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Phospholipase D Family Member 6 Is a Surface Marker for Enrichment of Undifferentiated Spermatogonia in Prepubertal Boars

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Spermatogonial stem cells (SSCs) have a functional ability to maintain self-renewal and sustain production of spermatozoa throughout the reproductive lifespan of a male. Studies on SSCs can thus better the understandings of spermatogenesis and unravel the mechanisms for self-renewal and differentiation of male germline stem cells. However, the rarity of SSCs in the testis and the lack of reliable surface markers obstruct the related study and further application of SSCs. This is especially the case in livestock animals. In this study, we identified that phospholipase D family member 6 (PLD6) is a surface marker for undifferentiated spermatogonia in boar testes. By magnetic-activated cell sorting, PLD6$^+$ cell fraction comprises 84.45% ± 0.35% of undifferentiated spermatogonia (marked by PLZF). Xenotransplantation of PLD6$^+$ cells into the recipient mouse testis revealed a ninefold increase of donor cell-derived colony formation compared with that in the unselected cell group, indicating the significant enrichment of SSCs. Furthermore, based on the sorted PLD6$^+$ cells with a high SSC content, we established a feeder-free culture system that could maintain porcine undifferentiated spermatogonia for 4 weeks in vitro with the expression of typical markers throughout the culture period. In conclusion, this study demonstrates that PLD6 is a surface marker of undifferentiated spermatogonia in testes of prepubertal boars and could be utilized to unprecedentedly enrich porcine undifferentiated spermatogonia. These data provide the basis for future studies on the refinement of germ cell culture and manipulation of porcine undifferentiated spermatogonia.

Keywords: PLD6, boar testis, undifferentiated spermatogonia, MACS, feeder-free culture

Introduction

Spermatogonial stem cells (SSCs), a subset of undifferentiated spermatogonia, are the foundation for spermatogenesis and essential to maintain the daily production of a vast number of spermatozoa throughout the reproductive lifespan of a male [1,2]. Manipulations of the male germline at the SSC stage will be maintained and transmitted to the offspring [3,4]. Thus, genetic manipulations of SSCs combined with germ cell transplantation present a novel approach for gene therapy and production of genetically modified animals [5,6]. In addition, numerous studies have demonstrated that SSCs can be transformed to pluripotent stem cells in vitro [7–10], providing a new and promising therapeutic prospect for regenerative medicine and treatment of human diseases without ethical issues [11–13]. Remarkable advances in rodents have laid the groundwork for advancements in the field of male reproductive biology, but the need for breakthrough remains in large animals to translate the technology to the agricultural and biomedical fields [14].

Unfortunately, the rarity of SSCs within the testis and the lack of reliable surface markers to enrich them are protruding limitation factors for the subsequent SSC culture and the other studies [15]. When initiating testicular culture, the spermatogonia can readily be overwhelmed by the contaminated somatic cells [2]. Kubota found that THY1$^+$ cells were highly enriched with undifferentiated spermatogonia, thereby greatly facilitating subsequent spermatogonial culture [16]. Moreover, given that somatic cells are easily transfected while SSCs are refractory to transfection/viral transduction [17,18], enrichment of undifferentiated spermatogonia can enhance genetic modification efficiency for SSCs. As a matter of fact, these are the two critical factors for generation of genetically modified animal models through SSCs.

To enrich spermatogonia, fluorescence-activated cells sorting (FACS) or magnetic-activated cells sorting (MACS) using spermatogonia-specific surface markers is the most efficient and precise way. Several surface markers, such as α 6/β 1-integrin [19], THY1 [5], GFRA1 [20], CD9 [21], and E-cadherin (CDH1) [22], have been adopted to identify and

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enrich undifferentiated spermatogonia in mice and rats. Among them, THY1 has been used to select undifferentiated spermatogonia in bulls [23], goats [24], and boars [25]. However, the outcomes are not comparable with those obtained in mice.

Phospholipase D6 (PLD6) is a member of phospholipase superfamily, primarily located on cell surface and the mitochondrial surface [26]. In mice, PLD6 is identified to be mainly expressed in testes and verified to show strong membrane expression pattern in undifferentiated murine SSCs both in vivo and in vitro. Therefore, PLD6 is considered a potential novel marker of mouse SSCs [27]. However, the cognition of PLD6 in any livestock species has not been reported.

In this study, we showed that PLD6 was expressed specifically in undifferentiated spermatogonia in boar testis tissue. This new cell surface marker enabled us to define and enrich porcine undifferentiated spermatogonia by MACS. Moreover, germ cell transplantation assay demonstrated that $PLD6^{+}$ cells were enriched for SSCs. Later, we developed a feeder-free culture condition that could maintain the sorted porcine undifferentiated spermatogonia for 4 weeks with the expression of undifferentiated spermatogonial markers.

Materials and Methods

Animals

Testis samples were obtained from 4- to 7-day-old Pig Improvement Company piglets when they were castrated in Besun farm, and transported in Dulbecco's phosphate-buffered saline (DPBS) to the laboratory within 2 h. Some aliquot tissues were fixed in Bouin's buffer for section. The experimental animals and procedures used in this study were approved by the Northwest A&F University's Institutional Animal Care and Use Committee.

Immunohistochemical analysis

Testis samples collected from 7-day-old and 2-month-old pigs were fixed in Bouin's solution overnight, and dehydrated and embedded in paraffin. Then 7-μm cross sections were adhered to glass slides.

For immunohistochemistry, sections were dewaxed, rehydrated, and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 15 min. Sections were retrieved by boiling in 0.01 M Tris-EDTA (pH = 9.0) for 20 min and treated with 3% H_2O_2 for 10 min. Subsequently, sections were incubated with 3% bovine serum albumin (BSA; XINTAI, Xian, China) for 2 h at 37°C. Tissues were then incubated with primary antibodies (shown in Table 1) at 4°C overnight. Next day, sections were washed in phosphate-buffered saline (PBS) and incubated with polymer auxiliary agent and horse radish peroxidase (HRP)-conjugated immunoglobulin G (ZSGB-BIO, Beijing, China) for 1 h at 37°C, respectively. Samples were stained with diaminobenzidine (CWBIO, Beijing, China). Finally, slides were sealed with neutral gum after hematoxylin (Sigma-Aldrich) counterstaining and dehydration.

For immunofluorescence, the sections were incubated with 3% BSA for 2 h at room temperature and incubated with primary antibodies (shown in Table 1) at 4°C overnight. The sections were washed with PBS and incubated with donkey antigoat, goat antirabbit, or goat antimouse

Table 1. Primary Antibodies Used in This Study

Antibody	*Species source*	*Suppliers*	*Dilution ratio*	
			IHC/ICC	*WB*
PLD6	Rabbit	Abcam	1:100	1:500
UCHL1	Mouse	Abcam	1:200	1:500
VASA	Mouse	Abcam	1:200	1:500
PLZF	Goat	Santa	1:200	1:400
SOX9	Goat	Santa	1:100	1:400
α-SMA	Mouse	Abcam	1:200	
GATA4	Goat	Santa		1:400
GAPDH	Mouse	Santa		1:1000

PLD6, phospholipase D family member 6.

(fluorescein isothiocyanate isomer I/Texas Red-conjugated) secondary antibody (1:400; Santa Cruz Biotechnology) at 37°C for 1 h, followed by nuclear staining with 4, 6-diamidino-2-phenylindole (DAPI, 1:1000; Bioworld Technology, Inc.). Digital images were captured with Nikon Eclipse 80i fluorescence microscope camera (Tokyo, Japan).

Isolation of porcine testis cells

The derivation of porcine germ cells was described in previous studies [6,25,28]. In brief, the testis tissue was minced into small pieces, incubated with collagenase Type IV (2 mg/mL; Invitrogen, Carlsbad, CA), and DNase I (0.1 mg/mL; Sigma) at 37°C for 15 min to obtain seminiferous tubules. The fragments of seminiferous tubules were treated with hemolytic lysate to remove red blood cells. Then the suspension of seminiferous tubules was obtained through incubation with 0.25% trypsin-EDTA (Invitrogen) at 37°C for 8 min. After filtration, the single-cell suspension was centrifuged and subjected to differential plating to remove the somatic cells. The number of cells was determined by hemocytometer with trypan blue.

Magnetic-activated cell sorting of $PLD6^{+}$ testis cells

The single cell suspensions were magnetically labeled according to the protocol of the supplier of the MACS cell separation system (Miltenyi Biotech, Germany) for the positive selection of intact cells containing PLD6 in the external membrane. In brief, aliquots of 2×10^6 cells were washed with DPBS and resolved in MACS buffer (DPBS with 2 mM EDTA and 0.5% BSA). The cells were incubated with rabbit anti-PLD6 antibody (1:20; Abcam, Cambridge, United Kingdom) at 4°C for 30 min and washed 2× with MACS buffer by centrifugation at 300*g* for 8 min followed by incubation with goat antirabbit IgG MicroBeads (1:5; Miltenyi Biotech) on ice for 20 min. Cells were subjected to another two washes in MACS buffer, $PLD6^{+}$ cells were collected by MACS (Miltenyi Biotech). Typical yields of $3.24 \pm 0.76 \times 10^5$ $PLD6^{+}$ cells were isolated from 2×10^6 cells.

Feeder-free germ cell culture

The sorted $PLD6^{+}$ cells were seeded with 10^5 cell/well at 12-well dishes (Falcon) coated with 10 μg/mL laminin in DMEM/F12 medium with 100 IU/mL penicillin, 100 mg/mL streptomycin, 1× L-Glutamax, 1× NEAA, 1× MEM vitamin,

2% B27 supplement, 100 μM β-mercaptoethanol, 1% fetal bovine serum (FBS), 40 ng/mL glial cell-derived neurotrophic factor (GDNF), 10 ng/mL basic fibroblast growth factor (bFGF), 10 ng/mL epidermal growth factor (EGF) and 20 ng/mL insulin-like growth factor 1 (IGF1). All cultures were maintained at 35°C in an atmosphere of 5% CO_2. The medium was refreshed every other day. Immunocytochemical staining for evaluation of UCHL1 and VASA markers was carried out from colonies collected at day 30.

Immunocytochemical analyses

Cells used for immunocytochemical staining were fixed with 4% paraformaldehyde (PFA) for 20 min and treated with 0.1% Triton X-100 for 10 min. Nonspecific antibody binding was blocked by incubation with 10% donkey serum for 2 h at room temperature. Then, cells were incubated with primary antibodies (shown in Table 1) at 4°C overnight, washed in PBS, and incubated with goat antimouse IgG, goat antirabbit IgGor, and donkey antigoat IgG (FITC/TR-conjugated; 1:200, Santa Cruz) at 37°C for 1 h. For negative controls, primary antibodies were omitted and the same staining procedure was followed. DAPI was added for nuclear counterstaining. Cells were observed under fluorescence microscope (BX51; Olympus, Japan) or carried with flow cytometric analysis by Flow Cytometer (CyFlow Cube; PARTEC, Germany).

Quantitative reverse transcription polymerase chain reaction analysis

Expression of specific genes of interest by MACS-isolated PLD6$^+$ cells, PLD6$^-$ cells, and unselected testis cells was examined using quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. Total RNA was isolated by Trizol reagent (Invitrogen) followed by treatment with DNase I (Sigma). For each sample, 1 μg of RNA was reverse transcribed with Revert Aid™ First Strand cDNA Synthesis Kit (Roche). FastStart Universal SYBR Green Master (Roche) was used for real-time quantitation of mRNA levels using an iQ5 detection system (Bio-Rad, Hercules, CA, USA). Specific primers for PCR amplification of the genes mentioned in this study are shown in Table 2. Data were analyzed using the comparative Ct-method with *Gapdh* serving as reference gene.

Western blot analysis

To determine SSC markers in MACS-isolated PLD6$^+$ testis cells and unselected total testis cell populations, total protein was extracted from the cells using RIPA Kit (HEART, Xian, China). Proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (Millipore) membranes followed by blocking with 5% nonfat milk powder dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature. Blots were then incubated with primary antibodies (shown in Table 1) for overnight at 4°C followed by washing in TBS-T. Then, membranes were incubated with rabbit antigoat IgG, goat antirabbit IgG, or goat antimouse IgG (HRP conjugated; 1:2000, CWBIO) for 2 h at room temperature followed by washing in TBS-T. Detections of protein were developed with Pierce ECL Western Blotting Substrate (Thermo) and viewed using a ChemiDox XRS (Bio-Rad). Digital images were captured. Expression of target protein was compared between different cell fractions by normalization of the target band density with that of GAPDH.

