corpus epididymis are required for sperm maturation.

Objectives—Microarray analysis identified that more than 17,000 genes are expressed in the epididymis; however, few of these genes demonstrate expression restricted to the epididymis. To analyze epididymis-enriched gene function *in vivo*, we generated knockout (KO) mutations in nine genes that are abundantly expressed in the caput and corpus region of the epididymis.

Corresponding authors: Martin M. Matzuk, MD, PhD, Director and Professor, Center for Drug Discovery and Department of Pathology & Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA, Phone: +1-713-798-6451; FAX: +1-713-798-5838, mmatzuk@bcm.edu, Masahito Ikawa, PhD, Professor, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 5650871, JAPAN, Phone: +81-6-6879-8375, FAX: +81-6-6879-8376, ikawa@biken.osaka-u.ac.jp.

[#]These authors contributed equally to this work.

Authors' Contributions

Designed the research: T.N., N.S., K.N., M.M.M., and M.I.; Performed the research: T.N., N.S., K.N., S.K., and D.J.D.; Analyzed the data: T.N., N.S., and K.N.; and Wrote the paper: T.N., N.S., K.N., M.M.M. and M.I.

COI statement:

The authors declare no conflict of interest.

Materials and Methods—KO mice were generated using the CRISPR/Cas9 system. The histology of the epididymis was observed with hematoxylin and eosin staining. KO males were caged with wild-type females for 3 to 6 months to check fertility.

Results—We generated individual mutant mouse lines having indel mutations in *Pate1*, *Pate2*, or *Pate3*. We also deleted the coding regions of *Clpsl2*, *Epp13*, and *Rnase13*, independently. Finally, the 150 kb region encoding *Gm1110*, *Glb112*, and *Glb113* was deleted to generate a triple KO mouse line. Histology of the epididymis and sperm morphology of all KO lines were comparable to control males. The females mated with these KO males delivered pups at comparable numbers as control males.

Discussion and Conclusion—We revealed that nine genes abundantly expressed in the caput and corpus epididymis are dispensable for sperm function and male fecundity. CRISPR/Cas9 mediated KO mice generation accelerates the screening of epididymis enriched genes for potential functions in reproduction.

Keywords

genome editing; genetically modified mice; sperm maturation

Introduction

Spermatozoa released from the testis are incapable of fertilizing eggs until they acquire capabilities necessary for fertilization competence (such as motility, capacitation, acrosome reaction and sperm-egg fusion capabilities) by translocating through the epididymis. This step is called “sperm maturation” (Robaire & Hermo 1988; Robaire, Hinton, and Orgebin-Crist 2006). The epididymis is mainly composed of 3 regions: the head (caput), body (corpus), and tail (cauda). In many mammalian species, the sperm journey from the caput region to the cauda region in about 10 days (Robaire & Hermo 1988). The timing of acquisition of fertilization competence by spermatozoa varies among mammalian species; however, almost all spermatozoa isolated from the proximal cauda epididymis have fertilizing ability (Robaire & Hermo 1988), suggesting that factors from the caput and corpus epididymis play important roles in sperm maturation.

Microarray analysis shows that more than 17,000 genes are expressed in the epididymis with gene expression patterns varying greatly among regions of the epididymis (Johnston *et al.*, 2005), suggesting that epididymis-specific or epididymis-enriched genes play important roles during sperm maturation. However, the physiological function of many of these genes remain unknown.

The emergence of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system opened a new era in mammalian genome editing (Cong *et al.* 2013; Wang *et al.* 2013; Yang *et al.* 2013). The guide RNA (gRNA)/Cas9 complex recognizes 20 nucleotides upstream of the protospacer adjacent motif (PAM) as the target sequences, then the Cas9 nuclease causes a blunt end double-strand break (DSB) between the 3rd and 4th nucleotides upstream of the PAM. After DSB formation, non-homologous end-joining (NHEJ) causes an indel mutation. We previously reported that indel mutations were efficiently obtained by

injecting single gRNA and Cas9 expressing plasmids into eggs (Mashiko *et al.*, 2013). In conjunction with conventional ES cell mediated gene-targeting, we also demonstrated that many testis-enriched genes are not essential for male fertility (Miyata *et al.*, 2016). Our previous work demonstrated that CRISPR/Cas9 mediated KO mice generation and phenotype screening is a cost-effective and labor-effective approach to quickly identify essential gene functions *in vivo* (Mashiko *et al.*, 2014).

In the present study, we have knocked out nine epididymis-enriched genes and discovered these genes are not essential for sperm fertilizing ability and male fecundity.

Materials and Methods

Animals

All mice used in this study were purchased from Japan SLC or CLEA Japan. Mice were acclimated to a 12 hour-light/12 hour-dark cycle. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (#Biken-AP-H30-01) and the Institutional Animal Care and Use Committee of Baylor College of Medicine (AN-716).

cDNA and RT-PCR

All tissues [brain, heart, kidney, liver, lung, spleen, thymus, ovary, uterus, testis, epididymis (caput, corpus, and cauda regions), prostate (mixture of dorsal, lateral and ventral regions), coagulating gland, and seminal vesicle] were collected from C57BL/6Ncr mice. These samples were homogenized in TRIzol (Ambion). The total RNA was reverse-transcribed to cDNA using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen). Five ng of cDNA was used for PCR with primer sets (Table S1) and KOD DNA Polymerase (KOD-FX Neo, TOYOBO).

Gene tree

The gene tree was made by GENETYX with the amino acid sequence (method: UPGMA) [for Colipase like 2 (CLPSL2): NP_001030043 (mouse), Q6UWE3 (human), XP_003311311 (chimpanzee), XP_022281476 (dog)] [for Epididymal protein 13 (EPP13): NP_001170887 (mouse), NP_001341587 (human), PNI23622 (chimpanzee), XP_024835189 (cow)] [for GM1110: XP_006510522 (mouse), ENSCAFT00000043177 (dog), ENSBTAT00000016226 (cow)] [for Galactosidase, beta 1-like 2 (GLB1L2): XP_006510360 (mouse), NP_612351 (human), XP_009422813 (chimpanzee), XP_022273772 (dog), XP_010811276 (cow)] [for GLB1L3: AAI32203 (mouse), AAH11001 (human), XP_024203264 (chimpanzee), XP_005620347 (dog), XP_024831720 (cow)] [for Prostate and testis expression 1 (PATE1): NP_001186882 (mouse), NP_612151 (human), XP_024203322 (chimpanzee), XP_022273825 (dog), XP_024843340 (cow)] [for PATE2: XP_011240865 (mouse), NP_997720 (human), XP_001148077 (chimpanzee), XP_005619644 (dog), XP_024843404 (cow)] [for PATE3: NP_001161064 (mouse), NP_001123355 (human), XP_024203289 (chimpanzee), XP_022273771 (dog), XP_024843215 (cow)] [for RNase A family 13 (RNASE13): XP_017171565 (mouse),

AAV87186 (human), XP_003804788 (chimpanzee), XP_013974975 (dog), AAI34657 (cow)].

