ORIGINAL ARTICLE

WILEY

Cell
Proliferation

p38 MAPK pathway is essential for self-renewal of mouse male germline stem cells (mGSCs)

Zhiwei Niu¹ | Hailong Mu² | Haijing Zhu² | Jiang Wu² | Jinlian Hua²

¹School of Basic Medical Sciences, Shanxi Medical University, Taiyuan, China

²College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering and Technology, Yangling, Shaanxi, China

Correspondence

Jinlian Hua, College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering and Technology, Northwest A&F University, Yangling, Shaanxi, China.
Email: jinlianhua@nwsuaf.edu.cn

Funding information

China National Basic Research Program, Grant/Award Number: JFYS 2016YFA0100203; Program of National Natural Science Foundation of China, Grant/Award Number: 31272518 and 31572399; Key Project of the Chinese Ministry of Science and Technology, Grant/Award Number: 2013CB967401; Youth Fund of Shanxi Medical University, Grant/Award Number: 02201501

Abstract

Objectives: Male germline stem cells (mGSCs), also called spermatogonial stem cells (SSCs), constantly generate spermatozoa in male animals. A number of preliminary studies on mechanisms of mGSC self-renewal have previously been conducted, revealing that several factors are involved in this regulated process. The p38 MAPK pathway is widely conserved in multiple cell types *in vivo*, and plays an important role in cell proliferation, differentiation, inflammation and apoptosis. However, its role in self-renewal of mGSCs has not hitherto been determined.

Materials and methods: Here, the mouse mGSCs were cultured and their identity was verified by semi-RT-PCR, alkaline phosphatase (AP) staining and immunofluorescence staining. Then, the p38 MAPK pathway was blocked by p38 MAPK-specific inhibitor SB202190. mGSC self-renewal ability was then analysed by observation of morphology, cell number, cell growth analysis, TUNEL incorporation assay and cell cycle analysis.

Results: Results showed that mouse mGSC self-renewal ability was significantly inhibited by SB202190.

Conclusions: This study showed for the first time that the p38 MAPK pathway plays a key role in maintaining self-renewal capacity of mouse mGSCs, which offers a new self-renewal pathway for these cells and contributes to overall knowledge of the mechanisms of mGSC self-renewal.

1 | INTRODUCTION

Male germline stem cells (mGSCs), also called spermatogonia stem cells (SSCs), are located in the base of the seminiferous tubules in the testes. They can produce spermatozoa incessantly in male animals. mGSCs have self-renewal and pluripotency capacities, and they pass genetic information to progeny.^{1,2} The study of mGSCs can help us understand the mechanism of spermatogenesis and may lay the foundation for therapies for male infertility. The self-renewal mechanism of mGSCs is one of the most important mGSC research areas.

To date, some initial research results about mGSC self-renewal mechanisms have been reported,^{3–5} but the precise mechanisms have not been determined, especially in livestock.⁵ Many factors

are involved in the regulation of mGSC self-renewal. These factors include cytokines, transcription factors and RNA-binding proteins, microRNA and signalling pathways. GDNF (glial cell line-derived neurotrophic factor) is the first cytokine confirmed by scientists to be necessary for maintaining mGSC self-renewal. GDNF is secreted by Sertoli cells and can bind with its specific receptor, Gfra1, on the surface of mGSCs. After binding with Gfra1, the transmembrane tyrosine kinase Ret, which is associated with Gfra1, is activated.⁶ Activated Ret can activate several pathways, including the PI3K pathway, MAPK pathway, SFK pathway and PLC- γ pathway, leading to downstream gene regulation. The ligand FGF2 can activate the FGF2 receptor (FGFR), which is located on the mGSC surface. The activated FGF2 receptor can further activate the Ras protein,

activating the MAPK pathway; thus, FGF2 and GDNF synergistically facilitate the self-renewal of mGSCs.⁷

Plzf was the first transcription factor determined to be essential for mGSC self-renewal.^{8,9} The mutation of Plzf causes mice to lose their spermatogenesis ability. Plzf is a necessary factor for maintaining mGSC self-renewal and antagonizes Sall4 during cell differentiation.¹⁰ Nanos2 is a RNA-binding protein that is essential for mGSC self-renewal. Undifferentiated mGSCs express Nanos2 and maintain their self-renewal capacity. Conditional knockout of Nanos2 in post-natal mice leads to exhaustion of their SSC reserve. Thus, Nanos2 is also one of the critical genes for mGSC self-renewal. In addition, Oct4, Oct6, Lhx1, Foxo1 and Etv5 are also important genes for the maintenance of mGSC self-renewal.^{11–16}