Table 2. Gene-Specific Primers Used for Polymerase Chain Reaction Amplification

Gene	*Primer sequences (from 5′ to 3′)*
Gapdh	F: AGGGCTGCTTTTAACTCTGGCAA R: GATGGTGATGGCCTTTCCATTG
Nanog	F: AACCAAACCTGGAACAGCCAGAC R: GTTTCCAAGACGGCCTCCAAAT
Plzf	F: GCGGAAGACCTGGATGACCT R: GTCGTCTGAGGCTTGGATGGT
Uchl1	F: TCCGGAAGACAGAGCAAAATGC R: CATGGTTCACCGGAAAAGG
Gfra1	F: TCGCCTACTCTGGGCTTATTG R: CAGGTCGTTTCCACTGTTGCT
Oct4	F: CGCGAAGCTGGACAAGGAGA R: CAAAGTGAGCCCCACATCGG
Sox9	F: AGGAAGTCGGTGAAGAACGG R: GAGAGTGCACCTCGCTCAT
Vimentin	F: GGATGTTTCCAAGCCTGACC R: GGCATCGTTGTTGCGGTTA
AMH	F: TCTGGGCTTGCTCTGACC R: TCCGTGTGAAGCAGCGAGAGT

Xenogeneic transplantation of porcine testis cells

Xenogeneic transplantation assay was conducted to compare SSC content of MACS-isolated PLD6$^+$ cells with the unselected total testis cell populations from prepubertal pig testis tissue as described previously [6,29]. In brief, recipient C57 mice were treated with a single intraperitoneal injection of busulfan (40 mg/kg; Sigma) to deplete endogenous spermatogenesis. Testes were removed after 1 month of busulfan treatment and processed for histology analysis to ascertain that treated mice are germ cell depleted in comparison with age-matched untreated control males.

After enzymatic digestion and MACS isolation, porcine PLD6$^+$ cells and unselected total testis cell populations were resuspended in DPBS and labeled with PKH26 red fluorescent cell linker dye (Sigma) following the manufacturer's instructions. Cells were then washed in DMEM/F12 with 10% FBS by centrifugation at 300*g* for 10 min to remove residual dye. For each sample, cells were resuspended in complete medium at a concentration of 1×10^7 cells/mL. Ten microliters of cell suspension was microinjected into the seminiferous tubules of each recipient testis ($n \geq 6$). As the control, the recipients were transplanted with the same concentration of freshly isolated unsorted testicular cells.

Approximately 8 weeks after transplantation, recipient mice testes were collected. The tunica albuginea was removed and the seminiferous tubules were gently dissected with collagenase Type IV (1 mg/mL; Invitrogen) to remove interstitial cells. Then the dispersed tubules were fixed in 4% PFA and analyzed for colonization of porcine germ cells using a fluorescent microscopy (Leica, Blenheim, Germany). Donor germ cells were identified by the specific PKH26 red fluorescent dye. Also, testes from mice that had

received donor cells were fixed in 4% PFA. And 5-μm histological sections were cut and stained with DAPI. Two to four cross sections of each testis were examined for the homing of donor cell colonies in the seminiferous tubules.

Statistical analyses

All experiments were repeated at least thrice. The statistical analysis was performed by Student's *t*-test and one-way analysis of variance using SPSS V22.0 statistical software (IBM). Data are presented as mean ± standard error of mean. Difference was considered to be significant at $P \leq 0.05$.

Results

PLD6 is expressed in a rare subpopulation of spermatogonia in porcine testes

In mice testis, immunohistological analysis showed that PLD6 was uniquely and highly expressed on the plasma membrane of SSCs [27]. Here, we examined the expression of PLD6 in the porcine testis. Immunohistochemical analysis of cross sections from porcine testis tissue revealed that PLD6 expression was primarily localized to spermatogonia along the basement membrane of seminiferous tubules in 7-day-, 2-month-, and 5/6-month-old testes ($n \geq 6$ isolations from different pigs, respectively, Fig. 1A). In adult testes (5/6-month-old), PLD6 was also expressed in the acrosome of spermatids (Fig. 1A). To examine the conservation of PLD6 expression, we also performed PLD6 staining on testis sections from other livestock species. Consistently, we found that PLD6 was expressed in a rare subpopulation of spermatogonia and spermatids in adult bulls and goats (Fig. 1B).

To further investigate the expression pattern of PLD6 in porcine testis tissue, the relative gene expression of *Pld6* in three types of testicular cells (germ cells, Sertoli cells, and Leydig cells) from 7-day-old piglets ($n = 3$ isolations from different piglets) was examined by quantitative real-time PCR (qPCR). qPCR analysis revealed that the expression of *Pld6* in the germ cells was 11-fold and 5-fold higher than that in Leydig cells and Sertoli cells, respectively (Fig. 1C). These results indicate that PLD6 is highly expressed in germ cells.

The PLD6$^+$ cell fraction of prepubertal boar testes is enriched for undifferentiated spermatogonia

In prepubertal porcine testes, the seminiferous tubules consist of spermatogonia, Sertoli cells, and spindle-shaped myoid cells that surround the seminiferous tubules. To determine the constitution of the PLD6$^+$ cell fraction, double immunofluorescent analysis was conducted to check the costaining of PLD6 with markers for testicular cells. Previous studies have revealed that VASA is a general marker of germ cells, and PLZF and UCHL1 are conserved markers for undifferentiated spermatogonia in most mammals including pigs. As expected, colocalization staining in 7-day-old and 2-month-old porcine testis tissue ($n \geq 6$ isolations from different pigs, respectively) showed that all of the PLD6-positve cells were VASA$^+$, SOX9 (a Sertoli cell marker)-negative and SMA (a myoid cell marker)-negative. Some VASA$^+$ cells were PLD6$^-$ (Fig. 2).