Egg collection

Pregnant mare serum gonadotropin (PMSG) (5 units, ASKA Pharmaceutical) or CARD HyperOva (0.1 mL, Kyudo) was injected into the abdominal cavity of B6D2F1 females, followed by human chorionic gonadotropin (hCG) (5 units, ASKA Pharmaceutical) and natural mating with B6D2F1 males 48 hours after PMSG or CARD HyperOva. After 20 hours, we collected fertilized eggs with 2 pronuclei for genome editing.

Pronuclear injection

Pronuclear injection with 5 ng/μL of gRNA/Cas9 expressing plasmid (for *Pate1*, *Pate2* and *Pate3*) was performed as previously reported (Mashiko *et al.*, 2013; Noda *et al.*, 2017). The crRNA and tracrRNA (Sigma) were diluted with nuclease free water (non-DEPC treated, Ambion). The mixture was denatured at 95°C for 1 minute and allowed to anneal by cooling gradually to room temperature (~1 hour). Each gRNA was mixed with Cas9 protein solution (Thermo Fisher Scientific) and T₁₀E_{0.1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4), and then incubated at 37°C for 5 minutes to prepare the gRNA/Cas9 RNPs. For multiple gene targeting, we prepared the gRNA/Cas9 RNP solution separately and then combined them [final concentration: 30 ng/μL (≈ 200 nM) Cas9 for 20 ng/μL (≈ 600 nM) of each gRNA (Table S2)]. After centrifugation at 20,000 g at 4°C for 10 minutes, the mixture was used for pronuclear injection (for *Cpsl2*, *Epp13*, *Rnase13*, *Gm1110*, *Glb112*, *Glb113*).

Electroporation

The gRNA was prepared as described above. The gRNA was mixed with Cas9 protein solution and opti-MEM media (Thermo Fisher Scientific), and then incubated at 37°C for 5 minutes to prepare the gRNA/Cas9 RNPs [final concentration: 50 ng/μL (≈ 330 nM) Cas9 for 20 ng/μL (≈ 600 nM) of each gRNA (Table S2)]. The gRNA/Cas9 RNP solution was placed between the electrodes with a 5 mm gap in the NEPA21 Super Electroporator (Nepagene). Fertilized eggs were arranged between the electrodes, and then the electroporation was done with the following conditions [resistance value: 550~600 Ω, poring pulse: 225 V (voltage), 2 ms (pulse amplitude), 50 ms (pulse interval), 4 (pulse number), 10% (attenuation), + (polarity), transfer pulse: 20 V (voltage), 50 ms (pulse amplitude), 50 ms (pulse interval), ± 5 (pulse number), 40% (attenuation), +/- (polarity)].

Egg transfer

Injected and electroporated embryos were transplanted into the uterus of pseudo-pregnant ICR recipients. After 19 days, offspring were obtained by natural birth or Caesarean section.

Epididymis histology and sperm morphology

Epididymides were fixed in Bouin's fluid (Polysciences) at 4°C overnight. Fixed epididymides were dehydrated by increasing ethanol concentrations, and then were embedded with paraffin. Paraffin sections (5-μm) were stained with Mayer hematoxylin solution for 3 to 5 minutes, counterstained with eosin Y solution [53% (v/v) ethanol, 0.3%

(v/v) eosin, and 0.5% (v/v) acetic acid] for 2 to 5 minutes, dehydrated in increasing ethanol concentrations, and finally mounted in Permount or Entellan new (Merck). The caudal epididymal spermatozoa were observed with phase contrast microscopy.

Mating test

KO male mice were caged with 2 or 3 B6D2F1 females (for *Pate1*, *Pate2*, *Pate3*, *Epp13*, *Rnase13*, and *Glb112-Gm1110* mutant mice), or 1 hybrid female (C57BL/6J × 129S5/SvEvBrd.) (for *Cpls12*) for 3 to 6 months. Frozen spermatozoa from *Pate1*-, *Pate2*-, *Pate3*-, *Cpls12*-disrupted males (B6D2-*Pate1*<em1Osb>, RBRC#09830, CARD#2450; B6D2-*Pate2*<em1Osb>, RBRC#09831, CARD#2451; B6D2-*Pate3*<em1Osb> *Pate3*<em2Osb>, RBRC#09832, CARD#2452; B6D2-*Cpls12*<em1Osb>, RBRC#10341, CARD#2711) will be available through RIKEN BRC (<http://en.brc.riken.jp/index.shtml>) and CARD R-BASE (<http://cardb.cc.kumamoto-u.ac.jp/transgenic/>).

Statistical analysis

All values are shown as the mean ± SD of at least three independent experiments. Statistical analyses were performed using Student's t-test (Figure 2 to 4 and 6) and Mann-Whitney test (Figure 5).

RESULTS

Selection of the target genes

We selected nine genes abundantly expressed in the epididymis and reconfirmed their expression by RT-PCR (Figure 1A). While *Glb113* showed detectable expression in testis, the remaining genes showed epididymis-enriched expression. *Cpls12*, *Epp13*, *Gm1110*, and *Rnase13* were strongly expressed in the caput region, while *Glb112*, *Glb113*, *Pate2*, and *Pate3* were strongly expressed in the corpus region, and *Pate1* was expressed in all regions at comparable levels. It should be noted that all genes examined in the study, except *Gm1110*, are conserved in human and various species (Figure 1B). Because *Gm1110*, *Glb112*, and *Glb113* located closely on the same chromosome and show high similarity in amino acid sequence (Figure S1), we decided to generate a null mice without these three genes.

Fertility of *Pate* family knockout mice

Four and thirteen *Pate* family genes form a cluster on human chromosome 11 and mouse chromosome 9 (Levitin *et al.*, 2008), respectively. While *Pate4* is abundantly expressed in mouse seminal vesicles and not expressed in the epididymis (Noda *et al.*, 2018), *Pate1*, *2*, and *3* are exclusively expressed in the epididymis (Figure 1A). Because we reported that *Pate4* KO mice are subfertile due to an impaired vaginal plug formation and semen leakage (Noda *et al.*, 2018), we knocked out the remaining members to determine their roles in fertility. We attempted to delete the region covering all *Pate1*, *Pate2*, and *Pate3*, by injecting sgRNA/Cas9 co-expressing plasmids into zygotes (Figure 2A). Although we could not delete the region [deletion/pups = 0/4 (sgRNA #1 + #3), 0/6 (sgRNA #1 + #4), Figure 2B], we obtained F0 indel mutant mice in *Pate1* and *Pate3* independently [*Pate1*: indel/pups = 1/4 (sgRNA #1 + #3), 2/6 (sgRNA #1 + #4), *Pate3*: indel/pups = 0/4 (sgRNA #1 + #3), indel/pups = 5/6 (sgRNA #1 + #4), Figure 2B]. We additionally generated *Pate2* F0 mutant mice

[*Pate2*: indel/pups = ¼ (sgRNA #2), Figure 2B]. Because small indels are difficult to detect by PCR and subsequent agarose gel electrophoresis (1 bp insertion in *Pate1*, 4 bp deletion in *Pate2*, 1 and 5 bp deletion in *Pate3*), we genotyped mutant mice by sequencing PCR amplicons (Figure 2C). KO mice were obtained by heterozygous F1 intercrosses. All the KO mice produced spermatozoa with normal morphology as shown in Figure 2D. When we examined 3 males for each KO lines, all KO males showed normal fecundity [littersize: 9.3 ± 0.7 for *Pate1^{em1/em1}*, 8.8 ± 0.8 for *Pate2^{em1/em1}*, and 7.9 ± 3.1 for *Pate3^{em1/em2}*, while control showed 8.5 ± 2.2 (Figure 2E)]. These results show that *Pate1*, *2*, *3* are dispensable for the male fecundity.