Notably, we found that the p38 MAPK pathway is also essential for mGSC self-renewal. p38 MAPK belongs to the mitogen-activated protein kinase (MAPK) family. MAPK proteins are part of the Ser/Thr protein kinase family. MAPK proteins exist in most eukaryotic cells and regulate many cell functions, including proliferation, differentiation, transformation, inflammation and apoptosis.¹⁷ The MAPK pathway can transduce extracellular stimuli into the nucleus and control gene expression to cause different biological effects. The MAPK signalling pathway is composed of three conserved signalling protein functions: the MAPKK kinase (MAPKKK, MAP3K), the MAPK kinase (MAPKK, MAP2K) and the MAPK.^{7,18} The MAPKKK→MAPKK→MAPK cascade leads to MAPK phosphorylation and activation.^{19–21}

There are four MAPK pathways that have been found in mammals. They are the ERK1/2, JNK, p38MAPK and ERK5 pathways. These four pathways are activated by diverse factors, leading to a variety of biological effects. These pathways also have crosstalk with each other and have synergic or antagonistic effects with each other.^{22–30}

The p38 MAPK protein is composed of 360 amino acids, with a molecular mass of 38 kDa. Four subtypes of p38MAPK (p38 α , p38 β , p38 γ and p38 δ) have been found. The distribution of the different subtypes is tissue-specific, and the proteins are involved in a variety of biological effects. In addition to proliferation, apoptosis and inflammation, the p38 MAPK pathway participates in cell stress, ischaemia-reperfusion injury, cell phenotypic differentiation and tumour malignancy. p38 MAPKs regulate various cellular physiological processes in diverse ways, demonstrating that they are basic and important pathways in cells.

A previous study found that the p38 MAPK pathway is expressed in mouse testis and male germ cell lines.^{31,32} p38 starts to be highly expressed in mice testis at 2 weeks and then decreases for 3–5 weeks. After 6 or 7 weeks, p38 expression begins to rise again. The expression changes of p38 hint that the pathway has a close relationship with the germ cell differentiation process. Wong and Cheng³³ reports that the MAPK signal pathway, including p38MAPK, is involved in spermatozoa production in mice. In human spermatozoa, p38 is mainly located in the posterior region of the acrosome and the spermatozoa tail and has negative regulatory effects on spermatozoa motility.³⁴ Furthermore, some studies have shown that p38 is related to the acrosome reaction induced by PMA. Blocking the p38 MAPK pathway could cause a decline in the acrosome reaction induced by PMA.³⁵ All this research

shows that the p38 MAPK pathway has a variety of specific biological functions in different cells at different times. Additionally, we noticed that the p38 MAPK pathway is present in male germ cells and plays an important role in male germ cell development. However, the relationship between the p38 MAPK pathway and the self-renewal mechanism of male germline stem cells is unclear.

In this study, murine mGSCs stored in our laboratory were first identified. Then, we used the p38 MAPK-specific inhibitor SB202190 to block the p38 MAPK pathway to explore its effects on the self-renewal capacity of murine mGSCs. The results showed that the p38 MAPK pathway plays a critical role in the self-renewal of murine mGSCs. This study offers a new pathway that can regulate the self-renewal of mGSCs.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Mouse mGSCs were stored in the Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University. The stored mouse mGSCs were cultured on Mitomycin C (Sigma, St Louis, MO, USA)-treated mouse embryonic fibroblasts (MEFs) in mESCs (mouse embryonic stem cells) culture medium, which consists of 83% DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 15% knockout serum replacement (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma) and 1000 U/ml^{−1} LIF (Millipore, Bedford, MA, USA). In this study, we transfer the mGSCs into 2i medium consisting of DM/F12, 1% N2 (Invitrogen), 2%B27 (Invitrogen), 2 mg/mL bovine serum albumin (Invitrogen), 1% non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 1 μ M PD0325901 (Sigma), 5 μ M SB216763 (Sigma). The mGSCs colonies were passaged by 0.05% trypsin (Invitrogen). The medium was changed every day.

