



# POSTN Promotes the Proliferation of Spermatogonial Cells by Activating the Wnt/ $\beta$ -Catenin Signaling Pathway

Caihong Li<sup>1,2</sup> · Dongkai Cheng<sup>2</sup> · Peng Xu<sup>2</sup> · Hongguang Nie<sup>1</sup> · Tao Zhang<sup>1</sup> · Xining Pang<sup>1,3</sup> 

Received: 30 December 2020 / Accepted: 22 April 2021 / Published online: 6 May 2021  
© Society for Reproductive Investigation 2021

## Abstract

The self-renewal of spermatogonial cells (SCs) provides the foundation for life-long spermatogenesis. To date, only a few growth factors have been used for the culture of SCs in vitro, and how to enhance proliferation capacity of SCs in vitro needs further research. This study aimed to explore the effects of periostin (POSTN) on the proliferation of human SCs. GC-1 spg cells were cultured in a medium with POSTN, cell proliferation was evaluated by MTS analysis and EdU assay, and the Wnt/ $\beta$ -catenin signaling pathway was examined. Thereafter, the proliferations of human SC were detected using immunofluorescence and RT-PCR. In this study, we found that CM secreted by human amniotic mesenchymal stem cells (hAMSCs) could enhance the proliferation capacity of mouse GC-1 spg cells. Label-free mass spectrometry and ELISA analysis demonstrated that high level of POSTN was secreted by hAMSCs. MTS and EdU staining showed that POSTN increased GC-1 spg cell proliferation, whereas CM from POSTN-silenced hAMSCs suppressed cell proliferation capacity. Then POSTN was found to activate the Wnt/ $\beta$ -catenin signaling pathway to regulate the proliferation of GC-1 spg cells. XAV-939, a Wnt/ $\beta$ -catenin inhibitor, partially reversed the effects of POSTN on GC-1 spg cell proliferation. We then analyzed human SCs and found that POSTN promoted human SC proliferation in vitro. These findings provide insights regarding the role of POSTN in regulating SC proliferation via the Wnt/ $\beta$ -catenin signaling pathway and suggest that POSTN may serve as a cytokine for male infertility therapy.

**Keywords** POSTN · hAMSCs · Spermatogonial cells · Proliferation

---

✉ Tao Zhang  
cmuzhangtao@126.com

✉ Xining Pang  
pangxining@126.com

<sup>1</sup> Department of Stem Cells and Regenerative Medicine, Shenyang Key Laboratory of Stem Cell and Regenerative Medicine, China Medical University, Shenyang 110013, Liaoning, China

<sup>2</sup> Center for Reproductive Medicine, Assisted Reproductive Technology Laboratory, Shenyang Jinghua Hospital, Shenyang 110005, Liaoning, China

<sup>3</sup> Shenyang Amnion Bioengineering and Technology R & D Center, Shenyang Liaoning Amnion Stem Cell and Regenerative Medicine Professional Technology Innovation Platform, Liaoning Human Amniotic Membrane Biological Dressing Stem Cell and Regenerative Medicine Engineering Research Center, Shenyang 110015, Liaoning, China

## Introduction

Spermatogenesis is a complex process of spermatogonial cell (SC) differentiation that is divided into three phases: mitosis, meiosis, and postmeiosis. SCs are undifferentiated unipotent stem cells located near the basement membrane of seminiferous tubules [1]. They show unique cell characteristics as stem cells and germ cells after being isolated from the testis and cultured in vitro [2]. Human SCs have great potential for cell-based, autologous organ regeneration therapy for various conditions, eliminating the need to use human embryonic stem cells (ESCs) [3]. However, the number of SCs in the human body is small; they might represent 0.03% of all germ cells in the body [4]. Currently, feeders are commonly used for the culture of SCs, but unknown factors secreted by feeders make the culture conditions difficult to control [5]. In addition, due to a lack of knowledge regarding culture conditions and the factors regulating and maintaining SCs in culture, no germ cell (GC) line has been established in mammalian species other

than mice [2]. Therefore, finding suitable factors for the enrichment of human SCs is important for their successful clinical application.

Mesenchymal stem cells (MSCs) have been shown to secrete various autocrine/paracrine factors, including growth factors and cytokines, which may be largely responsible for these cells' therapeutic effects [6]. Previous studies have shown that MSC-conditioned media can improve the proliferation of endothelial cells and keratinocytes [7]. To date, the secretion proteomes of various MSCs have been assessed [8]. Human amniotic membrane is routinely discarded after delivery as biological waste, which makes it an attractive source of amniotic MSCs (hAMSCs). In addition, it has many other advantages, such as anti-inflammatory, antimicrobial, and antiangiogenic characteristics and low immunogenicity. hAMSC-secreted factors are used in cell-free therapies for acute brain injury. In our previous work, we found many proteins related to cell proliferation in the conditioned medium of hAMSCs (hAMSC-CM) [9, 10]. However, the effect of hAMSC-CM on the proliferation of SCs is unclear.

POSTN, which is a 90 kDa extracellular protein of the fasciclin family that modulates cell-to-matrix interactions, is expressed in multiple compartments of the body, especially the aorta, stomach, lower gastrointestinal tract, placenta, and uterus [11]. POSTN is incorporated into the extracellular matrix and can exert its functions by signaling via cell-surface integrin receptors to promote cell adhesion, migration, and proliferation [12, 13]. High expression of POSTN is commonly detected in solid tumors, and POSTN expression correlates with tumor progression [14, 15]. This protein plays a key role in promoting the adhesion, migration, and proliferation of cancer cells. In the present study, we found that POSTN is expressed in hAMSC-CM. However, the role of POSTN in SC proliferation and the underlying mechanisms remain unknown.

In this study, we analyzed the effects of hAMSC-CM on GC-1 spg cells and identified high expression of POSTN in the proteomes of hAMSC-CM. Furthermore, we found that POSTN regulates GC-1 spg cell proliferation through the Wnt/ $\beta$ -catenin-mediated signaling pathway, activating cyclin-D1 transcription. The findings provide a better understanding of the mechanisms of SC proliferation and suggest that POSTN may serve as an attractive new cytokine for promoting spermatogenesis *in vitro*.

## Materials and Methods

### Cell Lines and Reagents

Mouse GC-1 spg (spermatogonia) cell lines were originally purchased from the American Type Culture Collection (Manassas, VA, USA). Recombinant POSTN was obtained from Sino Biological (Beijing, China). Recombinant THBS1

was obtained from OriGene Technologies (Rockville, MD, USA). XAV939 was purchased from MedChemExpress (Shanghai, China)

### Ethical Approval

All of the studies were approved by the ethics committees of The First Hospital of China Medical University and Reproductive Medicine Center of Shenyang Jinghua Hospital. Three obstructive azoospermia (OA) patients with normal spermatogenesis were recruited from Shenyang Jinghua Hospital. The written informed consent was obtained from all participants. All placenta samples (from cesarean section) were obtained from The First Hospital of China Medical University, and written informed consent was obtained from the affected individuals.