Fecundity of colipase like 2 (*Clpsl2*) knockout mice

CLPSL2 is conserved between mice and humans. Using RNAi methods, a previous paper suggested that *Clpsl2* is required for sperm maturation (Lu *et al.*, 2018). To evaluate these findings and determine the essential roles of CLPSL2 in the complete absence of the protein, we generated KO mice lacking the gene. Hereafter, we applied both sgRNA/Cas9 co-expressing plasmids and gRNA/Cas9 ribonucleoprotein (RNP) because the genome editing efficiency is comparable in various cell lines (Liang *et al.*, 2015). We also applied a zygote electroporation system because many eggs can be treated in a shorter time. When we injected 2 gRNA/Cas9 RNPs into zygotes, we could not obtain any F0 mutant mice (deletion/pups = 0/10); however, by electroporation, we could obtain F0 deletion mutants (deletion/pups = 2/16) (Figure 3A). The deletion of the coding region was confirmed by sequencing the PCR amplicons (Figure 3B). KO mice were obtained by heterozygous F1 intercrosses. Histological analysis of the epididymis and analysis of sperm morphology failed to reveal any apparent differences between *Clpsl2^{em1/em1}* and control males (Figure 3C and D). The *Clpsl2^{em1/em1}* males showed normal fecundity (litter size: 9.8 ± 0.8 for controls, 9.1 ± 0.5 for *Clpsl2^{em1/em1}*, Figure 3E). Thus, *Clpsl2* is not required for male fecundity.

Fertility of *Epp13* knockout mice

Epp13 is conserved between mice and humans (also known as *EDDM13*). We generated *Epp13* F0 mutant mice with pronuclear injection and electroporation of gRNA/Cas9 RNPs (injection: deletion/pups = 2/14, electroporation: deletion/pups = 2/30) (Figure 4A). The complete deletion of the coding region was confirmed by PCR and direct sequencing (Figure 4A and B). KO mice were obtained by heterozygous F1 intercrosses. There were no apparent differences between *Epp13^{em1/em1}* and control males in histology of epididymis and sperm morphology (Figure 4C and D). The *Epp13^{em1/em1}* males showed normal fecundity (litter size: 8.6 ± 0.3 for controls, 9.0 ± 0.5 for *Epp13^{em1/em1}*, Figure 4E). Thus, *Epp13* is not required for male fecundity.

Fertility of *Rnase13* knockout mice

Rnase13, conserved between mice and humans, is a member of the ribonuclease A superfamily members (Cho *et al.*, 2005), of which some members, *Rnase9* and *Rnase10*, have been shown to be required for sperm maturation (Krutskikh *et al.*, 2012; Westmuckett *et al.*, 2014). We generated *Rnase13* F0 mutant mice with 2 gRNAs (deletion/pups = 4/30) (Figure 5A). The deletion of the coding region was confirmed by PCR and direct sequencing

(Figure 5B). KO mice were obtained by heterozygous F1 intercrosses. There were no apparent differences between *Rnase13^{em1/em1}* and control males in histology of epididymis and sperm morphology (Figure 5C and D). The *Rnase13^{em1/em1}* males showed normal fecundity (litter size: 8.6 ± 0.2 for controls, 9.0 ± 1.6 for *Rnase13^{em1/em1}*, Figure 5E). Thus, *Rnase13* is not required for male fecundity.

Fertility of mice lacking *Glb112*, *Glb113*, and *Gm1110*

Glb112 and *Glb113* are categorized as galactosidases. *Gm1110* is located next to *Glb113* on mouse chromosome 9 (Figure 6A) (Zhen *et al.*, 2009). The homology rates between GLB1L2, GLB1L3, and GM1110 are high at the amino acid level (Figure S1, Gm1110 vs GLB1L2: 47.4%, Gm1110 vs GLB1L3: 44.9%), suggesting redundant functions of these genes on sperm maturation. *Gm1110* is identified as *Glb114* in rats and a previous report suggested that GLB1L4 is required for the sperm maturation (Zhen *et al.*, 2009). Thus, we generated 2 gRNAs against *Glb112* and *Gm1110* and deleted all 3 genes (injection: deletion/pups = 0/28, electroporation: deletion/pups = 2/27) (Figure 6A). The deletion was confirmed by PCR and direct sequencing (Figure 6B). KO mice were obtained by heterozygous F1 intercrosses. There were no apparent differences between triple KO (*Glb112-Gm1110*)^{del/del} and control males in histology of epididymis and sperm morphology (Figure 6C and D). The triple KO males showed normal fecundity [litter size: 8.2 ± 0.4 for controls, 8.5 ± 2.4 for (*Glb112-Gm1110*)^{del/del}, Figure 6E]. Thus, *Glb112*, *Glb113*, and *Gm1110* are not required for male fecundity.

Discussion

It is known that 4 and 13 genes of the *Pate* family members exist in human and mouse, respectively, and that *Pate1* to *Pate4* are conserved in both species (Levitin *et al.*, 2008). In human, PATE1 was localized to the sperm equatorial segment, and the anti-PATE1 antibody treatment decreased sperm motility (Liu *et al.*, 2015). Further, previous studies showed asthenozoospermia patients having reduced levels of PATE1 (Liu *et al.*, 2015), and that single nucleotide polymorphisms were found in idiopathic asthenozoospermia (Zhang *et al.*, 2016). These results suggest that human PATE1 is required for sperm function, but we revealed that mouse *Pate1* KO males are fertile (Figure 2D and E). In addition, we showed that mouse *Pate2* and *Pate3* are dispensable for male fecundity. Because more *Pate* family genes exist in mouse, we cannot rule out the possibility of gene complementation. Further studies will be required to fully uncover the role of PATE family protein in sperm functions.