2.2 | Alkaline phosphatase(AP) staining

Alkaline phosphatase (AP) activity was thought essentially for the pluripotent cells.³⁶ Briefly, cells were rinsed three times in PBS and fixed in 4% paraformaldehyde (PFA) for 10–15 min at room temperature. The fixed cells were washed for three times with PBS and stained with naphthol AS-MX phosphate (0.2 mg/mL, Sigma) and Fast Red TR salt (1 mg/mL, Sigma) in 100 mmol/L Tris-buffer, pH 8.2–8.4, for 10–30 min at room temperature, and washed again with PBS to terminate staining.³⁷

2.3 | Immunofluorescence staining

The mGSCs which need to detect were fixed in 4% PFA, treated with 0.1% Triton X-100 for 10 min at room temperature. After blocking with 1% BSA for 30 min, the cells were incubated with primary antibodies against Nestin (1:200; Chemicon, Temecula, CA, USA), β -III-tubulin (1:500; Santa Cruz Biotechnology, Inc., Dallas, Texas 75220, USA), cardiac α -actin (1:500; Sigma), Afp (1:500; Chemicon), SSEA-1 (1:200; Chemicon), Oct4 (1:200; C.S.T), Nanog (1:100; Santa Cruz),

TABLE 1 The primer sequences

Gene	Forward Primer(5'→3')	Reverse Primer(5'→3')	Products (bp)
β-III-tubulin	TAGACCCCAGCGCAACTAT	GTTCCAGGTTCCAAGTCCACC	127
Desmin	ACCGCTTCGCCAACTACAT	TCACTTTCTTAAGGAACGCGA	378
α-actin	GATTCTGGCGATGGTGAAGTCA	AGATTCCATACCAATGAAAGAGGG	354
Brachury	AAGGTGGCTGTTGGGTAGGGAGT	ATTGGGCGAGTCTGGGTGGATGT	451
Afp	CCCTCATCCTCCTGCTACATT	CGGAACAACTGGGTAAAGGT	146
Pdx1	CCCCAGTTTACAAGCTCGCT	CTCGGTTCCATTGCGGAAAGG	177
Oct4	GGCGTTCTCTTTGGAAAGGTGTTT	CTCGAACCACATCCTTCTCT	314
c-Myc	TGACCTAACTCGAGGAGGAGCTGGAATC	AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC	173
c-kit	GGCCTCACGAGTTCTATTTACG	GGGGAGAGATTCCCATCACAC	168
Klf4	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT	185
β-actin	GTGACGTTGACATCCGTAAGA	GCCGGACTCATCGTACTCC	245

overnight at 4°C, respectively. After washing three times in PBS, appropriate secondary antibodies were incubated for 1 hour at room temperature in the dark. Then the cell nucleus of samples was stained by Hoechst33342 (Sigma).³⁸ The images were captured with a Leica fluorescent microscope.

2.4 | In vitro differentiation of mGSCs

mGSCs were collected and plated on an uncoated cell culture suspension dish (Corning, NY, USA) with 3×10^5 cells per 3.5 cm dish in the 2i medium. After 3 days of culture, the cells had aggregated and formed 'embryoid bodies' (EBs). The resulting EBs, whose diameters were approximately 100 μm, were transferred in a 48-well culture plate (10–15 EBs per well) coated with 0.1% gelatin and were cultured for 7 days. Then, the EBs were harvested and analysed.

2.5 | RT-PCR

Total RNA was extracted with Trizol reagent (TaKaRa, Dalin, China) from mGSCs or treated EBs. Single-strand cDNAs were prepared from 2 μg of RNA using a reverse transcription kit (Fermentas), and specific gene expression was analysed. The RT-PCR primers used are described in Table 1 and are markers of stem cells, three germ layers or germ cells.^{39–46} PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, annealing at a temperature in accordance with the primer sequence for 30 sec, and then 72°C for 30 sec, with a final extension at 72°C for 10 min. The PCR products were analysed with 2% agarose (Invitrogen) gel electrophoresis, stained with ethidium bromide (Invitrogen) and visualized under UV illumination.

