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Dimethyloxaloylglycine promotes spermatogenesis activity of spermatogonial stem cells in Bama minipigs

Yaqi Cao †, **ZiFu Dai** †, **Huizhen Lao** †, **Huimin Zhao** *

College of Life Science and Technology, Guangxi University, Nanning 530005, China

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***Corresponding author:**
Huimin Zhao
College of Life Science and Technology, Guangxi University, Daxue Rd, Xixiangtang District, Nanning 530005, China.
Email: zhaohuimin-nn@126.com
https://orcid.org/0000-0002-7003-923X

†Yaqi Cao, ZiFu Dai and Huizhen Lao equally contributed to this work.

ABSTRACT

Background: The testis has been reported to be a naturally O_2-deprived organ, dimethyloxaloylglycine (DMOG) can inhibit hypoxia inducible factor-1alpha (HIF-1α) subject to degradation under normal oxygen condition in cells.
Objectives: The objective of this study is to detect the effects of DMOG on the proliferation and differentiation of spermatogonial stem cells (SSCs) in Bama minipigs.
Methods: Gradient concentrations of DMOG were added into the culture medium, HIF-1α protein in SSCs was detected by western blot analysis, the relative transcription levels of the SSC-specific genes were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Six days post-induction, the genes related to spermatogenesis were detected by qRT-PCR, and the DNA content was determined by flow cytometry.
Results: Results revealed that the levels of HIF-1α protein increased in SSCs with the DMOG treatment in a dose-dependent manner. The relative transcription levels of SSC-specific genes were significantly upregulated ($p < 0.05$) by activating HIF-1α expression. The induction results showed that DMOG significantly increased ($p < 0.05$) the spermatogenesis capability of SSCs, and the populations of haploid cells significantly increased ($p < 0.05$) in DMOG-treated SSCs when compared to those in DMOG-untreated SSCs.
Conclusion: We demonstrate that DMOG can promote the spermatogenesis activity of SSCs.

Keywords: Hypoxia; testis; stem cell; spermatogenesis

INTRODUCTION

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and together with oocytes, are essential for species continuity. The testis has been reported to be a naturally O_2-deprived organ, as the seminiferous tubules of the testes are poorly vascularized and under low oxygen tension [1]. Rat seminiferous tubules are thought to be under lower O_2 tension than normal interstitial O_2 tension conditions. SSCs are located on the basement membrane of seminiferous tubules and are almost completely surrounded by Sertoli cells, which form a microenvironment. The differentiation of SSCs and multipotent progenitors are affected by complex signals in the microenvironment, including hormones, temperature, and O_2 availability. A previous study suggested that hypoxic conditions in the range of 1%–5% O_2

ORCID iDs
Yaqi Cao
https://orcid.org/0000-0003-4646-142X
ZiFu Dai
https://orcid.org/0000-0003-3321-1245
Huizhen Lao
https://orcid.org/0000-0002-5715-4006
Huimin Zhao
https://orcid.org/0000-0002-7003-923X

Author Contributions
Conceptualization: Zhao H; Formal analysis: Cao Y; Investigation: Dai Z; Methodology: Lao H; Project administration: Zhao H; Resources: Zhao H; Software: Cao Y; Supervision: Zhao H; Validation: Dai Z; Visualization: Lao H; Writing - original draft: Cao Y; Writing - review & editing: Zhao H.

Conflict of Interest
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supported the maintenance of embryonic stem cells (ESCs) in a pluripotent state, thereby prevented their differentiation and even reprogrammed partially differentiated cells into a stem cell-like state [2]. A separate study showed that hypoxic conditions applied in the range of 0.1%–1% O_2 contributed to the differentiation of ESCs [3]. Although hypoxia is an important factor that determines the fate of cell, its effects on the spermatogenesis of SSCs under cell culture conditions *in vitro* remain unclear.

Hypoxia inducible factor-1alpha (HIF-1α) is a master transcriptional factor that responds to hypoxia [4]. HIF-1α is stabilized by dimerization with the β subunit, the dimer binds to target genes, and leads to the downstream activation of the target gene transcription. A previous study demonstrated that HIF-1α played an important role in spermatogenesis, wherein it was robustly expressed in SSCs of both juvenile and adult murine [5]. Reduced HIF-1α levels in testes block sperm production and cause infertility of the mice [6]. Under normoxic conditions, HIF-1*α* is hydroxylated, ubiquitinated, and degradated by the proteasomes. Prolyl hydroxylase (PHD) is greatly involved in the degradation of HIF-1α. In well-oxygenated cells, the PHD is catalyzed in the presence of Fe (II) and oxygen. Under normal hypoxia conditions when the PHD is blocked due to the lack of oxygen. Therefore, endogenous HIF-1α levels can be increased by the suppression of PHD activity, either by reducing cellular oxygen levels or by combining with Fe (II) competitively. Dimethyloxaloylglycine (DMOG) is a cell penetrant oxoglutarate analogue that inhibits PHD enzymes, thereby subjecting HIF-1α to cellular degradation under normal oxygen tension conditions, and has been demonstrated to stabilize HIF-1α both *in vitro* and *in vivo* [7]. The objective of the present study was to study the effect of DMOG on spermatogenesis capability and the proliferation of SSCs *in vitro*.

MATERIALS AND METHODS

Animal

The testes of 20-d-old piglets and 2-mon-old pigs were obtained through routine castration surgery performed at a local farm. Pigs that are castrated at this age to improve the meat quality and enhance growth rates. All procedures involving animals were performed in compliance with the Animal Care and Use Committee of the Germplasm Resource Center of Chinese Experimental Minipig and the Animal Care and Use Committee of Guangxi University (approval No. GXU2016-015).

Immunohistochemistry

Testicular samples from 20-d-old piglets and 2-mon-old pigs were subjected to immunohistochemical analysis. Briefly, testicular tissues were fixed in Bouin's fixative for 12 h, rinsed in water for 2 h, dehydrated, embedded in paraffin, and sliced into 5-μm-thick sections. Tissue samples were deparaffinized in xylene and rehydrated. Antigen retrieval was carried out by boiling sections in 10 mM sodium citrate (pH = 6.0) for 30 min, washed in phosphate-buffered saline (PBS; pH = 7.2) 3 times, cultured with 0.5% Triton X-100 for 5 min, blocked with 5% bovine serum albumin (BSA) in PBS for 30 min and incubated with the following primary antibodies overnight at 4°C: rabbit anti-human ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) (1:200; AbD Serotec, UK), rabbit anti-CD14 (1:100; Absin, China), rabbit anti-NSE (1:200; Absin), rabbit anti-HIF1-Alpha (1:100; Absin), and rhodamine-labeled *Dolichosbiflorus* agglutinin (DBA) (1:100; Vector Laboratories, Inc., USA). After washing again, the samples were incubated with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Invitrogen Molecular Probes, USA). To identify nuclei, samples were incubated with

Hoechst33342. Finally, sections were mounted with Vectashield mounting medium (Vector Laboratories, Inc.) and photographed under a fluorescence microscope (Eclipse 50i; Nikon, Japan). Primary antibodies were replaced with 1% BSA in PBS as the negative control.