Clpsl2 is conserved in mouse and human, and predominantly expressed in the caput epididymis (Figure 1). CLPSL2 from the caput epididymis binds to the sperm acrosome and principal piece of the sperm tail (Lu *et al.*, 2018). The previous study showed that knockdown of mouse *Clpsl2* by *in vivo* lentivirus-based RNAi resulted in decreased sperm motility, acrosome reaction, and sperm count in the cauda epididymis, leading to the male subfertility (Lu *et al.*, 2018). However, we could not observe any defects in *Clpsl2* KO (null) males. The discrepancy may be explained by experimental approaches; while KO mice specifically suppressed the target gene, RNAi interferes not only with the target gene, but

also genes carrying similar sequences. While one can claim that the gRNA may also have off-target effects, our data support that *Clpsl2* is not essential for male fertility.

RNASE9, 10, and 13 in the ribonuclease A superfamily were detected in the epididymis (Penttinen *et al.*, 2003; Westmuckett *et al.*, 2014) and show high homology rates at the amino acid level (Cho *et al.*, 2005). *Rnase9* KO males were fertile, but KO spermatozoa showed impaired sperm maturation (Westmuckett *et al.*, 2014). *Rnase10* KO males were severely subfertile due to the disruption of ADAM3 processing (Krutskikh *et al.*, 2012). However, we revealed that *Rnase13* is dispensable for male fecundity. We cannot rule out the possibility that other members complement RNASE13 function *in vivo*, so the additional KO approaches will be necessary to address this question. Because RNase A family genes are located on Chromosome 14, two gRNAs mediated deletion approaches as shown in Figure 6 are feasible.

Here, we showed that nine genes, which are abundantly expressed in epididymis and conserved among several mammalian species, are dispensable for sperm maturation and male fecundity. The CRISPR/Cas9 mediated KO approach is a powerful tool to determine whether genes of interests are essential for fertility. Screening for genes through a CRISPR/Cas9 mediated KO approach has accelerated the study of severed genes implicated in reproduction, especially of genes within gene families.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the Biotechnology Research and Development (NPO) for support to generate the mutant mice. And, the authors would like to thank Dr. Julio Castaneda for critical reading of the manuscript.

Funding information

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI grants (JP18K14612 to T.N., JP17H01394 and JP25112007 to M.I.), Japan Agency for Medical Research and Development (AMED) grant (JP18gm5010001), Takeda Science Foundation grants to M.I., the Eunice Kennedy Shriver National Institute of Child Health and Human Development (P01HD087157 and R01HD088412 to M.M.M. and M.I.), the Bill & Melinda Gates Foundation (Grand Challenges Explorations grant OPP1160866 to M.M.M. and M.I.), a Japan Society for the Promotion of Science Overseas Research Fellowships (20170633) to K.N., and a Training Interdisciplinary Pharmacology Scientists (TIPS) Program (T32 GM120011) predoctoral fellowship position to D.J.D.

References

- Cho S, Beintema JJ & Zhang J. (2005) The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories. *Genomics* 85, 208–220. [PubMed: 15676279]
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA & Zhang F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. [PubMed: 23287718]
- Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, & Turner TT. (2005). The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. *Biol Reprod* 73, 404–413. [PubMed: 15878890]

- Krutsikh AA, Poliandri A, Cabrera-Sharp V, Dacheux JL, Poutanen M & Huhtaniemi I. (2012). Epididymal protein Rnase10 is required for post-testicular sperm maturation and male fertility. *FASEB J* 26, 4198–4209. [PubMed: 22750516]
- Levitin F, Weiss M, Hahn Y, Stern O, Papke RL, Matusik R, Nandana SR, Ziv R, Pichinuk E, Salame S, Bera T, Vincent J, Lee B, Pastan I & Wreschner DH. (2008) PATE gene clusters code for multiple, secreted TFP/Ly-6/uPAR proteins that are expressed in reproductive and neuron-rich tissues and possess neuromodulatory activity. *J Biol Chem* 283, 16928–16939. [PubMed: 18387948]
- Liang XJ, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N & Chesnut JD. (2015) Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* 208, 44–53. [PubMed: 26003884]
- Liu FJ, Liu X, Han JL, Wang YW, Jin SH, Liu XX, Liu J, Wang WT & Wang WJ. (2015) Aged men share the sperm protein PATE1 defect with young asthenozoospermia patients. *Hum Reprod* 30, 861–869. [PubMed: 25637620]
- Lu X, Ding F, Lian Z, Chen L, Cao Z, Guan Y, Chen R, Cai D & Yu Y. (2018) An epididymis-specific secretory protein Clpsl2 critically regulates sperm motility, acrosomal integrity, and male fertility. *J Cell Biochem* 119, 4760–4774. [PubMed: 29323738]
- Mashiko D, Fujihara Y, Satouh Y, Miyata H, Isotani A & Ikawa M. (2013) Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci Rep* 3, 3355. [PubMed: 24284873]
- Mashiko D, Young SA, Muto M, Kato H, Nozawa K, Ogawa M, Noda T, Kim YJ, Satouh Y, Fujihara Y & Ikawa M. (2014) Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. *Dev Growth Differ* 56, 122–129. [PubMed: 24372541]
- Miyata H, Castaneda JM, Fujihara Y, Yu Z, Archambeault DR, Isotani A et al. (2016). Genome engineering uncovers 54 evolutionarily conserved and testis-enriched genes that are not required for male fertility in mice. *Proc Natl Acad Sci U S A* 113, 7704–7710. [PubMed: 27357688]
- Noda T, Fujihara Y, Matsumura T, Oura S, Kobayashi S & Ikawa M. (2018) Seminal vesicle secretory protein 7, PATE4, is not required for sperm function but for copulatory plug formation to ensure fecundity. *Biol Reprod*, [Epub ahead of print].
- Noda T, Oji A & Ikawa M. (2017) Genome Editing in Mouse Zygotes and Embryonic Stem Cells by Introducing SgRNA/Cas9 Expressing Plasmids. *Methods Mol Biol* 1630, 67–80. [PubMed: 28643250]
- Penttinen J, Pujianto DA, Sipila P, Huhtaniemi I & Poutanen M. (2003) Discovery in silico and characterization in vitro of novel genes exclusively expressed in the mouse epididymis. *Mol Endocrinol* 17, 2138–2151. [PubMed: 12920233]
- Robaire B & Hermo L. (1988) Efferent Ducts, Epididymis, and Vas Deferens: Structure, Functions, and Their Regulation In: *The Physiology of Reproduction* (eds Knobil E & Neill JD, pp. 999–1080. Raven Press.
- Robaire B, Hinton BT & Orgebin-Crist MC (2006). *The Epididymis In: Knobil and Neill's Physiology of Reproduction*. (eds Knobil E & Neill JD, pp. 1071–1148. Elsevier.
- Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F & Jaenisch R. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153, 910–918. [PubMed: 23643243]
- Westmuckett AD, Nguyen EB, Herlea-Pana OM, Alvau A, Salicioni AM & Moore KL. (2014) Impaired sperm maturation in RNASE9 knockout mice. *Biol Reprod* 90, 120. [PubMed: 24719258]
- Yang H, Wang H, Shivalila CS, Cheng AW, Shi L & Jaenisch R. (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154, 1370–1379. [PubMed: 23992847]
- Zhang S, Wang QM, Ding XP, Wang T, Mu XM & Chen ZY. (2016) Association of polymorphisms in PATE1 gene with idiopathic asthenozoospermia in Sichuan, China. *J Reprod Immunol* 118, 54–60. [PubMed: 27636828]

