

Ras/ERK1/2 pathway regulates the self-renewal of dairy goat spermatogonia stem cells

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Abstract

Spermatogonia stem cells (SSCs), also named the male germline stem cells (mGSCs), which is located at the base of the seminiferous tubules of testis, is the basis for generating sperm steadily in male animals. Currently, there are some preliminary study on the self-renewal and differentiation of SSCs, but further mechanism, especially in large animals, has not been clearly understood. Ras/ERK1/2 pathway is widely distributed in multiple cells *in vivo*. It plays an important role in cell proliferation, differentiation and so on. However, the study on the function for the self-renewal of dairy goats SSCs has not been investigated. In this study, the dairy goat SSCs characterization were evaluated by semi-RT-PCR, alkaline phosphatase (AP) staining, and immunofluorescence staining. Then, Ras/ERK1/2 pathway was blocked by specific MEK1/2 inhibitor PD0325901. We analyzed the proliferation by cell number, cell growth curve, Brdu incorporation assay, and cell cycle analysis. The results showed that the proliferation was significantly inhibited by PD0325901. Cell apoptosis induced by blocking the Ras/ERK1/2 pathway was analyzed by TUNEL. The expression of *ETV5* and *BCL6B*, the downstream gene of Ras/ERK1/2 pathway, was downregulated. This study suggest that the Ras/ERK1/2 pathway plays a critical role in maintaining the self-renewal of dairy goat SSCs via regulation of *ETV5* and *BCL6B*. This study laid a foundation for insights into the mechanism of SSCs self-renewal comprehensively.

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Introduction

Spermatogonia stem cells (SSCs), also named as the male germline stem cells (mGSCs), are located at the base of the seminiferous tubules of testis. It is the basis for generation of sperm steadily in mammalian animals. Besides the self-renewal and pluripotency, SSCs are the only one kind of adult stem cells of all the adult stem cells, which can pass their genetic materials to offspring *in vivo* (Izadyar *et al.* 2003, Oatley & Brinster 2008). SSCs are an excellent resource for exploring the mechanisms of spermatogenesis, and are used for treating infertility. It has the vital significance for the development of reproductive medicine, animal husbandry and veterinary medicine. Currently, there are some preliminary study about the self-renewal and differentiation mechanisms of mammalian SSCs (Oatley *et al.* 2006, He *et al.* 2009, Song & Wilkinson 2014); however, the precise mechanism has not been understood, especially in livestock (Cao *et al.* 2011). The Ras/ERK1/2 signaling pathway widely existed in eukaryotic cells is

one of the important pathways mediating cellular information transfer and is composed of three conservative signaling networks: MAPKK kinase (MAPKKK, MAP3K), MAPK kinase (MAPKK, MAP2K), and MAPK (Johnson *et al.* 2005, Ishii *et al.* 2012). The pathway mediates a variety of biological effects, including cellular proliferation, differentiation, transformation, inflammation, and apoptosis. In recent years, previous study found that Ras/ERK1/2 signaling pathway has a vital role in a variety of cellular functions, including cell proliferation, differentiation, and cell cycle progression (He *et al.* 2008, Ishii *et al.* 2012).

There are many factors that control the fate of mammalian SSCs, including key cytokines and transcriptional factors (Chen *et al.* 2005, He *et al.* 2007, 2008, Oatley & Brinster 2008, Zhu *et al.* 2012, Song & Wilkinson 2014). The cytokines play important roles in maintaining the self-renewal of SSCs via regulation of the downstream signaling genes especially. Glial cell line-derived neurotrophic factor (GDNF) was identified as the first necessary cytokine to maintain the self-renewal of

SSCs (Kanatsu-Shinohara *et al.* 2003). Knockout of GDNF can drastically reduce mouse SSCs in the seminiferous tubule (Meng *et al.* 2000). GDNF maintains the self-renewal of SSCs mainly via PI3K-AKT and Ras/ERK1/2 pathway (Lee *et al.* 2007, Sun *et al.* 2013). In SSCs, GDNF had regulative function by activating receptor *Gfra1* (He *et al.* 2007, Kanatsu-Shinohara & Shinohara 2013). Fibroblast growth factor 2 (bFGF2, FGF2) activate the Ras/ERK1/2 pathway by fibroblast growth factor receptor (FGFR). GDNF and bFGF2 are required to recapitulate the self-renewal of mammalian SSCs *in vitro*, and bFGF2 is dependent on Ras/ERK1/2 signaling to maintain the self-renewal of SSCs via upregulation of the *Etv5* and *Bcl6b* (Ishii *et al.* 2012). These two factors regulate the self-renewal of SSCs via different pathways, and suggested that the mechanisms of self-renewal of SSCs are very complex. The balance between the PI3K-AKT and Ras/ERK1/2 pathway is of great significance to maintain the self-renewal of SSCs (Kanatsu-Shinohara & Shinohara 2013).