### Human Spermatogonial Cells Isolation

The testicular tissue samples were obtained from OA patients with normal spermatogenesis according to the results of testicular biopsies. Biopsies were cut into small pieces of  $\times 1$  mm and subjected to collagenase type IV (2 mg/mL) (Sigma, St. Louis, MO, USA), DNase (8 mg/mL) (Sigma), and hyaluronidase (2 mg/mL) (Sigma) in a total volume of 4 mL for 20 mins in a 32 °C water bath shaker. Cells were precipitated by centrifugation (300 g, 10 mins) and suspended with 4 mL of TrypLE Select (Thermo Fisher Scientific, Waltham, MA, USA) for 10 mins in a water bath shaker at 32 °C. Cell suspension was centrifuged (300 g, 10 mins), and the precipitated cells were suspended in 200  $\mu$ L culture medium. Cells were cultured ( $4-5 \times 10^4$  cells/well/500  $\mu$ L) in DMEM/F12 medium (5% CO<sub>2</sub>, 37 °C) containing 10% fetal bovine serum (FBS, Hyclone, Marlborough, MA, USA) supplemented with different factors such as human rEGF (re-combinant epidermal growth factor) (20 ng/mL) (Biolegend, San Diego, CA, USA), human rGDNF (glial cell line-derived nerve growth factor) (10 ng/mL) (Biolegend), human rLIF (leukemia inhibitory factor) (10 ng/mL) (Biolegend), and human r-bFGF (basic fibroblast growth factor) (10 ng/mL) (Biolegend).

### Preparation of Human Amnion Mesenchymal Stem Cell-Derived Conditioned Medium

Amnion membranes were mechanically peeled from chorions of placentas obtained from women with an uncomplicated cesarean section. The human amnion mesenchymal stem cells (hAMSCs) were isolated and induced to osteogenic and adipogenic differentiation as previously reported [16]. hAMSCs conditioned medium was collected by culturing subconfluent hAMSCs in serum-free Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) for 48 h. The conditioned

medium was filtered using a 0.45- $\mu\text{m}$  filter and stored at  $-80^{\circ}\text{C}$  until use.

### Cell Proliferation Assay

Cell proliferation analysis was performed using EdU assay and MTS analysis. Cell proliferation assay was performed using the BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 647 (Beyotime). GC-1 spg cells were seeded in 6-well plates at a density of  $2.5 \times 10^5/\text{well}$  and were treated as described. After 48 h, cells were added EdU reagent for 2 h and then stained for EdU incorporation by immunostaining using an EdU staining kit (Beyotime, Shanghai, China). Hoechst 33342 was used to stain cell nuclei for 30 mins. Images were captured using Zeiss Observer.A1 microscope. The percentage of EdU-positive cells was defined as the proliferation rate. For MTS assay,  $2.5 \times 10^4$  GC-1 spg cells per well were seeded in 96-well plates and treated as described. Twenty microliters of MTS solution (Promega, Madison, WI, USA) was added at 24, 48, or 72 h after adding conditioned media and further incubated the cells at  $37^{\circ}\text{C}$  for 2 h. The absorbance was measured at a wavelength of 490 nm in a colorimeter, and their proliferation rate was quantified.

### Western Blot Analysis

We separated 20  $\mu\text{g}$  total protein lysate on a 10% polyacrylamide gel. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% skim milk for 1 h by rocking gently. Then, the membranes were incubated overnight at  $4^{\circ}\text{C}$  with antibodies against  $\beta$ -catenin, GSK3 $\beta$ , and GAPDH (Proteintech, Wuhan, China) and cyclin-D1 (Abcam, Cambridge, MA, USA). The membranes were then washed three times with PBST and incubated with the secondary horseradish-peroxidase-conjugated anti-rabbit antibody (Boster, Wuhan, China) at room temperature for 2 h. The protein bands were developed and visualized using enhanced chemiluminescent imaging system (Tanon, Shanghai, China).

### ELISA Assay

We measured the levels of POSTN and THBS1 proteins in the conditioned medium using ELISA assay with commercially available kits (MLBIO, Shanghai, China). Briefly, 50  $\mu\text{l}$  of conditioned medium was incubated with the detection antibody in the ELISA plate for 1 h at  $37^{\circ}\text{C}$ . After washing the plate, we added streptavidin and incubated the samples for 30 mins at  $37^{\circ}\text{C}$ . Then, after washing, we added the tetramethylbenzidine (TMB) substrate to the samples, incubated the plate for 10 mins at  $37^{\circ}\text{C}$ , and added the stop buffer. The samples were analyzed by quantifying the colorimetric reaction at 450 nm in a colorimeter.

### Immunofluorescence

Cells were fixed with 4% formaldehyde at room temperature for 20 mins and then treated with 0.3% Triton X-100 in PBS for 10 mins. After blocking with 5% BSA (Amresco, Houston, Texas, USA) for 1 h and cells were incubated with primary antibodies, including anti-ki67 (1:500, Abcam) and anti-OCT4 (1:500, Proteintech) at  $4^{\circ}\text{C}$  overnight. Antibodies binding were detected by Alexa Fluor-conjugated second antibody (Thermo Fisher Scientific) at room temperature for 1 h. DAPI was used for staining cell nuclei. The images were captured using the fluorescence microscope (Zeiss, Heidenheim, German).

### RNA Interference (RNAi) of POSTN

The POSTN-siRNAs and control siRNA were synthesized by GenePharma (Suzhou, China), and the sequences of siRNAs were as follows: si-POSTN-1, 5'-CCAUGGGAACCAGAUUGCAACAUAU-3'; si-POSTN-2, 5'-GGUCCUAAUCCUGAUUCTT-3'; si-NC, 5'-UUCUCCGACGUGUCACGUTT-3'. Cells were seeded at  $2 \times 10^5/\text{well}$  density to 6-well plates, and transfection was performed using lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instruction. The transfected cells were harvested at 48 h for mRNA analysis.

### RNA Extraction, RT-PCR, and Quantitative Real-Time PCR

Total RNA was extracted using RNAiso Plus reagent (Takara, Kusatsu, Japan), and the concentrations and quality of isolated RNA were determined by Nanodrop (Thermo Fisher Scientific). Reverse transcription (RT) was conducted by the GoScript Reverse Transcription Kit (Promega) according to the manufacturer's instruction. The primers of genes for RT-PCR and real-time PCR were listed in Table 1.

The PCR reactions were carried out using SapphireAmp Fast PCR Master Mix (Takara). The products were analyzed by 2% agarose gels and detected by automatic digital gel image analysis system (Tanon, Shanghai, China).

