Localization and expression patterns of prolactin-like protein J in mouse testis

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Abstract. Prolactin (PRL)-like protein J (PLP-J) is a member of the prolactin family, mainly expressed in the placental decidua tissues of females, and is involved in gestation. To the best of our knowledge, it has not previously been shown to be expressed in males. Preliminary experiments of the present study indicated that PLP-J is expressed in the testis of male mice and is implicated in the regulation of testicular function. To definitively address whether PLP-J is expressed in the mouse testis, the expression pattern and cellular localization of PLP-J in mouse testes during postnatal development were characterized in the current study using molecular and immunological methods. Reverse transcription (RT)-polymerase chain reaction (PCR) was performed to amplify gene fragments from mouse testis specimens, which yielded sequences matching those of the PLP-J gene in Genbank. Subsequently, *in situ* hybridization showed that PLP-J was localized in interstitial tissue of the mouse testis. Immunofluorescence results indicated that PLP-J and 3β-hydroxysteroid dehydrogenase 1 were colocalized in testis Leydig cells, confirming PLP-J expression in Leydig cells. In addition, PLP-J gene expression levels were examined at different stages of postnatal mouse development in male testis tissues using quantitative RT-PCR and western blotting. The results revealed that PLP-J expression levels were lowest in 18-day-old mice and highest in adults aged 4 months. Levels observed in 16-month-old individuals were lower than those observed in the 4-month-old mice, but remained significantly higher than the levels observed in 18-day-old mice. Furthermore, the roles of PLP-J in the murine testis TM3 Leydig cell line were studied. The results demonstrated that the upregulation of PLP-J expression in TM3 Leydig cells did not affect testosterone production or the cell cycle. In conclusion, this study demonstrated that PLP-J, a known member of the PRL family that was previously considered to be expressed solely in females, is also expressed in the testis of males with an age-dependent expression profile. Nevertheless, the physiological role of PLP-J in males remains unclear.

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Introduction

The prolactin (PRL) family is comprised of multiple proteins that regulate a plethora of biological functions (1-6). In rats and mice, the PRL gene family consists of at least 20 well-characterized genes, including placental lactogens, PRL-like proteins (PLPs), PRL-related proteins, proliferin and proliferin-related protein (7). The PRL family genes, located on chromosome 13 in mice and chromosome 17 in rats, are mainly expressed in the pituitary gland, uterus and placenta (1-6). These proteins demonstrate a unique spatio-temporal expression profile and strongly influence various aspects of gestation (1,7-9).

PLP-J, also termed PLP-I, Prlpj, Prlpi or Prl3c1, is a protein belonging to the PRL family. PLP-J was first reported in three independent studies (8,10,11). Using northern blotting, Toft and Linzer (10) displayed PLP-J expression in RNA samples isolated from maternal decidua but not in those from the heart, lung, kidney, liver, muscle, spleen, thymus and testis. They demonstrated that PLP-J mRNA production is limited to early gestation, (peaking on day 7, and beginning to decrease on day 9, with undetectable levels by day 11) using the technique of *in situ* hybridization in placental tissue with a PLP-J probe. Another study demonstrated that the decidual expression of PLP-J is independent of extra-embryonic or embryonic factors (12).

Alam *et al* (13) indicated that PLP-J promotes proliferation of uterine stromal cells but inhibits that of endothelial cells. PLP-J has also been demonstrated to interact with heparin-containing molecules, including syndecan-1 which is expressed in uterine stromal and endothelial cells. The restricted expression of PLP-J and its unique interaction with basal and endothelial cells suggest that this protein may be involved in the development of decidual cells and endometrial vasculature (13).

Preliminary experiments of the present study suggest that PLP-J may be present in the mouse testis. By characterizing the expression and cellular localization of PLP-J in relation to developmental changes in the mouse testis, the current study attempted to determine whether the testis is a source of PLP-J in the mouse. Molecular and immunological

approaches, including reverse transcription (RT)-polymerase chain reaction (PCR), immunofluorescence, *in situ* hybridization and western blotting were employed to investigate the mRNA and protein levels of PLP-J in the mouse testis. In addition, the effects of PLP-J on testosterone production, cell proliferation, and apoptosis were studied in the murine TM3 Leydig cell line.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM)/F-12 and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc., (Logan, UT, USA). The SYBR® PrimeScript™ RT-PCR kit (Perfect Real Time) was purchased from Takara Biotechnology (Dalian, China). The Bicinchoninic Acid (BCA) Protein Assay kit, Protein Extraction kit, BeyoECL Plus Western Blotting Detection kit, and Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Testosterone Immunoassay kits were obtained from USCNK (Wuhan, China). Antibody for 3β-HSD1 (sc-30821) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The *In Situ* Hybridization Detection kit was purchased from Haoyang (Tianjing, China). All other chemicals were purchased from Sangon Biotech (Shanghai, China).

Animals and tissue preparation. A total of 24 male Balb/c mice at 18 days, 4 months, and 16 months of age (n=8 in each age group) and a New Zealand white rabbit were obtained from the Chongqing Medical University Animal Center (Chongqing, China). Mice were sacrificed by intraperitoneal injection of 10% chloral hydrate solution at the dose of 8 ml/kg. Immediately after euthanasia, the testes were removed and washed with saline (0.9% NaCl). The left testis was fixed in a 4% paraformaldehyde solution for *in situ* hybridization and immunohistochemistry, while the right testis was used for protein and RNA extraction. The animal protocols conformed to those approved by the Chongqing Medical University Animal Care and Use Committee.

Cell culture. TM3 Leydig cells were obtained from The Cell Bank of the Chinese Academy of Sciences (Shanghai, China)) and 293FT cells were from Invitrogen (Carlsbad, CA, USA). TM3 Leydig cells were cultured in F12/DMEM supplemented with 10% FBS. 293FT cells were cultured in DMEM containing 10% FBS. All cells were placed in an incubator containing 95% air and 5% CO_2 at 37˚C, and the culture media were replaced every 2 days.

Preparation of the polyclonal antibody. A polyclonal anti-PLP-J antibody was prepared to detect PLP-J protein expression. Based on the amino acid sequence of PLP-J (NP_038794.1; http://www.ncbi.nlm.nih.gov/protein/7305407), the following peptide fragments were designed: RYDRKSNEEI, IQPGIEENNE, KTNEDLLK, KMYKILD, VLTHLGSYDGMM, RELRSSKKSK and FFYCLRKDTK. These fragments were joined, along with connecting residues, to yield the final sequence: RYDRKSNEEI GSIQPGIEENNEAKTNEDLLKKMYKILDGSVLTHLGS YDGMMGSRELRSSKKSKFFYCLRKDTK. The amino acid sequence was converted to a nucleotide sequence, which was generated via gene synthesis following incorporation of *Kpn*I and *Hin*dIII sites, and cloned into pET-30a (Novagen, Madison, WI, USA) via these two sites. The resulting plasmid was used to transform BL21-competent cells prior to the selection of positive clones. Protein production was facilitated by isopropyl β-D-1-thiogalactopyranoside induction.

To immunize a single New Zealand white rabbit, 200 μg of the recombinant protein was subcutaneously injected followed by three boosters of 100 μg recombinant protein at 21 days, 42 days and 63 days. Subsequent to the last booster, a blood sample was obtained from an ear vein for ELISA analysis to determine the titer. If the desired level (titer of antibody >12,800) was reached, blood was drawn from the carotid artery and centrifuged at 5,000 x g for 10 min to collect antiserum, which was subjected to protein G affinity purification. The purity of the antiserum was determined by western blot analysis. The antiserum was divided into aliquots, and the pre-immune serum was used as a control.

Quantitative RT-PCR. The expression of PLP-J mRNA in testis tissues was assessed by RT-PCR. The primer designs were based on the sequences of mouse PLP-J (NM_013766; http://www.ncbi.nlm.nih.gov/nuccore/7305406). The primer sequences were as follows: Forward, 5'-ACCAAGATGTGC CAAACCATTTCTA-3'; and reverse, 5'-CAGCTCTTGTCA TCATCCCATCA-3'. The predicted size of the RT-PCR product was 163 bp. For mouse β-actin amplification, the primer sequences were as follows: Forward, 5'-TCGTGCGT GACATCAAAGAG-3'; and reverse, 5'-CAAGAAGGAAGG CTGGAAAA-3'. The length of the RT-PCR product was 177 bp.

Briefly, total RNA from the mouse testis was extracted using TRIzol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized using the SYBR® PrimeScript™ RT-PCR kit (Perfect Real Time) in the thermal cycler (CFX96; Bio-Rad, Hercules, CA, USA). Each RT-PCR reaction was performed in triplicate in a total volume of 20 μl reaction mixture [2 μl cDNA, 6.8 μl H_2O, 10 μl SYBR® Premix Ex Taq™, 0.6 μl of each of the forward and reverse primers (10 μM)]. The PCR program consisted of 1 min at 95˚C, followed by 40 cycles of 10 sec at 95˚C and 30 sec at 60˚C. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (14). The expression level of PLP-J mRNA was normalized to the concurrent measurement of β-actin mRNA.

Probe synthesis and in situ hybridization. In concordance with the gene sequence of murine PLP-J, a probe with the sequence 5'-GGCAGTCACATATACCCACAGGAACAT-3' was designed in the current study, which was then synthesized at Sangon Biotech. To identify the cell type expressing PLP-J mRNA, *in situ* hybridization was performed on 5-μm tissue sections. Paraffin-embedded tissues were dewaxed and blocked in H_2O_2 solution for 20 min at room temperature to prevent endogenous catalase activity. After rinsing with 0.01 M phosphate-buffered saline (PBS) 3 times, acetylation solution was added dropwise until it covered the surface of the tissues, which were then incubated at room temperature for 10-30 min. The specimens were washed with PBS 3 times

and rinsed once with 0.2X saline sodium citrate (SSC) buffer. Prehybridization buffer was added dropwise until it covered the tissues, which were then incubated at 37˚C for 2 h. The samples were rinsed 3 times with 0.2X SSC for 5 min. Hybridization buffer was added dropwise until it covered the tissues, which were incubated at 37˚C for 8 h and then washed with SSC. Mouse Anti-Digoxin-Biotin Antibody solution was added and the samples incubated at 37˚C for 45 min. The specimens were rinsed 3 times with 0.01 M PBS for 5 min. A working solution of high-performance streptavidin peroxidase complex (3 ml) was added to the specimens, incubated at 37˚C for 45 min, and then washed with PBS. The slides were stained with 3,3'-diaminobenzidine and counter-stained with hematoxylin, which stained the nuclei blue. The slides were then observed with a BX51 microscope (Olympus, Tokyo, Japan).

Immunofluorescent staining of PLP-J. To examine whether PLP-J is expressed in Leydig cells, the cellular colocalization of PLP-J with 3β-hydroxysteroid dehydrogenase (HSD) 1 was examined in the testes of adult mice using a double immunofluorescence staining method. Briefly, the sections were incubated for 48 h at 4˚C with a mixture of rabbit anti-PLP-J IgG and goat anti-3β-HSD1 IgG. Subsequently, the sections were rinsed in 0.01 M PBS (pH 7.4) and then incubated for 24 h at 4˚C with a mixture of FITC-conjugated anti-rabbit IgG and tetramethyl rhodamine isothiocyanate-conjugated anti-goat IgG (ZSGB-Bio, Beijing, China). The tissues were then examined under the BX51 microscope.

For immunofluorescent staining of PLP-J in TM3 cells, the TM3 cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, followed by permeabilization in PBS containing 0.5% Triton X-100 at room temperature for 10 min. The cells were then immersed in blocking solution for 1 h. The cells were immunolabeled with rabbit anti-PLP-J primary antibody overnight at 4˚C. Following 3 washes with PBS, the cells were incubated with FITC-conjugated anti-rabbit IgG at room temperature for 1 h. The slides were then observed and analyzed using a TE-2000U fluorescence microscope (Nikon, Tokyo, Japan).

Western blotting. In order to isolate protein, tissues or cells were homogenized and lysed with radioimmunoprecipitation assay buffer. Insoluble material was removed by centrifugation at 12,000 x g for 10 min at 4˚C, and the supernatant was collected. The protein concentration was determined using the BCA assay. For western blotting, samples were subjected to SDS-PAGE and then transferred to a polyvinylidine fluoride membrane, followed by incubation with anti-PLP-J or anti-β-actin polyclonal antibody. Membranes were washed briefly in Tris-buffered saline with Tween 20 and incubated with secondary antibodies. The antigen-antibody complexes were then detected using the BeyoECL kit according to the manufacturer's instructions.

Lentiviral-mediated overexpression of PLP-J in TM3 cells. To investigate the biological activities of PLP-J *in vitro*, a PLP-J-expressing lentivirus was generated, which was transfected into TM3 cells to induce upregulation of PLP-J expression. The PLP-J sequence was obtained from Genbank (NM_013766.2; http://www.ncbi.nlm.nih.gov/nuccore/NM_013766.2), synthesized, and then cloned into the LV5 vector (GenePharma Co., Ltd., Shanghai, China). Lentiviruses were then produced in 293T cells, titrated, and used to transfect the TM3 cell line.

Testosterone measurement. To analyze the effect of PLP-J overexpression on testosterone production in TM3 cells, TM3-N cells (transfected with control lentivirus) and TM3-PLP-J cells (transfected with PLP-J lentivirus) were cultured for 48 h. The culture supernatants and cells were collected for testosterone analysis according to the manufacturer's instructions.

Flow cytometry for cell apoptosis analysis. Harvested TM3 cells were centrifuged at 300 x g for 10 min and the supernatant was removed. They were then washed once with 1X binding buffer. The cells were resuspended in 200 μl binding buffer prior to incubation with 5 μl Annexin V and 10 μl propidium iodide (PI) at room temperature for 15 min. Flow cytometric analysis was conducted using a FACSVantage SE flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometric analysis of the cell cycle. TM3 cells were trypsinized and centrifuged at 300 x g for 10 min. Following rinsing with PBS, the cells were fixed in 70% ethanol. TM3 cells were then centrifuged for 5 min and washed with 1X PBS. Next, the cells were incubated for 30 min in Hank's Balanced Salt Solution containing PI (10 μg/ml) and RNase (100 μg/ml) at room temperature, followed by analysis using the FACSVantage SE flow cytometer.

Statistical analysis. Data are expressed as the mean ± standard deviation. Significant differences were evaluated using one-way analysis of variance with the Newman-Keuls method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Preparation and validation of anti-PLP-J polyclonal antibodies. To evaluate the production of PLP-J, polyclonal anti-PLP-J antibodies were prepared. New Zealand white rabbits were immunized 4 times with a recombinant protein containing multiple PLP-J epitopes, and the resulting antiserum was shown by ELISA to have a titer of 1:25,600. Subsequent protein G affinity purification yielded an antibody concentration of 1.66 mg/ml, as revealed by UV spectroscopy. To verify the specificity of the anti-PLP-J antibody, 293FT cells were transfected with PLP-J-expressing lentivirus or control lentivirus and analyzed via western blotting using the polyclonal anti-PLP-J antibody. The results demonstrated that 293FT cells transfected with the PLP-J lentivirus produced a specific band with the expected size, which was absent in 293FT cells transfected with control virus, indicating that the prepared anti-PLP-J antibody specifically recognized its antigen (Fig. 1) and was suitable for subsequent experiments.

Localization of PLP-J mRNA and protein in murine testis and TM3 cells. The expression of PLP-J was first analyzed by RT-PCR, using RNA extracted from the testes of the mice. A

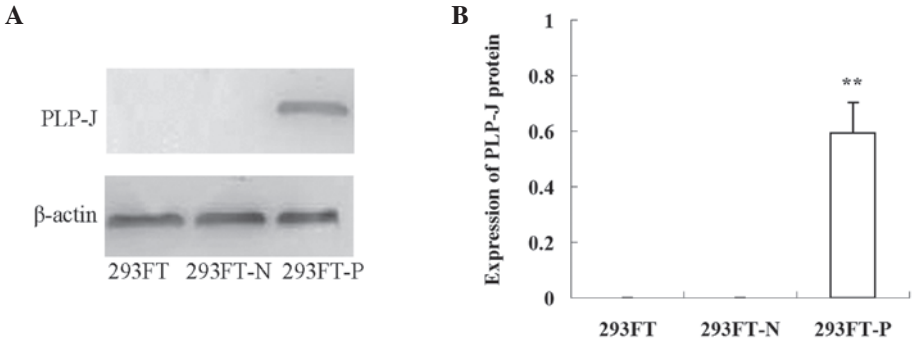

Figure 1. The prepared polyclonal anti-PLP-J antibody specifically recognized PLP-J. (A) Representative image of western blot of PLP-J in 293FT cells. No specific band was detected in 293FT or 293FT-N cells. (B) Relative expression of PLP-J in different groups following normalization to β-actin. Values represent the means ± standard deviation (n=3). **P<0.01 vs. the 293FT group. 293FT, parental 293FT cells; 293FT-N, 293FT cells transfected with negative control lentivirus; 293FT-P, 293FT cells transfected with PLP-J lentivirus; PLP-J, prolactin-like protein-J.

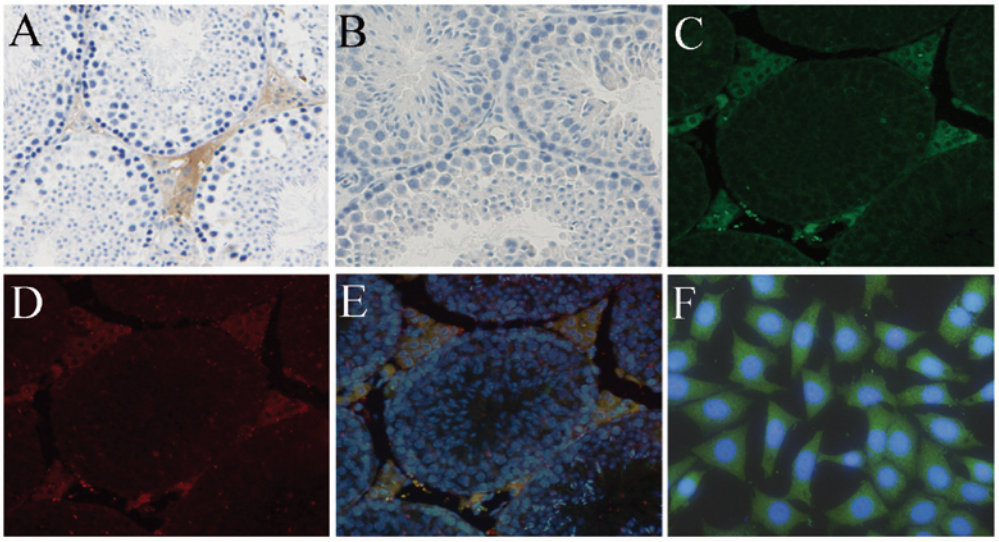

Figure 2. Localization of PLP-J mRNA and protein within mouse testis. (A) *In situ* hybridization was performed to reveal the spatial distribution of PLP-J in the mouse testis (magnification, x200). An antisense riboprobe for PLP-J was used; there is positive staining (brownish-yellow) in Leydig cells in the interstitial tissue. (B) Representative image of sense riboprobe signal for PLPL-J (magnification, x200). (C-E) Co-localization of PLP-J with 3β-HSD1 in mouse testis Leydig cells (magnification, x200). PLP-J is represented as green and 3β-HSD1 in red. (F) Immunofluorescent staining of PLP-J in the TM3 Leydig cell line (magnification, x400). PLP-J was present in the cytoplasm. Nuclei were stained blue with DAPI. PLP-J, prolactin-like protein-J; HSD1, hydroxysteroid dehydrogenase 1.

single amplification product of 163 bp was sequenced, which matched perfectly with the PLP-J nucleotide sequence (data not shown), indicating the presence of PLP-J mRNA in the testes.

In situ hybridization was used to elucidate the spatial distribution of PLP-J in the mouse testis. Positive signals (brownish-yellow) were detected in the interstitial tissues of the testis, but not in Sertoli and sperm cells (Fig. 2A). No signal was observed using the sense cRNA PLP-J probe (Fig. 2B).

The localization of PLP-J in the mouse testis was further studied by immunofluorescence using sections prepared from testes of adult mice. PLP-J and 3β-HSD1 were colocalized in the cytoplasm of Leydig cells (Fig. 2C-E). As 3β-HSD1 is known to be expressed in Leydig cells, their colocalization indicated that PLP-J was also present in the Leydig cells of testis interstitial areas. PLP-J immunofluorescence of TM3 Leydig cells further revealed that this protein was localized within the cytoplasm of Leydig cells (Fig. 2F).

PLP-J expression levels during development. Using RT-PCR and western blotting, PLP-J expression levels in the testes of male mice aged 18 days, 4 months, and 16 months were evaluated (Fig. 3). The RT-PCR results indicated that PLP-J was expressed at all the time points that were examined. PLP-J expression levels were significantly higher in testes of 4-month-old mice compared with levels in 18-day-old mice. In 16-month-old mice, PLP-J expression levels were lower than those observed in 4-month-old mice, but still significantly higher than levels observed in 18-day-old mice (Fig. 3A). Western blotting was then employed to further clarify the developmental changes in PLP-J protein expression using the polyclonal anti-PLP-J antibody. Similar to the observed mRNA expression pattern, expression levels of PLP-J protein were age-dependent (Fig. 3B and C).

Lentiviral-mediated PLP-J overexpression efficiently upregulates PLP-J expression in TM3 cells. To investigate the

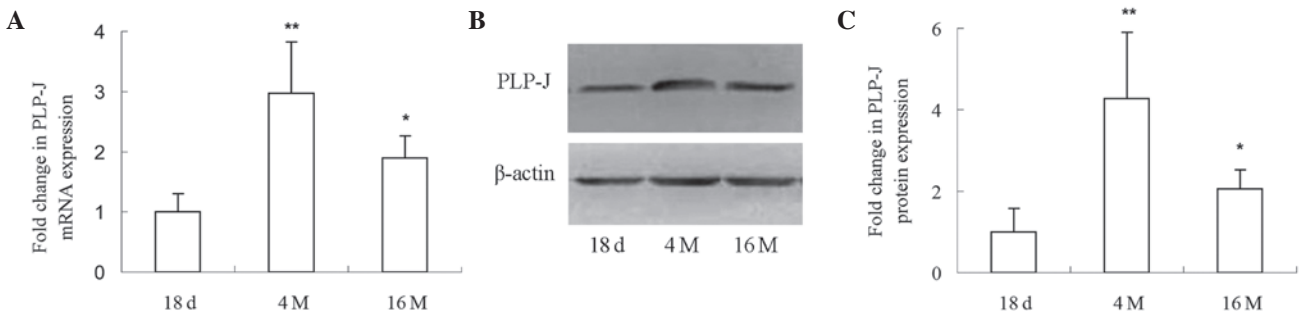

Figure 3. Developmental expression levels of PLP-J mRNA and protein in the postnatal mouse testis. (A) qPCR determination of PLP-J mRNA expression levels at various developmental stages. Levels of mRNA were normalized to β-actin mRNA. The PLP-J mRNA levels in 18-day-old mice were set to 1. (B) Representative image of western blot analysis of PLP-J expression in the mouse testis. (C) Relative expression of PLP-J protein in mice of different ages, following normalization to β-actin with the PLP-J protein level of 18-day-old mice set to 1. Values are expressed as the mean ± standard deviation of the 8 mice in each group. *P<0.05 vs. the 18-day-old group; **P<0.01 vs. the 18-day-old group. PLP-J, prolactin-like protein-J; qPCR, quantitative polymerase chain reaction.

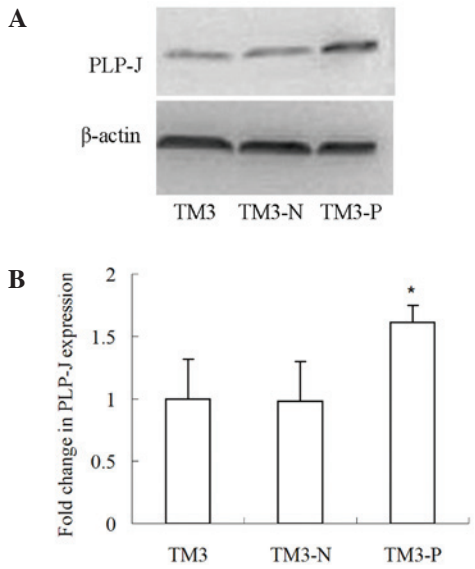

Figure 4. The PLP-J lentivirus increased the PLP-J protein levels in TM3 cells. (A) Representative western blotting for PLP-J in TM3 cells. (B) Relative expression of PLP-J protein in different groups following normalization to β-actin, with the PLP-J protein level in the TM3-N group set to 1. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. the TM3-N group. TM3, parental TM3 cells; TM3-N, TM3 cells transduced with negative control lentivirus; TM3-P, TM3 cells transduced with PLP-J lentivirus; PLP-J, prolactin-like protein-J.

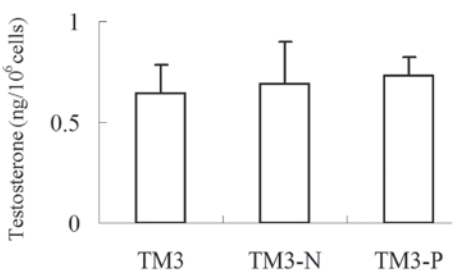

Figure 5. The levels of basal testosterone secretion in cultured TM3, TM3-N, and TM3-P cells. Cells were cultured for 48 h, then the supernatant was collected for testosterone analysis using a testosterone immunoassay kit. The data are expressed as the mean ± standard deviation (n=3). TM3, parental TM3 cells; TM3-N, TM3 cells transduced with negative control lentivirus; TM3-P, TM3 cells transduced with PLP-J lentivirus; PLP-J, prolactin-like protein-J.

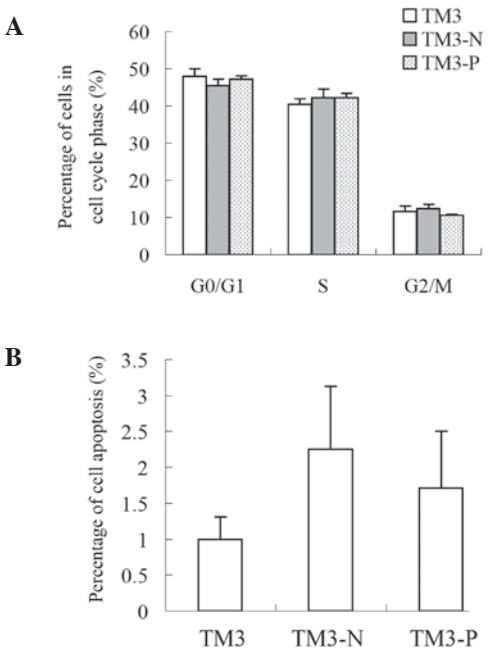

Figure 6. TM3 cells with upregulated expression of PLP-J exhibited no alterations in cell cycle progression or apoptosis. (A) The relative frequency of cells in different cell cycle phases. (B) Apoptosis levels of different cell groups. The data are expressed as the mean ± standard deviation (n=3). TM3, parental TM3 cells; TM3-N, TM3 cells transduced with negative control lentivirus; TM3-P, TM3 cells transduced with PLP-J lentivirus; PLP-J, prolactin-like protein-J.

function of PLP-J in TM3 cells, its expression in these cells was examined and it was discovered that the PLP-J expression levels were relatively low low (data not shown).. Thus, to accommodate the functional study, its expression was upregulated. A PLP-J-expressing lentivirus was concentrated via centrifugation to reach a titer of $1x10^9$ TU/ml, which was able to significantly boost PLP-J expression in TM3 cells (P<0.05; Fig. 4), further corroborating the specificity of the antibody.

PLP-J overexpression does not affect basal testosterone production. The basal testosterone secretion level in cultured TM3 Leydig cells was not significantly different between TM3, TM3-N, and TM3-P cells (P>0.05; Fig. 5). This result

implies that PLP-J is not directly involved in testosterone production *in vitro*.

PLP-J overexpression does not affect cell cycle progression and apoptosis in TM3 cells in vitro. The flow cytometry results demonstrated that PLP-J overexpression did not significantly affect cell cycle progression (Fig. 6A) or apoptosis (Fig. 6B).

Discussion

In female rats, the gestation process involves multiple physiological events that require numerous growth hormones and cytokines (15,16). These studies initially reported that PLP-J was a hormone of pregnancy, and its expression was only detected in the decidua of pregnant mice and not in other tissues or organs. Therefore, previous studies on this gene have focused solely on regulatory roles for PLP-J in female gestation.

To the best of our knowledge, the data presented in the current study are the first to demonstrate that PLP-J is also expressed in the postnatal mouse testis. This conclusion is based on the results of molecular and immunological experiments. Primers specific for PLP-J successfully amplified a gene fragment from the mouse testis, and its sequence matched perfectly with the PLP-J gene. *In situ* hybridization further revealed that PLP-J was expressed in the Leydig cells but not in Sertoli or germ cells. Expression levels of the PLP-J protein were examined by immunofluorescence and western blotting, which was facilitated by our production of a polyclonal anti-PLP-J antibody. The immunofluorescence results indicated that PLP-J and 3β-HSD1 were colocalized in Leydig cells of the testis, indicating that PLP-J is expressed in Leydig cells. Using TM3 Leydig cells, it was observed that PLP-J was localized in the cytoplasm.

To adapt to the physiological functions of different developmental stages, the testis exhibits a stage-specific gene expression profile (17-19). Therefore, the temporal expression pattern of PLP-J in the mouse testis during development was further explored in the current study. The results revealed that expression of the PLP-J protein and mRNA displayed similar patterns at different developmental stages: They were low in the 18-day-old mice and highest in the 4-month-old animals; and the levels in the 16-month-old mice were lower than those in the 4-month-old mice, but remained significantly higher than those in the 18-day-old mice.

Together, the characterization of PLP-J expression in the male mouse testis and the evaluation of its developmental expression pattern in the present study suggest that this protein has specific roles in male reproductive function.

In males, Leydig cells are the main producers of testosterone and also synthesize cytokines to support the development of Sertoli and germ cells. Therefore, this cell type is indispensable for maintaining normal reproductive activity (20).

A decrease in serum testosterone production is often associated with infertility, osteoporosis, diminished energy and muscle strength, poor cognition, reduced sexual behavior and occurrence of depression (21-25). Testosterone production by Leydig cells is regulated by complex interplay between multiple molecules including those involved in endocrine and paracrine signaling, and proper regulation is critical for optimum reproductive capacity. As a result, a number of Leydig cell-specific genes have been identified as important regulators of reproductive processes (26-30). As PLP-J is specifically expressed in Leydig cells of the testis and its expression peaks in the adult, similar to the expression pattern of testosterone, we hypothesized that PLP-J is also involved in testosterone biogenesis.

Since PLP-J exhibited low expression levels in TM3 cells, a strategy of PLP-J overexpression was adopted in the present study to analyze its function. Overexpression of PLP-J in TM3 Leydig cells by lentiviral transfection significantly increased the PLP-J protein levels. However, ELISA results revealed that overexpression of PLP-J did not influence basic testosterone production in TM3 cells, suggesting that this protein is not directly involved in testosterone biogenesis *in vitro*. Nevertheless, whether PLP-J participates in testosterone synthesis through other mechanisms remains to be examined.

In addition, experiments investigating the cell cycle and apoptosis in the present study revealed that PLP-J overexpression did not affect these two processes. Thus, the precise biological roles of this protein in the testis remain to be elucidated.

In summary, the current study demonstrated that PLP-J is expressed in the testis and that its mRNA and protein are expressed in an age-dependent manner. The present investigation of PLP-J function further revealed that PLP-J is not directly involved in testosterone production *in vitro*. To the best of our knowledge, the current study is the first to display that PLP-J is expressed in the male reproductive system. Expression of PLP-J in Leydig cells of the testis indicates a role for it in the male reproductive system, in addition to previously reported functions within the female reproductive system. Thus, the results may aid further elucidation of the mechanisms of the male reproductive system.

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