2.6 | Cell colony quantification

The number of mGSC colonies in the media with different concentrations of SB202190 was determined. The mGSCs were cultured in groups with different concentrations (0, 5, 10, 25 and 50 μM) of SB202190 for 72 h, and then the number of mGSCs colonies was

counted by microscope. Lif is usually used to culture pluripotent cells and SSCs. In this study, Lif was added to promote mGSC self-renewal, in contrast with SB202190. The number of mGSCs in the media with different concentrations of SB202190 was also counted. The mGSCs were cultured in several groups with different concentrations (0, 5, 10, 25 and 50 μM) of SB202190 for 72 h, and then cells were digested by trypsin and re-suspended. The number of mGSCs was counted.

2.7 | Cell passage capacity

The capacity to passage the mouse mGSCs was analysed. Briefly, cells were serially passaged at an initial seeding density of 2.7×10^5 cells per well in a six-well plate in triplicate. The mGSCs were passaged every 72 h, and the total cell number of per well was counted. Control media was 2i media; the experimental group culture media contained 2i and SB202190 (5 μM; Sigma). The proliferation ability of the cells was evaluated by cell counting at every passage. The cells were passaged persistently until the experiment group cell could not be passaged further.

2.8 | Cell cycle analysis

For cell cycle analysis, mGSCs were cultured in control media or media containing SB202190 (5 μM) for 48 h and were then re-suspended as single cells and washed in pre-cooling PBS. After that, the cells were re-suspended and incubated using the Cell Cycle Kit (LianKeBiology, Hangzhou, China) with 1 mL liquid A and 10 μL liquid B for 30 min, and cell cycle analysis was performed with a Beckman flow cytometer.⁴⁷

2.9 | Statistical analyses

The data were presented as mean ± S.E.M. and the S.E.M. in this study were calculated for at least three replicates for each of three independent experiments. Statistical comparisons were assessed using Student's *t* test. A *P* value of <0.05 was considered to be statistically significant difference and a *P* value of <0.01 was considered to be a highly significant difference.

FIGURE 1 The morphology and immunofluorescence staining of mGSCs colonies cultured in 2i media. A, The mGSCs colonies cultured in 2i media maintained typical nest-like colonies and AP positive similar to wild mESCs. B, Immunofluorescence staining of pluripotent markers SSEA-1, Oct4 and Nanog in the mGSCs stored in our laboratory. (Scale bar=100 μM)

3 | RESULTS

3.1 | Cultivation and identification of the mouse mGSCs

To avoid various factors in serum from impacting this research, we choose a culture medium whose ingredients were as simple as possible. We thus chose a chemically defined medium to culture mGSCs: 2i (two inhibitor, which includes the MEK-specific inhibitor PD0325901 and the GSK3-specific inhibitor CHIR99021) medium, which has been found to be useful for culturing mESCs.⁴⁸ In 2013, Leitch et al.⁴⁹ transformed primordial germ cells (PGCs) to embryonic germ cells (EGCs), which are similar to mESCs in some ways. Youn et al.⁵⁰ reported that PD0325901 could enhance the expression of Oct4 in mouse SSCs. Taking into account that mGSCs have some similar characteristics with ESCs, we tried to culture the mGSCs with 2i media. Mouse mGSCs cells stored in our laboratory were grown in 2i media and identified as described in previous studies.^{51,52} The mGSCs in 2i media maintained typical mESCs characteristics, with nest-like colonies similar to wild mESCs. The mGSCs were identified by AP staining (Figure 1A). The mGSCs in 2i media maintain higher AP activity, and

the colony morphology was more compact. Mouse mGSCs were also identified by immunofluorescence. The results showed that the pluripotent markers SSEA-1, Oct4 and Nanog were all present (Figure 1B).

After being in suspension culture for 3 days, these cells aggregated and formed into typical EBs (Figure 2A). After another 7 days, the EBs spontaneously differentiated and expressed specific markers of all three germ layers, including nestin and β -III-tubulin (ectoderm), cardiac α -actin (mesoderm) and Afp (endoderm), as analysed by immunofluorescence staining (Figure 2B). To further assess the differentiation potentiality of the mGSCs, we assayed the gene expression after 3 days of differentiation by semi-quantitative RT-PCR. The results confirmed that the EBs from mGSCs could differentiate into β -III-tubulin, Desmin-, cardiac α -actin-, Brachyury-, Afp- and Pdx1-positive cells (Figure 2C). These results indicate that mGSCs can form into EBs, with multi-lineage differentiation potential.

3.2 | The p38 MAPK pathway is critical for the proliferation of mouse mGSCs

After we added the specific p38MAPK inhibitor SB202190 to block the p38MAPK pathway, we found that the morphology of colonies

FIGURE 2 Analysis of the formed EBs from mGSCs. A, The mGSCs formed EBs after 3 days in suspension culture. B, Immunofluorescence analysis showed that EBs, after spontaneously differentiation, were positive for markers specific for all three germ layers: Nestin and β -III-tubulin (ectoderm markers), cardiac α -actin (mesoderm marker) and AFP (endoderm marker). Bar=200 μ m. F, RT-PCR analysis of the expression of the germ layer-specific markers in 3-day-old EB-derived mGSCs

cultured with SB202190 was more like the typical morphology of undifferentiated mGSC colonies than that of the control group (Figure 3A). The edge of the colonies cultured with SB202190 was also smoother. The morphology of the colonies cultured with SB202190 was more compact and closer to nest-like. Colonies cultured with SB202190

FIGURE 3 The number of mGSCs colonies with morphology typical for undifferentiated cells cultured with SB202190 is greater than in the control group. A, The morphology of mGSCs colonies with the p38 MAPK pathway blocked by SB202190. The two rows of pictures show that mGSCs colonies cultured with SB202190 are AP positive and that the colonies density is lower than in the other two groups. The colony morphology of cells cultured with SB202190 was more like the typical morphology of undifferentiated mGSC colonies than that of the control group. The second row of images show that the morphology of the colonies cultured with SB202190 was more compact and dense. B, the optimum concentration of SB202190 that can best maintain mGSC undifferentiated colonies. The study showed that the largest number of typical undifferentiated colonies was at a concentration of 5 μ M SB202190

were denser. We add different concentrations of SB202190 to determine the optimum concentration that could best maintain mGSC undifferentiated colonies. We found that the largest number of typical undifferentiated colonies was present at a concentration of 5 μ M SB202190 (Figure 3B). We further found that the number of mGSCs decreased with increasing concentrations of SB202190 (Figure 4A). These results indicate that blocking p38 can impede mGSC self-renewal, which seems in contradiction with the colony morphology and colony count studies. So we decided to do another experiment to explore the relationship between the p38 pathway and mGSC self-renewal. We passaged mGSCs consecutively, and determined the proliferative capacity of mGSCs cultured with SB202190, which was sharply decreased. The mGSCs cultured with SB202190 could not survive more than P3. In contrast, the control group without SB202190, the cells still show a slight surplus proliferation (Figure 4B).

FIGURE 4 The cell number and passage number of mGSCs cultured in different concentrations of SB202190. A, The number of mGSCs decreased with an increase in the concentration of SB202190. B, The maximum passage number of mGSCs cultured in different media. The maximum passage number of mGSCs cultured with SB202190 decreased

FIGURE 5 The cell cycle of mGSCs. The proportion of mGSCs in S phase was decreased in the experimental group cultured with 5 μM SB202190, compared with the control group (0 μM SB202190). The proportion of cells in S phase was 49.248% in the control group, while the proportion in the experimental group was 30.552%

3.3 | Cell cycle changes by blocking the p38 MAPK pathway

The cell cycle of mGSCs was analysed by flow cytometry, and the proportion of mGSCs in the S phase was decreased in

the experimental group cultured with 5 μM SB202190 compared with the control group. The proportion of mGSCs in S phase was 49.248% in the control group, while it was 30.552% in the experimental group (Figure 5). These results indicate that

the decrease in S phase cells and the increase in G1 phase cells might give rise to the decline of mGSC proliferation and viability.

FIGURE 6 The p38 pathway impairs the expression of pluripotent genes important for self-renewal, but does not increase apoptosis. A, The cellular apoptosis of mGSCs as determined by the TUNEL method. In contrast to the control group without SB202190, the number of TUNEL-positive cells culture with 5 μ mol/L SB202190 showed a marked decline. The positive cell count in the control group was 236, while in the 5 μ M group it was 144.6. B, Detection of pluripotency genes in mGSCs. *, $P < 0.05$; **, $P < 0.01$. The expression of Oct4 did not significantly change, while the expression of c-Myc increased, and klf4 and c-kit decreased

3.4 | The effects of the p38 MAPK pathway on mouse mGSCs apoptosis

In addition to the cell cycle, we also wanted to know whether the p38 MAPK pathway could impact cellular apoptosis. The cellular apoptosis of mGSCs cultured with 5 μ M SB202190 was analysed by the TUNEL method. In contrast to the control group without SB202190, the quantity of TUNEL-positive cells treated with 5 μ M SB202190 showed a marked decline. The number of positive cells in the control group was 236, while in the 5 μ M SB202190 group it was 144.6 (Figure 6A,B). These results show that the decline of cell proliferation ability is not caused by apoptosis.

