Supplementary material

SUPPLEMENTARY MATERIALS AND METHODS

Animal Material

Testes were acquired from 7-d-old male boars following castration. All animal experiments and procedures were approved by the Animal Protection Committee of Nanjing Agricultural University.

Isolation of porcine Sertoli cells

Sertoli cell isolation procedures were based on previous study (Wang et al., 2019), with some modifications. In brief, porcine Sertoli cells were isolated from 7-d-old porcine testes. We used two testes each time (30 testes in total). The tunica albuginea was removed, with the tissues snipped and then digested with 1 mg/mL of collagenase IV in an incubator at 37 °C for 30 min. After rinsing with D-Hanks solution and centrifugation at 600 *g* for 5 min at room temperature, red blood cell lysis buffer was added, followed by incubation at 4 °C for 15 min. After centrifugation at 600 *g* for 5 min at room temperature and rinsing with D-Hanks solution, the tissues were digested in 0.05% Trypsin-EDTA at 37 °C for 10 min. Digestion was monitored via microscopy. Medium containing 10% fetal bovine serum (FBS) was used to neutralize the reaction. The digested products were filtered with 70 μm Nylon mesh to remove large particles or cell clumps, followed by centrifugation at 600 *g* for 5 min at room temperature, after which the supernatant was discarded. The pellet was resuspended in DMEM/F12 medium (Gibco, USA) supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 U/mL penicillin and then cultured at 37 °C and 5% CO_2. After 4 h, hypotonic treatment (20 mmol/L Tris-HCl pH 7.4) was performed to remove germ cells. After purification, the number of Sertoli cells in 24-well plates was 1×10^6. Immunofluorescent staining of WT1, a Sertoli cell-specific marker, was employed, with the proportion of $WT1^+$ cells representing Sertoli cell purity. Statistical analysis revealed that the purity was over 80% (Figure 1C–G).

Treatment of Sertoli cells before co-culture

We used hypotonic treatment to purify Sertoli cells. Cells were cultured at 37 °C and 5% CO_2 for 24 h. When cell deformation was observed, we treated the cultured Sertoli cells with phosphate-buffered saline (PBS) or 50 μmol/L H_2O_2 for 24 h in 24-well plates in preparation for culture with spermatogonial stem cells (SSCs).

Co-culture of SSCs and Sertoli cells

Porcine SSCs were derived as reported previously (Zhang et al., 2017). Briefly, testes from 7-d-old porcines were cut into pieces and digested with collagenase type IV and then treated with red blood cell lysis buffer. After rinsing with D-Hanks and trypsin treatment, the supernatant was removed following centrifugation at 600 *g* for 5 min at room temperature. The pellet was resuspended in DMEM/F12 medium (Gibco) supplemented with 1% FBS, 2 mmol/L L-glutamine, 10% knockout serum replacement (Invitrogen), 5 mg/mL bovine serum albumin (MP Biomedicals), N_2 medium (Invitrogen), non-essential amino acid (Invitrogen), vitamins, 50 μmol/L β-mercaptoethanol, 10 μg/mL D-biotin (Sigma), 1 μg/mL DL-lactic acid, 100 nmol/L ascorbic acid, 20 ng/mL GDNF (Rat GDNF Peprotech 450–51), and 10 ng/mL bFGF (Human FGF2 Peprotech 100-18B). After two rounds of differential plating, porcine SSCs were transferred to Sertoli cells for co-culture at 37 °C with 5% CO_2. After purification, the number of SSCs was approximately 1×10^4. Follow-up H_2O_2 experiments were repeated at least three times.

Detection of reactive oxygen species (ROS) in Sertoli cells

Sertoli cells were treated with 0, 5, 10, 20, or 50 μmol/L H_2O_2 for 24 h. ROS levels were then

determined using a Reactive Oxygen Species Assay Kit (Beyotime). Rosup was used to treat Sertoli cells as a positive control (Figure 1I), and PBS-treated Sertoli cells were used as a negative control (data not shown). Sertoli cells were incubated in DMEM/F12 medium with DCFH-DA (1:1 000) for 20 min at 37 °C in a 5% CO_2 incubator. After rinsing with PBS for three times, fluorescent signals were examined using FACSCalibur (BD Biosciences) and fluorescence microscopy.

Analysis of apoptotic cells

Sertoli cells were treated with 0, 5, 10, 20, or 50 μmol/L H_2O_2 for 24 h. Apoptosis was examined using an Annexin V-FITC Apoptosis Detection Kit (Beyotime). Briefly, cells were rinsed with PBS once and then incubated with Annexin V-FITC and propidium iodide (PI) in Annexin V-FITC binding buffer for 20 min in the dark at room temperature. Fluorescent signals were detected using fluorescence microscopy. Three replicates were performed for the experiments.

Immunofluorescence staining

Cells cultured on 24-well plates were rinsed three times with PBS and fixed with Carnoy for 20 min at −20 °C. Subsequently, cells were rinsed with PBS three times and then blocked with 10% goat serum in PBS for 30 min at room temperature. Cells were then incubated with anti-WT1 (Abcam, ab89901) antibody in PBS at 4 °C overnight. After rinsing with PBS three times, cells were incubated with secondary antibody Alexa Flour532 goat anti-rabbit (Invitrogen, A-11009) for 30 min at 37 °C . DAPI was used to counterstain the nucleus and samples were detected using fluorescence microscopy. Three replicate experiments were carried out for staining.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Tiangen, DP405) and converted into cDNA with a PrimeScript RT Master Mix (Takara, RR036A). To analyze gene expression, PCR was performed with Taq DNA polymerase (Takara, R001WZ) under the following conditions: 5 min denaturation at 95 °C, 35 cycles at 95 °C for 30 s , annealing for primers at 55 °C or 62 °C for 30 s, and final extension for 5 min at 72 °C. Three replicates were performed. Primer information is listed in Supplementary Table S1.

Supplementary Table S1 Information on primers used in current study

Gene	Primer Sequence (5'−3')	GenBank Accession No.	Product size (bp)	Annealing temp (°C)
Cx43	F: CACCAGGTGGACTGTTTCCT R: TCTTTCCCTTCACACGATCC	NM_001244212.1	151	55
WT1	F: TGAGCGAAGGTTTTCTCGTT R: GCTGAAGGGCTTTTCACTTG	NM_001001264.1	166	55
SOX9	F: AGCAGACGCACATCTCTCCCA R: CGCCCCTCTCGCTTCAGGTCA	NM_213843.2	190	62
AMH	F: AAGCTCCTCATCAGCCTGTCT R: ATTGGGGCGATCGGGTTTG	NM_214310.3	145	62

Western blotting

For Western blotting, protein samples were run on SDS PAGE gel at 80–120 V and then transferred to nitrocellulose membranes. After blockage with 5% skim milk for 1 h, nitrocellulose membranes were

incubated with primary antibodies β-tubulin (Anbo, P07437), WT1 (Abcam, ab89901), CX43 (Cell Signaling, 3512), and ITGB1 (BD, 610467) at 4 °C overnight. Peroxidase-conjugated goat anti-rabbit or mouse IgG (Santa Cruz, sc-2004, sc-2005) were used as secondary antibodies. Enhanced chemiluminescence (ECL) was used to visualize the immunoreactive bands and finally expose the film. Three replicates were performed.

Statistical analysis

At least three replicates were performed for all experiments. Data were analyzed with Prism 8 and presented as means±*SEM*. Statistical significance was determined with t-tests. A *P*-value of <0.05 was considered significant. The FACSCalibur results were analyzed with FlowJo 7.6.

REFERENCES

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