Isolation of SSCs and Sertoli cells

The testes from 20-d-old piglets were used for the isolation of SSCs and Sertoli cells. Briefly, tunica albugineae were removed, and testicular tissues was minced, digested with enzymes mixture solution (DNase I and collagenase IV in PBS), and filtered through a 40 μm nylon mesh. Red blood cells (RBCs) were removed by RBC lysis buffer (Sigma-Aldrich, USA). Next, dissociated cells were seeded in 0.2% (w/v) gelatin-coated plates and cultured in Dulbecco's Modified Eagle Medium: Nutrient F-12 (DMEM/F12) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) at 31°C and 5% CO_2. After cultivating for 12 h, the floating cells were collected for SSC isolation using fluorescence-activated cell sorting (FACS). Briefly, the floating cells were incubated with rabbit anti-CD14 (1:100; Absin) for 60 min at 37°C, washed with PBS, and incubated with Alexa Fluor 594- conjugated donkey anti-rabbit IgG for 15 min at 37°C. Cell sorting was performed using a FACSAriaII apparatus (BD Bioscience, USA). Adherent cells were used for the identification of Sertoli cells.

Cultivation of SSCs and Sertoli cells

Immediately after cell sorting, $CD14^+$ SSCs were seeded onto sandos inbred mice mouse embryo-derived thioguanine- and ouabain-resistant (STO) feeder layers in a 6-well plate at a density of 2×10^5 cells per well, then cultured in SSC medium, The media was prepared by supplementing DMEM/F12 with 3 mg/mL BSA, 0.05 mg/mL pyruvic acid sodium (Sigma-Aldrich), 0.5 mg/mL L-glutamine, and the following the growth factors: 20 ng/mL GDNF, 10 ng/mL bFGF, and 100 ng/mL GFRα1. Cells were subsequently were cultured at 37°C in 5% CO_2. The Sertoli cells were cultured in DMEM/F12 supplemented with 10% FBS at 37°C in 5% CO_2.

Transduction of shRNA of *HIF-1α*

In order to inhibit HIF-1α expression, the shRNA of *HIF-1α* (forward oligo: 5′-TCCAGTTGAATCTTCAGATATTCAAGAGATATCTGAAGATTCAACTGGTTTTTTC-3′, reverse oligo: 5′-TCGAGAAAAAACCAGTTGAATCTTCAGATATCTCTTGAATATC TGAAGATTCAACTGGA-3′) was constructed with lentivirus vector (Neuron Bio, China). For transduction, SSCs were seeded in a 12-well plate and infected with the vector at a multiplicity of infection of 50. Positive cells were sorted by flow cytometry and cultured for further analysis.

Western blot

Western blot was carried out to determine the effects of DMOG on HIF-1α protein expression in SSCs. HIF-1α protein expression in *shHIF-1α* SSCs was also analyzed using western blot, and the SSCs infected with empty vector as a negative control. SSCs were treated with gradient concentrations (0, 200, 400, 800, and 1,000 μM) of DMOG in SSC medium for 2 d, the *shHIF-1α* SSCs and the negative control were treated with 1,000 μM DMOG in SSC medium for 2 d. Then, cells were harvested, homogenized in lysis buffer and incubated on ice for 20 min. The cellular debris was separated by centrifugation at 10,000 × g at 4°C. The protein content was quantified by BCA assay. Then, 50 μg protein was loaded onto a 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to a polyvinylidene fluoride membrane, blocked in 5% milk in TBS-T for 2 h at room temperature, incubated with anti-HIF-1α primary antibody (1:800) overnight, and incubated corresponding secondary antibody was employed at (1:5,000) dilution for 60 min. Membranes were scanned with an Odyssey Scanner (Li-COR Biosciences, USA) and quantified with Odyssey v3.0.

Effect of DMOG on SSC proliferation

In order to evaluate the effects of DMOG on SSC proliferation, SSCs were preconditioned with different concentrations of DMOG (Sigma-Aldrich) (200, 400, 800, and 1,000 μM) in SSC medium. The *shHIF-1α* SSCs were preconditioned with 1,000 μM DMOG. After culturing for 2 d, the medium was replaced with fresh SSC medium. SSC proliferation was measured using a Cell Counting Kit-8 (CCK-8; Beyotime, China) following the manufacturer's instructions. The optical density was measured using a microplate reader at 450 nm. DMOG-untreated SSCs cultured in SSC medium as a control.

In vitro spermatogenesis

Normal and *shHIF-1α* SSCs were seeded respectively onto Sertoli cells, treated with 800 μM DMOG in differentiation medium that consisted of minimum essential media alpha (MEM α; Life Technologies, USA) with 10% knockout serum replacement (10828028; Thermo Fisher Scientific), 3 ng/mL retinoic acid, 100 ng/mL activin A (R&D Systems, USA), 20 ng/mL BMP4 (R&D Systems), 200 ng/mL FSH (Sigma-Aldrich) and 10 μm T (Sigma-Aldrich). Cells were cultured at 37°C in 5% CO_2. After culturing for 2 d, the medium was replaced with fresh differentiation medium without DMOG. Six d post-induction, cells were collected for analysis. DMOG-untreated SSCs were cultured in differentiation medium as a control.

Immunocytochemistry

Cultured cells were fixed in 1 mL 4% paraformaldehyde, washed for 5 min in 1 mL PBS 3 times, permeabilized with 1 mL 0.5% Triton X-100 in PBS for 10 min, and incubated with 1 mL 10% normal goat serum in DMEM/F12 for 30 min at room temperature to block non-specific binding. Next, cells were incubated with the following primary antibodies at 4°C overnight to identify SSC clusters: rabbit anti-human UCHL1 (1:200; AbD Serotec), rabbit anti-CD14 (1:100; Absin), rabbit anti-HIF1α (1:100; Absin), and DBA (1:100; Vector Laboratories, Inc.). The primary antibodies used to identify Sertoli cells were rabbit anti-WT1 (1:200; Sigma-Aldrich) and rabbit anti-NSE (1:200; Sigma-Aldrich). The primary antibodies used to identify differentiated cells were rabbit anti-mouse Stra8 (1:200, ab49602; Abcam) and rabbit anti-human SCP3 (1:100, ab15093; Abcam).