Zhen W, Li P, He B, Guo J & Zhang YL. (2009) The novel epididymis-specific beta-galactosidase-like gene *Glb114* is essential in epididymal development and sperm maturation in rats. *Biol Reprod* 80, 696–706. [PubMed: 19092116]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

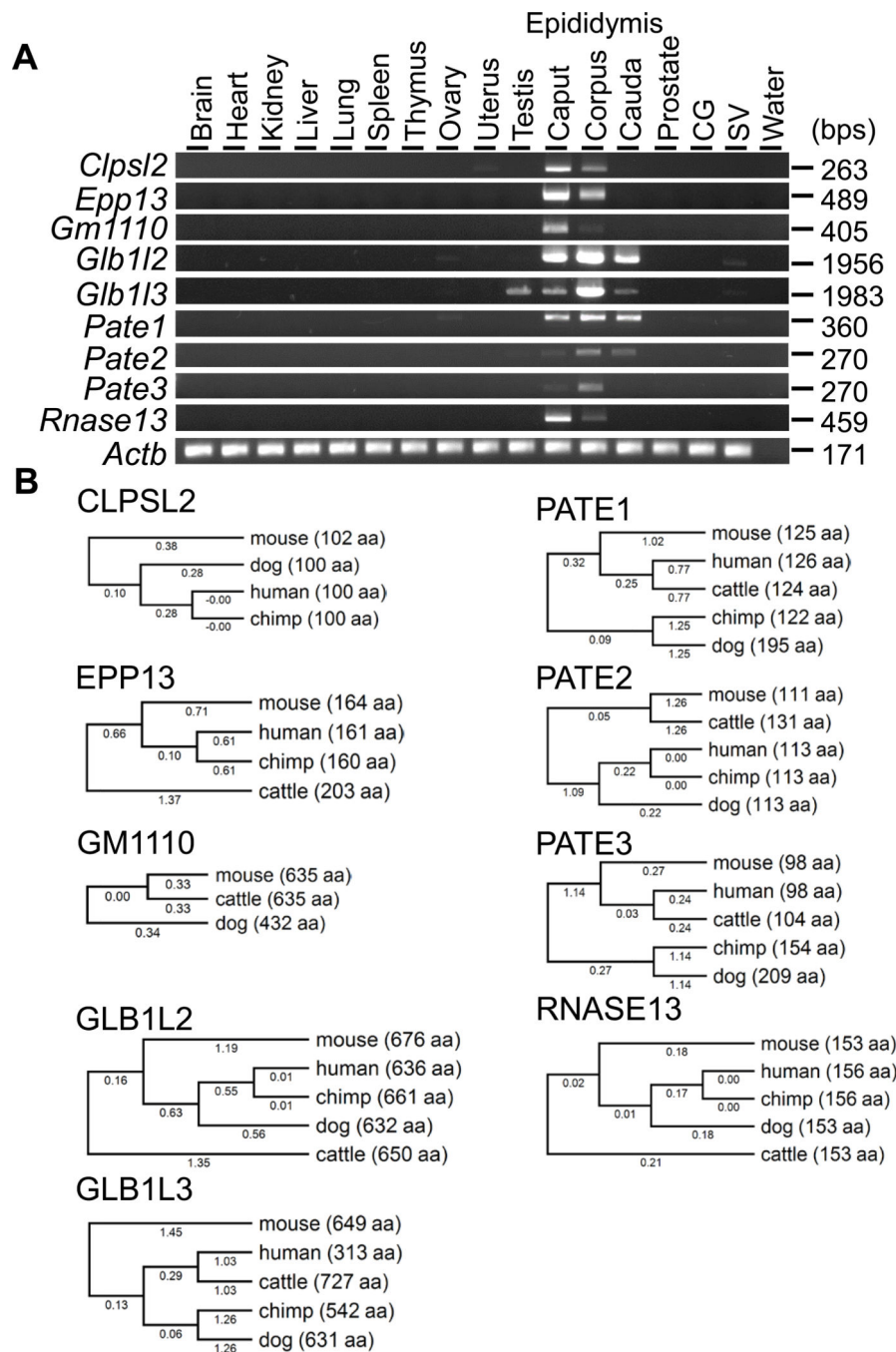


Figure 1. Tissue expression analysis and conservation of target genes between some mammalian species.
 (A) RT-PCR analysis. Actin beta (*Actb*) was used as the control. (B) Phylogenetic trees. The values under branches and parentheses show the distances and the lengths of the amino acid sequences, respectively.