These results suggest that PLD6 expression is restricted to a subpopulation of germ cells in the prepubertal porcine testis. The coimmunofluorescence of PLD6 and UCHL1 showed three types of germ cells in the basement membrane: cells that are stained for both UCHL1 and PLD6, cells that are stained for only UCHL1, and cells that only showed PLD6 staining (Fig. 2). The results suggest that the expression of PLD6 partly overlaps that of UCHL1, indicating that PLD6-positive cells represent a subset of undifferentiated spermatogonia. Coimmunofluorescence of PLD6 and PLZF showed that the majority of PLD6$^+$ cells were PLZF$^+$, and only a few PLD6$^+$ cells were PLZF$^-$ (Fig. 2).

We next attempted to isolate a pure population of spermatogonia from the 2-month-old porcine testis tissue using MACS with an antibody directed against PLD6. After two-step enzymatic digestion and differential plating, the testicular cells consisted of 90% viable cells as determined by trypan blue dye exclusion. To further improve the purity of germ cells, MACS was conducted using PLD6 as a surface

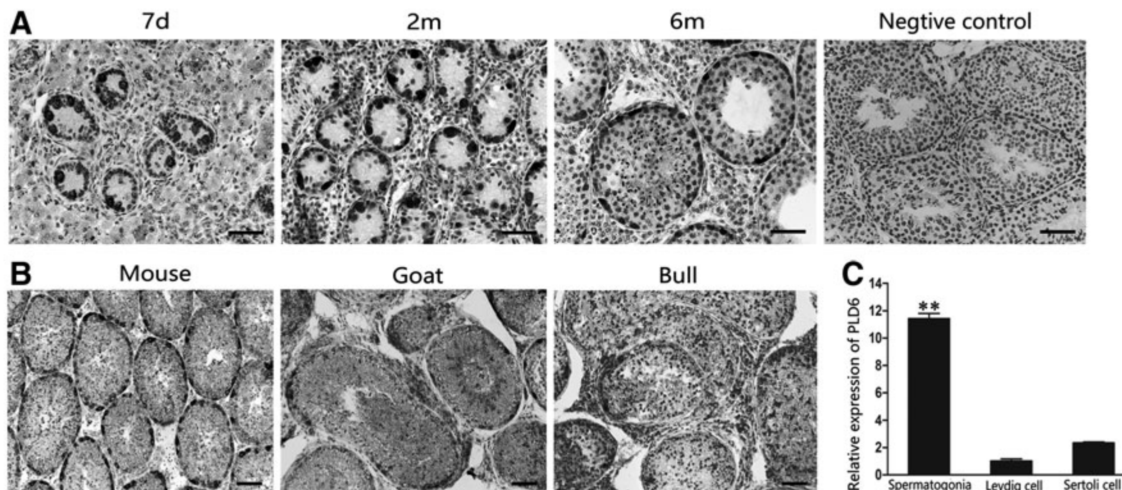

FIG. 1. Expression of PLD6 in testes of various species. **(A)** Immunohistochemical staining of PLD6 in neonatal, prepubertal, and mature porcine testes. **(B)** Immunohistochemical staining of PLD6 in testes of mouse, bull, and goat, respectively. **(C)** Differential expression of PLD6 gene among spermatogonia, Leydig cells, and Sertoli cells of porcine testis. Scale bars = 50 μm. $^{**}P < 0.01$. PLD6, phospholipase D family member 6.

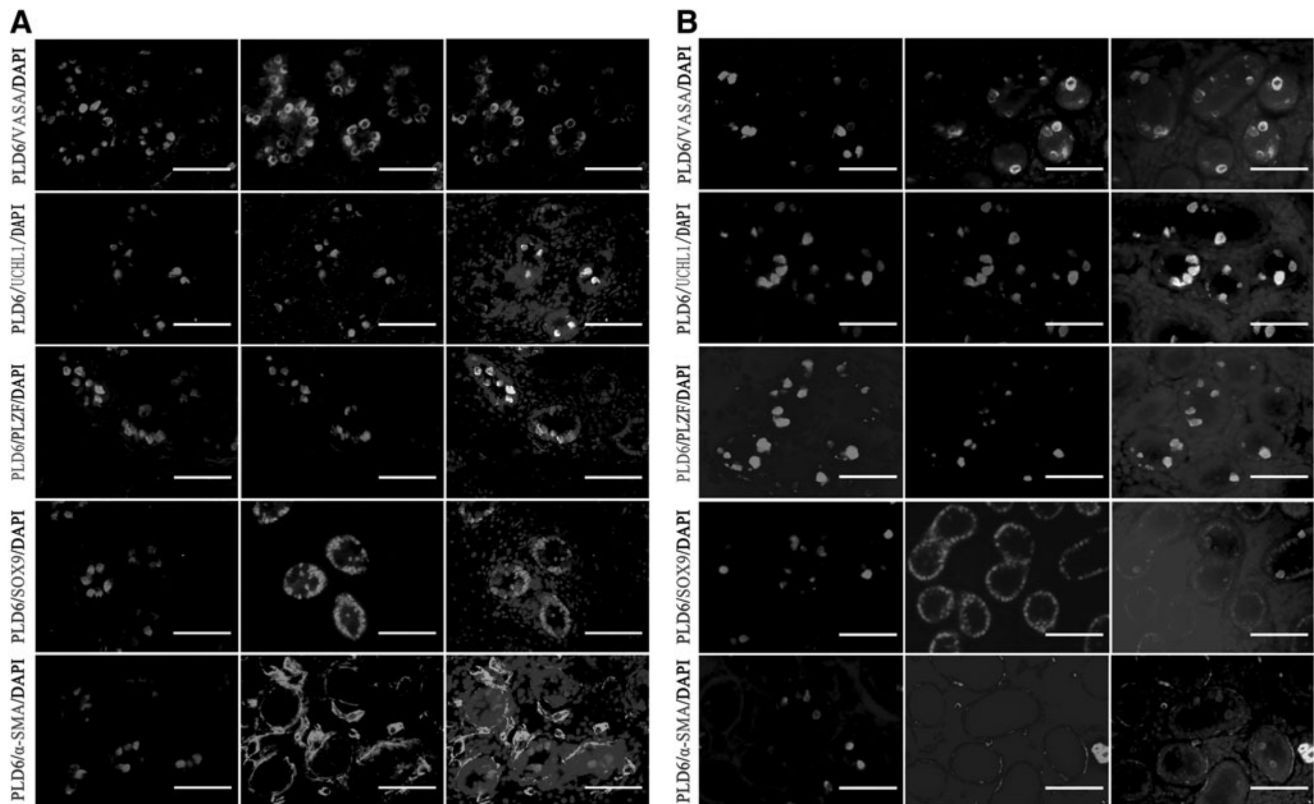

FIG. 2. Coimmunofluorescence examination of the expression of PLD6 with VASA, UCHL1, PLZF, SOX9, and α-SMA in neonatal **(A)** and prepubertal **(B)** porcine testes. Scale bars = 50 μm.

marker. The 2×10^6 isolated cells yielded $3.24 \pm 0.48 \times 10^5$ PLD6$^+$ cells. The sorted PLD6$^+$ cells were examined for expression of VASA, UCHL1, and PLZF by FACS. The FACS results revealed that the MACS-isolated PLD6$^+$ cell population consisted of 91.31% ± 0.56% of VASA$^+$, 86.04% ± 0.63% of UCHL1$^+$, and 84.45% ± 0.35% of PLZF$^+$ cells ($n = 3$ different cell preparations, Fig. 3A). This represented about 8.4-fold (84.45%/10.50%) enrichment for undifferentiated spermatogonia (PLZF$^+$) in the sorted fractions.

Next, we examined the protein and mRNA expression in the PLD6$^+$ cell fraction isolated from prepubertal porcine testis tissue. Western blot analysis revealed that the expression of germ cell/undifferentiated spermatogonial markers (VASA, PLD6, UCHL1, and PLZF) in the PLD6$^+$ cell fraction was significantly higher than that in the unselected total testicular cells and PLD6$^-$ cells, whereas the expression of testis somatic cell markers (GATA4 and SOX9) was substantially lower than that in the unselected total testicular cells and PLD6$^-$ cells (Fig. 4A). Similarly, examination of SSC-related gene expression by qRT-PCR analysis revealed a significant increase ($n = 3$ different cell preparations, at least 3.9-fold and up to 8.1-fold) in the PLD6$^+$ cell fraction compared with the unselected testicular cell population. Consistently, the expression of somatic cell markers *Vim*, *Amh*, and *Sox9* was significantly lower than that in the unsorted cell population (Fig. 4B).

Collectively, PLD6 was expressed in a rare subpopulation of undifferentiated spermatogonia, and PLD6 can be used as a surface marker to enrich undifferentiated spermatogonia from the prepubertal porcine testis.

The PLD6$^+$ cell fraction in prepubertal porcine testis tissue is enriched for SSCs

To determine whether SSCs are enriched in the sorted fraction, we carried out spermatogonial transplantation, the golden functional assay for SSCs. The sorted PLD6$^+$ cell fraction and unsorted total testicular cell population were transplanted into the seminiferous tubules of the busulfan-treated recipient mouse testis tissue, respectively. To make the donor cells discernible, the cells were prelabeled with a red fluorescent dye PKH26, as previously described [6]. After 8 weeks, the number of fluorescently labeled porcine germ cell colonies was evaluated in dispersed seminiferous tubules of recipient testis tissue (Fig. 5).

Colonies along the periphery of tubules were generally smaller in the group of unselected cells (Fig. 5A). Quantification of germ cell colonies showed that SSCs were significantly enriched in PLD6$^+$ cells ($n \geq 6$, 210.88 ± 10.45 colonies per testis). Overall, the PLD6$^+$ cells generated over ninefold more colonies than the unselected total testicular cell population (Fig. 5B). As expected, PLD6$^+$ cells were significantly enriched for cells capable of colonization, suggesting that PLD6$^+$ population is enriched foe SSCs. These results indicate that the prepubertal porcine undifferentiated spermatogonia were enriched efficiently by MACS using PLD6 as a surface marker.

Feeder-free culture of porcine undifferentiated spermatogonia

Although we have reported a porcine spermatogonial culture system recently, the system included autologous Sertoli cell

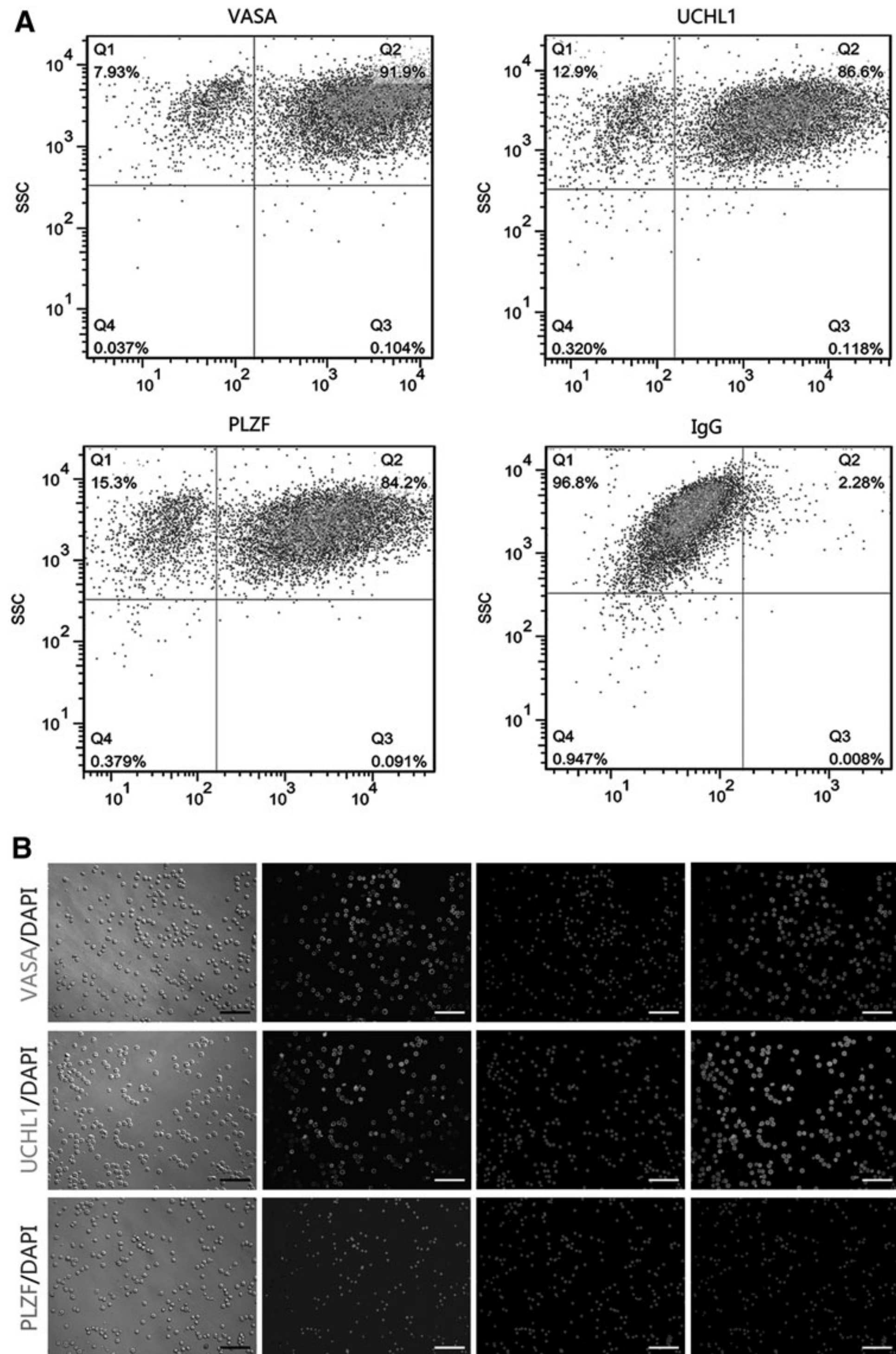

FIG. 3. Analysis of MACS-isolated PLD6$^+$ cell fraction from prepubertal pigs. **(A)** Flow cytometric analysis for the percentage of VASA$^+$, UCHL1$^+$, and PLZF$^+$ cells in PLD6-positive cell fraction. **(B)** Representative images of immunofluorescence of cells expressing VASA, UCHL1, and PLZF in PLD6-positive cell fractions from MACS, respectively. Scale bars = 100 μm. MACS, magnetic-activated cell sorting.

feeders that confound studies on SSC self-renewal [6]. Thus, maintenance of the undifferentiated spermatogonia in a feeder-free culture environment would benefit research on SSC self-renew and differentiation. Here, based on the sorted cells with a relatively high SSC content, we attempted to establish a feeder-free culture system for porcine undifferentiated spermatogonia.

The MACS-sorted PLD6$^+$ cell fraction from prepubertal porcine testis tissue was added to plastic culture wells coated with laminin and cultured under the similar condition as reported [6]. Colonies appeared at day 5 of culture and further expanded with the extension of culture (Fig. 6A). However, the total number of cells dropped, probably as cell differentiation/apoptosis prevails over proliferation. The clumps gradually diminished within 3–4 weeks. Nevertheless, immunostaining in the colonies revealed that the expression of VASA, UCHL1, and GFRA1 was maintained in feeder-free culture (Fig. 6B), indicating the maintenance of the undifferentiated spermatogonial phenotype.

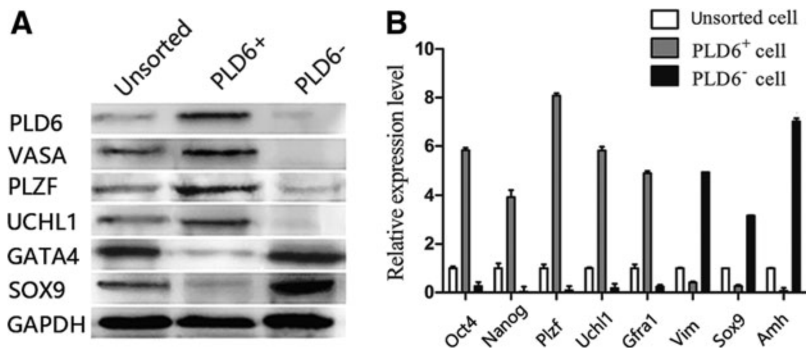

FIG. 4. Examination of the MACS-isolated cell fractions from prepubertal porcine testes for expression of genes restricted to the undifferentiated spermatogonia and testicular somatic cell. Western blot analysis **(A)** and real-time quantification **(B)** of undifferentiated spermatogonial markers and somatic cell markers in unsorted total testes cells, PLD6$^+$ and PLD6$^-$ cells. Data are presented as mean ± SEM ($n \geq 3$). SEM, standard error of mean.

Discussion

The pig is an economically important livestock for production of meat. It is also being considered as a nonrodent animal model with increasing importance for biomedical and pharmacological research [14,30]. However, the existing methods to generate transgenic pig models are costly and inefficient [31]. Genetic manipulation of SSCs combined with germ cell transplantation represents a promising alternative for the current means. Identification and isolation of SSCs are prerequisites for this respect as well as for basic research on male germ cell biology. Although a great deal of progress has been made in isolation and characterization of undifferentiated spermatogonia/SSCs in rodents, little is achieved in pigs due to the lack of reliable markers to distinguish the porcine undifferentiated spermatogonia from the testicular cells.

A previous study in mice showed that PLD6 could be a surface molecular marker of the undifferentiated spermatogonial population [27]. Here, to probe whether PLD6 could be a marker of porcine undifferentiated spermatogonia, we investigated PLD6 expression in porcine testis tissue. Immunohistochemical staining showed that PLD6 protein expression was present in a rare subpopulation of spermatogonia along the basement membrane seminiferous tubules in the neonatal, prepubertal, and mature porcine testis tissue, similar to what was reported previously in mice [27]. Moreover, qRT-PCR analysis validated that *Pld6* was highly expressed in spermatogonia among prepubertal porcine testicular cells. These results suggest that PLD6 could be a marker for porcine spermatogonia. Furthermore, we found that PLD6 is also located in goats and bulls. The consistent expression of PLD6 implies that PLD6 may be a conserved marker for undifferentiated spermatogonia in mammal species, especially in livestock.

Remarkable progress has been made in the identification of SSCs in rodents and nonhuman primates. Alpha 6-integrin, β 1-integrin, THY1, GFRA1, CD9, UCHL1, PLZF, CDH1, and Lin28 have been proven to be reliable markers for mouse and rat SSCs/progenitors [14,32]. Nevertheless, in livestock, information regarding the characterization of undifferentiated spermatogonia remains limited. In this study, coimmunofluorescence analysis revealed that PLD6 colocalized with germ cell marker VASA but not Sertoli cell marker SOX9 or myoid cell marker α-SMA. Moreover, the PLD6$^+$ cells were a subpopulation of UCHL1$^+$ spermatogonia and highly overlapped PLZF$^+$ spermatogonia. These results suggest that PLD6$^+$ cells could be a subset of spermatogonia, and PLD6 expression could be more restricted to undifferentiated spermatogonia. Therefore, PLD6 could serve as a reliable marker for undifferentiated spermatogonial population in boars.

Identification of surface markers to enrich undifferentiated spermatogonia would provide highly enriched and pure

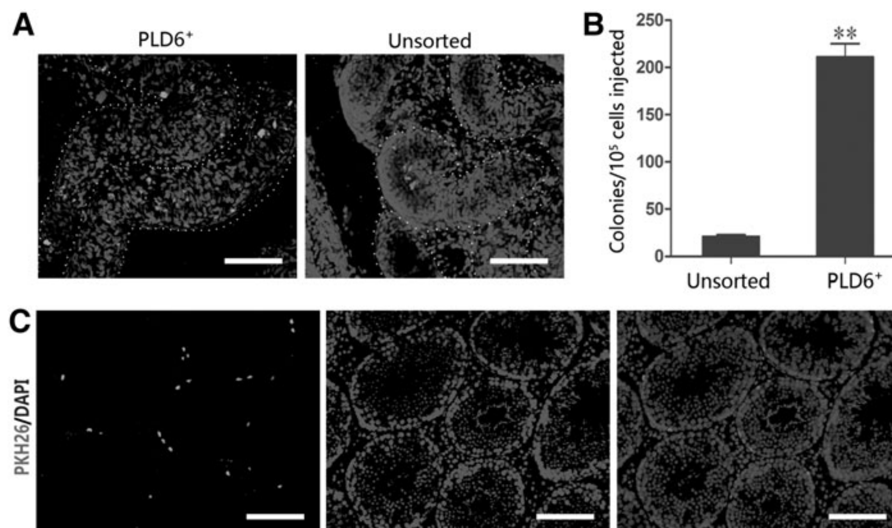

FIG. 5. Functional analysis of colonizing ability in PLD6-selected testis cells and unselected total testis cells after transplantation. **(A)** Cell colonies of MACS-isolated PLD6 cell and unsorted total testicular cell in recipient mouse. **(B)** Quantitative comparison of cell colonies with PKH26 in the seminiferous tubules per testis. Data are mean ± SEM for three independent experiments. $^{**}P < 0.01$. **(C)** PKH26-positive cells located on the basement membrane of seminiferous tubules of MACS-isolated PLD6$^+$ cell in the recipient mouse. Scale bars = 100 μm.

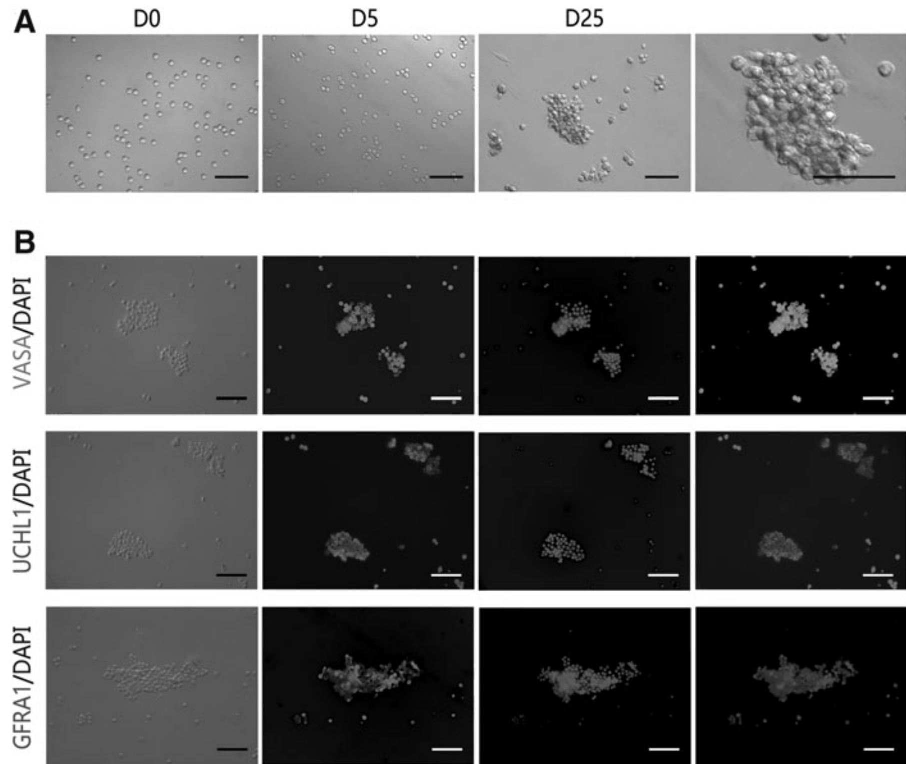

FIG. 6. Feeder-free culture of PLD6^{+} spermatogonia. **(A)** The germ cell clumps at different stages of culture. **(B)** Representative images of immunocytochemical staining for expression of the undifferentiated spermatogonial marker (VASA, UCHL1, and GFRA1) in the cultured germ cell clumps after 4 weeks of culture. Scale bars = 100 μm.

populations of spermatogonia for subsequent transplantation or cell culture. Several groups have attempted to enrich undifferentiated spermatogonia from prepubertal rather than postpubertal testis tissue of large animals [14], in that in large animals, postpubertal testicular tissue is refractory to enzymatic dissociation, and typically the collected cells are greatly damaged during this process. Also, there are much more cell types in postpubertal seminiferous tubules than in prepubertal seminiferous tubules, which makes it difficult for sorting of undifferentiated spermatogonia. Moreover, the rare population of the undifferentiated spermatogonia in postpubertal testis tissue makes the output inefficient. For these reasons, it is standard practice to use prepubertal testis tissue for spermatogonial isolation [14].

Of all surface markers, THY1 is first used to enrich spermatogonia from livestock [23]. Using MACS, the THY1^{+} cell fraction contained 64.4% PLZF^{+} cells in bulls [23] and 57.33% PLZF^{+} cells in goats [24]. Our previous study for the first time reported the enrichment of porcine spermatogonia by MACS and THY1. Nevertheless, the THY1^{+} cell fraction yielded only 55.0% UCHL1^{+} spermatogonia [25], even incomparable to the outcome of optimized differential plating and density gradient centrifugation [6,33]. A possible reason could be that THY1 was also expressed in Leydig cells in rats and pigs, in addition to germ cells [34,35]. Apart from THY1, the common markers used for rodent spermatogonia, such as α6-integrin, β1-integrin, or CD9, have been demonstrated to be ineffective in enrichment of undifferentiated spermatogonia from nonrodent species [2,14]. Only few of the α6-integrin-selected (2.3%), β1-integrin-selected (2.0%), and CD9-selected (1.4%) cells are positive for UCHL1 (an undifferentiated spermatogonia marker) in pigs [36]. Purification of porcine undifferentiated spermatogonia based on the glial cell-derived neurotrophic factor receptor α-1 (GFRA1) yielded unsatisfactory outcome as well [37]. Therefore, a novel surface marker that can enable efficient enrichment of porcine undifferentiated spermatogonia is needed. PLD6 has been identified as a surface marker for mouse SSCs [27].

Using live cell immunolabeling in combination with subcellular fractionation, we observed that PLD6 can be expressed on the surface (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd) and in the cytoplasm of spermatogonia. Hence, we used PLD6-MACS to enrich porcine undifferentiated spermatogonia from prepubertal boar testis tissue. Surprisingly, the MACS-isolated PLD6^{+} cell fraction contained 84.45% of PLZF^{+} spermatogonia, with 8.4-fold of enrichment than the total unselected testicular cell suspension. Moreover, transplantation assay showed that the number of cell colonies was ninefold higher in the PLD6^{+} fraction than that in the unsorted fraction, demonstrating the high SSC content in the PLD6^{+} population. Therefore, PLD6 is a surface marker for enrichment of the undifferentiated spermatogonial population from the testis of prepubertal boars. For more convincing detection of PLD6 at the surface, a reliable plasma membrane isolation procedure will be tested.

Despite several attempts, the cell culture of SSCs from domestic species is still in its infancy [6,28,38]. One of the main reasons might be the lack of approaches to separate the rare SSCs from the large number of testicular somatic cells. To this end, we attempted to culture the sorted PLD6^{+} spermatogonia on laminin-coated dishes under similar culture conditions for feeder-based culture as described in our previous report [6]. The typical grape-like SSC colonies did appear on day 5 of culture and further expanded during the 21-day of culture, although the total number of cells

dropped at later time points of the 21 days culture period, which indicating that a portion of the cultured spermatogonia were proliferating. The decrease of the number of cells might be due to the fact that differentiation/apoptosis prevails over cell proliferation [2,3].

Nevertheless, this study for the first time describes an in vitro feeder-free condition that supports the establishment and maintenance of boar undifferentiated spermatogonia. Furthermore, our presented results are comparable with those achieved in bulls [38]. Yet, the pure spermatogonia could not be maintained in vitro for a long time without feeder layers, suggesting that some undefined factors secreted by feeder cells are still indispensable for in vitro self-renewal of undifferentiated spermatogonia. Hence, the primary feeder-free condition described here remains to be optimized to provide platforms for investigations of SSC self-renewal in vitro.

In this study, PLD6 was identified as a new marker for enrichment of undifferentiated spermatogonia from the prepubertal porcine testis. The highly enriched spermatogonia can be used for RNA sequencing to unravel the mechanisms for SSC self-renewal and differentiation once in conjunction with in vitro differentiation. In addition, as undifferentiated spermatogonia are resistant to cryopreservation than the differentiating counterparts [39], the highly enriched undifferentiated spermatogonia can be cryopreserved, thereby preserving the economically valuable species [2]. In future, it would also be appealing to investigate PLD6 in more extensive mammalian species, including nonhuman primates and humans. The enrichment of human undifferentiated spermatogonia by MACS and PLD6, if any, would greatly facilitate the clinical application of SSC autotransplantation as transplantation efficiency is highly relied on the number of transplanted SSCs [13].

In contrast, PLD6 is a highly divergent PLD superfamily member most closely related to a subfamily of prokaryotic PLDs, which function as endonucleases [40]. By cleaving the mitochondrial-specific lipid cardiolipin to generate the canonical PLD superfamily product phosphatidic acid [41], PLD6 has two functional roles in nongerm cells, one involving relatively low expression that influences mitochondrial morphology and the other involving high expression that leads to mitochondrial aggregation [42]. In mouse germ cells, PLD6 plays a conserved role in the piRNA biogenesis pathway and links lipid metabolism/signaling on the mitochondrial membrane to small RNA biogenesis [26,42]. However, the reported roles of PLD6 are more limited to the mitochondrial and the unique expression pattern of PLD6 on the undifferentiated spermatogonia in boars, as revealed by this study, implying its undefined function in SSC proliferation and self-renewal. Further function studies can help to reveal the role of PLD6 in undifferentiated spermatogonia.

In conclusion, this study reveals that PLD6 is a marker for enrichment of undifferentiated spermatogonia in the prepubertal boars. This finding not only helps elucidate the unique characteristics of porcine germ cells but also facilitates culture and manipulation of the germ cells. Furthermore, this achievement could contribute to the development of germ cell applications in pigs. As the pig is an important nonrodent research model, the data presented in this study are also useful for studying spermatogenesis in other species.

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Author Disclosure Statement

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