SETDB1 plays an essential role in maintenance of gonocyte survival in pigs

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Abstract

Histone methyltransferase SETDB1 suppresses gene expression and modulates heterochromatin formation through H3K9me2/3. Previous studies have revealed that SETDB1 catalyzes lysine 9 of histone H3 tri-methylation and plays essential roles in maintaining the survival of embryonic stem cells and spermatogonial stem cells in mice. However, the function of SETDB1 in porcine male germ cells remains unclear. The aim of the present study was to reveal the expression profile and function of SETDB1 in porcine germ cells. SETDB1 expression gradually increased during testis development. SETDB1 was strongly localized in gonocytes. Knockdown of SETDB1 gene expression led to gonocyte apoptosis and a decrease in H3K27me3, but no significant change in H3K9me3. These observations suggested that SETDB1 is a novel epigenetic regulator of porcine male germ cells, and contributes to the maintenance of gonocyte survival in pigs, probably due to the regulation of H3K27me3 rather than H3K9me3. These findings will provide a theoretical basis for the future study of epigenetic regulation of spermatogenesis.

Reproduction (2017) **154** 23–34

Introduction

Gonocytes, also known as prospermatogonia (Gaskell *et al.* 2004), resume cell division after birth. During their proliferation, they differentiate into spermatogonia and initiate spermatogenesis (Culty 2009). Spermatogenesis is a complicated process, by which spermatozoa is continually produced in the adult mammalian testes. At the age of 2 months, gonocytes and spermatogonia co-exist in the porcine testes (Goel *et al.* 2008). However, gonocytes disappear in the testes when boars reach puberty (Manku & Culty 2015). This process of spermatogenesis requires a precisely ordered regulation of gene expression (Eddy 2002).

Gene expression is regulated by histone modifications (Spencer & Davie 1999), which is widely related with both activation and repression of gene expression (Kouzarides 2002). Three distinct methylation states, which are mono- (me1), di- (me2), and tri- (me3) methylation that occur at lysine (K) of histone H3 and H4 (such as K4, K9, and K27 of histone H3 and K20 of histone H4), are present in mammalian cells (Du & Huang 2007). Generally, H3K4 and H3K36 are related to transcriptional activation, whereas H3K9, H3K27, and H4K20 are associated with transcriptional repression (Martin & Zhang 2005, Du & Huang 2007). These histone methylations are modulated by methyltransferases and demethylases.

The methyltransferase SETDB1 (Set domain, bifurcated 1), also known as ESET, suppresses gene expression and modulates heterochromatin formation through H3K9me2/3 (Schultz *et al.* 2002). Previous studies demonstrated that SETDB1 plays essential roles in the maintenance of embryonic stem (ES) cells (Bilodeau *et al.* 2009, Yuan *et al.* 2009, Lohmann *et al.* 2010), articular cartilage and survival of neurons (Tan *et al.* 2012, Lawson *et al.* 2013). As conventional knockout of the *Setdb1* resulted in mouse embryonic lethality (Dodge *et al.* 2004), the roles of SETDB1 for spermatogenesis have not been examined extensively. Spermatogonial stem cells, as unique adult stem cells, are the cornerstone of male fertility. Previous study indicated that SETDB1 maintained the survival of spermatogonial stem/progenitor cells via catalyzing H3K9me3 in mice (An *et al.* 2014). On the other hand, it is with growing interest to use swine as non-rodent species in biomedicine and pharmaceutical research because of the similarities in anatomy and physiology between pigs and humans (Swindle *et al.* 2012). However, the roles of SETDB1 in porcine male germ cells remain unknown. The objective of the present study was to address the role of SETDB1 in porcine gonocytes. We found that SETDB1 was strongly expressed in the gonocytes, and that knockdown of SETDB1 led to gonocyte apoptosis and a decrease in H3K27me3, but no change in

H3K9me3. These data suggest that SETDB1 contributes to the maintenance of gonocyte survival in pigs.