Real-time PCR reactions were performed using SYBR Premix Ex TaqII (Takara) according to the manufacturer's instructions. The relative expression levels of indicated genes were analyzed via the using the  $2^{-\Delta\Delta\text{CT}}$  method for relative quantification of each target gene, normalized to the house-keeper control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Cell Cycle Assay

Cells were harvested and fixed in 70% ice-cold ethanol for 24 h at  $4^{\circ}\text{C}$ . The cells were incubated with propidium iodide

**Table 1** Primer sequences of genes used for RT-PCR and quantitative real-time PCR

Genes primer sequences products	Primer sequences	Product size (bp)
Primer sequences of genes used for RT-PCR		
OCT4	F: 5'-AATTTGCCAAGCTCCTGAAG-3' R: 5'-CGTTTGGCTGAATACCTTCC-3'	104
PLZF	F: 5'-AAGGCTGCAGTGGACA-3' R: 5'-CTGCATCATCATCTCCGTCTT-3'	144
VASA	F: 5'-TGGAAACAGAGATGCTGGTG-3' R: 5'-CCTCTGTTCCGTGTTGGATT-3'	183
SALL4	F: 5'-TCCCAAACACCAGTTTCCTC-3' R: 5'-TGTGTCTGCATTGCTCCTTC-3'	90
ACTB	F: 5'-CGGGAAATCGTGCGTGAC-3' R: 5'-TGGAAGGTGGACAGCGAGG-3'	434
Primer sequences of genes used for quantitative real-time PCR		
POSTN	F: 5'-CTCATAGTCGTATCAGGGGTCG-3' R: 5'-ACACAGTCGTTTTCTGTCCAC-3'	138
GAPDH	F: 5'-CAACTTGGTATCGTGAAGG-3' R: 5'-GCCATCACGCCACAGTTTC-3'	101

in the dark at 37 °C for 30 mins. The cell cycle was analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

### Statistical Analysis

All data were presented as mean  $\pm$  SD. Statistical analyses were performed by Student's *t*-test or one-way ANOVA using GraphPad Prism 9 Software. *p*-values less than 0.05 ( $p < 0.05$ ) were considered to indicate a significant difference.

## Results

### Identification of hAMSCs

The hAMSCs exhibited spindle-shaped morphology upon culture (Fig. 1b). FACS analysis showed that the hAMSCs expressed mesenchymal markers CD73, CD44, and CD29 but did not express HLA-DR, CD31, or CD45 (Fig. 1a). Moreover, the cells had the potential to differentiate into osteocytes and adipocytes (Fig. 1c, d).

### Conditioned Media From hAMSCs Promoted the Proliferation of GC-1 spg Cells

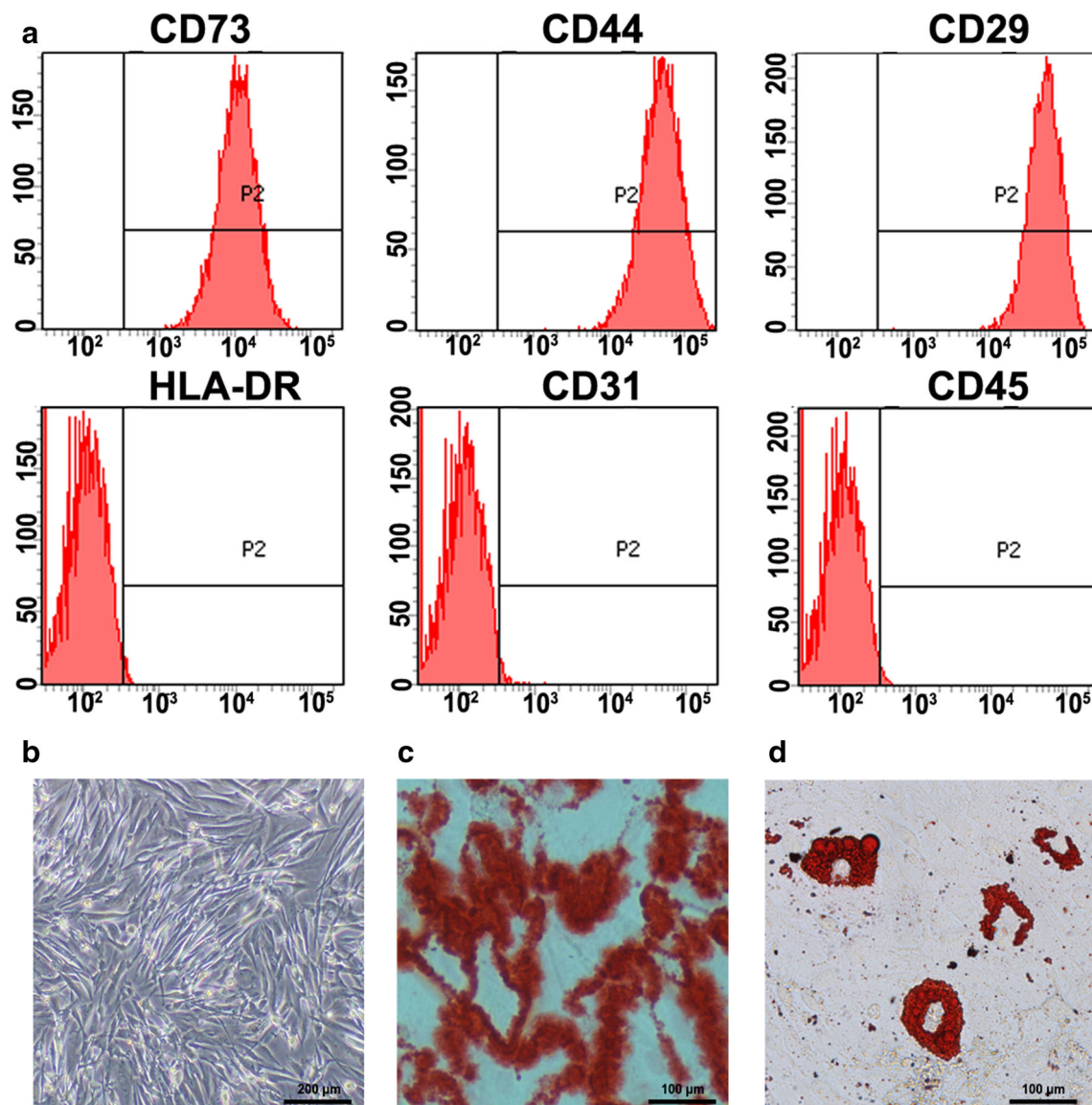
We analyzed the proliferation characteristics of GC-1 spg cells grown in 0%, 25%, 75%, or 100% hAMSC-CM using MTS analysis (with the cells cultured in 0% hAMSC-CM serving as the control group). The MTS results showed that cell proliferation was significantly increased in the groups treated with 75% and 100% hAMSC-CM compared with the control group

(Fig. 2a). Then, EdU assay was used to study the effect of hAMSC-CM on cell proliferation. The data showed that the percentages of EdU-positive cells in the 75% and 100% hAMSC-CM groups were significantly higher than the percentage in the control group (Fig. 2b, c).

