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Full Length Research Paper

Glycogen synthase kinase 3 (GSK3) inhibitor 6-bromoindirubin-3'-oxime (BIO) promotes the proliferation of mouse male germline stem cells (mGSCs) under serum- and feeder-free conditions

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The 6-bromoindirubin-3'-oxime (BIO), as one of glycogen synthase kinase 3 (GSK3) inhibitors, is a key regulator of many signaling pathways with the capacity to maintain the pluripotency of human and mouse embryonic stem cells (ESCs). Whether BIO can maintain the pluripotency of mouse male germline stem cells (mGSCs) remains unclear at present. In this study, with BIO and retinol (RE) both in Knock-out serum replacement (KSR) medium, we got a relatively optimal feeder- and serum-free system for mGSCs *in vitro*. After continuous culturing, the proliferation efficiency of undifferentiated mGSCs and differentiation capacity of mGSC-induced embryoid bodies (EBs) were examined as well. Results show that in the presence of LIF or retinol, BIO significantly increased the number of mGSCs and alkaline phosphatase (AP) positive colonies, and the mitosis index through the BrdU assay. BIO also increased the expression of pluripotent markers including Oct4, Nanog, PCNA and c-Myc analysed by real-time polymerase chain reaction (PCR). The mGSCs cultured in RE medium containing BIO can form embryoid bodies (EBs), which consist of three embryonic germ layers analysed by immunofluorescence. When these cells were transplanted into infertile mice, they could well repair the capacity of making male germ cells. Our results demonstrate the combined treatment of BIO and RE could significantly promote the proliferation of mouse mGSC colonies and maintain the undifferentiated status.

Key words: Male germline stem cells (mGSCs), BIO, retinol (RE), feeder- and serum-free, mouse.

INTRODUCTION

Spermtogonial stem cells (SSCs) are a unique population of adult stem cells that have self-renewal potential and could differentiate into three germ layers, thus being valuable for restoration of fertility, germline gene therapy, regenerative medicine and efficient production of transgenic animals (Izadyar et al., 2003; de Rooij, 2006; Kubota and Brinster, 2006; de Rooij and Mizrak, 2008).

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SSCs are rare *in vivo* and could be successfully cultured *in vitro* for long periods of time, they were also named the dramatically expanding cells-germline stem cells (GSCs) (Kanatsu-Shinohara et al., 2003, 2004).

GSCs, isolated from mouse neonates or p53-deficient adult mice, could form colonies of ES-like cells called multipotent germline stem cells after culturing for 4 to 7 weeks (Kanatsu-Shinohara et al., 2004). Using differentg assays, Guan et al. (2006) and Seandel et al. (2007) also obtained ES-like cells from adult mouse testis named multipotent adult germline stem cells (maGSCs) and multipotent adult spermatogonial-derived stem cells (maSSCs), respectively (Guan et al., 2006; Seandel et

al., 2007). These three types of cells appeared to have the multipotent characters of the ES-like cells, due to their potentials to form teratoma, EBs and chimaeras with the ability to form endodermal, mesodermal and ectodermal derivatives (de Rooij and Mizrak, 2008). Recently, Ko et al. (2009) reported a novel protocol for obtaining germline-derived pluripotent stem (gPS) cells from adult unipotent GSCs. Pluripotency of gPS cells was confirmed by *in vitro* and *in vivo* differentiation, germline contribution in chimeras, and germline transmission to the next generation, indicating a novel protocol to establish pluripotent cell lines without the introduction of exogenous reprogramming factors (Ko et al., 2009).

Since many groups have obtained the ES-like cells, it seems that the purity of the starting population of GSCs and finding a very specific approach to isolate and transform GSCs are not of crucial importance (de Rooij and Mizrak, 2008). Nevertheless, the transformation and proliferation of the ES-like cells from GSCs and whether GSCs could be directly differentiated into other cell lineages remain to be further elucidated. What's more, the transformed ES-like cells were mostly cultured under conditions with serum and/or feeder cells in these laboratories (Kanatsu-Shinohara et al., 2004, 2005; Guan et al., 2006; Seandel et al., 2007; Ko et al., 2009). Both serum and feeder cells provide complex and undefined materials with considerable variation, making it un-controllable to investigate the molecular mechanism. Recently, Kanatsu-Shinohara et al. (2011) developed a serum- and feeder-free culture system including glial cell line-derived neurotrophic factor (GDNF), fetuin and lipid-associated molecules for SSC proliferation *in vitro*. However, the medium needs to be refined and the mechanism should be further investigated.

Previous reports showed that BIO is the first pharmacological reagent to maintain self-renewal in human and mouse ESCs (Meijer et al., 2003; Sato and Brivanlou, 2006). BIO, as an inhibitor of GSK3β, could activate Wnt signaling pathway to sustain pluripotency of both human and mouse ESCs (Sato et al., 2004; Dravid et al., 2005; Anton et al., 2007; Sineva and Pospelov, 2010). Whether BIO could maintain pluripotent stem cell properties and participate in maintaining the pluripotency of mGSCs is still an issue (Golestaneh et al., 2009b; Wen et al., 2010). To establish a culture system without serum and feeder cells, firstly we isolated mouse male germline stem cells (mGSCs) from mouse adult testis and BIO was then added to culture the cells in serum- and feeder-free conditions. The proliferated cells were assessed for ESC-like properties by AP staining, BrdU staining, real-time PCR, immunofluorescence staining and differentiation potentiality.