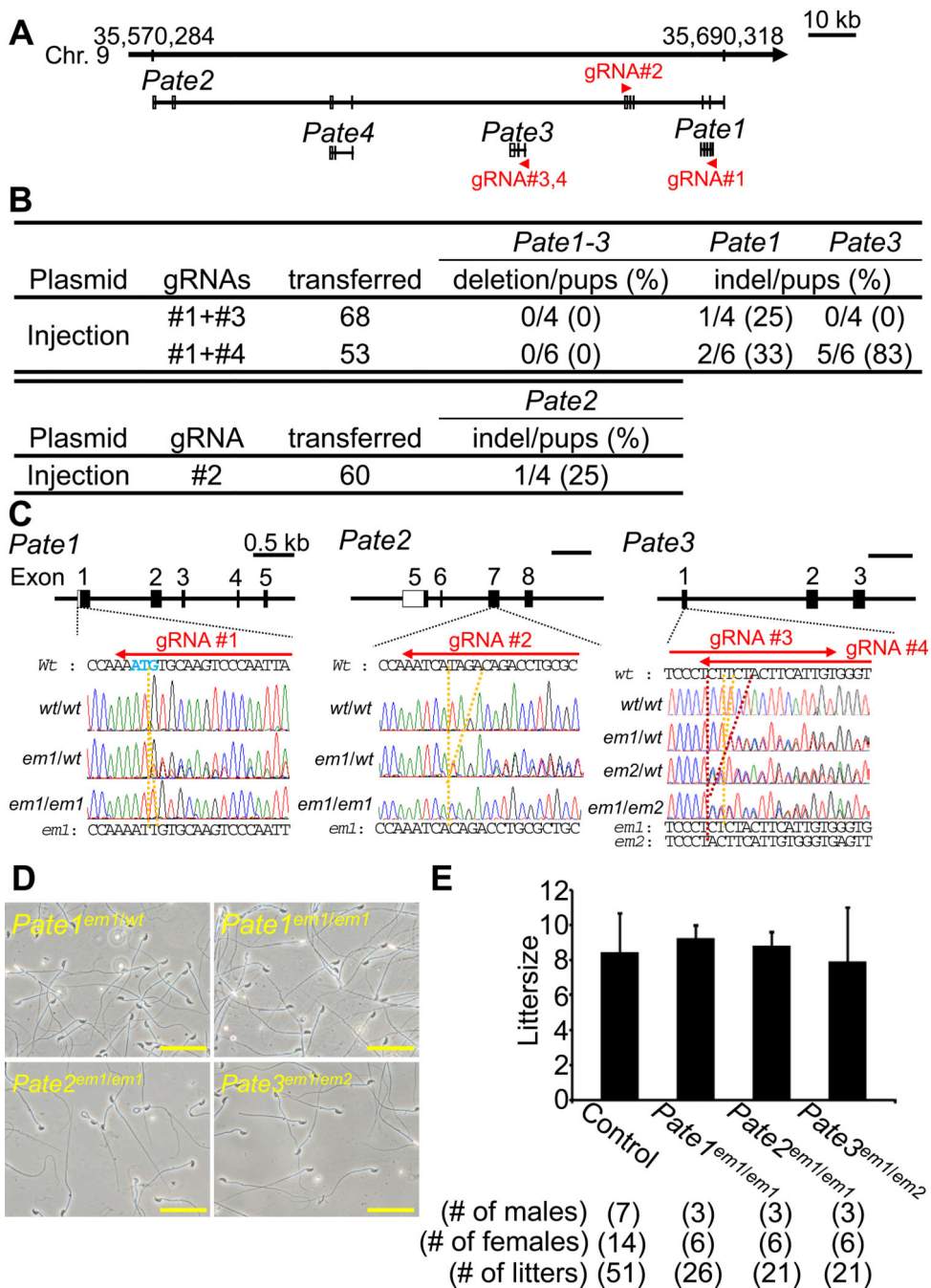


Figure 2. Fecundity of KO males of *Pate* family genes.

(A) *Pate* family genes within murine genomic locus [chromosome 9qA4]. (B) Genome editing efficiency of injecting gRNA/Cas9 expressing plasmids into eggs. (C) DNA sequencing of KO mice of *Pate* family genes. Enzyme mutation (*em*) 1 for *Pate1*: 1 base “T” insertion; *em1* for *Pate2*: 4 base “TAGA” deletion; *em1* and *em2* for *Pate3*: 1 base “T” and 5 base “CTTCT” deletion. Black boxes: coding region, Blue colored letters: initial methionine. (D) Sperm morphology observed under phase contrast. (E) Male fecundity. There was no

difference in average litter size between control and KO males of each gene (*Pate1*: $p = 0.57$, *Pate2*: $p = 0.80$, *Pate3*: $p = 0.76$). Heterozygous males of each gene were used as controls.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

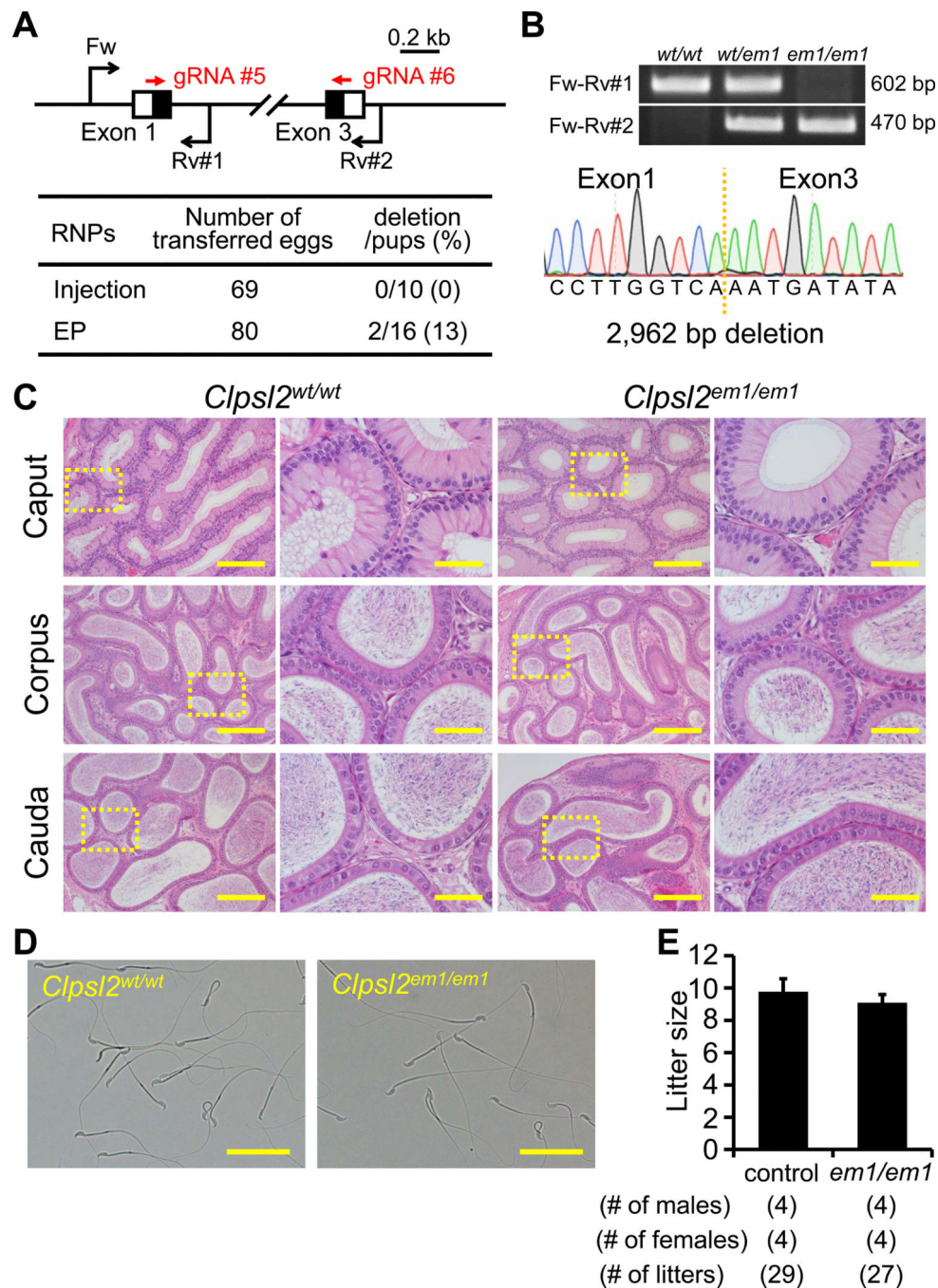


Figure 3. Fecundity of *Clpsl2* KO males.

(A) Genome editing efficiency with gRNA/Cas9 RNPs. Black arrows: primers for genotyping, Black boxes: coding region, EP: electroporation. (B) Genotyping with PCR and DNA sequencing. Three primers (Fw, Rv#1, and Rv#2) were used for the PCR (also see panel A). *em1*: 2,962 bp deletion. (C) Histological analysis with H&E staining. Dashed areas in left panels were enlarged (right panels). Scale bars on the left and right panels are 200 μ m and 50 μ m, respectively. (D) Sperm morphology observed under phase contrast.

