Ppp2ca knockout in mice spermatogenesis

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Abstract

Protein phosphatase 2A (PP2A) is a ubiquitous serine/threonine phosphatase involved in meiosis, mitosis, sperm capacitation, and apoptosis. Abberant activity of PP2A has been associated with a number of diseases. The homolog PPP2CA and PPP2CB can each function as the phosphatase catalytic subunit generally referred to as PP2AC. We generated a *Ppp2ca* conditional knockout (CKO) in C57BL/6J mice. Exon 2 of *Ppp2ca* was knocked out in a spatial or temporal-specific manner in primordial germ cells at E12.5. This *Ppp2ca*-null mutation caused infertility in male C57BL/6J mice. These CKO mice provide a powerful tool to study the mechanisms of *Ppp2ca* in development and disease.

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Introduction

Protein phosphatase 2A (PP2A) is a ubiquitous serine/threonine phosphatase involved in meiosis, mitosis, sperm capacitation, and apoptosis. Several studies have demonstrated a link between PP2A and a number of diseases (Janssens *et al.* 2005, Rivera & Losada 2006, Krapf *et al.* 2010, Brestovitsky *et al.* 2011, Ruediger *et al.* 2011). Its catalytic subunit PP2AC plays a critical role in embryonic development and human diseases, such as Alzheimer's disease, cancer, and diabetes (Goedert *et al.* 1995, Zolnierowicz 2000, Kowluru 2005, Eichhorn *et al.* 2009, Liu & Wang 2009). The homologues PPP2CA and PPP2CB can each function as the catalytic subunit of PP2A and are related to the male and female reproductive systems respectively. While the amino acid sequence of the two encoded proteins are very similar, PPP2CA is ten times more abundantly expressed than PPP2CB (Khew-Goodall *et al.* 1991). The PP2A phosphatase participates in the regulation of several cell-signaling pathways and cellular functions, including proliferation, cytoskeletal rearrangement, apoptosis, and cell migration (Janssens & Goris 2001, Kong *et al.* 2004, 2007). It exists primarily as a heterotrimeric holoenzyme consisting of a structural A subunit, a regulatory B subunit, and a catalytic C subunit. Previous studies have specifically linked dysregulation of the catalytic subunit with several pathologies, including Alzheimer's disease, cancer, diabetes, and sperm capacitation. For example, SFK-mediated PP2AC phosphorylation inactivates PP2A and leads to mammalian

sperm capacitation (Goto & Harayama 2009, Krapf *et al.* 2010).

Interestingly, dysregulation of both PP2CA and PP2CB is associated with infertility. For example, *Ppp2cb* mRNA is upregulated in meiosis arrest female 1 (MARF1)-mutant mice, which results in meiotic arrest and female infertility (Su *et al.* 2012). Furthermore, mutant mice could undergo meiotic reversal if *Ppp2cb* was inhibited. Therefore, meiotic arrest in MARF1 mutant oocytes is dependent on upregulated *Ppp2cb* gene expression. The amino acid sequence of PPP2CA is 97% identical with PPP2CB and appears to play a role in embryonic development because null mutations lead to early embryonic lethality at E6.5 (Götz *et al.* 1998). It is possible that *Ppp2ca* is involved in WNT signaling, which controls epithelial–mesenchymal transition during vertebrate development (Peifer *et al.* 1994). As PP2A plays an important role in sperm capacitation, and *Ppp2ca*-null mutations are embryonic lethal, we bred a *Ppp2ca* conditional knockout (CKO) mouse with a loxP system to study the function of *Ppp2ca* in the male reproductive system.

Mvh, also known as DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Ddx4*), is exclusively expressed in testicular germ cells. The MVH protein is localized in the cytoplasm of spermatocytes and round spermatids containing a perinuclear granule. The protein is also expressed in germ cells colonized in embryonic gonads, but is not detected in pluripotent embryonic cells, such as stem cells and germ cells (Fujiwara *et al.* 1994). A *Ddx4-Cre* mouse line displays active expression in

primordial germ cells by E12.5 and maintains expression in germ cells for life (Tanaka *et al.* 2000). In this study, we employed *Ddx4-Cre* transgenic mice to specifically delete *Ppp2ca* in embryonic gonad tissues. These tissue-specific CKO mice were used to demonstrate that *Ppp2ca* is required for testicular development.

Materials and methods

Construction of targeting plasmid

Bacterial artificial chromosome (BAC) DNA extracted from DH10B cells was electroporated into SW106 cells, and then SW106 colonies containing the BAC were selected and confirmed with Ampicillin and BamHI digestion respectively. Two primer pairs (A/B; Y/Z) were used to amplify the homology arms from the BAC. These two homology arms were ligated into PL253, forming a target vector that was confirmed by PCR (primers A/B; Y/Z). The target vector was electroporated into the SW106 cells containing the BAC, and Ampicillin-resistant colonies were selected by PCR and BamHI and SalI digestion. The homology arms were amplified from the BAC by PCR (primers C/D; E/F), and then ligated into PL452 in a loxP-Neo/kanamycin (Kan)-loxP cassette to produce the first loxP (1stloxP) plasmid. This plasmid was electroporated into 0.1% arabinose-treated SW106 cells, and kanamycin-resistant colonies were used for plasmid propagation following restriction enzyme mapping. The second LoxP site was inserted similarly into the gene-targeting vector (primers G/H; I/J) and mapped by restriction enzyme digests (Fig. 1C). The completed targeting plasmid contained exon 2 between the two loxP sites and was verified by sequencing. The sequences of the primers used are listed below:

PPP2CA-A: AATAATGCGGCCGCGCAGCCATCCCTTTTACTC-CTG; PPP2CA-B: AATAATAAGCTTCTCTCTACTGTGTTAC-CCTTC; PPP2CA-Y: AATAATAAGCTTGGTTGGTTAGTTATTT-AGGTGGC; PPP2CA-Z: AATAATACTAGTTGATGGTTTGTG-TATGCTTGG; PPP2CA-C: AATAATGCGGCCGCAACCCCAAC-AACAACCACA; PPP2CA-D: AATAATGAATTCCTAGGCTCTC-TCAAAAAGAAACACCTGC; PPP2CA-E: AATAATGGATCCCAG-CCTTCTCTTCTCCTTTG; PPP2CA-F: AATAATGTCGACCCT-CGCCCCATTTTCATCTAT; PPP2CA-G: AATAATGCGGCCGCA-GGGCTTTTGTCTCTTCTGT; PPP2CA-H: AATAATGAATTC-GTTAGGTATTTGCTCACTCTTGG; PPP2CA-I: AATAATGGA-TCCGGCATCGCACATCACCTA; PPP2CA-J: AATAATGTCGAC-ACCATCTACTCTAACTCTCCACTT.

Generation of *Ppp2ca*^{flax/+} mice and *Ppp2ca*^{flax/-} knockout mice

The gene-targeting vector was linearized by restriction digest with NotI and then electroporated into C129 mouse embryonic stem (ES) cells. The electroporated ES cells were cultured with G418, and the resistant clones were screened for a successful recombination event by long-range PCR. The PCR primers used were specific for the neomycin cassette and upstream (Ppp2ca-FS5/ES-1R3) or downstream (Neo-F/pp2ca-RS2) of the respective 5' or 3' homology arms (Fig. 1D) resulting in PCR products ~7 kb in length. The presence of the 3' loxP site was also

Figure 1 Generating a conditional null *Ppp2ca* allele. (A) The PPP2CA protein is made of 309 amino acids and the catalytic domain (light orange region) spans residues 23–293. The knockout region (dark orange region, crossed) spans amino acids 35–105. (B) Generation of *Ppp2ca*^{flax} mice. The *Ppp2ca* allele spans nearly 30 kb from exon 1 to exon 7 (first row, scale). The targeted region spans exon 2 to exon 6 (second row). The targeting construct contains a silent mutation with a NotI site, and inserts a neo cassette flanked by frt sites (open triangles) into intron 2. This introduces a loxP site (black triangles) upstream of exon 2 but downstream of the neo cassette (third row). ES cells were screened for the *Ppp2ca*^{flax} allele using primers Ppp2ca-FS5/ES-1R3 and Neo-F/pp2ca-RS2 (fourth row). *Ppp2ca*^{flax} mice were identified with primers Ppp2ca-loxp1-L-F/Ppp2ca-loxp2-R-R (fifth row). (C) BamHI and SalI double digestion: M, marker; lane 1, PL253-Ppp2ca fragment; lane 2, PL253-Ppp2ca-1stloxP fragment; lane 3, PL253-Ppp2ca-1stloxP-popout fragment; lane 4, PL253-Ppp2ca-targeting vector fragment. (D) ES clones were screened for a successful recombination event by a long-range PCR. (left panel) PCR screening with primer pair Ppp2ca-FS5/ES-1R3 yields a 7094-bp product upstream of the 5' homology arm. (right panel) PCR screening with primer pair Neo-F/pp2ca-RS2 yields a 7851 bp product downstream of the 3' homology arm. M, marker. (E) Primer pair Ppp2ca-FR distinguishes *Ppp2ca*^{flax/flax} mice (322 bp) from WT mice (408 bp).

Figure 2 Decreased *Ppp2ca* expression in mutant mice. (A) *Ppp2ca*^{flox/+}; *Ddx4-Cre* is identified by PCR. Lanes 2 and 3: *Ddx4-Cre* is identified with primer CRE-F/R (364 bp). Lanes 4 and 5: flox is identified with primer Ppp2ca-F/R (322 bp). Lanes 6 and 7: knockout fragment between the two loxP sites is identified with primer Ppp2ca-loxp1-L-F/Ppp2ca-loxp2-R-R (1024 bp). M, marker. (B) Testes of WT mice, *Ppp2ca*^{flox/+} mice, and *Ppp2ca*^{flox/-} littermates were tested for PP2Ac protein expression by western blotting analysis using PP2Ac antibody. There was a dramatic reduction in the expression of PP2Ac in mutant mice, and any protein detected may have been the product of the homolog *Ppp2cb*. GAPDH was used as the loading control. (C) Gene expression in the testes of *Ppp2ca*^{flox/-}, *Ppp2ca*^{flox/+}, and WT mice was determined by reverse transcription PCR. The mutant mice had shorter transcripts than control mice, indicating successful knockout. Background *Ppp2ca* expression in the mutant samples could be from nontesticular cells present in our samples.

validated using PCR primers that flanked the loxP site. Of the 20 G418-resistant clones, one had a homologous recombination event. ES cells from this clone (C2) were microinjected into C57BL/6J blastocysts to generate chimeric mice. The chimeric mice (*Ppp2ca-loxp1-L-F/ES-1R3*; *Neo-F/Ppp2ca-loxp2-R-R*) were then bred with C57BL/6J mice to verify germline transmission of the targeted allele. The *Ppp2ca*^{flox/+} mice were genotyped by PCR (*Ppp2ca-F/R*). Subsequently, we bred the *Ppp2ca*^{flox/+} mice to obtain homozygous *Ppp2ca*^{flox/flox} mice. To achieve germ cell-specific deletion of *Ppp2ca*, female homozygous flox mice were crossed with male *Ddx4-Cre* transgenic mice (germ cell-specific Cre line, #006954, Jackson Laboratory, Bar Harbor, ME, USA). The male heterozygous progeny that bears the genotype *Ppp2ca*^{flox/+}; *Ddx4-Cre* (*Cre-F/R*; *Ppp2ca-F/R*) was mated with the female *Ppp2ca*^{flox/flox} mice to obtain *Ppp2ca*^{flox/-}; *Ddx4-Cre* mice (*Cre-F/R*; *Ppp2ca-F/R*; *Ppp2ca-loxp1-L-F/Ppp2ca-loxp2-R-R*). This study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Number: NJMU-IACUC-20100601001). The sequences of the primers used are listed below:

Ppp2ca-FS5: CCCCCCTCTTCTCCTCCT; ES-1R3: AAGGGTTATTGAATATGATCGGA; Neo-F: CGTTGGCTACCGTGATATTAGAGCTTGCAGAACCTTCG; *Ppp2ca*-RS2:

GGTTGAAGACCTAGTAACCTCATACAAG; *Ppp2ca-loxp1-L-F*: AATAATGCGGCCGCAACCCCAACAACAACCACA; *Ppp2ca-loxp2-R-R*: AATAATGTGACACCATCTACTCTAACTCTCACTT *Cre-F*: TTGCCTGCATTACCGGTGCGATGC; *Cre-R*: TTGCACGTTACCGGCATCAACG.

Western blotting analysis

Western blot analysis of endogenous proteins from organ tissues was performed as described previously (Brestovitsky *et al.* 2011). The following antibodies were used: rabbit monoclonal PP2Ac antibody (Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich), and goat anti-rabbit IgG-HRP antibody (Sigma-Aldrich). Visualization was achieved with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

RNA preparation and RT-PCR

RNA was extracted from testicular cells using RNAiso Plus following the manufacturer's protocol (TaKaRa, Dalian, China). To obtain cDNA, the PrimeScript™ Reverse Transcription Reagent Kit with gDNA Eraser (TaKaRa) was used with random primers (Invitrogen). The sequences of the specific primers used for PCR are listed below:

Mpp2ca-P5F: GGTCAGAGCCTCTGCGAGAA; *Mpp2ca*-P5R1: CCGGTCATGGCACCAGTTAT.

DNA content assay

The testicular cells of mice at P38 and P56 were analyzed by flow cytometric analysis to quantify the number of haploid, diploid, and tetraploid cells.

Figure 3 Phosphatase function is required for normal spermatogenesis. (A) Mice testes at 4 weeks. Left to right: WT, heterozygous, and mutant. The mutant testes were significantly reduced in size compared with the WT. (B) Mutant tubules with vacuolation (*) and disrupted morphology as observed by H&E staining of the seminiferous tubules.

Figure 4 Decreased *Ppp2ca* expression at 8 weeks. (A) The testes on the left is from an 8-week-old control mouse, and the one on the right is from an 8-week-old mutant mouse. (B) The weight of testes from mutant mice (Homozygote) and normal mice at 4 and 8 weeks. (C) Mutant testes were almost completely lacking in mature spermatozoa, as observed by H&E staining of the seminiferous tubules and epididymides. The mutant tubules exhibited vacuolation (*), incorrect placement of cell types (arrow), and lacked elongated spermatozoa. The mutant epididymides contained some round cells (^) of different sizes and shapes, and no normal sperm. Results are mean $\pm$ S.E.M. ($n=3$ animals/genotype), *** $P<0.001$ vs controls.

Sperm smear

The epididymides of killed mice were minced and then placed in tubes with 200 μ l of PBS for a 5 min incubation in a 37 $^{\circ}$ C water bath. A sample of the supernatant (10 μ l) was pipetted dropwise on to a glass slide, covered with a coverslip, and allowed to dry at room temperature. The slides were then fixed in 4% paraformaldehyde for 40–60 min and rinsed in PBS for 5 min, followed by staining with hematoxylin and eosin (H&E).

Fertility testing

To test for fertility, 8-week-old male mice were randomly divided into control and experimental groups according to genotype. Each male mouse was housed with two 8-week-old C57/B6 female mice for 1 week. Following co-housing, the female mice were killed, and the number of embryos within the uterine horns was determined. Live embryos were taken as an indication of male fertility.

Results

Generating a conditional null *Ppp2ca* allele

There are seven exons in the mouse *Ppp2ca* gene, spanning $\sim$ 30 kb (Khew-Goodall *et al.* 1991). In one reported *Ppp2ca* knockout more than 0.5 kb of the promoter, exon 1, and 0.3 kb of intron 1 are replaced by a neomycin (neo) resistance cassette, which leads to early-embryonic lethality at E6.5 (18). The catalytic domain of PPP2CA spans amino acid 23–293. We designed a conditional targeting vector spanning $\sim$ 2.2 kb to disrupt exon 2, which encodes amino acids 35–105 (Fig. 1A). To construct the conditional gene-targeting vector, we used recombination to introduce loxP sites at each end of exon 2 in a vector containing positive and negative markers for selection (Fig. 1B). Positive clones were identified by PCR and enzyme digestion (Fig. 1C). The plasmid containing the loxP sites and positive and negative selection markers was then electroporated into ES cells. Long-range PCR was then used to verify the 24 selected colonies and colony C2 that carried the *Ppp2ca* CKO allele (Fig. 1D). C2 clones were microinjected into C57BL/6J blastocysts to generate chimeric animals. The heterozygous *Ppp2ca*^{flox/+} offspring were viable, fertile, and did not display any obvious abnormalities. Next, we bred *Ppp2ca*^{flox/+} mice together to obtain *Ppp2ca*^{flox/flox} mice (Fig. 1E), which were also fertile and did not exhibit any obvious abnormalities.

Decreased *Ppp2ca* expression in mutant mice

To examine whether *Ppp2ca* plays a role in the male reproductive system, we applied a Cre-loxP system to ablate *Ppp2ca* expression in developing testes. Floxed *Ppp2ca* mice were bred with the *Ddx4-Cre* line. By utilizing this Cre line, we were able to examine

Table 1 Decreased levels of *Ppp2ca* expression caused male infertility.

Male		Female		Average number of pups
Genotype	Number	Genotype	Number	
Control	7	WT	14	7.93 ± 2.08
Mutant	7	WT	14	0

Seven control mice and seven mutant mice were used for comparison; the seven mutant males produced no pups.

the functions of *Ppp2ca* in the male reproductive system. From this, we obtained the *Ppp2ca*^{-/+}; *Ddx4-Cre* (heterozygous) lines and the *Ppp2ca*^{flx/-}; *Ddx4-Cre* (mutant) lines (Fig. 2A). The mutant mice were infertile; therefore, *Ppp2ca* was not expressed in testes. Next, we examined the protein expression of PPP2CA in the testes of the different mouse strains. Compared with control testes, there were low levels of expression in the testes of mutant mice (Fig. 2B) using a PP2Ac antibody. As mutational protein expression was not observed, expression at the RNA level was determined. Due to the Cre-lox recombination, *Ppp2ca* transcripts were shorter in mutant mice than in control mice (Fig. 2C).

Phosphatase function is essential for normal spermatogenesis

The testicular morphology of mutant mice and WT mice were completely different at 4 weeks. For example, there was a significant reduction in the size and weight of the testes (Fig. 3A and B). The mutant testes were ~20% the weight of the control testes, but there was no significant reduction in the overall weight of the mutant mice (Fig. 4B). Normal tubules containing spermatogonia, primary spermatocytes, and secondary spermatocytes were not observed in the mutant testes. Instead, the mutant tubules contained a large number of vacuolation structures, and any spermatogonia or primary spermatocytes were arranged in a disorderly manner. However, there was no difference in the testicular morphology and size between the heterozygous and WT mice at 4 weeks (Fig. 3B).

By 8 weeks, the mutant testes were approximately half the weight of the control testes (Fig. 4A and B), and there were fewer vacuole-like structures in the tubules. Compared with the control, the germ cells in the mutant tubules were disorganized and lacked elongated spermatozoa (Fig. 4C). Based on these results, the function of *Ppp2ca* is essential for normal spermatogenesis.

Decreased expression of *Ppp2ca* caused male infertility

At 8 weeks, the seminiferous tubules of normal mice contain spermatogonia, primary and secondary spermatocytes, spermatids, and sperm. In the mutant mice, however, the cells were similar to primary spermatocyte-stage cells, chromatin agglutination was abnormal and

scattered, and there was a lack of normal spermatozoa in the testes (Fig. 4B). Overall, the process of spermatogenesis was disordered in the mutant testes; at all levels, reproductive cells were arranged in a disorderly manner or were completely absent. In normal mice, the epididymis tubules at the tail of the epididymis contained many sperm. The mutant epididymides contained some round cells of different sizes and shapes, but nothing resembling normal sperm (Fig. 4B). The male mutant mice infertility was determined by fertility testing (Table 1). Therefore, *Ppp2ca* deficiency can cause male infertility.

Ppp2ca-deficient mice were incapable of generating normal sperm

It remained uncertain at which stage *Ppp2ca* influenced sperm production. First, the haploid, diploid, and tetraploid cells in the testes of mice at P38 were investigated by flow cytometric analysis. Compared with the control group, meiosis was blocked at the diploid stage in cells from mutant mice (Fig. 5A1). This result indicated that the gene influences meiosis at younger ages. We also examined the haploid, diploid, and tetraploid cells in testes of adult mice at P56. Surprisingly, the number of haploid and diploid cells in testes were almost the same (Fig. 5A2). This

Figure 5 Decreased *Ppp2ca* expression reduced normal sperm count in the epididymides. (A) Flow cytometric analysis of haploid, diploid, and tetraploid cells in P38 and P56 testes. M1, M2, and M3 populations correspond to 1C, 2C, and 4C cells respectively. (B left panel) Sperm from control (WT) epididymides. (B right panel) Normal sperm were not observed in the mutant sperm smear. Experiments had been repeated.

demonstrated that meiosis was still blocked in the diploid stage, but some cells could proceed to the next stage as the mouse aged. However, normal sperm were not observed in a sperm smear collected from the mutant epididymides at P56 (Fig. 5B1 and B2). Although there were still haploid cells in testes of mice at P56, these sperm were not normal.

Discussion

It is well known that PP2Ac is expressed abundantly in testis (Hatano *et al.* 1993) and plays an important role in embryonic development and sperm capacitation. Based on these facts, we set out to determine the homologue responsible in these mechanisms. Therefore, we generated conditional *Ppp2ca* knockouts to determine whether the loss of expression in mouse testes caused testicular morphological changes or infertility.

Previously, our laboratory demonstrated that knockout of exons 3–5 of *Ppp2ca* resulted in testicular morphological changes and infertility. In comparison, the knockout of exon 2 delayed morphological changes, and the vacuole-like structures increased significantly. We have yet to determine whether these delayed changes are the result of exon 2 deletion or whether the rest of PP2Ac delays the changes.

Disruption of PP2A activity will lead to meiotic arrest. Specifically, PP2A prevents the cleavage of centromeric cohesin during meiosis I by counteracting CK1-mediated phosphorylation of REC8, which activates separase (Ishiguro *et al.* 2010). Our results suggest that deletion of *Ppp2ca* causes male infertility. Therefore, we surmise that an arrest in meiosis leads to infertility. We plan to investigate whether meiosis is blocked in *Ppp2ca*-knockout cells, and which stage of meiosis is blocked.

PP2AC is the catalytic subunit critical for the function of PP2A. *Ppp2ca* is an important homolog that can function as the PP2AC subunit. We hypothesized that the loss of *Ppp2ca* would affect testicular morphology. However, *Ppp2ca* is also known to perform other critical functions as well. For instance, decreased *Ppp2ca* expression has been associated with Alzheimer's disease and other tauopathies (Planel *et al.* 2007, Whittington *et al.* 2011), which indicates that it plays a role in regulating phosphorylation in the brain. In addition, PP2A was shown to play a role in cardiomyocyte cell death by regulating calcium ion (Ca^{2+}) signaling (Ferron *et al.* 2011). Further investigations are necessary to determine the importance of *Ppp2ca* gene function in other organs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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