### POSTN Promotes the Proliferation of GC-1 spg Cells

We performed mass spectrometry to analyze the proteins secreted in hAMSC-CM previously in our laboratory [10]. The enrichment analysis showed that hAMSCs-CM contained high levels of proteins related to cell proliferation. We then performed ELISA to determine the levels of POSTN and THBS1; the results indicated that the concentrations of POSTN and THBS1 proteins were respectively 75 and 135 (ng/mL) in hAMSC-CM (Fig. S1). MTS assay was used to analyze cell proliferation by culturing GC-1 spg cells with one of several concentrations (10, 50, 100, and 200 ng/mL) of POSTN or THBS1. The results showed that compared to THBS1, POSTN significantly increased GC-1 spg cell proliferation (Fig. 3a, b). Then, EdU assay was applied to investigate the effects of various concentrations of POSTN. The results showed that 100 and 200 ng/mL POSTN significantly increased cell proliferation compared with that in the control group (Fig. 3c, d). For there is no significant difference between the effect of 100 and 200 ng/mL POSTN on the proliferation capacity, the concentration of POSTN was used as 100 ng/mL for further investigation.

To determine whether POSTN was required for the proliferation of GC-1 spg cells under treatment with hAMSC-CM, hAMSCs transfected with POSTN-specific or negative



**Fig. 1** Basic characterization of hAMSCs. **a** Flow cytometry analysis showed surface expression of CD73, CD44, CD29, HLA-DR, CD31, and CD45 on the hAMSCs. **b** Representative phase-contrast bright field images (scale bar: 200  $\mu\text{m}$ ) displayed cultures of hAMSCs. **c**

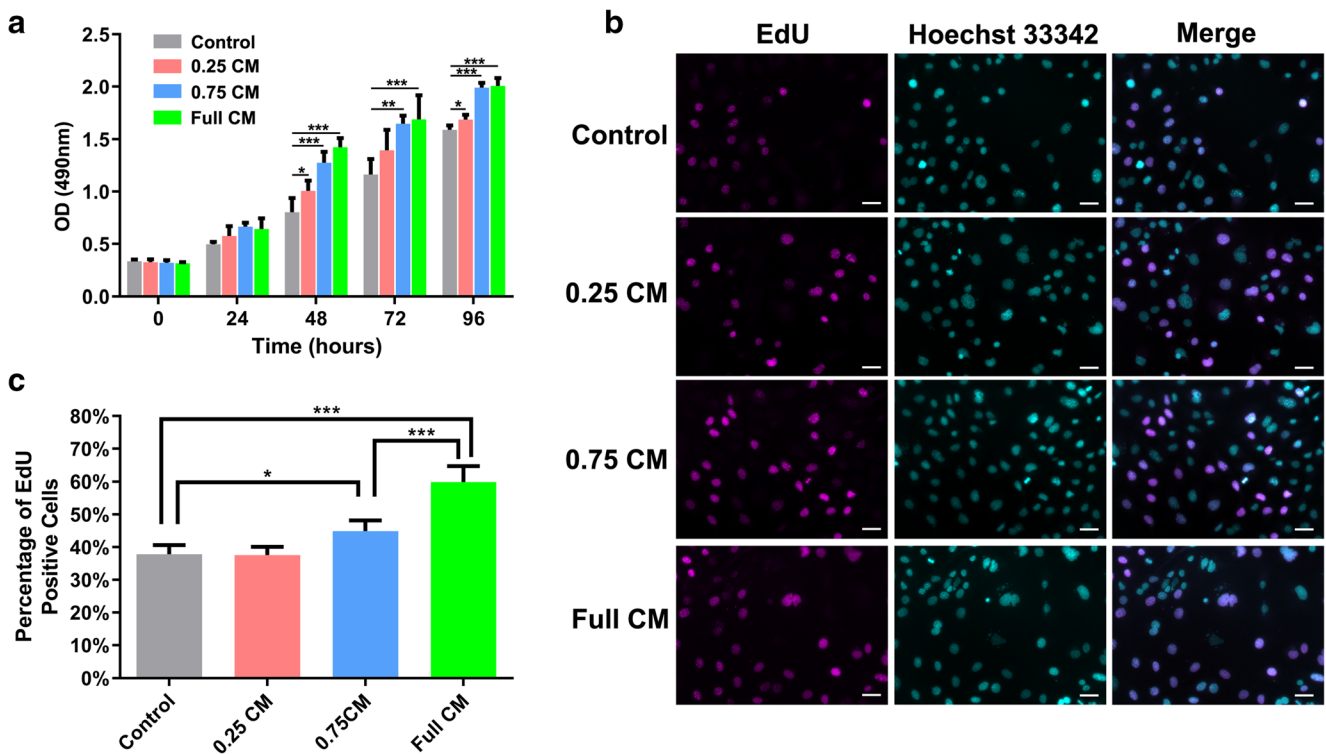
Representative images demonstrated alizarin red (scale bar: 200  $\mu\text{m}$ ) staining. **d** Representative images showed oil red O (scale bar: 100  $\mu\text{m}$ ) staining

control siRNAs for 24 h (Fig. S2) and then collected the conditioned medium. The concentration of POSTN in the CM was decreased with POSTN-silenced (Fig. 3e). We used si-POSTN-1 for the further experiment. MTS analysis and EdU assay results demonstrated that GC-1 spg cells cocultured with CM-siPOSTN showed significantly reduced proliferation relative to that of cells incubated with CM-siNC (Fig. 3f–h).

### The Wnt Signaling Pathway Is Involved in POSTN-Induced GC-1 spg Cell Proliferation

In the postnatal testis, the Wnt/CTNNB1 pathway mediates proliferation of spermatogonial cells and progenitor cells [17]. To determine whether POSTN promotes the proliferation of

GC-1 spg cells through the Wnt/ $\beta$ -catenin pathway, the Wnt/ $\beta$ -catenin signaling inhibitor XAV939 (40  $\mu\text{M}$ ) was added to the cell culture. MTS assay showed that treatment with XAV939 significantly suppressed the proliferation of GC-1 spg cells (Fig. 4a). EdU analysis was used to study the effects of XAV939. The results indicated that the number of EdU-positive cells in the XAV939 group was significantly lower than that in the POSTN group (Fig. 4b, c). Furthermore, western blot analysis revealed that under POSTN addition, the protein expression of both  $\beta$ -catenin and cyclin D1 was increased, whereas that of Gsk3 $\beta$  was inhibited. When XAV939, a Wnt/ $\beta$ -catenin inhibitor, was added, the protein expression of  $\beta$ -catenin and cyclin D1 was decreased, whereas that of Gsk3 $\beta$  was increased (Fig. 4d). These results



**Fig. 2** In vitro culturing with hAMSCs-CM promotes proliferation of GC-1 spg cells. **a** Cell viability was assessed by MTS assay at various time points (0, 24, 48, and 72 h) in different dilutions of hAMSCs-CM. **b**, **c** EdU incorporation assay showed the percentages of EdU-positive cells

treated with different concentrations of hAMSCs-CM. Scale bars = 50  $\mu$ m. The values are shown as means  $\pm$  SD from three independent experiments. \*\*\* $p$  < 0.001; \*\* $p$  < 0.01; \* $p$  < 0.05

demonstrated that POSTN regulates GC-1 spg cell proliferation via the Wnt/ $\beta$ -catenin pathway. Cell cycle analysis results found that S phase cell rates increased in POSTN group compared with the control group (Fig. 4e).