MATERIALS AND METHODS

Kunming strain mice were used in the study. They were maintained under standard conditions with free access to food and water at the Animal Facilities in our lab. All of the feeding and experimental procedures on animals were in accordance with the guidelines of the local laws.

The effects of BIO and retinol on the proliferation of mouse mGSCs

During the primary culture, the mGSC colonies were obtained following an established protocol (Pan et al., 2010; Zhang et al., 2011). They were cultured by feeder-free condition, combined with ESGRO Complete Clonal Grade Medium (Clonal medium, Chemicon), which is a commercial medium without serum. To further study whether these mGSC colonies could grow well in feeder- and serum-free condition with non-commercial medium *in vitro*, they were dissociated mechanically or by TrypLE (Invitrogen), or Accutase (Millipore). The subcultured GSCs were cultured in feeder- and serum- free system with BIO addition, including (1) KSR control medium: DMEM containing 15% Knock-out serum replacement (KSR, Invitrogen), 4 mM Glutamine, 0.1 mM 2-mercaptoethanol (Invitrogen), 1% non-essential amino acids (Invitrogen); (2) different concentrations of BIO medium: supplemented 1.25, 2.5, 5 μM BIO (Sigma, USA) separately in KSR medium; (3) Clonal medium. On the 3rd and 7th day, the total number of mGSCs by using hemocytometer and the number of AP positive clones in different media were counted. With the same methods, we calculated the number of mGSCs in KSR, different concentrations of BIO in combination with 1000 IU/ml LIF, and the optimal concentration of BIO with 1000 IU/ml LIF or 5×10^{-6} M RE addition (Zhang et al., 2011).

Characterization of mouse mGSCs cultured *in vitro* with BIO + RE medium

Alkaline phosphatase staining

Alkaline phosphatase (AP) was detected following fixation of cells with 4% paraformaldehyde (PFA) and the fixed cells were washed three times with PBS, then stained in naphtol AS-MX phosphate (200 μg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM tris-buffer (pH 8.2) for 10 to 15 min at room temperature. Staining was terminated by washing cultures with PBS to evaluate the characteristics and count the number of AP positive colonies.

BrdU incorporation assay

The proliferation of mGSCs was assayed by BrdU incorporation (Dyce et al., 2004). In brief, mouse mGSCs were treated with 50 μg/ml BrdU (Sigma) for 4 h and then subjected to BrdU fluorescence staining. Cells were fixed in 4% PFA for 15 min at room temperature and washed with PBS for three times, then incubated with 0.1% Triton-100 for 5 min and the cells were washed three times in PBS at room temperature. Anti-BrdU (1:100; Santa Cruz) dissolved in 0.1 M PBS (pH 7.4) containing 4% normal goat serum was added and the cells were incubated overnight at 4°C. Cells were washed in PBS for three times, and then incubated with the secondary antibody (FITC, 1:500, Millipore) for 1 h at room temperature. After washed three times, cells were visualized under fluorescent microscope and analysed for BrdU staining.

Real-time PCR (QRT-PCR)

Real-time PCR was performed to assess the expression of marker genes in mGSCs cultured in KSR, RE, BIO+RE, LIF, BIO+LIF, and

Table 1. The primers used for QRT-PCR.

Name	Sense primer	Antisense primer	Product size
Oct4	5'- tatgcaaatcggagaccctg- 3'	5'-aagctgattggcgatgtgag-3'	143
Nanog	5'-gattcttctaccagtcccaaac- 3'	5'-atgcgtt caccagat agcc 3'	285
PCNA	5'-agtggagaacttggaaatggaa-3'	5'-gagacagtggagtggcttttgt-3'	154
c-Myc	5'-acttctccaccgccgatcag- 3'	5'-aggctggtgctgtctttgcg-3'	211

Clonal medium for 48 h after subculture. Pluripotency and proliferation-related genes (Oct4, Nanog, PCNA, c-Myc) were analysed, and β-actin was used as internal control. Total RNA was extracted by using the Trizol method (Invitrogen) and the cDNA was synthesized based on 500 ng RNA with a commercially available kit (Fermentas). Then real-time PCR was performed using SYBR Green kit (Bioer), and the program was initiated with denaturation at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 20 s at 58°C, while fluorescence was collected from 65°C to 95°C, with the frequency 0.5°C up by each time, and the data were analysed by Linegene 9660 software. The following genes were amplified using the primers indicated in the Table 1.

Immunofluorescence staining

The 10th passage mGSC colonies cultured in RE medium containing BIO were fixed with 4% PFA for 10 min at room temperature, followed by three washes in cold PBS for 5 min each. Then the cultures were treated with blocking solution (PBST + 1% BSA) for a minimum of 30 min and incubated in primary antibodies specific for Oct4 (1:500, Chemicon), SSEA1 (1:200, Chemicon), CD49f (1:500, Chemicon), GFRα1 (1:100, Santa Cruz), which are markers of mGSCs (Kossack et al., 2009). The appropriate FITC-conjuated secondary antibodies (1:500, Chemicon) were used according to the manufacturer's manual. The nuclei of cells were stained by Hoechst33342.