Materials and methods

Testis samples preparation

Porcine testes from 7-day-old, 2-month-old and adult Landrace hybrid boars were obtained from Yangling pig farm by standard castration procedures. After castration, the testes were maintained in ice-cold PBS during transfer to the laboratory. Small pieces of testis tissue were fixed in Bouin solution overnight, washed three times in PBS and processed for histology.

Preparation and enrichment of gonocytes

The tunica albuginea was removed from the testis tissues before mincing with scissors. The minced tissue was digested with collagenase type IV (2 mg/mL, Invitrogen) at 37°C for 20 min, and washed with PBS to remove interstitial cells and red blood cells. The resulting seminiferous tubules were incubated in 0.25% trypsin-EDTA (Invitrogen) at 37°C for 5 min. The reaction was stopped by fetal bovine serum (FBS; Gibco). After filtration with 40-µm nylon mesh, the single-cell suspension was centrifuged at 600g for 5 min at room temperature, and the pellets were suspended in DMEM/F12 (Invitrogen) with 2% FBS (Gibco). Gonocytes were enriched through differential plating. In brief, the cell suspension was plated into 10-cm dishes with 2×10^7 cell/dish and cultured at 37°C for 6 h. The plates were pipetted gently to remove the cells that were weakly attached to the surface. The cells were transferred into new dishes, cultured overnight at 37°C in a CO₂ incubator. Next day, the supernatant cells were harvested by gently pipetting. The harvested cells were analyzed using antibody for glial cell-derived neurotrophic factor receptor alpha-1 (GFRA1), which is a surface marker for gonocytes and undifferentiated spermatogonia in pigs (Lee *et al.* 2013). The adherent cells were analyzed using antibody for SRY-box containing gene 9 (SOX9), which is a marker for Sertoli cells (Frojdman *et al.* 2000, Franca *et al.* 2016).

RNAi lentivector, siRNA and transient transfection

Small hairpin RNA (shRNA) targeting the porcine *SETDB1* gene was designed and cloned into pCDH-U6-MCS-EF1-GreenPuro. A sequence specific to porcine *SETDB1* cDNA was as follows: shRNA-1 sequence GGTGATGAGTACTTTGCCA, shRNA-2 sequence GGGATTGCCATTAATCAA and a scramble control NC-shRNA sequence GATGAAATGGGTAAGTACA. These plasmids were co-transfected HEK 293T cells in 6-well plates with *SETDB1* overexpression vector (70–80% cell density per well). All cells were collected after 48 h culture and then subjected to mRNA analysis using RT-qPCR. In addition, HEK 293T cells were co-transfected with pCDH-U6-*SETDB1*/NC-shRNA and pGag/Pol, pRev, pVSV-G plasmids to produce lentivirus.

Transfection of small interfering RNA (siRNA) (25 nM) corresponding to the shRNA was performed using

Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol.

Immunohistochemistry

The 6-µm-thick sections were de-paraffinized with xylene and re-hydrated with ethanol series from 100% (v/v) to 70% (v/v). The slides were washed three times, each for 5 min in PBS, followed by 0.5% Triton X-100 in ultrapure water for 10 min at room temperature. The slides were washed and boiled in a solution of 0.01-M Tris-EDTA (pH=9) for 15 min for antigen retrieval, and cooled at room temperature for 40 min. The sections were incubated with 3% H₂O₂ to block endogenous peroxidase, washed three times with PBS, and blocked with 10% goat serum for 2 h at room temperature. Subsequently, the slides were incubated with the primary antibodies against SETDB1 (1:50; Santa Cruz Biotechnology), or H3K9me3 (1:50; Millipore) at 4°C overnight. While some slides were incubated with blocking solution as a negative control. Next day, the slides were rinsed four times in PBS, incubated with biotinylated secondary antibody for 1 h at 37°C and then washed three times with PBS. The slides were exposed to the horse radish peroxidase at 37°C for 1 h, followed by incubation in 3,3'-diaminobenzidine (CWBIO, Beijing, China) for staining and counterstained with Mayer hematoxylin solution (Sigma-Aldrich), and washed three times with PBS for 5 min each. Digital images were captured with the Nikon Eclipse 80i microscope camera.

Immunohistofluorescence analysis

The procedure was similar to that in immunohistochemistry section with minor modification. Briefly, the slides were incubated with 10% donkey serum for blocking nonspecific reactions at room temperature for 2 h and incubated with the primary antibodies against GFRA1 (1:50; Santa Cruz Biotechnology), SETDB1 (1:50; Santa Cruz Biotechnology), H3K9me3 (1:50; Millipore) and c-KIT (1:50; Santa Cruz Biotechnology) respectively, overnight at 4°C. Next day, the sections were washed four times with PBS and then incubated with either donkey anti-Goat (1:200; Abcam) or donkey anti-Rabbit (1:100; Santa Cruz Biotechnology) antibodies for immunofluorescence labeling. Slides were washed and counterstained with DAPI (CWBIO). Fluorescent images were captured with the Nikon Eclipse 80i fluorescence microscope camera.

Immunocytochemistry assay

Gonocytes were seeded into a 96-well plate and treated with siRNA for additional 48 h. The cells were fixed with 4% paraformaldehyde for 30 min at 4°C and permeabilized for 10 min using 0.5% Triton-X100 solution. The cells were incubated with 10% donkey serum for blocking nonspecific reactions at room temperature for 2 h and then incubated with the primary antibody overnight at 4°C. Next day, the cells were washed three times in PBS, incubated with either donkey anti-Rabbit (1:100; Santa Cruz Biotechnology) or donkey anti-Goat (1:200; Santa Cruz Biotechnology) antibodies

for immunofluorescence labeling. Cells were washed and counterstained with DAPI (CWBIO). Fluorescent images were taken using the fluorescence microscope (Olympus).

Western blot

Testicular cells were lysed with RIPA (Beyotime Institute of Biotechnology, Jiangsu, China) that contained 20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and protease inhibitors, and were centrifuged at 12,000g for 10 min at 4°C. Cell lysates were mixed with SDS loading buffer and boiled at 100°C for 5 min. Cell lysates were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore). The membranes were incubated with the following primary antibodies: rabbit anti-H3K9me3 (1:1000; Millipore; 07-442), rabbit anti-H3K27me3 (1:1000; Millipore; 07-449), rabbit anti-SETDB1 (1:500; Santa Cruz Biotechnology; sc-66884), rabbit anti-EZH2 (1:1000; Cell Signaling Technology; 4905), mouse anti-beta-ACTIN (1:2000; CWBIO; CW0096M) and rabbit anti-H3 histone (1:2000; Millipore; 06-755). Secondary antibodies were horseradish peroxidase-linked anti-rabbit or anti-mouse antibody (1:5000; Abcam). The membranes were visualized on a Bio-Rad Chemidoc XRS using a Western Bright ECL Kit (Bio-Rad).

Co-immunoprecipitation (Co-IP) assay

Overexpression SETDB1 and EZH2 plasmids were co-transfected into HEK 293T cells using Turbofect Transfection Reagent (Thermo Fisher Scientific). After 48 h, the cells were lysed with RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and protease inhibitors). Cell lysates were immunoprecipitated using anti-SETDB1 (Santa Cruz Biotechnology; sc-66884) or anti-EZH2 (Cell Signaling Technology; 4905) antibody and added with protein G agarose beads (Millipore) overnight with constant rotation at 4°C. Finally, the protein-antibody-bead complex was washed and analyzed by Western blot.

Plasmid construction and bimolecular fluorescence complementation (BiFC) assay

SETDB1 protein contains the tudor domain, which mediates the protein-protein interactions (Yang *et al.* 2002). We cloned the porcine EZH2 gene and SETDB1 tudor domain to produce plasmids pBiFC-EZH2-VN173 and pBiFC-SETDB12001-VC155 for BiFC assay. Briefly, DNA fragments of EZH2 and SETDB1-2001 were amplified by PCR, and cloned into the NotI/KpnI sites of pBiFC-VN173 and EcoRI/KpnI sites of pBiFC-VC155 respectively. PCR-amplified primers were listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

The plasmids pBiFC-EZH2-VN173 and pBiFC-SETDB12001-VC155 constructs were co-transfected into HEK 293T cells using Turbofect Transfection Reagent (Thermo Fisher Scientific). After 48 h, the fluorescence intensity was captured using a fluorescence microscope (Olympus).

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from the cells or tissues with TRIzol (Invitrogen) according to the manufacturer's protocol. The reverse transcriptions were conducted using PrimeScript RT reagent Kit with gDNA Eraser (Takara). SYBR premix Ex Taq II (Takara) was used for Real-time PCR using an IQ5 (Bio-Rad). Reactions were run in triplicate in three independent experiments. The primer sequences are shown in [Supplementary Table 1](#). The transcript abundance of each gene was normalized to the geometric mean of housekeeping gene GAPDH. Fold change in transcript abundance was analyzed according to the $2^{-\Delta\Delta C_t}$ method.

TUNEL staining

To detect apoptotic cells, TdT-mediated dUTP nick end labeling assays were carried out with TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Jiangsu, China) according to the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde at 4°C for 25 min and washed with PBS. After being permeabilized with proteinase K (20 µg/mL) for 5 min at room temperature, the cells were incubated with 50 µL TdT incubation buffer at 37°C for 60 min in darkness. In the end, cells were washed and observed with the fluorescence microscope (Olympus).

Statistical analysis

All the experiments were repeated at least three times. Statistical evaluation of the data was performed with one-way ANOVA with a least significant difference Tukey's test (SPSS). Data were calculated as mean ± standard deviations (s.d.) and considered significant when the *P* value was less than 0.05 (*), 0.01 (**) or 0.001 (***).

Results

Expression profiles of SETDB1 in the developmental testes

The porcine SETDB1 sequence was cloned. The resultant sequence was consistent with it in NCBI. To analysis the conservation of SETDB1 across species, the amino acids of pig (XP_005663543), mouse (NP_001157113) and human (NP_001138887) SETDB1 were aligned. The amino acid sequence of porcine SETDB1 was 95.52% homologous to human and 90.18% to mouse ([Supplementary Fig. 1](#)). These observations demonstrate that SETDB1 is highly conserved in these species studied.

The testis tissue from 7-day-old, 2-month-old and adult Landrace hybrid boars were used to reveal the expression profiles of SETDB1 during testis development. RT-PCR and Western blot assay showed that the expression of SETDB1 was gradually increasing. It achieved the highest level at adult stage ([Fig. 1A](#) and [B](#)). However, H3K9me3 level was contrary to the SETDB1 expression. Western blot assay showed that the abundance of H3K9me3 was

Figure 1 Expression profiles of *SETDB1* and H3K9me3 during porcine testis development. (A) Transcripts of *SETDB1* were examined by qRT-PCR in the testes from different ages (7-days, 2-months and adult) of pigs. The levels of the transcription were normalized against *GAPDH*. (B) Western blot detection of SETDB1 and H3K9me3 protein levels during postnatal development of the testes. (C) Intensity analysis of SETDB1 and H3K9me3 were calculated by ImageJ. Data are presented as the mean $\pm$ s.d., *** P < 0.001.

the highest in the neonatal piglet testis tissue, and then decreased (Fig. 1B and C).

Distribution of *SETDB1*, H3K9me3 and H3K27me3 in porcine testes

The immunohistochemistry was performed to localize the distribution of SETDB1, H3K9me3 and H3K27me3 in porcine testes. H3K9me3 and H3K27me3 distributed in gonocytes/undifferentiated spermatogonia, Sertoli cells and interstitial cells, whereas SETDB1 was localized in gonocytes/undifferentiated spermatogonia and interstitial cells in 1-week-old and 2-month-old testis tissues. SETDB1, H3K9me3 and H3K27me3 were localized in the spermatogonia, spermatocyte, Sertoli cells and interstitial cells in the seminiferous tubules of adult testis tissue (Fig. 2).

We further detected the distribution of SETDB1 and H3K9me3 in undifferentiated and differentiated germ cells. SETDB1 localized strongly in cytoplasm and weakly in nuclei in GFRA1-positive spermatogonia (Fig. 3A and B). H3K9me3 showed extensive perinuclear distribution in gonocytes/undifferentiated spermatogonia (GFRA1 as a marker for gonocytes and undifferentiated spermatogonia) in the 7-day-old and 2-month-old testis tissue (Fig. 3A and B). Subsequently, SETDB1 was localized in the nuclei of undifferentiated spermatogonia (GFRA1-positive cells) and differentiated spermatogonia (c-KIT as a marker for differentiated spermatogonia) in adult testes (Fig. 4A). Interestingly, H3K9me3 showed perinuclear localization in GFRA1 positive cells, while displaying intranuclear distribution in differentiated spermatogonia (c-KIT positive cells, Fig. 4B).

Expression of *SETDB1* in gonocytes

The interstitial cells and red blood cells were removed by velocity sedimentation during the digestion. The harvested seminiferous tubules from 1-week-old boar testes are mainly composed of Sertoli cells and gonocytes. Therefore, the interstitial cells were excluded from the

recovered cells, and would not interfere in the accuracy for identifying gonocytes through immunocytochemistry assay of GFRA1 expression. The purity of the enriched gonocytes reached $76.5\% \pm 3.9\%$ (GFRA1 positive cells, Fig. 5A and B). The *SETDB1* expression in gonocytes was 8.9 times higher than that in Sertoli cells at mRNA level (Fig. 5C). *SETDB1* was mainly expressed in gonocytes (Fig. 5D, E and Supplementary Fig. 2).

SETDB1 knockdown induced gonocyte apoptosis

The results of SETDB1 expressed in gonocytes revealed a unique role of *SETDB1* in gonocytes. Gonocytes were transfected with high interference efficiency siRNA-1 (Supplementary Fig. 3) designed against *SETDB1*-coding sequence. As shown in Fig. 6A and B, the expression of *SETDB1* in *SETDB1*-KD gonocytes was significantly decreased at both RNA and protein level. Unexpectedly, the level of H3K9me3 did not change in the *SETDB1*-KD group. Surprisingly, *SETDB1* interference resulted in a dramatic decrease of H3K27me3 level (Fig. 6C and D). In addition, immunofluorescence assay showed that the fluorescence intensity of SETDB1 significantly weakened in the *SETDB1*-KD gonocytes, but no change in H3K9me3 (Fig. 6E and F). These observations indicate that *SETDB1*-KD lead to a decrease of H3K27me3, but little or no change on H3K9me3 level.

Interestingly, knockdown of *SETDB1* induced apoptosis (Fig. 7A). The proportion of apoptotic gonocytes was $48.7\% \pm 5.7\%$ in the treatment group, whereas $25.4\% \pm 1.2\%$ in NC control group (Fig. 7B). On the contrary, the depletion of *SETDB1* in Sertoli cells did not induce cell apoptosis. The number of apoptotic Sertoli cells was similar between *SETDB1*-KD group and the control (Supplementary Fig. 4).

Furthermore, we detected the apoptotic related proteins to confirm the apoptosis using Western blot. As shown in Fig. 7C, knockdown of *SETDB1* in gonocytes led to an increase of cleaved CASP3, and a decrease of anti-apoptotic protein BCL2. The depletion of *SETDB1* made neither change for the pro-apoptotic protein BAX nor the total CASP3.

Figure 2 Immunohistochemistry detection of SETDB1, H3K9me3 and H3K27me3 levels during porcine testis development. Asterisks: gonocytes; arrows: Sertoli cells; arrowheads: pachytene spermatocytes. Bars = 50 μ m.

In addition, to verify the interaction between SETDB1 and EZH2 that regulates apoptosis via depositing H3K27me3, the Co-IP assay with antibodies for SETDB1 and EZH2 showed that co-immunoprecipitated SETDB1 or EZH2 could be detected by either EZH2 antibody or SETDB1 antibody (Fig. 7D). In addition, BiFC assay revealed that the interaction of SETDB1 and EZH2 produced an observable fluorescence compared to the EZH2-Mock control (Fig. 7E).

Discussion

The methyltransferase SETDB1 plays an importance role in the maintenance of ES cells through repressing the expression of genes encoding developmental regulators and restricting extraembryonic trophoblast lineage potential (Bilodeau *et al.* 2009, Yeap *et al.* 2009, Yuan *et al.* 2009). Our previous study showed that SETDB1 was required for the survival of spermatogonial

Figure 3 Cellular distribution of SETDB1 and H3K9me3 in the seminiferous tubules during porcine testis development. (A) Co-immunofluorescence detection of glial cell-derived neurotrophic factor receptor alpha-1 (GFRA1) (a marker for gonocytes/undifferentiated spermatogonia) and SETDB1 or H3K9me3 in the neonatal piglet testes. (B) Co-immunofluorescence detection of GFRA1 and SETDB1 or H3K9me3 in the 2-month-old pig testes. White boxes were shown at higher magnification. Bars=20µm.

Figure 4 Cellular distribution of SETDB1 and H3K9me3 in porcine adult testis tissue. (A) Co-immunofluorescence staining of SETDB1 and GFRA1 (a marker for undifferentiated spermatogonia) or c-KIT (a marker for differentiated spermatogonia). GFRA1: green; c-KIT: green; SETDB1: red; DAPI: blue (counterstaining of DNA). (B) Co-immunofluorescence staining of H3K9me3 and GFRA1 or c-KIT. GFRA1: red; c-KIT: red; H3K9me3: green; DAPI: blue (counterstaining of DNA). White boxes were shown at higher magnification. Bars=50 μm.

Figure 5 Enrichment of gonocytes and expression of *SETDB1* in gonocytes and Sertoli cells. (A) Enrichment of gonocytes by different plating. Immunofluorescence of GFRA1 (red) and DAPI (blue) were performed to calculate the number of gonocytes. Bars = 50 μ m. (B) Proportion of gonocytes (GFRA1-positive cells) before and after enrichment. After enrichment, the purity of gonocytes was 76.5% $\pm$ 3.9% while it was 12.3% $\pm$ 2.2% before the enrichment. *** P < 0.001. (C) qPCR analysis of *SETDB1* mRNA level in gonocytes or Sertoli cells. The transcriptional level of *SETDB1* in gonocytes is 8.9-fold that of Sertoli cells. *** P < 0.001. (D) Western blot detection of SETDB1 expression in gonocytes and Sertoli cells. (E) Co-immunofluorescent staining of GFRA1 and SETDB1 in the cells before enrichment of gonocytes. SETDB1 mainly expressed in gonocytes (GFRA1-positive cells). GFRA1: red; SETDB1: green; DAPI: blue (counterstaining of DNA). Bars = 50 μ m.

Figure 6 Knockdown of *SETDB1* decreased protein expression. (A) qRT-PCR detected the expression of *SETDB1* in SETDB1-KD, NC and Mock group. *** P < 0.001. (B) Western blot detected *SETDB1* expression in treated group. (C) H3K27me3 level was detected by Western blot. (D) H3K27me3 intensity analysis was calculated by ImageJ. *** P < 0.001. (E and F) Immunofluorescent staining of SETDB1 and H3K9me3 in SETDB1-KD gonocytes. GFRA1: red; SETDB1: green; H3K9me3: green; DAPI: blue (counterstaining of DNA). Bars = 50 μ m.

Figure 7 Knockdown of *SETDB1* induced gonocyte apoptosis. (A) TUNEL-positive cells apparently increased in *SETDB1*-deficient gonocytes compared to the control. Red: TUNEL positive cells. Bars = 50 μ m. (B) The statistical analysis of TUNEL-positive gonocytes in treated group. *** P < 0.001. (C) Western blot detected apoptosis-related proteins. (D) Co-IP assay was performed to detect the interaction of *SETDB1* and *EZH2* by Western blot using anti-*SETDB1* and anti-*EZH2* primary antibodies. (E) BiFC assay. Plasmids expressing *SETDB1*-VC and *EZH2*-VN were co-transfected into HEK 293T cells. The fluorescence was detected at 48 h post-transfection. Bars = 50 μ m.

stem/progenitor cells in mice (An *et al.* 2014). Here, for the first time, we demonstrate that *SETDB1* plays an essential role in maintenance survival of porcine gonocytes, which are the progenitor cells of spermatogonial stem cells.

In the present study, we revealed that *SETDB1* was strongly expressed in gonocytes, which suggests that *SETDB1* may have a specific role for maintenance of the gonocyte pool. *SETDB1* was expressed in both cytoplasm and nuclei, in porcine gonocytes, which is consistent with the previous reports in HeLa and NIH3T3 cells (Loyola *et al.* 2006, Cho *et al.* 2013). Previous reports suggested that human and mouse *SETDB1* have nuclear export signals (NESs) and nuclear localization signals (NLSs), making the nucleocytoplasmic shift (Cho *et al.* 2013, Kang 2015, Tachibana *et al.* 2015). We speculate that porcine *SETDB1* may contain these motifs. In addition, by using proteasome inhibitor and nuclear export inhibitor leptomycin B, Tachibana *et al.* (2015) found that the intranuclear *SETDB1* undergoes protease degradation and exports from the nuclei to

the cytoplasm (Tachibana *et al.* 2015). Thus, *SETDB1* is extensively distributed in cytoplasm. Moreover, *SETDB1*, as a H3K9 monomethyltransferase on free/non-nucleosomal H3, also mediates H3K9me1 in cytoplasm (Towbin *et al.* 2012, Mozzetta *et al.* 2015). Furthermore, we detected the distribution of H3K9me3 in porcine germ cells, and found that H3K9me3 was distributed in the perinuclear region of gonocytes and undifferentiated spermatogonia, which is in line with the previous studies in mice (Payne & Braun 2006, An *et al.* 2014). Previous studies revealed that H3K9me3 staining patterns shift from a perinuclear to a punctate distribution during SSC differentiation (Payne & Braun 2006). This perinuclear distribution can be a novel marker for male germline stem cells (Payne & Braun 2006), and ensures a robust spatial separation of active and inactive chromatin domains (Towbin *et al.* 2012).

Knockdown of *SETDB1* induced gonocyte apoptosis. However, unexpectedly, interference *SETDB1* in porcine gonocytes led to no change in H3K9me3 level, which is in agreement with the previous report in ES

cells (Dodge *et al.* 2004). These findings suggest that other histone methyltransferases are also involved in catalyzing H3K9 trimethylation. In addition to SETDB1, protein G9A (also known as EHMT2), G9A-like protein 1 (GLP; also known as EHMT1) and SUV39H1 (also known as KMT1A) also catalyze methylation at H3K9. G9a and GLP can catalyze H3K9 from zero to mono-, di- and trimethylation *in vitro* (Collins *et al.* 2005, Kubicek *et al.* 2007). In euchromatic regions, G9a and GLP are mainly involved in H3K9me1 and H3K9me2 (Tachibana *et al.* 2005). The H3K9me3 levels are maintained by SUV39H1 and/or SETDB1 (Peters *et al.* 2003, Rice *et al.* 2003). SUV39H1 can also methylate unmethylated H3K9 (Rea *et al.* 2000), but more likely to catalyze H3K9me1 to H3K9me2 and H3K9me3 marks (Loyola *et al.* 2006). Therefore, knockdown of *SETDB1* does not absolutely result in a reduction of H3K9me3.

The previous studies have shown that the reciprocal action between SETDB1 and Polycomb Repressive Complex 2 (PRC2) regulate ES cell pluripotency and differentiation (Fei *et al.* 2015). Knockdown of *SETDB1* reduces the combination of EZH2 (a component of PRC2) as well as the level of H3K27me3 in mouse ES cells (Fei *et al.* 2015). Moreover, Liu *et al.* (2014) reported that SETDB1 played an essential role in establishing and/or maintaining H3K27me3 and H3K9me3 at some retrotransposition loci in the prenatal germline (Liu *et al.* 2014). In addition, increasing evidence demonstrated that EZH2 regulated cell proliferation and apoptosis via depositing H3K27me3 (Girard *et al.* 2014, Zhang *et al.* 2014, He *et al.* 2015, Ren *et al.* 2015). Thus, we detected the global H3K27me3 level and found that knockdown of *SETDB1* induced the decrease of H3K27me3 level in porcine gonocytes, which is consistent with that in murine primordial germ cells (Liu *et al.* 2014) and neurons (Fei *et al.* 2015).

Furthermore, bimolecular fluorescence complementation (BiFC) has been used to study the protein interactions in cells (Hu *et al.* 2002). The Co-IP and BiFC assay indicated that porcine SETDB1 indeed interacted with porcine EZH2. Knockdown of *SETDB1* resulted in apoptosis and the decrease of H3K27me3 level in porcine gonocytes, which is consistent with that in murine primordial germ cells (Liu *et al.* 2014) and neurons (Fei *et al.* 2015), but did not affect the expression of EZH2 (Supplementary Fig. 5). Thus, *SETDB1*-KD induced gonocyte apoptosis via interacting with EZH2 to regulate H3K27me3 level. Further investigation is needed to elucidate the mechanisms by which SETDB1 regulates H3K27me3 and apoptosis in porcine gonocytes.

Finally, the seminiferous tubules of 1-week-old boar testis tissue are mainly composed of Sertoli cells and gonocytes. Depletion of *SETDB1* induced apoptosis of gonocytes, but did not lead to apoptosis in Sertoli cells. Therefore, although the purity of gonocytes was

76.5%, the contaminated Sertoli cells probably did not affect the observations obtained in the present study. To rule out the possible effect from the Sertoli cells, future study is needed to enrich the gonocytes through magnetic-activated cell sorting or fluorescence-activated cell sorting.

In conclusion, knockdown of *SETDB1* induced gonocyte apoptosis. SETDB1 plays an important role in maintaining the gonocyte pool, probably due to the regulation of H3K27me3 rather than H3K9me3. We speculate that *SETDB1* may maintain the stem cell characteristics and survival of gonocytes/SSCs through repressing the transcription of differentiation related and pro-apoptotic genes. The findings from this study may provide some insight on the establishment of a successful culture system for porcine germline stem cells.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0107>.

Declaration of interest

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

Funding

This study was supported in part by National Basic Research Program of China (973 program; 2014CB943100), the National Natural Science Foundation of China (Grant No. 31272439, 31230048) and the Doctoral Program Foundation of Higher Education of China (Grant No. 20130204110017) to W Z.

Acknowledgements

The authors thank Dr Xiaodong Xu for kindly providing pBiFC-VN173 and pBiFC-VC155 plasmids, and Drs Wuzi Dong and Chuanying Pan for suggestions and comments. We thank Dr Huayan Wang for proofreading the manuscript.

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Received 20 February 2017
 First decision 16 March 2017
 Revised manuscript received 27 March 2017
 Accepted 18 April 2017