### POSTN Promoted the Proliferation of Human SCs

Cells were isolated from testicular biopsies from patients with obstructive azoospermia. For OCT4 plays a critical role in SC maintenance in culture and for colonization activity following cell transplantation [18], we chose OCT4 as SC proliferation marker. Compared with control treatment, the addition of POSTN protein to the culture medium promoted the proliferation of human SCs, which were expressing stem cell marker OCT4 (Fig. 5a, b). RT-PCR results showed that mRNA expression levels of OCT4, PLZF, SALL4, and VASA were increased following the addition of POSTN (Fig. 5c).

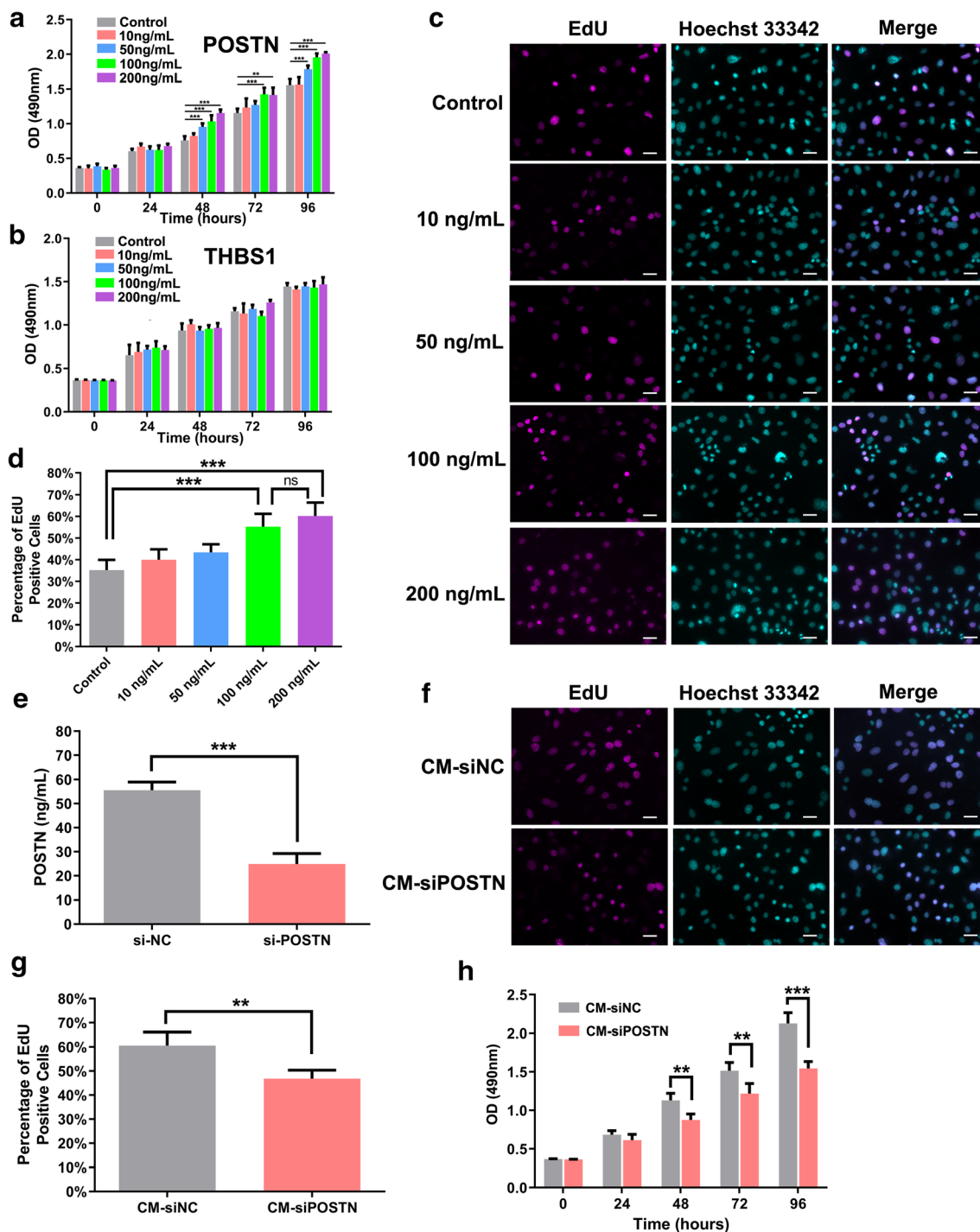
### Discussion

At present, only a few growth factors have been used in the in vitro culture of SCs. Therefore, it is urgent to identify additional factors for enhancing SC proliferation. The results of this study provide insight into the involvement of hAMSC-secreted factors in enhancing the proliferative capability of

SCs. These factors may have promise for future applications, such as SC differentiation in vitro or transplantation into the male infertile patients.

Our study shows that hAMSC-CM enhances the proliferation of GC-1 spg cells in vitro. The results are consistent with a previous study by Zhaleh et al., which showed that the CM of bone marrow MSCs can enhance cell proliferation and growth and suppress cell death in various cell lines [19]. Previous studies from our lab have found that hAMSC-CM and hAEC-CM contain many kinds of factors related to wound healing both in vitro and in vivo [10, 20]. In addition, it has been reported that human amniotic epithelial cells (hAECs) can maintain mouse spermatogonial cells in an undifferentiated state [3]. Furthermore, hAEC-CM has been shown to promote the proliferation of keratinocytes [21]. For we have found that POSTN levels are higher in hAMSC-CM than in hAEC-CM (data not shown), we speculate that proteins secreted from hAMSCs are beneficial for SC proliferation in vitro. The different impacts of hAMSC-CM and hAEC-CM on cell proliferation ability warrant further investigation.

Another key finding of the present study was that the POSTN secreted by hAMSCs could increase GC-1 spg cells and human SC proliferation in vitro. We previously used label-free mass spectrometry to analyze the proteins in hAMSC-CM. The enrichment analysis showed that POSTN

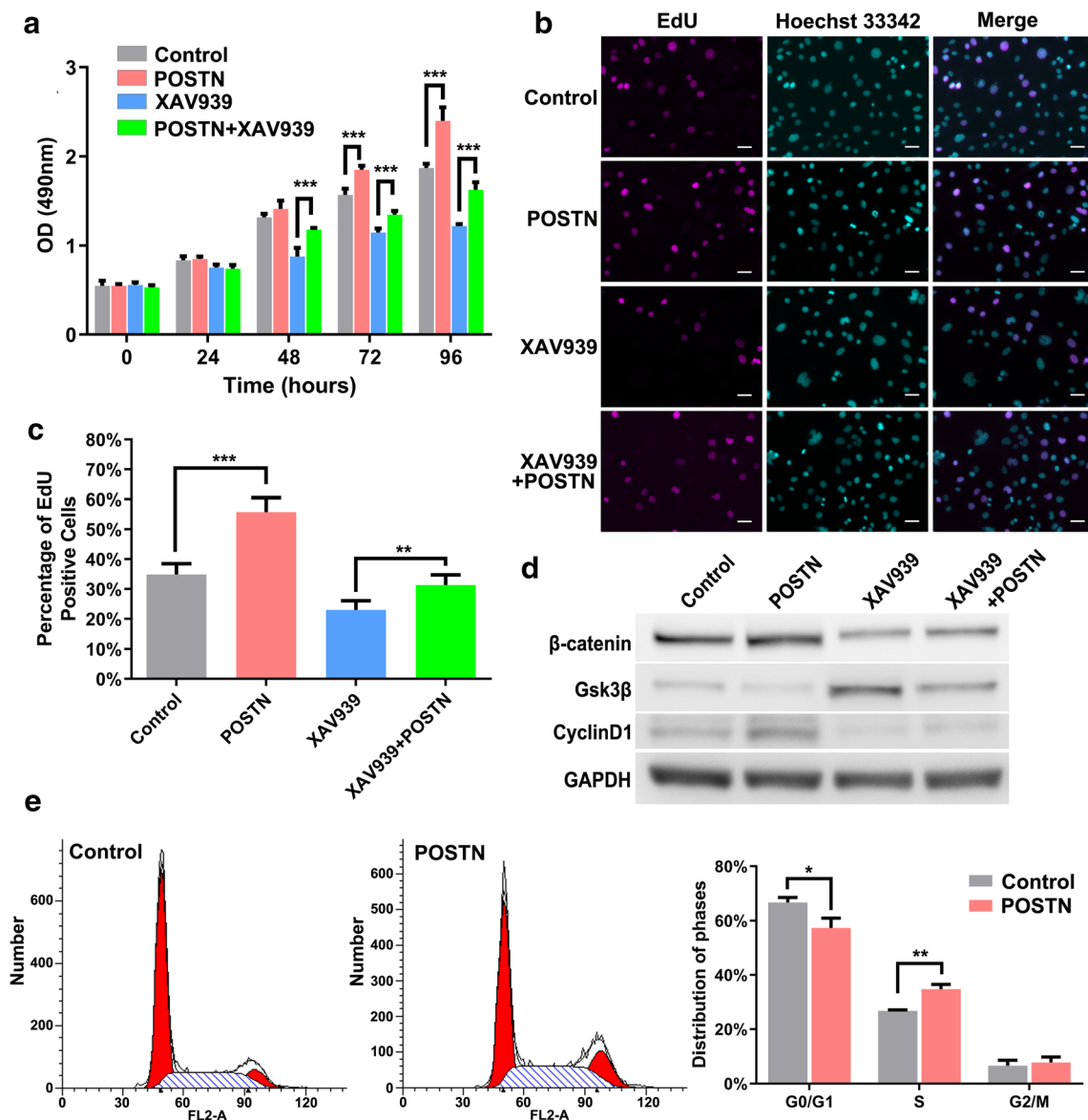


**Fig. 3** POSTN promotes proliferation of GC-1 spg cells. **a** The histogram plot showed cell density treated with 0, 10, 50, 100, and 200 ng/mL POSTN at 0, 24, 48, 72, and 96 h. **b** The histogram plot revealed cell density treated with 0, 10, 50, 100, and 200 ng/mL THBS1 at 0, 24, 48, 72, and 96 h. **c, d** EdU incorporation assay showed the percentages of EdU-positive cells treated with different concentrations of POSTN. Scale bars = 50  $\mu$ m. Values are means  $\pm$  SD from three independent experiments. **e** ELISA assay displayed POSTN protein levels (ng/mL)

in the CM obtained from hAMSCs transfected with si-NC, si-POSTN-1. **f, g** EdU incorporation assay demonstrated the percentages of EdU-positive cells in the CM from hAMSCs with POSTN knockdown. **h** Representative images showed the results of the proliferation assay at various time points (0, 24, 48, and 96 h) on the hAMSCs in si-NC-hAMSCs-CM and si-POSTN-hAMSCs-CM. The values are means  $\pm$  SD. \*\*\* $p$  < 0.001; \*\* $p$  < 0.01

and THBS1 are highly expressed proteins in hAMSC-CM and are related to cell proliferation. The ability of POSTN to

promote cell growth in cancer cells has been well studied; exposure to POSTN, transfection of the POSTN gene, or



**Fig. 4** POSTN promotes the proliferation of GC-1 spg cells through the Wnt/β-catenin pathway GC-1 spg cells were cultured and treated with POSTN and/or XAV939 (β-catenin inhibitor). **a** MTS assay showed the proliferation capacity of cells. **b, c** EdU incorporation assay demonstrated the percentages of EdU-positive cells. **d** Western blot analysis was carried

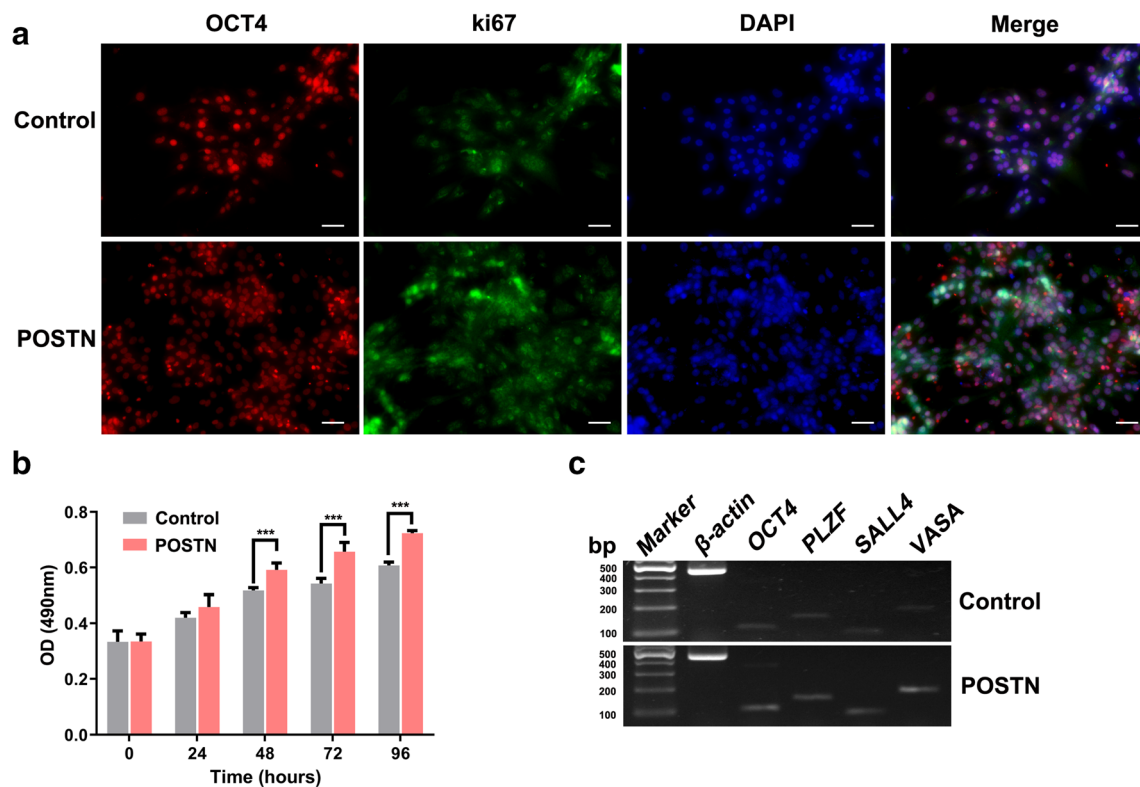
out to detect the expression level of β-catenin, Gsk3β, and cyclin-D1. **e** The proportion of cells in each phase of the cell cycle was evaluated by flow cytometry after 48 h of treatment. The values are expressed as means ± SD. \*\*\**p* < 0.001; \*\**p* < 0.01; \**p* < 0.05

coexistence of POSTN-producing cells can enhance the proliferation of cancer cells [22]. Although THBS1 is a major regulator of TGF-β activation, it also has TGF-β-independent functions in hemostasis, cell adhesion, migration, and the regulation of growth factors (EGF, VEGF, and FGF) [23]. Our results showed that compared to THBS1, POSTN significantly increased the proliferation of GC-1 spg cells, suggesting that THBS1 and its subsequent pathways do not play leading roles in the self-renewal of GC-1 spg cells.

POSTN might be an important factor for addition to the cultural medium of SCs. For only a very small piece of human testicular tissue can be obtained in each operation, the number

of SCs it contains is very small. In addition, it is difficult to study the mechanisms of spermatogenesis, mainly due to the scarcity of SCs relative to other germ cell populations in testicular biopsies. Thus, it is important to develop methods to increase SC numbers in vitro prior to differentiation [24]. Although Huleihel achieved human spermatogenesis in vitro using a 3D culture system, it is difficult in such cases to perform additional examinations to confirm protein/expression results, such as the ploidy of the cells [25]. To date, only a few factors/signaling pathways facilitating the in vitro proliferation of SCs have been identified, and only GDNF, IGF-1, and FGF2 signaling are believed to be essential [26, 27]. Thus,





**Fig. 5** POSTN promotes the proliferation of human SSCs. **a** Immunofluorescence revealed cellular localization of OCT4 and Ki67 in human SSCs. Fluorescent signals of OCT4 (red), Ki67 (green), and DAPI (blue) were imaged individually and merged under fluorescence

efforts to identify additional effective factors for SC expansion *in vitro* are urgently needed. The discovery of POSTN in the present study is of great significance for SC culture *in vitro*.

We sought to identify the pathways downstream of POSTN signaling for SC proliferation. The findings demonstrated that POSTN promotes the proliferation of mouse GC-1 spg cells via the Wnt/ $\beta$ -catenin signaling pathway. This finding is consistent with our understanding of the canonical Wnt signaling pathway. During development, Wnt/ $\beta$ -catenin signaling is required for the specification of primordial germ cells and proper development of the male fetal reproductive tract [28]. Hinako found that Wnt/ $\beta$ -catenin signaling is involved in spermatogonial stem/progenitor cell regulation *in vivo* and revealed the mode of action [29]. Ilaria et al found that POSTN can recruit Wnt ligands and thereby increase Wnt signaling in cancer stem cells [30]. Moreover, research by Golestaneh demonstrated that Wnt3A pathways play important roles in the regulation of SCs of the mouse C18-4 cell line and of human spermatogonia [28]. In summary, POSTN promoted SC proliferation through activation of  $\beta$ -catenin and cyclin D1, whereas inhibition of Gsk3 $\beta$ .

In summary, the present study demonstrated that POSTN in hAMSC-CM may be an extrinsic factor promoting GC-1 spg cell proliferation *in vitro* through activation of the Wnt/ $\beta$ -catenin pathway. The findings reveal that POSTN is to be

microscope. **b** MTS assay showed that POSTN significantly increased SSC proliferation compared with that in the control group. **c** RT-PCR demonstrated the transcripts of OCT4, PLZF, SALL4, and VASA after POSTN treated. The values are expressed as means  $\pm$  SD. \*\*\* $p < 0.001$

closely associated with SC proliferation and provides a potential therapeutic option for treating male infertility.

**Abbreviations** SC, spermatogonial cell; hAMSCs, human amniotic mesenchymal stem cells; CM, condition medium; hAECs, human amniotic epithelial cells; ESCs, human embryonic stem cells

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s43032-021-00596-1>.

**Funding** This research was funded by Shenyang Science and Technology Plan, grant number 20-204-4-31, and National Key Research and Development Program of China, grant number 2016YFC1000600, and The APC was funded by 20-204-4-31.

## References

- Toolee H, Rastegar T, Solhjoo S, Mortezaee K, Mohammadipour M, Kashani IR, et al. Roles for Kisspeptin in proliferation and differentiation of spermatogonial cells isolated from mice offspring when the cells are cocultured with somatic cells. *J Cell Biochem.* 2019;120(4):5042–54. <https://doi.org/10.1002/jcb.27780>.
- Sahare MG, Suyatno, Imai H. Recent advances of *in vitro* culture systems for spermatogonial stem cells in mammals. *Reprod Med Biol.* 2018;17(2):134–42. <https://doi.org/10.1002/rmb2.12087>.