In vitro differentiation of mGSCs

The mGSC colonies cultured in BIO + RE medium were dissociated mechanically into small clumps and re-suspended in DMEM containing 15% Fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin at a density of 300 to 500 cells/20 µl for 72 h to form EBs. For further differentiation, the EBs were cultured in Petri dishes coated with 0.1% gelatin and DMEM containing 15% FBS, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen) and 0.1 mM non-essential amino acids for 3 to 14 days to investigate the potentiality of spontaneous differentiation.

To prove the differentiation potential to male germ cells, some EBs were induced by 10^{-6} M retinoic acid (RA, Sigma) (Geijsen et al., 2004; Kerkis et al. 2007; Nayernia et al., 2006; Ohta et al., 2010), and the cells were cultured for 10 to 20 days in this medium before they were analysed by immunofluorescence staining.

Transplantation of mGSCs into germline-deficient mouse models

Germline-deficient mouse models were made by intraperitoneal injection of busulfan (40 mg/kg). These models were nearly the same as normal mice, except with the deficiency in male germ cell production (Wang et al., 2010). Then mGSCs, subcultured in BIO + RE medium for 15 passages *in vitro*, were transplanted into one side testis of these mouse models, and the other side was injected with medium as control. Both sides were analysed by immunofluorescence staining one month after transplantation. All the presented experiments were replicated at least 3 times and the data were analysed by t-test.

RESULTS

BIO contributes to the proliferation of mouse mGSCs

The formation of compact colonies was observed after 4 to 7 days of culture in Clonal medium. Then they grew large enough to form typical mGSC colonies with well-demarcated morphology and strongly expressed AP.

To observe the effect of BIO on mGSC proliferation, these colonies were dissociated mechanically or by TrypLE, or Accutase. The subcultured mGSCs were cultured in KSR medium with different concentrations of BIO (1.25, 2.5, 5 µM) or Clonal medium. After 7 days, the number of cells cultured with BIO alone was significantly less than that in Clonal medium, indicating that BIO alone could not obviously improve mGSC proliferation (Figure 1 A).

Then we added 1000 IU/ml LIF each on the basis of BIO, and the mGSCs were cultured in KSR medium with LIF or LIF in combination with different concentrations of BIO (1.25, 2.5, 5 µM), or Clonal medium. By counting the number of cells and AP positive colonies, we found that BIO conbined with LIF could promote the proliferation of mGSCs much better than LIF alone (Figure 1B, D). To some extent, a higher concentration of BIO could promote mGSCs grow faster relatively. Compared with BIO alone (Control), colonies in medium with LIF or LIF conbined with BIO were more typical and tight (Figure 1C). Clonal medium, as positive control, was the most efficient medium to promote mGSC proliferation and could obtain more AP positive colonies, while the morphology of colonies was not very typical and compact (Figure 1C). In general, according to the proliferation ability and morphology, BIO could promote the proliferation of mGSCs in the presence of LIF, and we