3.5 | The p38 pathway impairs the expression of pluripotency genes

To determine why the mGSC proliferation ability decreased, we assayed five classic pluripotency genes in the mGSCs. Although the expression of Oct4 did not significantly change, the expression of c-Myc was increased, while klf4 and c-kit expression levels were reduced (Figure 6C). These results suggest that the decline in the self-renewal ability of mGSCs may be caused by the reduced expression of the pluripotent genes Klf4 and c-kit, caused by the blockade of the p38 pathway.

4 | DISCUSSION

4.1 | 2i medium for the short-term culture of mGSCs

Murine spermatogonial stem cells can survive for a long time in vitro.^{53,54} The in vitro cultivation system of mGSCs derived from ESCs relies on serum and feeders made by mouse embryonic fibroblasts and also needs the addition of GDNF, bFGF, Lif and EGF.^{51-53,55} The feeder and serum compositions are not clear, and their preparation process is complex. Consequently, there is a sizable difference between each batch produced, and they are difficult to standardize. This makes the cultivation of mGSCs untoward and complicated when attempting to perform research on the mechanism of mGSC self-renewal. Therefore, researchers have tried to establish an mGSC culture system without serum and feeder.⁵⁵⁻⁵⁷

In 2008, Ying et al.⁴⁸ reported a three inhibitor (3i) culture system for embryonic stem cells. They also used 2i cultivation systems, which

were simpler and replaced the 3i system. Leitch et al.⁴⁹ reported that PGCs could be cultured in 2i medium. In the same year, Youn et al.⁵⁰ reported that they could enhance the expression of the pluripotency gene Oct4 in SSCs with PD0325901. In this study, we successfully used the 2i medium to culture mGSCs for a short period of time. Then, we used this 2i culture system as the experimental platform to research the role of the p38 MAPK pathway in mGSC self-renewal *in vitro*.

4.2 | Blocking the p38 MAPK pathway could impact mGSCs self-renewal

The p38 MAPK-specific inhibitor SB202190 was added to the media to block the p38 MAPK pathway. We found that, compared with the control and Lif groups, the morphology of the mGSC colonies treated with SB202190 was more like that of typical undifferentiated mGSC colonies.^{51,52} We first hypothesized that blocking the p38 MAPK pathway could promote to maintain the undifferentiated state of the mGSCs. In Figure 3B, we only counted the number of mGSCs colonies which could keep the typical undifferentiated state to determine the optimal concentration of SB202190 that could best keep the mGSCs in an undifferentiated state. However, we found that although the number of morphologically undifferentiated, typical colonies increased, the total number of colonies in the Petri dish had decreased (Figure 4A).

We found, by cell count and passage number, that SB202190 could impede mGSC proliferation (Figure 4A,B). For determining cellular self-renewal ability, the cell number and the passage number are more convincing than morphological observations. Although blocking p38 activation made the morphology of mGSCs colonies closer to the undifferentiated state, superficially appearing to promote self-renewal, it actually hindered cellular proliferation and the self-renewal.

4.3 | The decrease in mGSC self-renewal was caused by a decrease in S phase cells instead of an increase in cellular apoptosis

We analysed the cell cycle by flow cytometry and found that blocking the p38 MAPK pathway caused the proportion of mGSCs in the S phase to decrease. At the same time, mGSC apoptosis was evaluated. We found that the percentage of apoptotic cells decreased after p38 blockade. We thus speculated that this combination of effects is why cellular proliferation was observed to slow down and the number of cells decreased. These data suggest that these effects were due to the restrained expression of self-renewal-related genes after p38 blockade instead of being caused by increased cellular apoptosis.

ACKNOWLEDGEMENTS

This work was supported by grants from the China National Basic Research Program (JFYS 2016YFA0100203), the Program of National Natural Science Foundation of China (31272518, 31572399), the Key Project of the Chinese Ministry of Science and Technology (2013CB967401), and the Youth Fund of Shanxi Medical University (02201501).

CONFLICT OF INTEREST

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

REFERENCES

1. Izadyar F, Den Ouden K, Stout TA, et al. Autologous and homologous transplantation of bovine spermatogonial stem cells. *Reproduction*. 2003;126:765–774.
2. Oatley JM, Brinster RL. Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol*. 2008;24:263–286.
3. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc Natl Acad Sci USA*. 2006;103:9524–9529.
4. He Z, Kokkinaki M, Dym M. Signaling molecules and pathways regulating the fate of spermatogonial stem cells. *Microsc Res Tech*. 2009;72:586–595.
5. Song HW, Wilkinson MF. Transcriptional control of spermatogonial maintenance and differentiation. *Semin Cell Dev Biol*. 2014;30:14–26.
6. He Z, Jiang J, Hofmann M-C, Dym M. Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. *Biol Reprod*. 2007;77:723–733.
7. Ishii K, Kanatsu-Shinohara M, Toyokuni S, Shinohara T. FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of ETV5 and Bcl6b through MAP2K1 activation. *Development*. 2012;139:1734–1743.
8. Buaas FW, Kirsh AL, Sharma M, et al. Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet*. 2004;36:647–652.
9. Costoya JA, Hobbs RM, Barna M, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet*. 2004;36:653–659.
10. Hobbs RM, Fagoonee S, Papa A, et al. Functional antagonism between Sall4 and Plzf defines germline progenitors. *Cell Stem Cell*. 2012;10:284–298.
11. Hobbs RM, Seandel M, Falcatori I, Rafii S, Pandolfi PP. Plzf regulates germline progenitor self-renewal by opposing mTORC1. *Cell*. 2010;142:468–479.
12. Looijenga LH, Stoop H, de Leeuw HP, et al. POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res*. 2003;63:2244–2250.
13. Ohbo K, Yoshida S, Ohmura M, et al. Identification and characterization of stem cells in prepubertal spermatogenesis in mice. *Dev Biol*. 2003;258:209–225.
14. Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. *J Clin Invest*. 2011;121:3456–3466.
15. Falender AE, Freiman RN, Geles KG, et al. Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. *Genes Dev*. 2005;19:794–803.
16. Chen C, Ouyang W, Grigura V, et al. ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature*. 2005;436:1030–1034.
17. Huang D, Ding Y, Luo W-M, et al. Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis *in vivo*. *Cancer Res*. 2008;68:81–88.
18. Johnson GL, Dohlman HG, Graves LM. MAPK kinase kinases (MKKKs) as a target class for small-molecule inhibition to modulate signaling networks and gene expression. *Curr Opin Chem Biol*. 2005;9:325–331.
19. Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P. Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. *J Biol Chem*. 2001;276:40225–40233.

20. Yoon S, Seger R. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors*. 2006;24:21–44.
21. Kanatsu-Shinohara M, Shinohara T. Spermatogonial stem cell self-renewal and development. *Annu Rev Cell Dev Biol*. 2013;29:163–187.
22. Boilly B, Vercoutter-Edouart A, Hondermarck H, Nurcombe V, Le Bourhis X. FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev*. 2000;11:295–302.
23. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol*. 2007;19:142–149.
24. Kamata H, S-i Honda, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. 2005;120:649–661.
25. Wu J-C, Yan H-C, Chen W-T, et al. JNK signaling pathway is required for bFGF-mediated surface cadherin downregulation on HUVEC. *Exp Cell Res*. 2008;314:421–429.
26. Ravanti L, Toriseva M, Penttinen R, et al. Expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts is induced by transforming growth factor β via p38 mitogen-activated protein kinase. *FASEB J*. 2001;15:1098–1100.
27. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002;298:1911–1912.
28. Hayashi M, Lee J-D. Role of the BMK1/ERK5 signaling pathway: Lessons from knockout mice. *J Mol Med*. 2004;82:800–808.
29. Hayashi M, Fearn C, Eliceiri B, Yang Y, Lee J-D. Big mitogen-activated protein kinase 1/extracellular signal-regulated kinase 5 signaling pathway is essential for tumor-associated angiogenesis. *Cancer Res*. 2005;65:7699–7706.
30. Mehta P, Jenkins B, McCarthy L, et al. MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. *Oncogene*. 2003;22:1381–1389.
31. MA Z, Yang Y, Wang Y. Expression of p38 mitogen -activated protein kinase in male mouse testicular cell lines. *Chongqing Med*. 2009;38:295–296.
32. Zheng Y, Li J, Guo J, et al. Cloning of spermatogenesis related gene-p38 MAPK gene from testis tissue of mouse. *Acta Anat Sin*. 2001;32:260–263.
33. Wong C-H, Cheng CY. Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: A review of recent data. *Dev Biol*. 2005;286:1–15.
34. Almog T, Lazar S, Reiss N, Etkovitz N, Milch E, Rahamim N, et al. Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality. *J Biol Chem*. 2008;283:14479–14489.
35. Almog T, Naor Z. The role of Mitogen activated protein kinase (MAPK) in sperm functions. *Mol Cell Endocrinol*. 2010;314:239–243.
36. Piedrahita JA, Moore K, Oetama B, et al. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. *Biol Reprod*. 1998;58:1321–1329.
37. Niu Z, Hu Y, Chu Z, et al. Germ-like cell differentiation from induced pluripotent stem cells (iPSCs). *Cell Biochem Funct*. 2013;31:12–19.
38. Harichandan A, Sivasubramanian K, Hennenlotter J, Schwentner C, Stenzl A, Bühring H-J. Isolation of adult human spermatogonial progenitors using novel markers. *J Mol Cell Biol*. 2013;5:351–353.
39. Hübner K, Fuhrmann G, Christenson LK, et al. Derivation of oocytes from mouse embryonic stem cells. *Science*. 2003;300:1251–1256.
40. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature*. 2004;427:148–154.
41. Hua J, Sidhu KS. Coaxing HESC to form oocyte-like structures by co-culture with testicular extract and hormones. *Open Stem Cell J*. 2011;3:34–45.
42. Hua J, Sidhu K. Recent advances in the derivation of germ cells from the embryonic stem cells. *Stem Cells Dev*. 2008;17:399–412.
43. Dyce PW, Wen L, Li J. In vitro germline potential of stem cells derived from fetal porcine skin. *Nat Cell Biol*. 2006;8:384–390.
44. Hua J, Yu H, Liu S, et al. Derivation and characterization of human embryonic germ cells: Serum-free culture and differentiation potential. *Reprod Biomed Online*. 2009;19:238–249.
45. Nayernia K, Nolte J, Michelmann HW, et al. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. *Dev Cell*. 2006;11:125–132.
46. Clark AT, Bodnar MS, Fox M, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet*. 2004;13:727–739.
47. Cao H, Chu Y, Lv X, et al. GSK3 inhibitor-BIO regulates proliferation of immortalized pancreatic mesenchymal stem cells (iPMSCs). *PLoS ONE*. 2012;7:e31502.
48. Ying QL, Wray J, Nichols J, et al. The ground state of embryonic stem cell self-renewal. *Nature*. 2008;453:519–523.
49. Leitch HG, Nichols J, Humphreys P, et al. Rebuilding pluripotency from primordial germ cells. *Stem Cell Rep*. 2013;1:66–78.
50. Youn H, Kim SH, Choi KA, Kim S. Characterization of Oct4-GFP spermatogonial stem cell line and its application in the reprogramming studies. *J Cell Biochem*. 2013;114:920–928.
51. Guan K, Nayernia K, Maier LS, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature*. 2006;440:1199–1203.
52. Kanatsu-Shinohara M, Inoue K, Lee J, et al. Generation of pluripotent stem cells from neonatal mouse testis. *Cell*. 2004;119:1001–1012.
53. Kanatsu-Shinohara M, Ogonuki N, Inoue K, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod*. 2003;69:612–616.
54. Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL. Culture of mouse spermatogonial stem cells. *Tissue Cell*. 1998;30:389–397.
55. Kanatsu-Shinohara M, Miki H, Inoue K, et al. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. *Biol Reprod*. 2005;72:985–991.
56. Piravar Z, Jeddi-Tehrani M, Sadeghi MR, Mohazzab A, Eidi A, Akhondi MM. In vitro Culture of Human Testicular Stem Cells on Feeder-Free Condition. *J Reprod Infertil*. 2013;14:17–22.
57. He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M. Isolation, characterization, and culture of human spermatogonia. *Biol Reprod*. 2010;82:363–372.