Scale bars: 50 μm . (E) Male fecundity. There was no difference in average litter size between WT and KO males ($p = 0.20$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

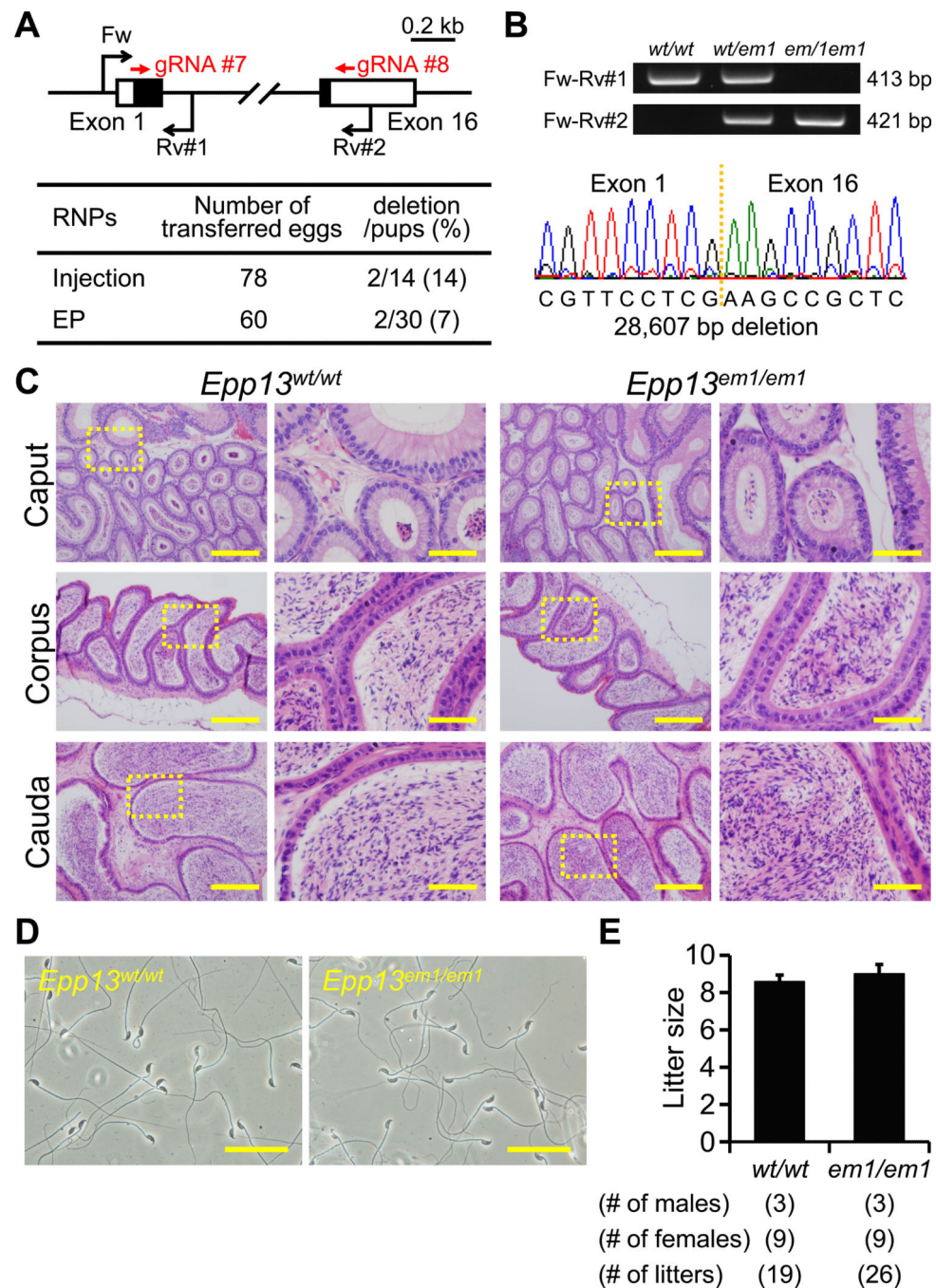


Figure 4. Fecundity of *Epp13* KO males.

(A) Genome editing efficiency with gRNA/Cas9 RNPs. Black arrows: primers for genotyping, Black boxes: coding region, (B) Genotyping with PCR and DNA sequencing. Three primers (Fw, Rv#1, and Rv#2) were used for the PCR (also see panel A). *em1*: 28,607 bp deletion. (C) Histological analysis with H&E staining. Dashed areas in left panels were enlarged (right panels). Scale bars on the left and right panels are 200 μ m, respectively. (D) Sperm morphology observed under phase contrast. Scale bars: 50 μ m. (E)

Male fecundity. There was no difference in average litter size between WT and KO males ($p = 0.28$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