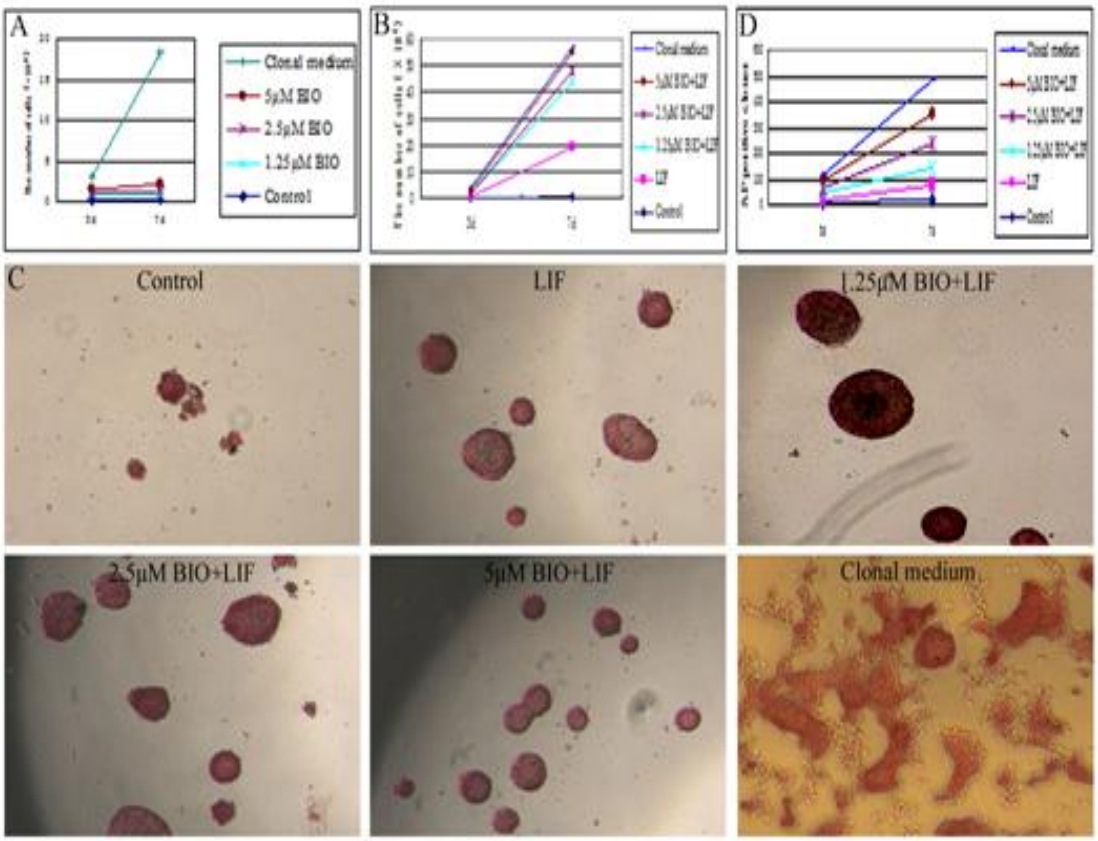

Figure 1. Differentiation potential of mGSCs *in vitro* and *in vivo*. Immunofluorescence staining of three germ layers by EB spontaneous differentiation *in vitro* (A, B, C). A, β-Ⅲ tubulin (ectoderm), Bar=200 μm; B, AFP (endoderm), Bar=20 μm; C, Islet (mesoderm), Bar=20 μm. Restoration of germline-deficient mouse models by transplantation of mGSCs cultured in RE medium containing BIO *in vivo* (D-F′). After transplantation for one month, compared with the untransplanted sides, testis of the transplanted sides were well repaired, and there were multi-layers of spermatogenic cells within the seminiferous tubules. D, E, F - the untransplanted sides; D′, E′, F′ - the transplanted sides. D, D′, H.E. staining, Bar=200 μm; E, E′, immunofluorescence staining for Stra8 and Dazl, Bar=400 μm; F, F′, immunofluorescence staining for Stra8 and Scp3, Bar = 400 μm.

employed 2.5 μM BIO as an optimal concentration for further study.

BIO combined with retinol promotes the proliferation of mouse mGSCs

Our previous study has shown that retinol (RE), the alcohol form of Vitamin A, could replace LIF to some extent to support the self-renewal of mGSCs (Zhang et al., 2011). Therefore, we tested whether BIO combined with RE could replace the effect of LIF. The same concentration of BIO (2.5 μM) was added in medium, combined with LIF or RE, respectively. The analysis of proliferative cell calculation and AP positive colony calculation showed that 2.5 μM BIO + RE is superior to RE alone and even 2.5 μM BIO + LIF (Figure 2A and B). Brdu incorporation assay also demonstrated that the cell proliferation rate in 2.5 μM BIO + RE medium was similar to that in 2.5 μM BIO + LIF medium, with the similar morphology (Figure 2C and D). Real-time PCR analysis indicated that 2.5 μM BIO + RE could significantly up-regulate the pluripotency and proliferation-related genes including Oct4, Nanog, PCNA and c-Myc compared with RE (Figure 3A to D). In brief, 2.5 μM BIO combined with RE could promote the proliferation of mGSCs very well.

Characterization of mouse mGSCs cultured in 2.5 μM BIO + RE medium

The cells were passaged every 2 to 3 days and cultured consecutively for more than 20 passages in 2.5 μM BIO + RE medium. Even in higher passages, mGSC colonies still highly expressed AP and pluripotent markers of embryonic stem cells (ESCs), such as Oct4 and SSEA1 (Figure 3E and F). The mGSC colonies were also positive for specific markers such as GFRα1 and CD49f (Figure G and H). These results indicate that the mouse mGSCs express the surface markers and the transcription factors,