After incubating with primary antibodies, the samples were washed in PBS 3 times and exposed to 300 μL Alexa Fluor 594-labeled donkey anti-rabbit IgG (1:500) for 30 min at room temperature. After a final wash with 1 mL PBS, cells were stained with 300 μL 10 μg/mL Hoechst33342 to visualize nuclei, mounted with Vectashield mounting medium (Vector Laboratories, Inc.), and photographed under a fluorescence microscope (Eclipse 50i; Nikon). Primary antibodies were replaced with 3% BSA in PBS as the negative control.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was performed to identify sorted cells by FACS. After isolation, the total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, USA) following the manufacturer's instructions. Isolated RNA was transcribed to cDNA using a Quantitect RT kit (Qiagen) and purified using a QIAquick PCR purification kit (Qiagen). For each RT-PCR reaction, 20 ng cDNA template was used in a 25 mL reaction volume with HotStar Taq Plus (Qiagen) and the associated primers. All targets were amplified for 30 cycles. Amplification products were identified by size on a 2% agarose gel.

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Table 1. The primers used in reverse transcription polymerase chain reaction

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
UCHL1	GATTGAAGAGCTGAAGGGAC	TCAGAACCGATCCATCCTCA
CDH1	CGACGGTGTGGTTACAGTCA	GTCACCTTGGTGGACAGCTT
OCT4	AAGCTGGACAAGGAGAAGCT	TCGTTTGGCTGAACACCTTC
CD14	ACCACCCTCAGACTCCGTAAT	ATAGGTCCAGGGTGGTGAGAG
NANOS2	AATCTCGCCACGTGTACTCC	CGGGCAGTACTTGAGTGTGT
DDX4	TCAAAGGAACAGCGCCAAAC	AACGACCAGTACGCCCAATTC
ID4	TGCCTGCAGTGCGATATGAA	GGCAGGATCTCCACTTTGCT
Stra8	CTCTTCAGCAACCTCAGGAA	CATCCTCCAGGTTGAAGGAT
Tnp1	CAGAAAGTACAATGTCGACC	TTGCGATTGGCATCATCGCA
ACROSIN	CATCTTGCTGAACTCGCACT	CAACAAATCTCTCCTGCAGG
GAPDH	CTCTGGCAAAGTGGACATTG	TCTCGCTCCTGGAAGATGGT

SSC-related and spermatogenesis-genes were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR), which was carried out using SYBR Premix Ex Taq I (Tli RNaseH Plus, RR820A; Takara, China) and a LightCycler 96 instrument (Roche Diagnostics, Switzerland). The total 20 L reaction volume was composed of SYBR Premix Ex Taq II (10 μL), forward primer (1 μL, 2 μM), reverse primer (1 μL, 2 μM), cDNA (2 μL), and ddH_2O (6 μL). The qPCR reaction was conducted as follows: 95°C for 30 sec, 40 cycles at 95°C for 5 sec, and 60°C for 30 sec. Melting curve analysis was conducted following instrument-specific procedures. The relative quantitative data were calculated by the $2^{-\Delta\Delta Ct}$ method. All values were normalized to the house-keeping gene, *GAPDH*. The primers used in this study are listed in **Table 1**.

Flow cytometry analysis

Flow cytometry analysis was performed to determine the cell content of *in vitro* inducted cell mixtures, the testicular mixtures of 20-d-old piglets, and 2-mon-old pigs. Briefly, the cells were collected by gently pipetting, re-suspended in PBS, fixed in 75% alcohol, permeabilized with 0.5% Triton X-100, incubated in 40 μg/mL RNase A, stained with 10 μg/mL Hoechst33342 and analyzed by flow cytometry.

Statistics

Statistical analyses were calculated using GraphPad software (GraphPad software, USA). All the experiments were repeated for three times. Significant differences between experimental samples were determined by a one-way analysis of variance followed by Tukey's *post hoc* test. A p value of < 0.05 was considered statistically significant.

RESULTS

Immunohistochemical staining of testicular sections

Immunohistochemical staining was performed to identify the cells in testis tissue sections. In testicular tissue sections of 20-d-old piglets, UCHL1 expression was observed in SSCs located on the basement membrane of seminiferous tubules and varied in different SSCs measured by fluorescence intensity. Roughly 17% of SSCs showed low UCHL1 expression (**Fig. 1A**). The $CD14^+$ staining was observed in the cytomembrane of germ cells (**Fig. 1B**). The NSE-positive staining was also observed in seminiferous tubules (**Fig. 1C**). In testicular tissues of 2-mon-old pigs, HIF-1α was highly expressed in SSCs and spermatocytes (**Fig. 1D**), HIF-1α-positive SSCs showed strong DBA-positive staining (**Fig. 1E**, arrow), while HIF-1α-positive spermatocytes showed weak DBA-positive staining (**Fig. 1E**, triangle).

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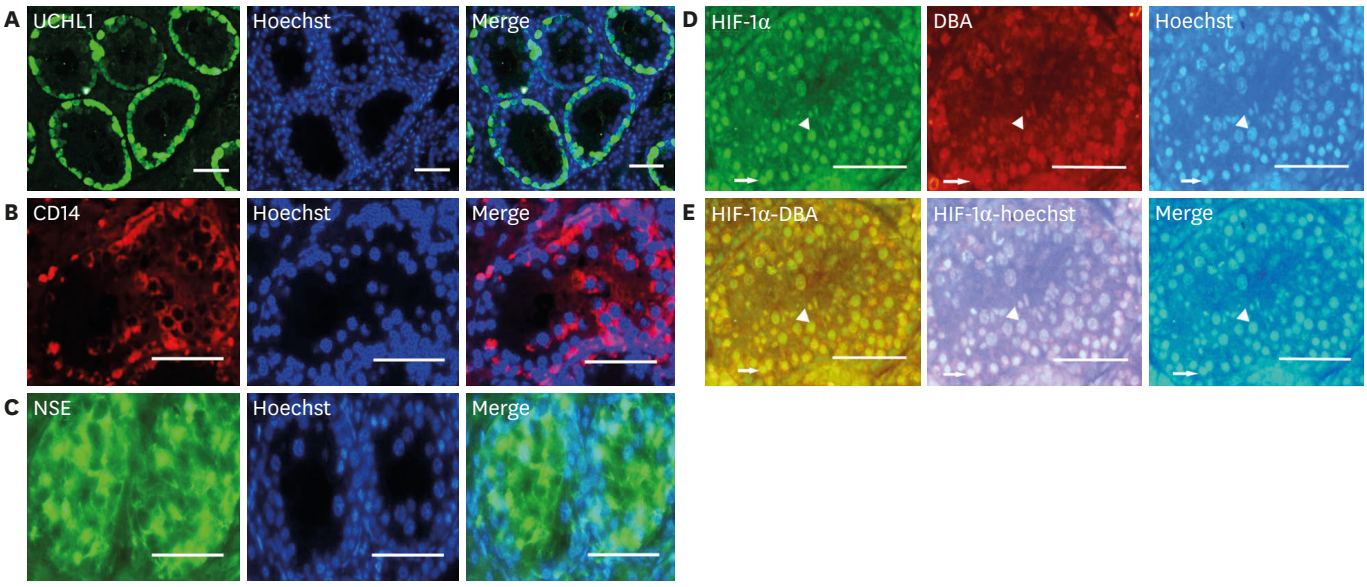