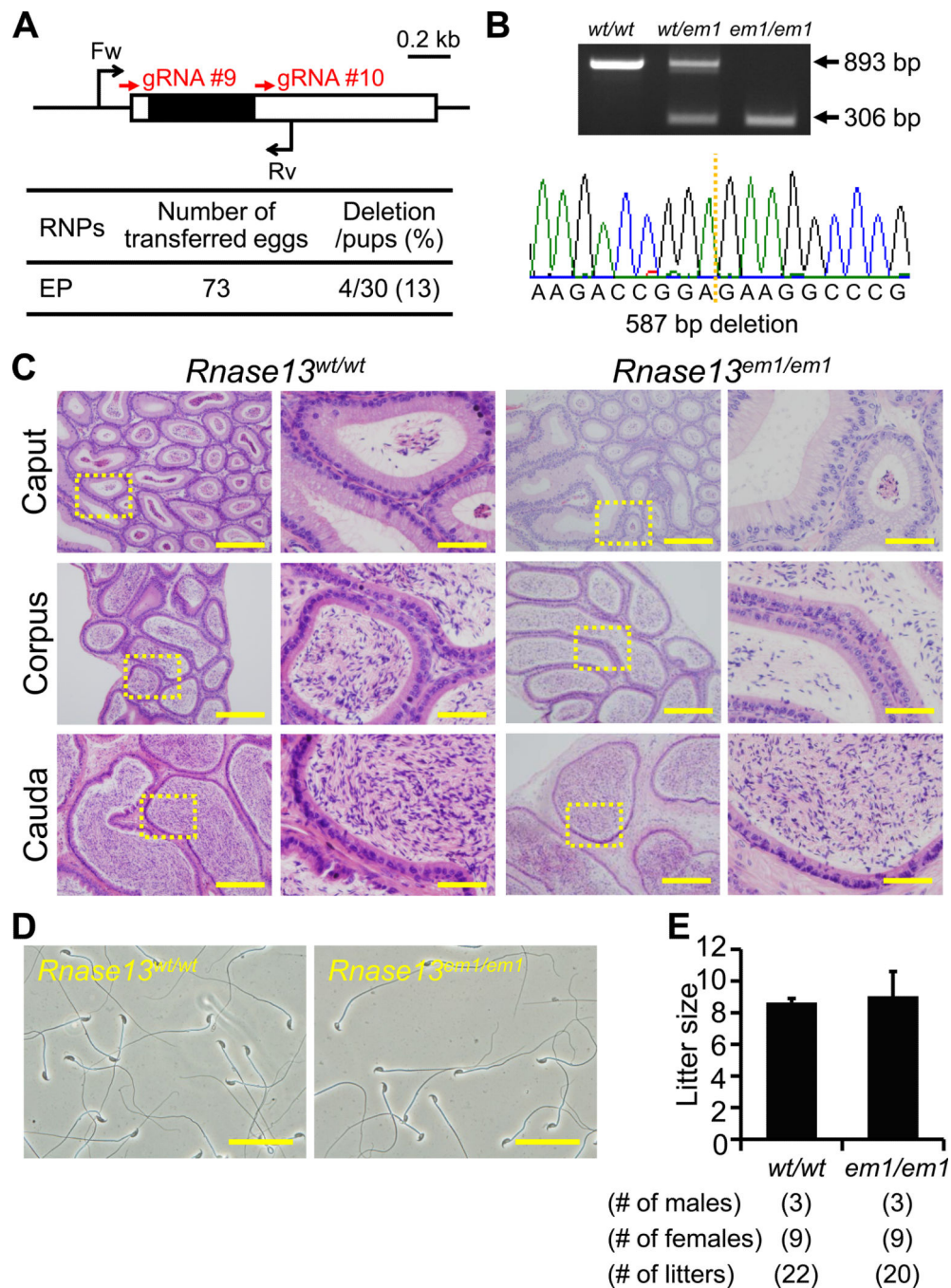


Figure 5. Fecundity of *Rnase13* KO males.

(A) Genome editing efficiency with gRNA/Cas9 RNPs. Black arrows: primers for genotyping, Black boxes: coding region. (B) Genotyping with PCR and DNA sequencing. Two primers were used for PCR (also see panel A). *em1*: 587 bp deletion, (C) Histological analysis with H&E staining. Dashed areas in left panels were enlarged (right panels). Scale bars on the left and right panels are 200 μ m and 50 μ m, respectively. (D) Sperm morphology observed under phase contrast. Scale bars: 50 μ m. (E) Male fecundity. There was no difference in average litter size between WT and KO males ($p = 0.51$).

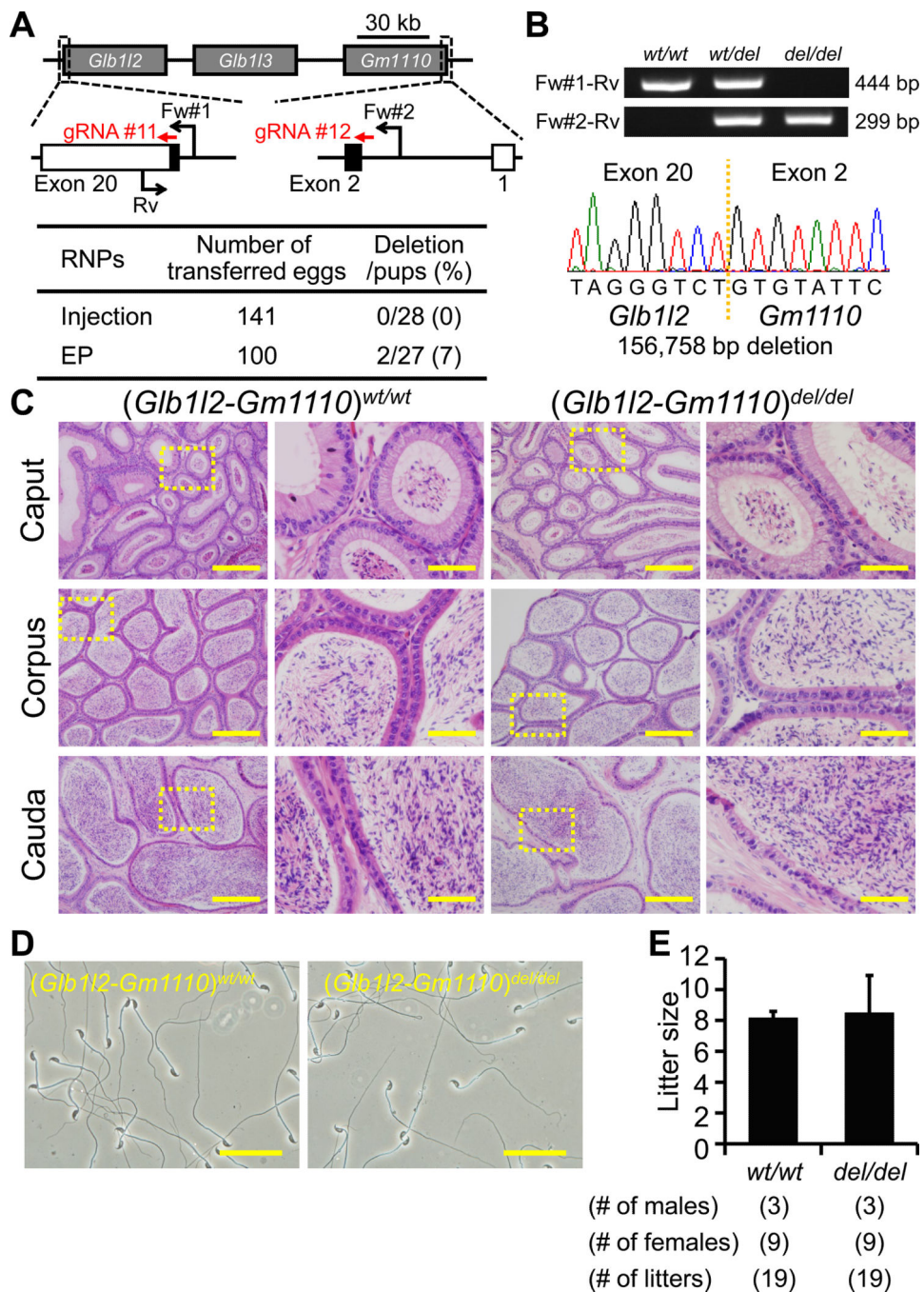


Figure 6. Fecundity of *Glb1l2-Gm1110* KO mice.

(A) Genome editing efficiency with gRNA/Cas9 RNPs. Black arrows: primers for genotyping, Black boxes: coding region. (B) Genotyping with PCR and DNA sequencing. Three primers (Fw#1, Fw#2, and Rv) were used for the PCR (also see panel A). *emI*: 156,758 bp deletion. (C) Histological analysis with H&E staining. Dashed areas in left panels were enlarged (right panels). Scale bars on the left and right panels are 200 μ m and 50 μ m, respectively. (D) Sperm morphology observed under phase contrast. Scale bars: 50

µm. (E) Male fecundity. There was no difference in average litter size between WT and KO males ($p = 0.83$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript