PMCA4 gene expression is regulated by the androgen receptor in the mouse testis during spermatogenesis

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Abstract. The present study aimed to investigate the expression of ATPase Ca^{++} transporting plasma membrane 4 (PMCA4) in mouse testis and to determine its role in spermatogenesis. Reverse transcription-quantitative PCR, western blotting and immunofluorescence were performed to evaluate the expression levels of PMCA4 in mouse testes at various weeks postnatal in wild type mice, and in testes from Sertoli cell-specific androgen receptor knockout and androgen receptor knockout (ARKO) mice. Luciferase assay, androgen receptor (AR) overexpression and AR antagonist experiments were used to confirm that AR regulated the expression of PMCA4. The results demonstrated that PMCA4 was highly expressed in mouse testes at 3-8 weeks postnatal. PMCA4 expression levels in ARKO mouse testes were decreased compared with wild type. In addition, activation of AR by testosterone administration resulted in an increase in the activity of the PMCA4 promoter. Cells transfected with an AR-overexpressing plasmid exhibited increased expression levels of the PMCA4 protein. Finally, the increase in PMCA4 protein levels induced by testosterone was prevented by pre-treatment with the AR antagonist flutamide. The present results confirmed that PMCA4 was upregulated during mouse testis development and that PMCA4 mRNA and protein expression levels were regulated by androgens and AR. The present findings suggest that PMCA4 may be involved in the regulation of spermatogenesis.

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Introduction

Androgens and androgen receptors (ARs) have a crucial role in maintaining spermatogenesis (1). Increasing evidence has demonstrated that low testosterone levels and mutations in the AR gene could result in spermatogenesis failure and male infertility (2-4). A previous study using a Sertoli cell-specific AR knockout (SCARKO) mouse model revealed spermatogenesis arrest at the diplotene stage (5), indicating that the function of androgens in Sertoli cells is critical for spermatogenesis.

Free testosterone binds to cytoplasmic AR in Sertoli cells, inducing a conformational change in AR and the nuclear translocation of the testosterone/AR complex. Subsequently, the complex binds to the androgen response element (ARE) in the chromatin, to induce target gene transcriptional activation or repression (1). At present, reproductive homeobox 5 is the only target gene that has been identified (6). Therefore, additional AR target genes, that may be physiologically relevant to spermatogenesis, need to be identified.

Our previous study (7) showed that ATPase Ca^{++} transporting plasma membrane 4 (ATP2B4, also known as PMCA4) was one of the 2,276 downregulated genes in AR knockout (ARKO) mice compared with WT mice. PMCA4 belongs to the family of P-type primary ion transport ATPases, which have key roles in intracellular Ca^{2+} homeostasis (8). Schuh *et al* (9) demonstrated that PMCA4 is required for sperm motility and male fertility in mice, while Patel *et al* (10) showed that PMCA4 is critical for murine sperm maturation. Olli *et al* (11) found that deletion of PMCA4 gene resulted in decreased sperm motility and infertility and suggested the potential involvement of PMCA4 mutations in human asthenospermia. However, the underlying mechanism responsible for the decreased sperm motility resulting from the absence or knockdown of PMCA4 has not been fully elucidated (11). The present study aimed to explore the expression pattern and function of PMCA4 during mouse spermatogenesis.

Materials and methods

Animals. A total of 30 C57BL/6 male mice were purchased from Guangdong Experimental Animal Center (Foshan,

China). SCARKO (n=3) and ARKO (n=3) mice were provided by Dr Yaoting Gui, at the Peking University Shenzhen Hospital (Shenzhen, China). Experimental protocols involving animals were reviewed and approved by the ethics committee of The People's Hospital of Longhua (Shenzhen, China; approval no. LHRY-1907014). ARKO mouse testes were collected from 8-9-week-old mice. WT postnatal testes were collected from C57BL/6 mice aged 1-8 weeks (3 mice in each group). Samples from other organs mentioned in the present study were from C57BL/6 adult mice (age, 8-9 weeks; n=6). Mice were maintained for at least 5 days under standard conditions with water and chow ad libitum and a 16-h light/8-h dark cycle at 22-25˚C and 50-60% relative humidity. Euthanasia was performed by cervical dislocation after CO_2 sedation.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from mouse testes using TRIzol (Thermo Fisher Scientific, Inc.) and cDNA synthesis was performed using the PrimeScript RT Master Mix kit (Takara Bio, Inc.) according to the manufacturer's protocol. RT-qPCR reactions were performed with the SYBR® Premix EX TaqTMII PCR kit (Takara Bio, Inc.), with primers specific for mouse PMCA4 (forward, 5'-CTGAGGGAATGGACGAGAT-3' and reverse, 5'-CAACTGCTGCGGAATAGGA-3'; product size, 204 bp), with GAPDH (forward, 5'-AGTGGCAAAGTGGAGATT-3'; and reverse, 5'-GTGGAGTCATACTCCAACA-3'; product size, 116 bp) used as the endogenous control. The annealing temperature was 60˚C, with 40 cycles. Data were calculated using $2^{-\Delta\Delta Cq}$ method (12).

Western blot analysis. Protein samples were extracted from tissue and cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), then 20 μg protein samples (determined by bicinchoninic acid) were loaded and run on 10% SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 10% (w/v) non-fat milk in TBS buffer (Dalian Meilun Biology Technology Co., Ltd.) with 0.05% Tween-20 (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. After incubation overnight at 4˚C with primary antibodies targeting PMCA4 (140 kDa, Abcam; cat. no. ab2783; 1:1,000 dilution), GAPDH (37 kDa, Cell Signaling Technology, Inc.; cat. no. 5174; 1:1,000 dilution), or AR (110 kDa, Santa Cruz Biotechnology, Inc., cat. no. sc-816; 1:1,000). The membranes were treated with horseradish peroxidase-labeled secondary antibody (anti-rabbit and anti-mouse IgG; both 1:1000; cat nos. 7074 and 7076, respectively; both Cell Signaling Technology, Inc.) for 1 h at room temperature. Positive bands were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). The densitometry was determined by ImageJ 1.48 software (imagej.net/).

Sperm collection. Mouse epididymides for sperm preparation were collected from C57BL/6 mice as described previously (13). Briefly, cauda epididymis was dissected and placed in Quinn's Advantage Fertilization (HTF) Medium (pH 7.4; SAGE®Media; cat. no. ART-1020; Cooper Surgical, Inc.) at 37˚C for 10 min following shearing, to allow the sperm to be dispersed. After a wash with fresh medium, the sperm was centrifuged at 800 x g for 10 min at room temperature, and then resuspended in fresh Quinn's medium ($1x10^7$ cells/ml) for the next step of experiments.

Immunofluorescence. Mouse testes were dissected, fixed with 4% paraformaldehyde for 24 h at 4˚C, then embedded in paraffin. The paraffin-embedded testicular tissue was cut in 2-μm sections. After dewaxing and rehydrating, the slides were subjected to antigen retrieval, by immersing in 10 mM sodium citrate (pH 6.0) and microwaving at 1,000 W for 30 min. The sections were blocked in 10% bovine serum albumin at 37˚C for 30 min, followed by cooling to room temperature (RT). An anti-PMCA4 antibody (1:100; cat. no. ab2783; Abcam) was added on to the slides and kept at 4˚C overnight. The following day, after three washes with PBS, the sections were incubated with an anti-mouse Alexa Fluor 594 antibody (1:500; cat. no. A-11005; Thermo Fisher Scientific, Inc.) for 1 h at RT. The sections were counterstained with Hoechst 33342 (1:2,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min at RT. Following two additional washes, the sections were mounted with SlowFade (Invitrogen; Thermo Fisher Scientific, Inc.) and observed using a fluorescent microscope (magnification, x200; Zeiss GmbH).

Cell transfection. Murine Sertoli cell line TM4 was purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (v/v) and 100 μg/ml each penicillin and streptomycin at 37˚C with atmospheric conditions of 95% air and 5% CO_2. Plasmids pcDNA3.1-AR and pcDNA3.1 were constructed by IGE Biotechnology Ltd. Cells were plated in 10-cm culture dishes; upon reaching 50% confluence, cells were transfected (37˚C; 24-48 h) with 2.5 μg plasmid using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). Cells were harvested 24-48 h after transfection.

Dual-luciferase assay. At 24 h post-transfection, TM4 cells were cultured in serum-free medium [0.1% BSA in DMEM (cat. no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.)] for 6 h before addition of 10 nM testosterone (Dalian Meilun Biology Technology Co., Ltd.) or ethanol. Following 6 h incubation at 37˚C and removal of the medium, cells were lysed by addition of 100 μl passive lysis buffer (Promega Corporation). Luciferase activity was measured following the manufacturer's instructions (cat. no. E1910; Promega Corporation). The activity of luciferase reporter was normalized to that of Renilla luciferase.

Statistical analysis. All experiments in the present study were repeated at least three times. Statistical analysis was performed using Sigmaplot 16.0 (Systat Software, Inc.). Data were expressed as the mean ± standard error of mean. Statistical significance was evaluated by one -way ANOVA followed by Fisher's protected least-significant difference post hoc test, unless otherwise specified. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of PMCA4 during mouse testes development. First, the protein expression levels of PMCA4 were

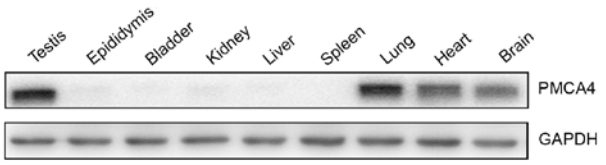

Figure 1. Expression of PMCA4 in different mouse tissues. Western blot analysis of PMCA4 protein expression in various tissues form adult C57BL/6 mice. GAPDH was used as in internal control. PMCA4, ATPase Ca^{++} transporting plasma membrane 4.

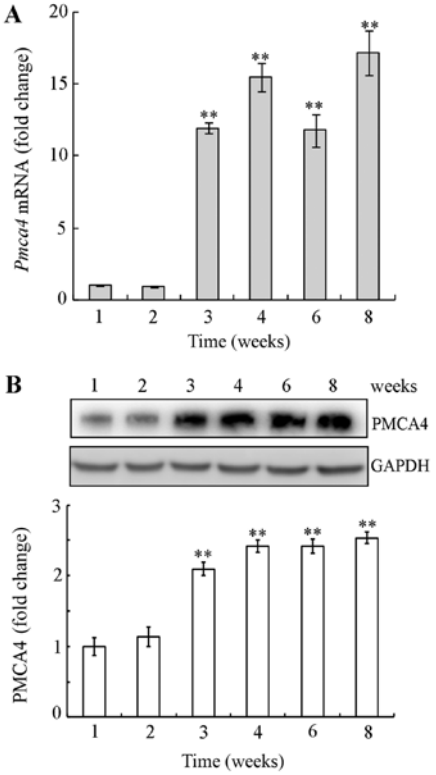

Figure 2. Temporal expression of PMCA4 during mouse testis postnatal development. (A) mRNA expression levels were evaluated at 1, 2, 3, 4, 6 and 8 weeks postnatal. mRNA levels were normalized to GAPDH and plotted relative to those of the 1-week-old mice. (B) Protein expression levels were detected by western blotting, with GAPDH used an internal control. Data were analyzed by one-way ANOVA followed by post hoc Bonferroni's test and are presented as mean ± standard error of the mean (n=4). **P<0.01 vs. 1 week. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; w, week.

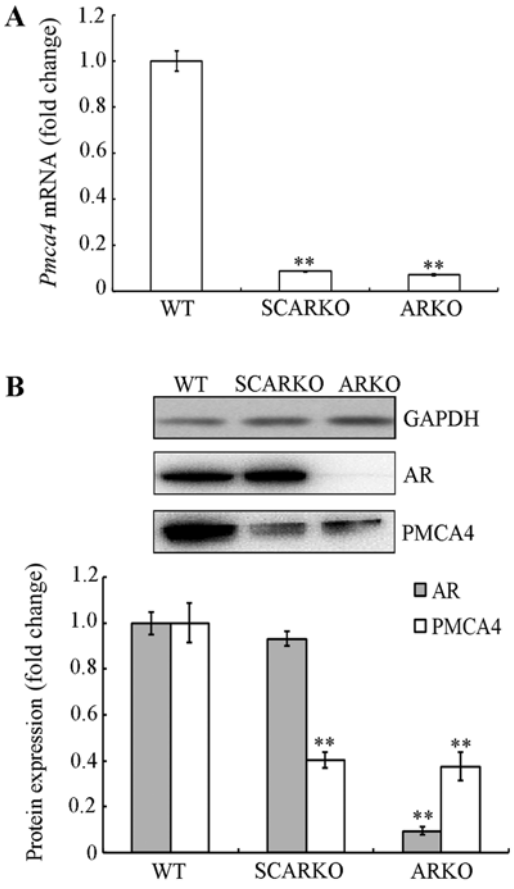

Figure 3. PMCA4 expression is decreased in testes from ARKO mice. (A) mRNA and (B) protein expression levels of PMCA4 were evaluated in WT, SCARKO and ARKO mouse testes. Data are presented as the mean ± standard error of the mean relative to the WT group (n=4). **P<0.01 vs. WT. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; AR, androgen receptor; WT, wild type; SCARKO, Sertoli cell-specific AR knockout; ARKO, AR knockout.

examined in various tissues from adult mice. Western blotting results revealed that the PMCA4 protein was expressed in the testes, as well as in the lung, heart and brain (Fig. 1). No protein expression of PMCA4 was observed in epididymis, bladder, liver, kidney and spleen tissue (Fig. 1).

Next, the mRNA and protein expression levels of PMCA4 were examined by RT-qPCR and western blotting, respectively, in mouse testicular tissues during postnatal development. PMCA4 expression levels were significantly increased at week 3-8 postnatal compared with week 1, both at the mRNA and the protein level (Fig. 2). These findings suggest that PMCA4 was upregulated during mouse testes postnatal development.

PMCA4 expression is decreased in the testes of AR-knockout mice. To confirm the results from our previous gene expression analysis (8), PMCA4 mRNA and protein expression levels were examined in the testes of SCARKO, ARKO and WT mice. Compared with WT mice, PMCA4 mRNA levels were decreased in testicular tissues from SCARKO (0.087±0.002; P<0.001) and ARKO (0.075±0.004; P<0.001) mice (Fig. 3A). Similar results were obtained for the protein expression levels, as shown in Fig. 3B. These findings indicated that PMCA4 expression was decreased in AR knockout mice, thus, it was hypothesized that PMCA4 may be regulated by AR in mouse testes.

PMCA4 expression during spermatogenesis. To further explore the potential role of PMCA4 in spermatogenesis, the subcellular localization of PMCA4 during testes development was investigated by immunofluorescence staining. As presented in Fig. 4, PMCA4 immunostaining was absent before 3 weeks postnatal. In addition, the staining pattern in the microscopy images revealed that PMCA4 expression was located in the elongated spermatids (Fig. 4).

Next, immunofluorescence staining for PMCA4 was performed in testicular tissues from WT and ARKO mice. In the testis from WT mice, high intensity fluorescence (red) was observed in the lumen of the testicular tubule and was co-localized with the mature sperm (Fig. 5). Consistent with

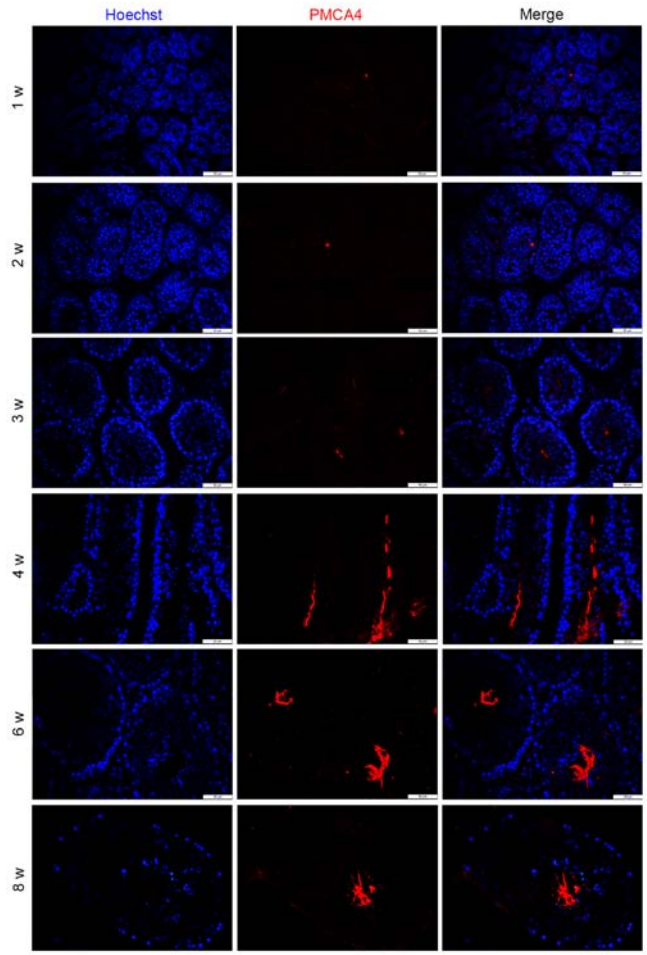

Figure 4. Immunofluorescence staining for PMCA4 in mouse testis at different postnatal weeks. Representative microscopy images show PMCA4 staining (red) and nuclear staining (Hoechst 33342; blue) (n=4). Scale bar, 50 μm. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; w, week.

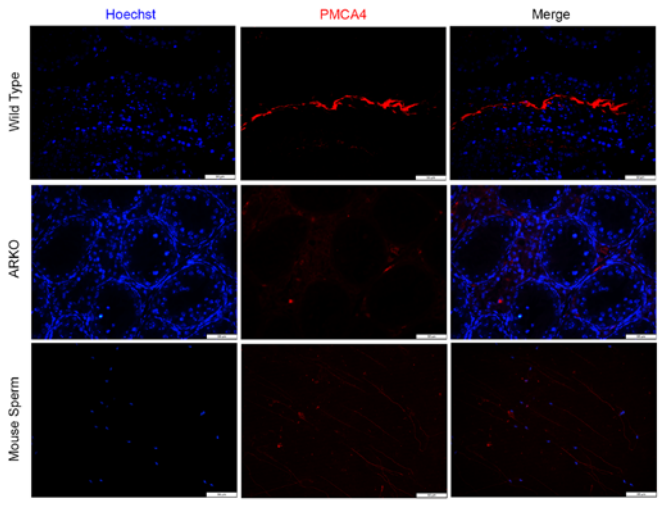

Figure 5. Immunofluorescence staining for PMCA4 in mouse testis from wild type and ARKO mice, and in wild type mouse sperm. Representative microscopy images show PMCA4 staining (red) and nuclear staining (Hoechst 33342; blue) (n=4). Scale bar, 50 μm. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; ARKO, AR knockout.

the western blotting data in Fig. 3B, minimal florescence signal was observed in the testis from ARKO mice (Fig. 5).

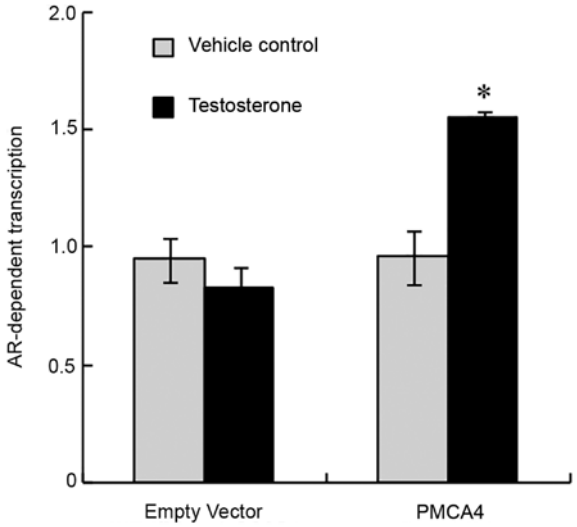

Figure 6. PMCA4 promoter activity is increased by testosterone treatment in TM4 cells in vitro. Dual-luciferase assays were used to detect the effects of testosterone on the PMCA4 promoter activity in TM4 cells. Cells were transfected with empty vector as a control. Results are plotted as fold-change of testosterone-treated relative to vehicle-treated. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. PMCA4 vehicle control. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; AR, androgen receptor.

Immunostaining of PMCA4 in healthy mouse sperm revealed that the protein was located at the sperm tail. The present data suggested that PMCA4 may be involved in the movement and motility of the sperm.

PMCA4 promoter is activated by testosterone in vitro. To evaluate whether testosterone and AR affect the transcription of PMCA4, a dual-luciferase assay was used to determine the PMCA4 promoter-driven luciferase activity. As shown in Fig. 6, following treatment of TM4 cells with testosterone or ethanol (vehicle control), cells transfected with the control empty vector exhibited no difference in luciferase activity following exposure to testosterone. By contrast, cells transfected with the PMCA4 promoter-driven vector exhibited significantly increased luciferase activity following testosterone treatment (Fig. 6). The present data indicated that testosterone and AR regulated the promoter activity of PMCA4.

PMCA4 protein expression is regulated by AR in vitro. To further confirm that AR regulates PMCA4 protein expression, TM4 cells were transfected with pcDNA3.1-AR or pcDNA3.1 (empty vector). A significant increase in PMCA4 and AR protein expression levels was observed by western blotting in the AR-overexpressing cells compared with the control-transfected cells (Fig. 7A). In Fig. 7B, cells were pre-incubated for 6 h with 30 μM flutamide, an AR antagonist (14), and then were exposed to 10 nM testosterone for 24 h. As expected, testosterone induced an increase in PMCA4 and AR protein expression levels in the negative control group, which were treated with vehicle for flutamide (Fig. 7B). Flutamide pre-treatment, however, significantly decreased the testosterone-mediated induction in PMCA4 and AR protein expression levels (Fig. 7B).

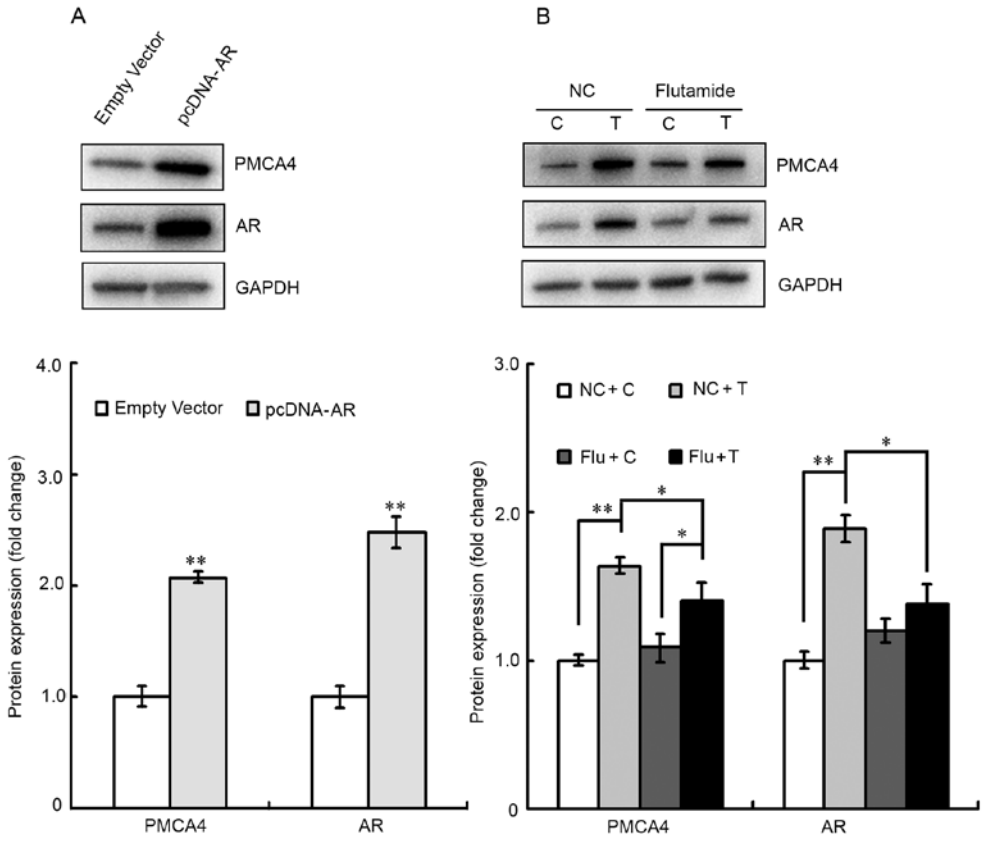

Figure 7. PMCA4 expression is upregulated by androgens and AR in TM4 cells. (A) TM4 cells were transfected with either empty vector or pcDNA-AR, and subsequently the AR and PMCA4 protein expression levels were evaluated via western blotting. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean (n=3). **P<0.01 vs. empty vector group. (B) Cells were pre-treated with flutamide for 6 h, prior to 10 nM testosterone administration for 24 h. AR and PMCA4 protein expression levels were then evaluated by western blotting. Testosterone increased PMCA4 and AR protein expression, and this increase was blocked by the AR antagonist flutamide. Data are presented as the mean ± standard error of the mean (n=3). The statistical significance of the differences between groups was determined by one-way ANOVA followed by all pairwise Holm-Sidak test. *P<0.05 and **P<0.01, with comparisons shown in brackets. PMCA4, ATPase Ca^{++} transporting plasma membrane 4; AR, androgen receptor; NC, negative control for Flu; C, control for T and ethanol; T, testosterone; Flu, flutamide.

Discussion

Ionic homeostasis has a key role in sperm maturation, capacitation and gamete communication (15); the balance of ion transport systems is central to sperm motility (16). Thus, there is little doubt as to the importance of calcium homeostasis in sperm motility and fertilization (17,18).

AR is capable of transmitting testosterone signals by at least two known mechanisms, the classical and non-classical pathways. In the non-classical Ca^{2+} influx pathway, androgen interacts with a Gq coupled G-protein coupled receptor (GPCR) in the plasma membrane. Phospholipase C (PLC) is then activated to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (19). Decreased PIP2 concentration inhibits ATP-sensitive potassium channels causing membrane depolarization and Ca^{2+} entry via L-type Ca^{2+} channels (20). Voltage dependent L-type Ca^{2+} channels open allowing the influx of Ca^{2+}, which can regulate multiple cellular processes (20). However, the physiological functions regulated by the testosterone-mediated influx of Ca^{2+} have not been identified (19).

PMCA represents a family of enzymes that extrude Ca^{2+} from the cytoplasm across the plasma membrane of eukaryotic cells. PMCA4 is the most common PMCA isoform in sperm (21), and has two major splice variants 4a and 4b. The latter one is thought to be a key regulator in calcium clearance in murine sperm (22,23), where PMCA4 variant 4b deletion disrupts calcium homeostasis and results in the loss of both progressive and hyperactivated sperm motility, ultimately leading to male infertility (9,24). A study conducted by Chen *et al* (25) reported that PMCA4 was downregulated in seminoma, the most common testicular malignant germ cell tumor, suggesting that PMCA4 has an important role in the spermatogenesis and male fertility; however, the molecular mechanism remains unclear. Therefore, the present study aimed to explore the expression pattern and function of PMCA4 during mouse spermatogenesis.

Results from western blot analysis in different murine tissues revealed that PMCA4 was expressed in the testes, lungs, heart and brain. PMCA4 mRNA and protein expression levels were markedly increased in mice testis at 3 weeks postnatal, which is the time point when the first wave of spermatogenesis occurs (26), suggesting that PMCA4 expression may be primarily restricted to post-meiotic germ cells. This was further confirmed by immunofluorescence results showing that PMCA4 was located at the lumen and inner layers of seminiferous tubules. Previous reports using

rats (22), as well as bovines (27), have demonstrated that PMCA4 is expressed in the epididymis epithelium. The present findings are in agreement with a previous study published by Patel *et al* (10), which reported that PMCA4 protein is expressed in the testis and throughout the epididymis. However, the molecular mechanism by which PMCA4 influences spermatogenesis has not been reported to date. In the present study, PMCA4 mRNA levels in testis tissues from SCARKO and ARKO mice were significantly decreased compared with WT controls, and PMCA4 protein expression levels were decreased in ARKO mice compared with WT controls. These findings suggested that AR may regulate PMCA4 expression in mouse testes. By using a luciferase activity assay in vitro, the present study confirmed that activation of AR by testosterone administration increased the activity of the PMCA4 promoter. Cells overexpressing AR in vitro also had higher expression levels of the PMCA4 protein, and the increase in the PMCA4 protein expression induced by testosterone was prevented by pre-treatment with the AR antagonist flutamide.

It was previously reported that PMCA4 and the nitric oxide synthases (NOSs) are interacting partners that have been identified in a quaternary complex including Caveolin-1 (11). A previous study also found that in mouse Sertoli cells AR localizes in the plasma membrane by association to Caveolin-1 (28). The aforementioned evidence supports the hypothesis that AR regulates PMCA4 expression. In general, androgens and AR affect the development of spermatogenic cells in Sertoli cells (5,29). The present results demonstrated that PMCA4 was almost exclusively localized in the sperm tail. A study by Lestari *et al* (30) reported that PMCA4 expression in the normozoospermia group was much higher than in the asthenozoospermia group due to impaired sperm structure and function, suggesting that PMCA4 defects may be a factor in sperm dysfunction and infertility.

In conclusion, the present study demonstrated that AR regulates PMCA4 expression and that the expression levels of PMCA4 mRNA and protein are downregulated in testes form AR knockout mice compared with WT mice. The present data indicated that PMCA4 may serve a potentially important role in spermatogenesis, and likely in male reproduction. Further studies are needed to better understand the molecular mechanisms by which AR regulates the PMCA4 expression and function in spermatogenesis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RS, QD, HG and ZW performed the experiments. HL and QD analyzed the data and wrote the manuscript. QD obtained funding and revised the manuscript. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental protocols involving animals were reviewed and approved by the Ethics Committee of The People's Hospital of Longhua (Shenzhen, China; approval no. LHRY-1907014).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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