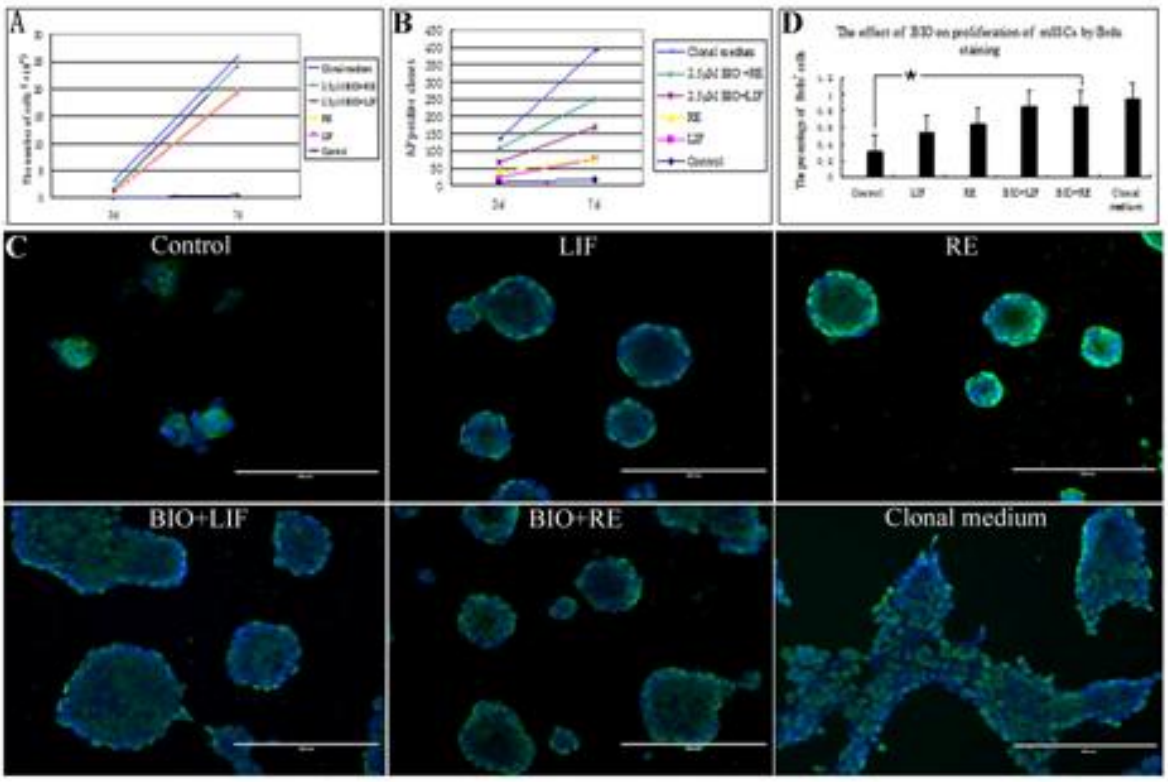

Figure 2. The effects of BIO on the proliferation of mGSCs. BIO alone could not obviously increase the number of mGSCs without LIF (A); In the presence of LIF, BIO could significantly promote mGSC proliferation (B); The morphology of mGSC colonies cultured with BIO remained typical morphology analysed by AP staining (C), while higher concertrations of BIO increased AP positive colonies in the presence of LIF (D).

which are characteristics of undifferentiated ES cells and typical mGSCs (Conrad et al., 2008).

To determine whether mouse mGSCs cultured in 2.5 µM BIO + RE medium could differentiate into three germ layers *in vitro*, we applied a common method (EB induction) to induce spontaneous differentiation of mouse mGSCs into ectoderm, mesoderm and endoderm. Many cell types were spread from the attached EBs, and immunofluorescence staining showed that the differentiated cells derived from mGSC-induced EBs were positive for B-III-tubulin (an ectoderm marker), AFP (an endoderm marker) and Islet1 (a mesoderm marker) (Figure 4A to C). These results proved the multipotency of mGSCs.

Since mGSCs were isolated from germline, then we tested the ability of mGSCs to produce germ cells. Cultured mGSCs were transplanted into testis of busulfan-treated mouse models. One month later, H.E. staining and immunofluorescence staining showed that compared with untransplanted sides, mGSCs could effectively restore the damage caused by busulfan with the expression of germ cell markers such as Stra8 and Scp3 (Figure 4D to F). For differentiation ability *in vitro*, mGSC-induced EBs was treated with RA for 10 to 20 days. Immunofluorescence staining results showed that these EBs could also express meiosis- and male germ cell-specific markers, such as Vasa, Stra8, c-Kit, Dazl and Acr (Figure 5). In summary, mGSCs cultured in 2.5 µM BIO + RE medium possessed the ability to differentiate into germ cells *in vivo* and *in vitro*.

DISCUSSION

Mouse mGSCs could be cultured for long periods and retain the capacity to differentiate into multiple cell lineages in the presence of serum or feeder cells *in vitro* (Kanatsu-Shinohara et al., 2003, 2005; Hermann et al., 2009; Kossack et al., 2009). Basic techniques to isolate and culture mGSCs are well established, and many existing mGSC lines have been cultured by using mouse embryonic fibroblast (MEF) feeder cells and (or) serum or other animal-sourced medium components. These mGSCs shared morphology and phenotypical characteristics similar to mouse ES cells, so they might potentially replace ESCs to solve many diseases, and meantime avoid the ethical controversy (Kanatsu-Shinohara et al., 2003, 2005; Turnpenny et al., 2006; Conrad et al., 2008; Kanatsu-Shinohara et al., 2008; Mizrak et al., 2010). However, feeders and animal-