Fig. 1. In testes sections of 20-d-old piglets, UCHL1 positive-staining was observed on cytoplasm of SSCs (A), CD14 expressed in cytomembrane of SSCs (B), NSE expression was observed in seminiferous tubules (C). Double immnolabelling of testes sections of 2-mon-old pig testes was carried out by DBA and HIF-1α. HIF-1α-positive staining was observed in SSCs (D, arrow) and spermatocyte (D, triangle). HIF-1α-positive SSCs also showed DBA specific affinity (E, arrow). Nuclear counterstaining was carried out by Hoechst33342. Scale bars = 50 μm.
SSC, spermatogonial stem cell; DBA, *Dolichosbiflorus* agglutinin; HIF-1α, hypoxia inducible factor-1 alpha.

Isolation of SSCs

The SSCs used in this study were isolated from 20-d-old piglets. After Immunohistochemical staining of testicular sections, we found that CD14 was a surface marker of SSCs, which was consistent with the finds of a previous study [8]. Therefore, we hypothesized that CD14 was a marker that can be used for isolating SSCs by FACS. Immunofluorescence staining revealed that only 0.5% ± 0.1% of testicular cells expressed CD14. After isolation, sorted cells were analyzed by RT-PCR. Results revealed that the sorted cells expressed SSC specific markers such as DEAD box polypeptide 4 (*DDX4*), POU transcription factor (*Oct4*), *UCHL1*, *CD14,* and *NANOS2*; *CDH1* was not detected (**Fig. 2**). The results demonstrated that the sorted cells were SSCs.

The effects of DMOG on SSC proliferation

The effects of DMOG on SSC proliferation were measured by CCK-8 (**Fig. 3**). Results revealed that 200 μM DMOG increased the SSC proliferative ability to 32% ± 3%, and 400 μM DMOG increased the SSC proliferative ability to 60% ± 2%, when compared to the control. SSC proliferation increased to stable levels when the DMOG concentration reached 400 μM. After treatment with 800 and 1,000 μM DMOG, the cells proliferation did not significantly differ

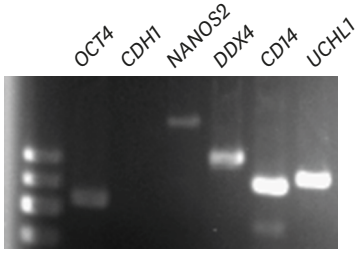

Fig. 2. *OCT4*, *NANOS2*, *DDX4*, *CD14*, and *UCHL1* genes transcription were detected. *CDH1* transcription was not detected.

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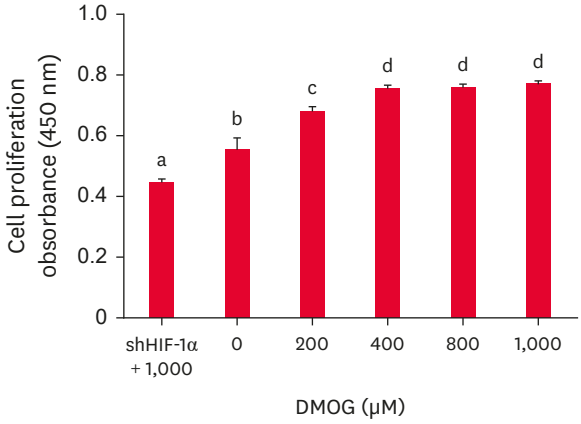

Fig. 3. Cell proliferation was evaluated using CCK-8. Different letters showed that the difference among the groups was significant, the same letters showed that the difference among the groups was not significant (p<0.05). DMOG, dimethyloxaloylglycine.

when compared to 400 μM DMOG, while the proliferation of *shHIF-1α* SSCs treated with 1,000 μM DMOG was significantly decreased ($p < 0.05$) when compared to the control.

Western blot

Western blot analysis was carried out to evaluate the effects of different concentrations of DMOG on the expression of HIF-1α in SSCs. The results revealed that DMOG increased the HIF-1α protein levels in SSCs dose dependent (**Fig. 4A**). HIF-1α expression increased by 3-, 7-, 10-, and 11-fold under 200, 400, 800, and 1,000 μM DMOG treatment. The increased HIF-1α expression was not significant when comparing 1,000 to 800 μM DMOG (**Fig. 4B**). HIF-1α protein was not detected in *shHIF-1α* SSCs, however detected in the control, which was infected with empty vector (**Fig. 4C**). The results demonstrated HIF-1α expression in *shHIF-1α* SSCs was inhibited by shRNA of *HIF-1α*.

Immunocytochemical analysis of SSCs and Sertoli cells

Immunocytochemical analysis was performed to identify the cultured SSC, and Sertoli cell, the results of which showed that the cultured cell clusters expressed SSC specific factors, such as CD14, UCHL1, and HIF-1α, and showed DBA specific binding (**Fig. 5A-D**). which demonstrated that the cell clusters were formed of SSCs. Sertoli cell specific markers, such as NSE and WT1, were detected in the cytoplasm and nucleus of the adherent cells, respectively (**Fig. 5E and F**). The results demonstrated that the adherent cells were Sertoli cells. Therefore, the in vitro cultured cells, including SSCs and Sertoli cells, remained the cell characters as in testis, and could be used for following experiments.