3. Liu T, Guo L, Liu Z, Cheng W. Human amniotic epithelial cells maintain mouse spermatogonial stem cells in an undifferentiated state due to high leukemia inhibitor factor (LIF) expression. *In Vitro Cell Dev Biol Anim.* 2011;47(4):318–26. <https://doi.org/10.1007/s11626-011-9396-5>.
4. Koruji M, Shahverdi A, Janan A, Piryaei A, Lakpour MR, Gilani SM. Proliferation of small number of human spermatogonial stem cells obtained from azoospermic patients. *J Assist Reprod Genet.* 2012;29(9):957–67. <https://doi.org/10.1007/s10815-012-9817-8>.
5. He Y, Chen X, Zhu H, Wang D. Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells. *CYTOTECHNOLOGY.* 2015;67(6): 921–30. <https://doi.org/10.1007/s10616-015-9850-4>.
6. Meirelles LS, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev.* 2009;20(5-6):419–27. <https://doi.org/10.1016/j.cytogfr.2009.10.002>.
7. Saheli M, Bayat M, Ganji R, Hendudari F, Kheirjou R, Pakzad M, et al. Human mesenchymal stem cells-conditioned medium improves diabetic wound healing mainly through modulating fibroblast behaviors. *Arch Dermatol Res.* 2020;312(5):325–36. <https://doi.org/10.1007/s00403-019-02016-6>.
8. Ge L, Jiang M, Duan D, Wang Z, Qi L, Teng X, et al. Secretome of olfactory mucosa mesenchymal stem cell, a multiple potential stem cell. *Stem Cells Int.* 2016;2016:1243659–16. <https://doi.org/10.1155/2016/1243659>.
9. Pischiutta F, Brunelli L, Romele P, Silini A, Sannali E, Paracchini L, et al. Protection of brain injury by amniotic mesenchymal stromal cell-secreted metabolites. *Crit Care Med.* 2016;44(11):e1118–31. <https://doi.org/10.1097/CCM.0000000000001864>.
10. He D, Zhao F, Jiang H, Kang Y, Song Y, Lin X, et al. LOXL2 from human amniotic mesenchymal stem cells accelerates wound epithelialization by promoting differentiation and migration of keratinocytes. *Aging (Albany NY).* 2020;12(13):12960–86. <https://doi.org/10.18632/aging.103384>.
11. Idolazzi L, Ridolo E, Fassio A, Gatti D, Montagni M, Caminati M, et al. Periostin: the bone and beyond. *EUR J INTERN MED.* 2017;38:12–6. <https://doi.org/10.1016/j.ejim.2016.11.015>.
12. Lambert AW, Wong CK, Ozturk S, Papageorgis P, Raghunathan R, Alekseyev Y, et al. Tumor cell-derived periostin regulates cytokines that maintain breast cancer stem cells. *Mol Cancer Res.* 2016;14(1): 103–13. <https://doi.org/10.1158/1541-7786.MCR-15-0079>.
13. Kormann R, Kavvadas P, Placier S, Vandermeersch S, Dorison A, Dussaulte JC, et al. Periostin promotes cell proliferation and macrophage polarization to drive repair after AKI. *J Am Soc Nephrol.* 2020;31(1):85–100. <https://doi.org/10.1681/ASN.2019020113>.
14. Venning FA, Wullkopf L, Erler JT. Targeting ECM disrupts cancer progression. *Front Oncol.* 2015;5:224. <https://doi.org/10.3389/fonc.2015.00224>.
15. Morra L, Moch H. Periostin expression and epithelial-mesenchymal transition in cancer: a review and an update. *Virchows Arch.* 2011;459(5):465–75. <https://doi.org/10.1007/s00428-011-1151-5>.
16. Wu Q, Fang T, Lang H, Chen M, Shi P, Pang X, et al. Comparison of the proliferation, migration and angiogenic properties of human amniotic epithelial and mesenchymal stem cells and their effects on endothelial cells. *Int J Mol Med.* 2017;39(4):918–26. <https://doi.org/10.3892/ijmm.2017.2897>.
17. Chassot AA, Le Rolle M, Jourden M, Taketo MM, Ghyselink NB, Chaboissier MC. Constitutive WNT/CTNBB1 activation triggers spermatogonial stem cell proliferation and germ cell depletion. *Dev Biol.* 2017;426(1):17–27. <https://doi.org/10.1016/j.ydbio.2017.04.010>.
18. Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, Porteus MH. Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells.* 2008;26(11):2928–37. <https://doi.org/10.1634/stemcells.2008-0134>.
19. Zhaleh H, Bidmeshki PA, Azadbakht M. Mesenchymal stem cell condition medium enhanced cell viability in morphine-treated cells. *Bratisl Lek Listy.* 2020;121(4):263–70. [https://doi.org/10.4149/BLL\\_2020\\_040](https://doi.org/10.4149/BLL_2020_040).
20. Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M. A signaling principle for the specification of the germ cell lineage in mice. *CELL.* 2009;137(3):571–84. <https://doi.org/10.1016/j.cell.2009.03.014>.
21. Zhao B, Liu JQ, Zheng Z, Zhang J, Wang SY, Han SC, et al. Human amniotic epithelial stem cells promote wound healing by facilitating migration and proliferation of keratinocytes via ERK, JNK and AKT signaling pathways. *Cell Tissue Res.* 2016;365(1): 85–99. <https://doi.org/10.1007/s00441-016-2366-1>.
22. Gonzalez-Gonzalez L, Alonso J. Periostin: a matricellular protein with multiple functions in cancer development and progression. *Front Oncol.* 2018;8:225. <https://doi.org/10.3389/fonc.2018.00225>.
23. Murphy-Ullrich JE, Suto MJ. Thrombospondin-1 regulation of latent TGF-beta activation: a therapeutic target for fibrotic disease. *Matrix Biol.* 2018;68-69:28–43. <https://doi.org/10.1016/j.matbio.2017.12.009>.
24. Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, et al. Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. *Proc Natl Acad Sci U S A.* 2009;106(51):21672–7. <https://doi.org/10.1073/pnas.0912432106>.
25. Abofoul-Azab M, AbuMadighem A, Lunenfeld E, Kapelushnik J, Shi Q, Pinkas H, et al. Development of postmeiotic cells in vitro from spermatogonial cells of prepubertal cancer patients. *Stem Cells Dev.* 2018;27(15):1007–20. <https://doi.org/10.1089/scd.2017.0301>.
26. Wang S, Wang X, Wu Y, Han C. IGF-1R signaling is essential for the proliferation of cultured mouse spermatogonial stem cells by promoting the G2/M progression of the cell cycle. *Stem Cells Dev.* 2015;24(4):471–83. <https://doi.org/10.1089/scd.2014.0376>.
27. Sharma M, Braun RE. Cyclical expression of GDNF is required for spermatogonial stem cell homeostasis. *DEVELOPMENT.* 2018;145(5). <https://doi.org/10.1242/dev.151555>.
28. Golestaneh N, Beauchamp E, Fallen S, Kokkinaki M, Uren A, Dym M. Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells. *REPRODUCTION.* 2009;138(1):151–62. <https://doi.org/10.1530/REP-08-0510>.
29. Takase HM, Nusse R. Paracrine Wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. *Proc Natl Acad Sci U S A.* 2016;113(11):E1489–97. <https://doi.org/10.1073/pnas.1601461113>.
30. Malanchi I, Santamaria-Martinez A, Susanto E, Peng H, Lehr HA, Delaloye JF, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *NATURE.* 2011;481(7379): 85–9. <https://doi.org/10.1038/nature10694>.