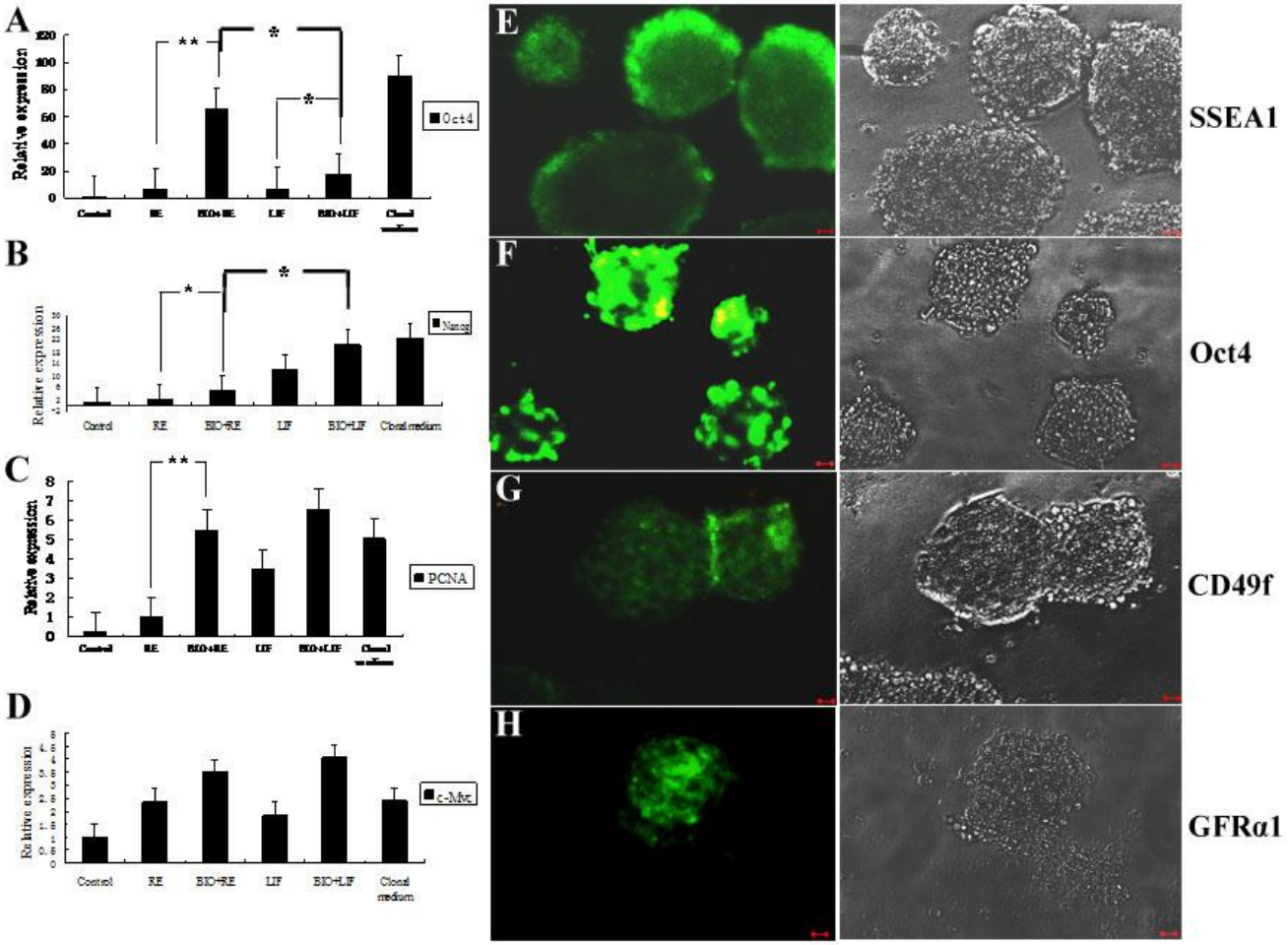

Figure 3. The effects of BIO combined with LIF or RE on the proliferation of mGSCs. The comparison of BIO, RE and LIF analysed by the number of cells (A), AP positive colonies (B) and BrdU staining (D). On the basis of BIO, RE could replace LIF and significantly promote the proliferation of mGSCs. The BrdU staining of mGSCs cultured in BIO + LIF (RE) medium for 48 h (C), Bar = 200 μm.

derived components in mGSC culture may also hinder the clinical applications of mGSCs, due to: (a) the presence of immunogenic material; (b) the risk of transmitting animal virus or prion materials; and (c) difficulty with quality control of these undefined components.

Very recently, Kanatsu-Shinohara et al. (2011) developed a serum- and feeder-free culture system including glial cell line-derived neurotrophic factor (GDNF), fetuin and lipid-associated molecules for SSC proliferation *in vitro*. They demonstrated that fetuin promotes cell attachment while lipid signaling is nece-ssary for germline stem cell proliferation (Kanatsu-Shinohara et al., 2011). However, the medium needs to be improved and the molecular mechanism remains largely unknown.

Retinol, the alcohol form of vitamin A, was shown to promote self renewal of mouse ES Cells by activating PI3K/Akt Signaling Pathway via IGF-1 Receptor which was independent of LIF-Jak-STAT3 signaling pathway (Chen and Khillan, 2010). Our group also demonstrated that retinol can maintain self-renewal of mGSCs under serum- and feeder-free conditions (Zhang et al., 2011). However, the proliferation rate is still limited, and the molecular mechanism needs to be further investigated.