DMOG improved SSC-related gene expression

After treatment with different concentrations of DMOG (0, 200, 400, 800, and 1,000 μM), the SSC-related genes were detected by qRT-PCR (**Fig. 6**). The results revealed that DMOG markedly increased ($p < 0.05$) the expression levels of *UCHL1*, *CD14*, *NANOS2*, *OCT4*, *DDX4*, and *ID4* in a dose-dependent manner; the enhancement was not significant between 800 and 1,000 μM DMOG. Thus, the results indicated that 800 μM DMOG was the most appropriate concentration for maintaining the stemness of SSCs. Therefore, 800 μM DMOG was selected for subsequent experiments. The relative expression levels of *UCHL1*, *CD14*, *NANOS2*, *OCT4*, *DDX4*, and *ID4* decreased in shHIF-1α SSCs cultured in the medium adding 1,000 μM DMOG

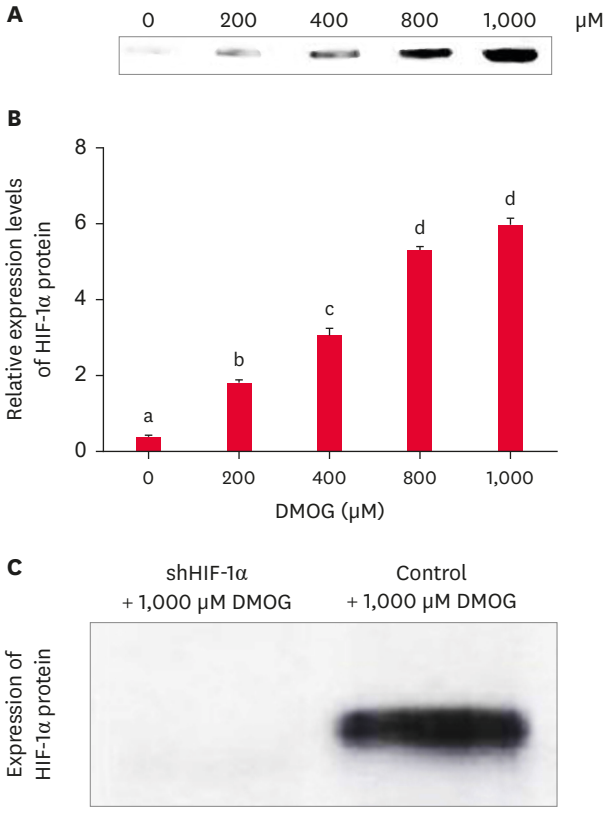

Fig. 4. The results of western blot analysis showed that DMOG significantly increased the protein levels of HIF-1α (A). The results of statistics suggested that the increase expression of HIF-1α in SSCs by DMOG was in a dose-dependent manner, there was no notable increase between the groups adding 800 μM and 1,000 μM DMOG, respectively (B). Different letters showed that the difference among the groups was significant, the same letters showed that the difference among the groups was not significant. HIF-1α protein was detected in *shHIF-1α* SSCs, and detected in SSCs transfected with empty vector (C) ($p<0.05$).
DMOG, dimethyloxaloylglycine; HIF-1α, hypoxia inducible factor-1alpha; SSC, spermatogonial stem cell.

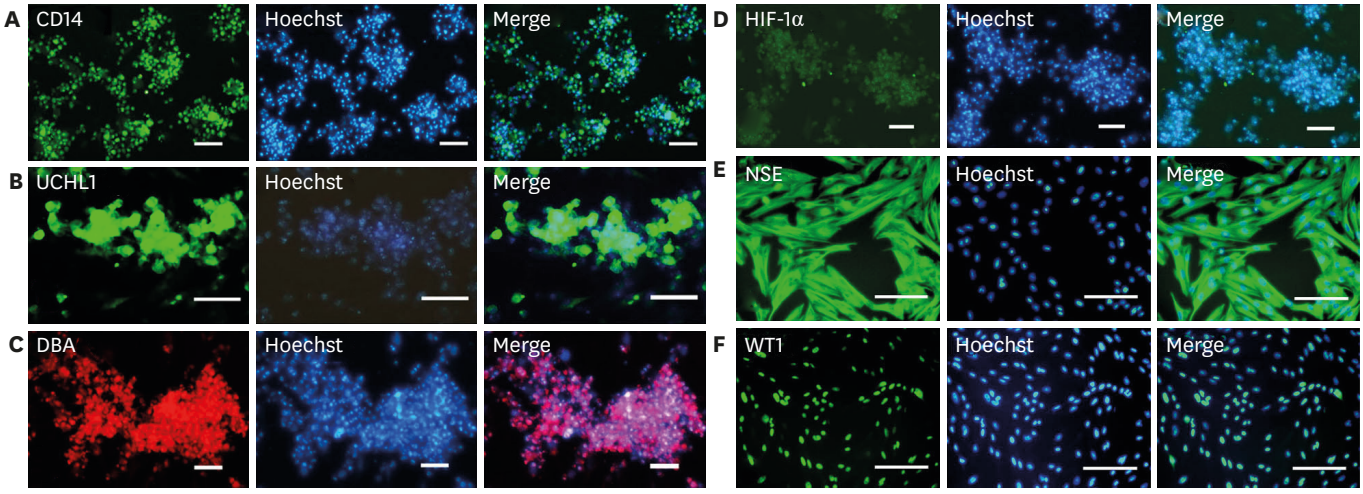

Fig. 5. The results showed the SSCs were positive staining for CD14 (A), UCHL1 (B), DBA (C), and HIF-1a (D). The Sertoli cells expressed NSE in cytoplasm (E), and WT1 in nuclear (F). Scale bar = 50 μm.
DBA, *Dolichosbiflorus* agglutinin; HIF-1α, hypoxia inducible factor-1alpha.