Here, we studied the effects of BIO, retinol and LIF on the proliferation of mGSCs. We found that in serum- and feeder-free medium, BIO combined with LIF or RE could better maintain the proliferative capacity of mGSCs and its pluripotency to generate cells of all three germ layers-endoderm, mesoderm and ectoderm. And these germ layer cells could further differentiate into many specific cell lineages. Previous studies have showed that the inhibitor of GSK3β may severely impair differentiation of mouse ESCs into three germ layers (endoderm, ectoderm and mesoderm) through activation of β-catenin signaling (Doble et al., 2007). Wnt/β-catenin pathway, especially WNT3A, may play an important role in the regulation of proliferation and pluripotency of mouse and human mGSCs (Katoh, 2007; Golestaneh et al., 2009a).

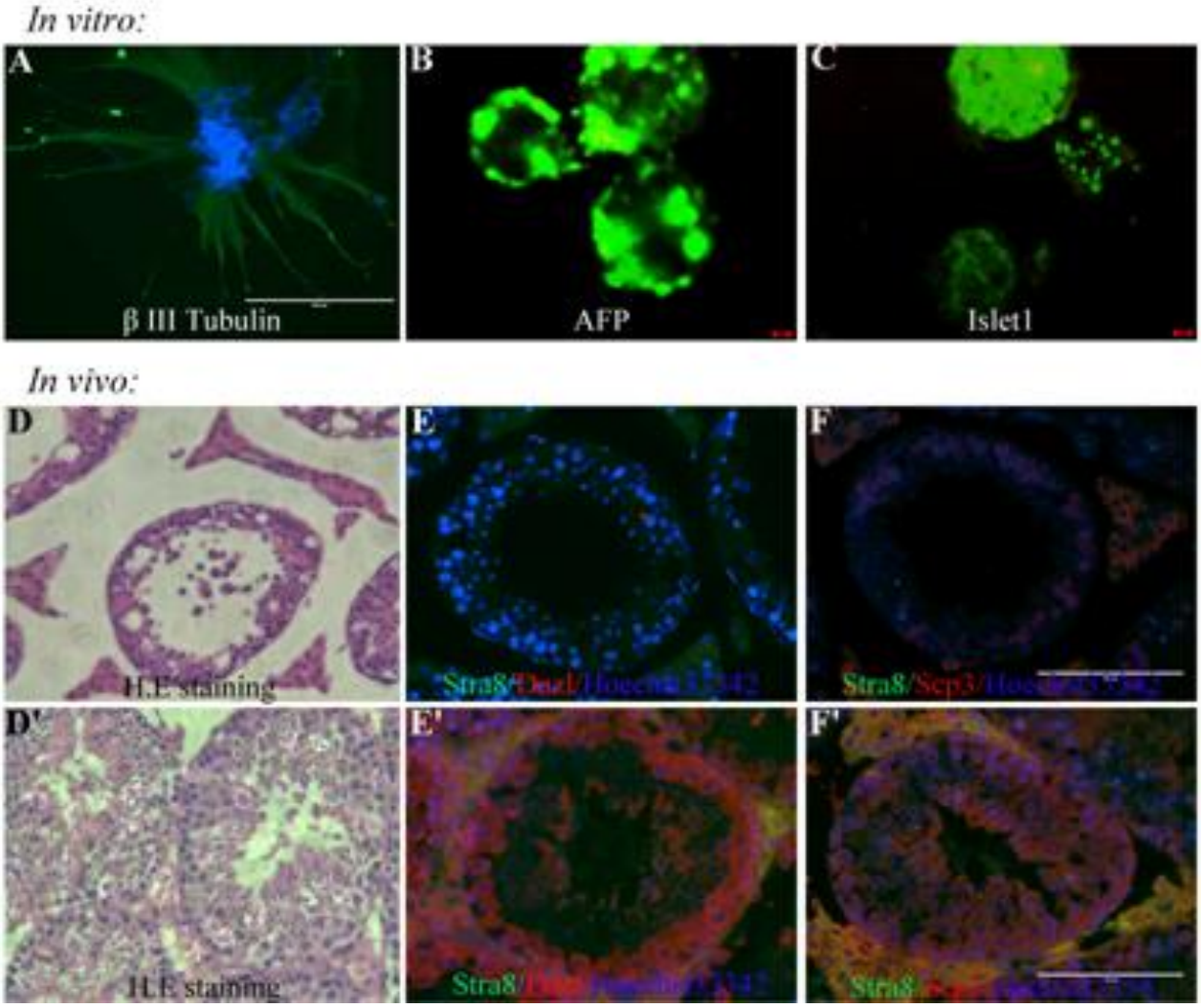

Figure 4. Real-time PCR and immunofluorescence analysis of mGSCs. The expression of pluripotent genes Oct4 (A), Nanog (B), PCNA (C) and c-Myc (D) of mGSCs cultured with different media for 48 h were analysed by real-time PCR. *, $P < 0.05$; ** $P < 0.01$. Immunofluorescence staining of mGSC specific markers SSEA1 (E), Oct4 (F), CD49f (G) and GFRα1 (H), Bar = 20 μm.