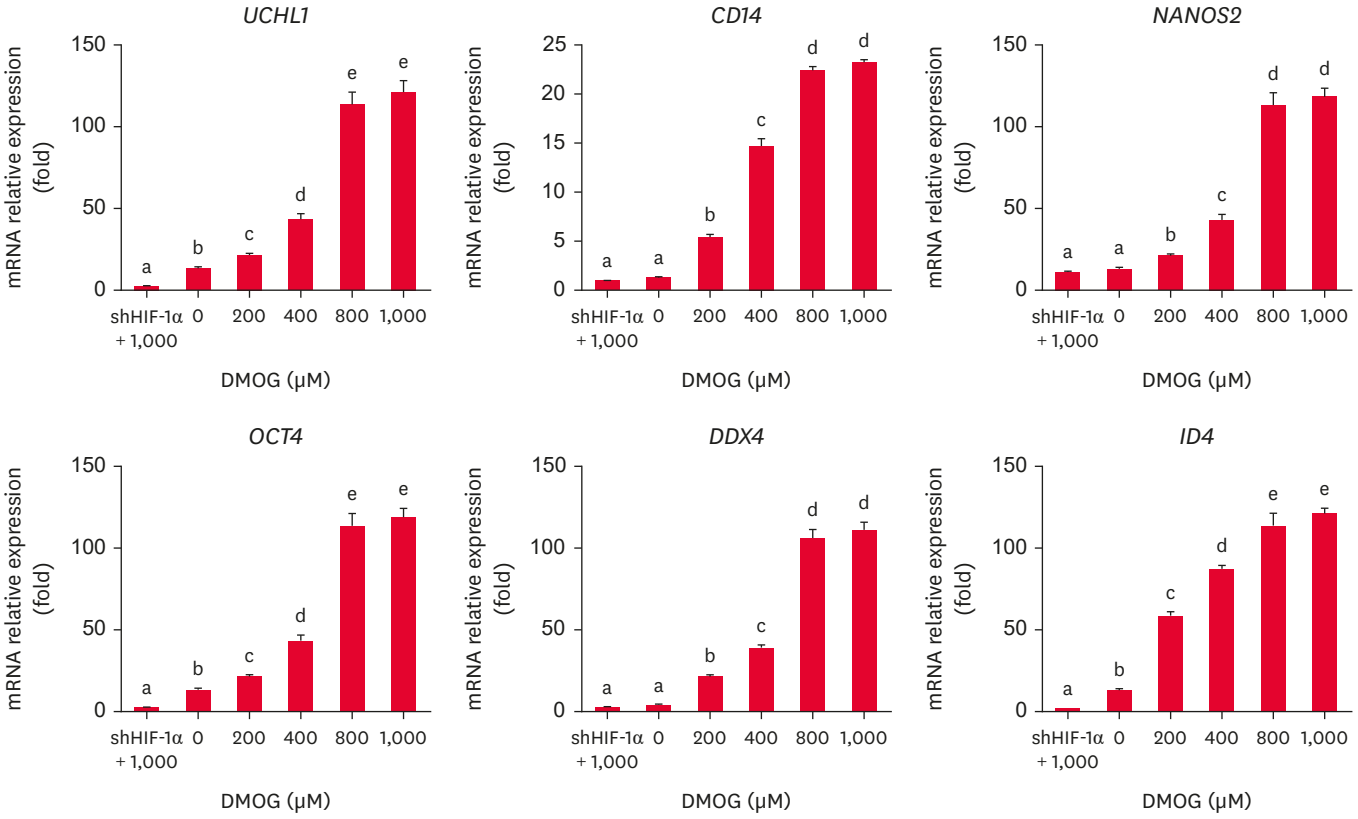

Fig. 6. Expression levels of *UCHL1*, *CD14*, *NANOS2*, *OCT4*, *DDX4*, and *ID4* in SSCs were enhanced in response to DMOG in a dose-dependent manner. However, the relative expression levels of these genes decreased in shHIF-1α SSCs exposed to 1,000 μM DMOG. Different letters showed that the difference among the groups was significant, the same letters showed that the difference among the groups was not significant ($p<0.05$).
DMOG, dimethyloxaloylglycine; SSC, spermatogonial stem cell.

when compared to the control, suggesting that the increased transcription of these genes can be attributed to the overexpression of HIF-1α stabilized by DMOG.

DMOG improved the spermatogenesis potential of SSCs

Next, we determined the effects of 800 μM DMOG on the differentiation potential of SSCs and determined the genes related to spermatogenesis by qRT-PCR (**Fig. 7**). The results showed that 800 μM DMOG significantly increased ($p < 0.05$) the transcript levels of *Stra 8*, *Tnp1*, and *ACROSIN* in SSCs when compared to the control. The transcript levels of *Stra 8* and *Tnp1* decreased in shHIF-1α SSCs treated with 800 μM DMOG, while *ACROSIN* significantly decreased ($p < 0.05$) when compared to the control. The results of immunocytochemical staining showed that the inducted cells expressed Stra 8 and Scp3 protein in the cytoplasm and nucleus, respectively (**Fig. 8**). Flow cytometry analysis showed that the haploid peak was not observed in the testicular cell mixture of 20-d-old piglets (**Fig. 9A**), but was observed in adult testicular cell mixture (**Fig. 9B**), the haploid peak appeared in the DMOG-untreated SSCs (**Fig. 9C**). The populations of haploid cells significantly increased ($p < 0.05$) in DMOG-treated SSCs (**Fig. 9D**), compared to those in DMOG-untreated SSCs.

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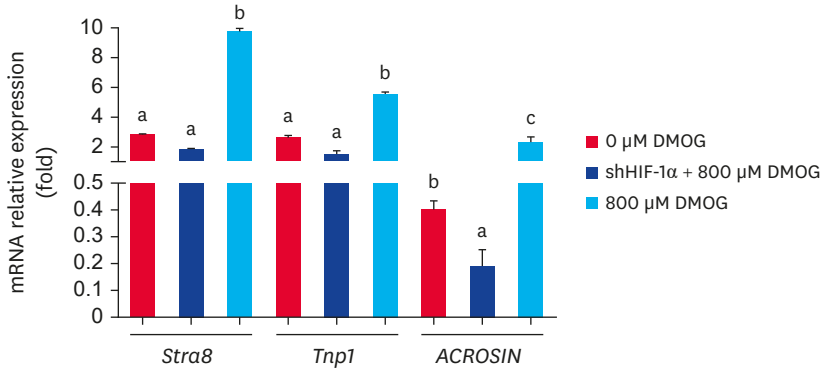

Fig. 7. The expression levels of *Stra8*, *Tnp1*, and *ACROSIN* were significantly enhanced in SSCs in response to 800 μM DMOG, however the expression levels of these genes decreased in shHIF-1α SSCs treated with 800 μM DMOG. Different letters showed that the difference among the groups was significant, the same letters showed that the difference among the groups was not significant ($p<0.05$).
SSC, spermatogonial stem cell; DMOG, dimethyloxaloylglycine.

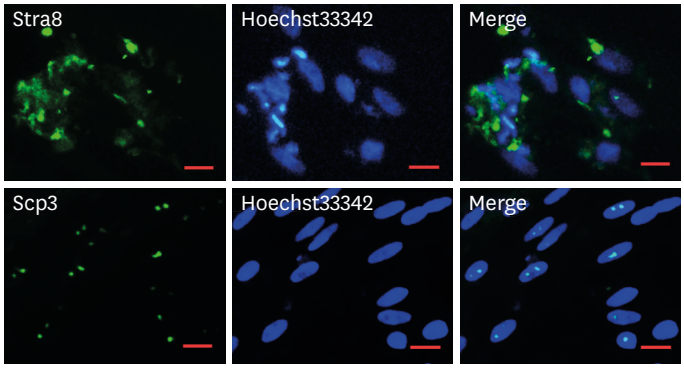

Fig. 8. The inducted cells expressed Stra8 in cytoplasm and Scp3 in nuclear. Nuclear counterstaining was carried out by hoechst33342. Scale bars = 50 μm.

DISCUSSION

SSC is the unique kind of cell that can transmit genetic information to next generation. Genetic manipulation of SSCs and differentiation of the transgenic SSCs into spermatoblast is a potential strategy to produce transgenic animal. In testes, physiological hypoxia maintains SSC self-renewal and spermatogenesis. To date, the SSCs had been mostly cultured in ambient air, and are unlikely to be optimally maintained for their spermatogenesis potential. A previous study suggested that the derivation of novel stem cell populations could be enhanced by culturing in the range of 3%–5% O_2 [9]. Thus, we speculated that moderate hypoxia was crucial for the *in vitro* culturing of SSCs.

DMOG is a proly1-4-hydroxylase inhibitor that upregulates HIF-1α protein levels under normoxic condition. HIF-1α activates a broad array of genes and therefore modulates cell proliferation, differentiation, and pluripotency. In this study, we demonstrated that HIF-1α was expressed in SSCs and spermatocyte in Bama minipig testes, which suggests that HIF-1α plays an important role in SSC proliferation and differentiation.

Enriching SSCs is the first step toward establishing SSC cell lines. Differential plating is a widely used technique for SSC enrichment, however, the SSCs enriched by this method can

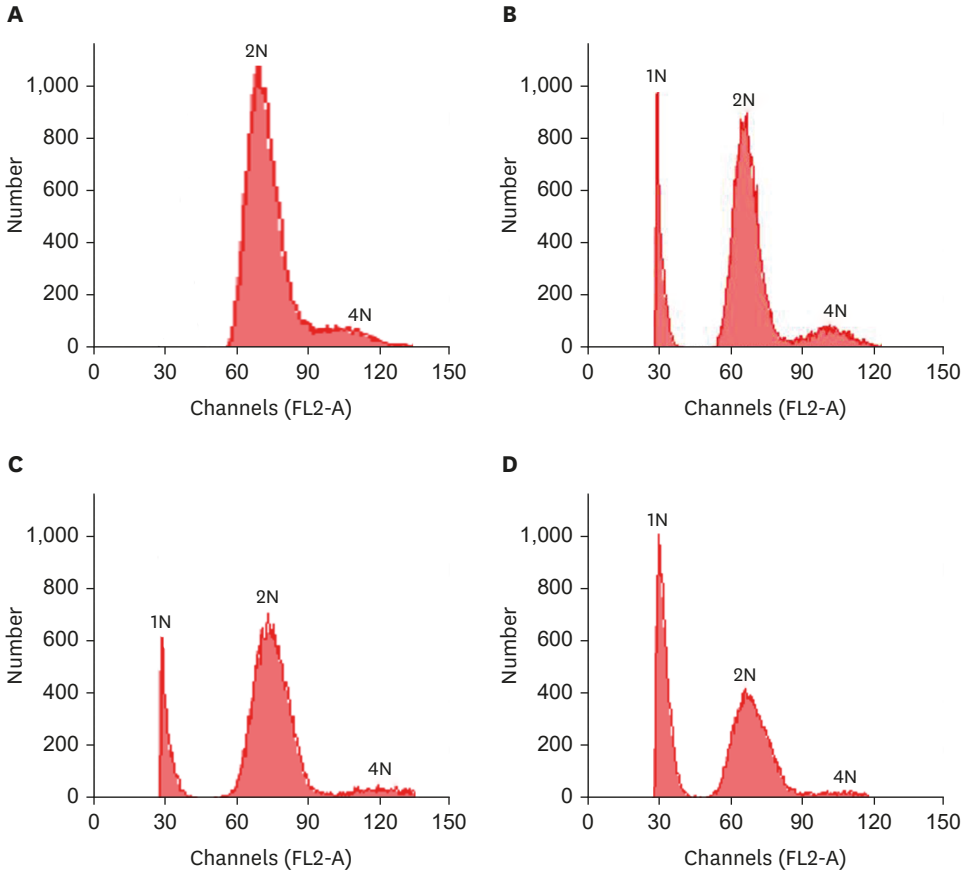

Fig. 9. DNA content of the testicular cell mixture from piglets was measured by flow cytometry (A). Haploid peak (1N) was not observed in the cell mixture, diploid peak (2N) and tetraploid peak (4N) were observed. DNA content of the testicular cell mixture from 2-mon-old pig was determined by flow cytometry (B). Haploid peak (1N), diploid peak (2N), and tetraploid peak (4N) were observed. DMOG-untreated SSCs were inducted and the cell content was measured (C). DMOG-treated SSCs were inducted and the cell content was measured, the populations of haploid cells were significantly increased in DMOG-treated SSCs, when compared to DMOG-untreated SSCs (D).
SSC, spermatogonial stem cell; DMOG, dimethyloxaloylglycine.

be contaminated with testis somatic cells. Recently, CD14 was found to be a membrane marker in pig SSCs [8], and we confirmed this result in the present study, which made it possible to isolate pig SSCs by FACS. In this study, $CD14^+$ SSCs were isolated using FACS, which guaranteed the high purity of SSCs.

It had been demonstrated that DMOG had no obvious cytotoxic effects on adipose-derived stem cells [10]. Therefore, DMOG was chose in this study to mimic hypoxia microenvironment of seminiferous tubules. We found that DMOG could enhance SSC proliferation, this result was in consistent with the findings of a previous study [11]. However, the proliferation of *shHIF-1α* SSCs decreased when the cells cultured in the medium adding DMOG, which indicated that the SSC proliferation was attributed to accumulation of HIF-1α protein.

A number of SSC-specific genes had been found so far. *UCHL1* was found to be a conservative SSC marker of different species [12-15]. *DDX4* is conserved in the germ cell development of many species [16]. *NANOS2* is an intrinsic regulator that maintains undifferentiated state of SSCs in mice [17]. *ID4*, a key regulator of mammary stem cell self-renewal, was found to be a marker of SSCs in mice [18]. *OCT4*, a pluripotency marker, was also detected in SSCs [19].

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Recently, *CD14* had been discovered to be a membrane marker of pig SSC. In the present study, the transcriptional levels of aforesaid genes were significantly improved in DMOG-treated SSCs, the results demonstrated that DMOG could enhance the stemness activity of SSCs. However, the transcriptional levels of these genes were inhibited in DMOG-treated *shHIF-1α* SSCs, indicating that DMOG enhanced the expression of these genes by activating *HIF-1α* expression.