It exerts the effects accompanied with the activation of a series of key downstream factors that finally resulted in the activation of transcriptional genes, such as Oct4, Sox2 and Nanog, which are intrinsic pluripotent transcriptional factors to maintain the undifferentiated status of mammalian ESCs (Cole et al., 2008; Giorgetti et al., 2010; Wen et al., 2010). The function of GSK3 inhibitor BIO in the culture of pluripotent stem cells has also been previously described (Dravid et al., 2005; Anton et al., 2007; Sineva and Pospelov, 2010). In our study, we found that BIO could stimulate spermatogonial stem cells to form AP positive mGSC colonies and these cells could be cultured up to 2 months in the presence of BIO. BrdU incorporation assay further proved that BIO could promote more mGSCs to enter mitosis stage (Mussmann et al., 2007). BIO combined with RE also up-regulate the expression of some key ES cell transcriptional factors including Oct4, Nanog, and proliferative markers: PCNA, c-Myc in mGSC culture. PCNA is a distinct marker for the proliferative spermatogonia (Wrobel et al., 1996; Costoya et al., 2004), which was up-regulated in BIO + RE medium. More important, these cultured mGSCs well maintained the capacity of making male germ cells while they were transplanted into infertile mice. To our knowledge, this is the first study that proved that BIO can promote the proliferation and stemness of mouse mGSCs.

Conclusion

Our results demonstrated that BIO combined with retinol can promote the proliferation of mGSCs, maintaining the undifferentiated status and differentiation potential in

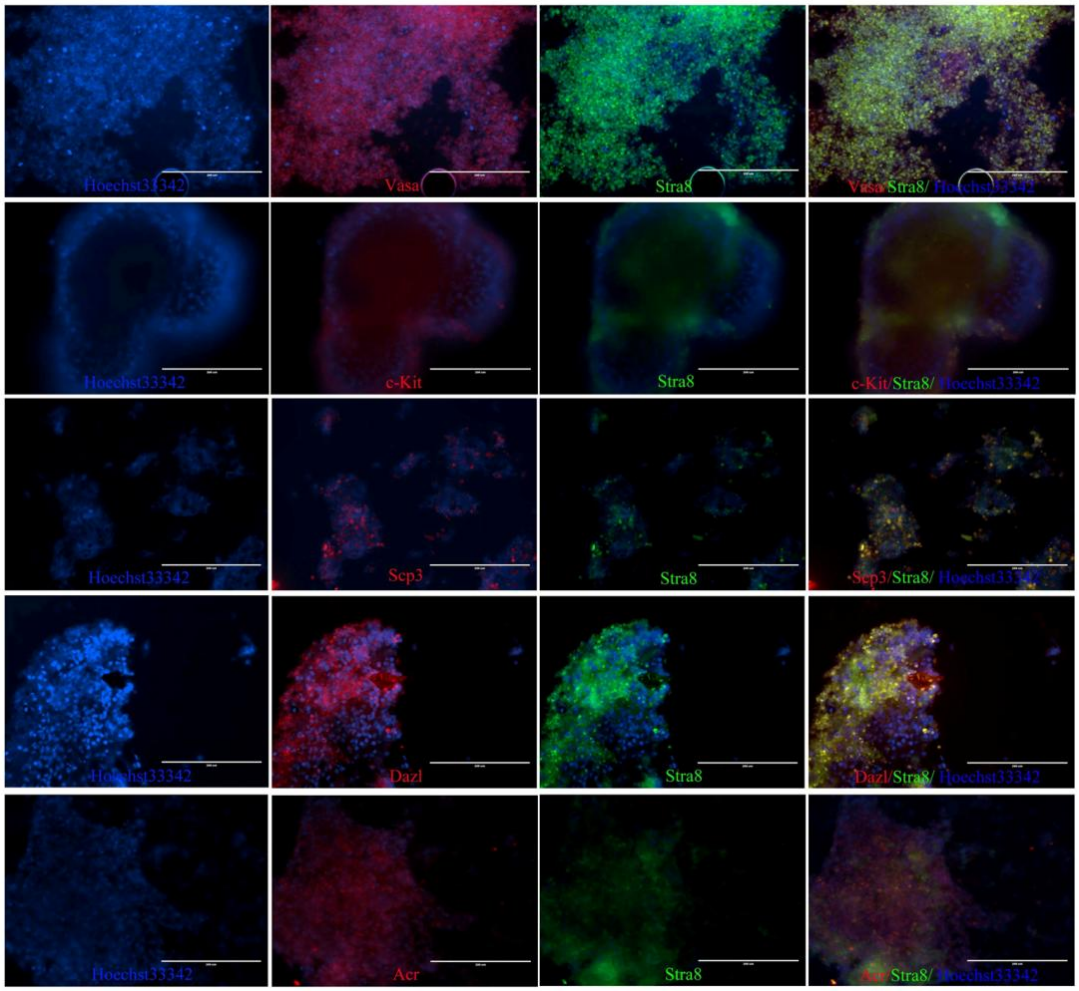

Figure 5. Differentiation of mGSCs cultured in RE medium containing BIO differentiated into male germ cells. Immunofluorescence staining of male germ cell-specific markers after induction of mGSC-induced EBs with 1μM RA for 10-20 days *in vitro* (Fig.5). Primary antibodies were Vasa, c-Kit, Scp3, Dazl, Acr; Bar=200 μm. These induced EBs expressed many germ cell markers, even late spermatogenesis-related genes, such as Acr.

serum- and feeder-free system.

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