In the present study, we demonstrated that the SSC proliferation ability increased to stable levels when the DMOG concentration reached 400 μM, while the expression of SSC-related genes increased to stable levels under 800 μM DMOG. This result indicated that the optimal concentrations of DMOG for activating different signaling pathways varied. Thus, we speculated that the higher expression levels of SSC-related genes reflected the enhanced stemness activity of SSCs. Therefore, 800 μM of DMOG was selected for the differentiation experiment, the results of which revealed that 800 μM DMOG could improve the transcriptional levels of *Stra8*, *Tnp1*, and *ACROSIN*. These 3 genes were respectively expressed in early, intermediate, and late stages of spermatogenesis. *Stra8* is a meiosis gatekeeper [20]. *Tnp1* is a post-meiotic gene and participates in nuclear transition [21]. *ACROSIN* is expressed in the head of elongated sperm. Upregulation of these 3 genes indicated that DMOG improved SSC spermatogenesis capability, and the cell content analysis also confirmed this result. However, the transcriptional levels of these 3 genes decreased when shHIF-1α SSCs were inducted in the medium adding 800 μM DMOG, which indicated that DMOG enhanced spermatogenesis capability was attributed to HIF-1α protein.

In conclusion, we demonstrated that DMOG increased the expression of SSC-marker genes and improved the spermatogenesis capability of SSCs by stabilizing HIF-1α. This provides evidence for further applications of DMOG in SSC cultivation and differentiation.

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REFERENCES

1. Kastelic JP, Rizzoto G, Thundathil J. Review: Testicular vascular cone development and its association with scrotal thermoregulation, semen quality and sperm production in bulls. Animal. 2018;12(s1):s133-s141.
PUBMED | CROSSREF
2. Ezashi T, Das P, Roberts RM. Low O_2 tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci U S A. 2005;102(13):4783-4788.
PUBMED | CROSSREF
3. Francis KR, Wei L. Human embryonic stem cell neural differentiation and enhanced cell survival promoted by hypoxic preconditioning. Cell Death Dis. 2010;1(2):e22.
PUBMED | CROSSREF
4. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. Hypoxia-inducible factor (HIF-1)α: its protein stability and biological functions. Exp Mol Med. 2004;36(1):1-12.
PUBMED | CROSSREF
5. Takahashi N, Davy PM, Gardner LH, Mathews J, Yamazaki Y, Allsopp RC. Hypoxia inducible factor 1 alpha is expressed in germ cells throughout the murine life cycle. PLoS One. 2016;11(5):e0154309.
PUBMED | CROSSREF

6. Tang X, Chang C, Hao M, Chen M, Woodley DT, Schönthal AH, et al. Heat shock protein-90alpha (Hsp90α) stabilizes hypoxia-inducible factor-1α (HIF-1α) in support of spermatogenesis and tumorigenesis. Cancer Gene Ther. 2021;28(9):1058-1070.
PUBMED | CROSSREF
7. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science. 2001;292(5516):468-472.
PUBMED | CROSSREF
8. Park HJ, Lee WY, Park C, Hong K, Song H. CD14 is a unique membrane marker of porcine spermatogonial stem cells, regulating their differentiation. Sci Rep. 2019;9(1):9980.
PUBMED | CROSSREF
9. Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol. 2008;9(4):285-296.
PUBMED | CROSSREF
10. Ding H, Gao YS, Wang Y, Hu C, Sun Y, Zhang C. Dimethyloxaloylglycine increases the bone healing capacity of adipose-derived stem cells by promoting osteogenic differentiation and angiogenic potential. Stem Cells Dev. 2014;23(9):990-1000.
PUBMED | CROSSREF
11. Wang J, Xue X, Fan K, Liu Q, Zhang S, Peng M, et al. Moderate hypoxia modulates ABCG2 to promote the proliferation of mouse spermatogonial stem cells by maintaining mild ROS levels. Theriogenology. 2020;145:149-157.
PUBMED | CROSSREF
12. Vansandt LM, Livesay JL, Dickson MJ, Li L, Pukazhenthi BS, Keefer CL. Conservation of spermatogonial stem cell marker expression in undifferentiated felid spermatogonia. Theriogenology. 2016;86(4):1022-1035.e3.
PUBMED | CROSSREF
13. Sharma A, Shah SM, Tiwari M, Roshan M, Singh MK, Singla SK, et al. Propagation of goat putative spermatogonial stem cells under growth factors defined serum-free culture conditions. Cytotechnology. 2020;72(3):489-497.
PUBMED | CROSSREF
14. Valli H, Sukhwani M, Dovey SL, Peters KA, Donohue J, Castro CA, et al. Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. Fertil Steril. 2014;102(2):566-580.e7.
PUBMED | CROSSREF
15. Zhao H, Li T, Yang H, Mehmood MU, Lu Y, Liang X, et al. The effects of growth factors on proliferation of spermatogonial stem cells from Guangxi Bama mini-pig. Reprod Domest Anim. 2019;54(12):1574-1582.
PUBMED | CROSSREF
16. Gassei K, Sheng Y, Fayomi A, Mital P, Sukhwani M, Lin CC, et al. DDX4-EGFP transgenic rat model for the study of germline development and spermatogenesis. Biol Reprod. 2017;96(3):707-719.
PUBMED | CROSSREF
17. Zhou Z, Shirakawa T, Ohbo K, Sada A, Wu Q, Hasegawa K, et al. RNA binding protein Nanos2 organizes post-transcriptional buffering system to retain primitive state of mouse spermatogonial stem cells. Dev Cell. 2015;34(1):96-107.
PUBMED | CROSSREF
18. Sun F, Xu Q, Zhao D, Degui Chen C. Id4 marks spermatogonial stem cells in the mouse testis. Sci Rep. 2015;5(1):17594.
PUBMED | CROSSREF
19. Cai H, Jiang Y, Zhang S, Cai NN, Zhu WQ, Yang R, et al. Culture bovine prospermatogonia with 2i medium. Andrologia. 2021;53(6):e14056.
PUBMED | CROSSREF
20. Niu C, Guo J, Shen X, Ma S, Xia M, Xia J, et al. Meiotic gatekeeper STRA8 regulates cell cycle by interacting with SETD8 during spermatogenesis. J Cell Mol Med. 2020;24(7):4194-4211.
PUBMED | CROSSREF
21. Alrahel A, Movahedin M, Mazaheri Z, Amidi F. Study of Tnp1, Tekt1, and Plzf genes expression during an *in vitro* three-dimensional neonatal male mice testis culture. Iran Biomed J. 2018;22(4):258-263.
PUBMED | CROSSREF