In this study, the dairy goat SSCs were isolated and identified, further we used MEK1/2 specific inhibitor PD0325901 to block the Ras/ERK1/2 pathway, and explore its effects on the self-renewal of dairy goat SSCs. The results showed that the Ras/ERK1/2 pathway plays a critical role in dairy goat SSCs' self-renewal via regulation of *ETV5* and *BCL6B*, which are both the downstream genes of Ras/ERK1/2 pathway. This study laid a promising platform for exploring the mechanism of SSCs' self-renewal comprehensively.

Materials and methods

Collection and isolation of dairy goat testis

Guanzhong dairy goat testes were collected from Yaoan slaughterhouse in Yangling Hi-tech area, and the testes were collected from the dairy goats at different ages. The goats were killed by standard method for experimental research, and all the procedures were approved by Shaanxi Centre of Stem Cells Engineering and Technology, Northwest A&F University. The goat testes were washed five to ten times with PBS supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. The seminiferous tubules were stripped from each testis and then dissected into small pieces using forceps. The seminiferous epithelial cells were dissociated using three enzyme cocktails: CDD, 2 mg/ml collagenase (Invitrogen), 20 µg/ml DNase (Sigma) and 2 mg/ml dispase (Invitrogen). All the enzyme cocktails were dissolved in Dulbecco's PBS. The digestion was conducted at 37 °C for 15 min by pipetting every 5 min for the first step. After centrifugation at 100 g for 5 min, the fragments of seminiferous tubules were collected and digested with TD (0.25% trypsin and 10 mg/ml DNase I) for the second step for 10 min at 37 °C. Then the cells were collected and centrifuged at 100 g for 5 min, resuspended by DMEM/F12 containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). They were then transferred to the culture dish coated with gelatin and cultivated for 4 h. The non-adherent cells were

collected and transferred to new dishes. Then, the cells were purified by MASC technique to obtain Thy1-positive cells required for culture (Wu *et al.* 2013).

Cell culture

Dairy goat SSCs were purified and cultured in the plates coated with Matrigel (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the medium consisted of DMEM/F12 (Invitrogen), 1% FBS, 10% KSR (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 2 ng/ml bFGF (Millipore, Bedford, MA, USA), 10 ng/ml GDNF (Reproach), 50 ng/ml *Gfra1* (Sino Biological, Inc., Beijing, China), and 20 ng/ml epidermal growth factor (EGF, Sino Biological, Inc.). The dairy goat SSCs were passaged by Tryple (Invitrogen). The medium was changed every day (Zhu *et al.* 2013, Niu *et al.* 2014).

Cell growth curve

The growth curve of dairy goat SSCs was analyzed according to the protocol described previously (Lv *et al.* 2012). Briefly, cells were serially subcultured at an initial seeding density of 1×10^4 cells per well in a 24-well plate in triplicates and the total cell number of per well was counted after 48-h cultured in the control medium or media containing PD0325901 (0, 2.5, and 10 µM, Sigma). The proliferation ability of the cells was evaluated by cell count number at an interval of 48 h. The number of cells was determined for 8 consecutive days (Cao *et al.* 2012).

Alkaline phosphatase staining

Alkaline phosphatase (AP) activity was determined essentially as described previously (Piedrahita *et al.* 1998). 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 (200 µg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM Tris-buffer, pH 8.2–8.4, for

Table 1 The sequence of primers and RT-PCR conditions.

Name	Primer (5' → 3')	Product size (bp)	T _m
NANOG	Forward: CCCGAAGCATCCAACCTC	297	58
	Reverse: CTGCCTCTGAAATCTGTCAAT		
VASA	Forward: GGAGATGAAGATTGGGAAGCA	1124	58
	Reverse: GTTGGTGCTACAATAATACACTCTG		
GFRA1	Forward: CGCCTTGCGGATTTTTTACC	286	58
	Reverse: ACACGGTCACATCGGAGCCA		
PLZF	Forward: CACCGCAACAGCCAGCACTAT	127	58
	Reverse: CAGCGTACAGCAGGTATCCAG		
CD49F	Forward: CGAAGCACGAATCCCCGAGAC	235	58
	Reverse: TGCTCTACACGAACAATCGCTTT		
GAPDH	Forward: CGGCTCTCAAGGGCATTCTAGGC	158	58
	Reverse: TGAGGTCCACCACCCTGTGTGCTG		

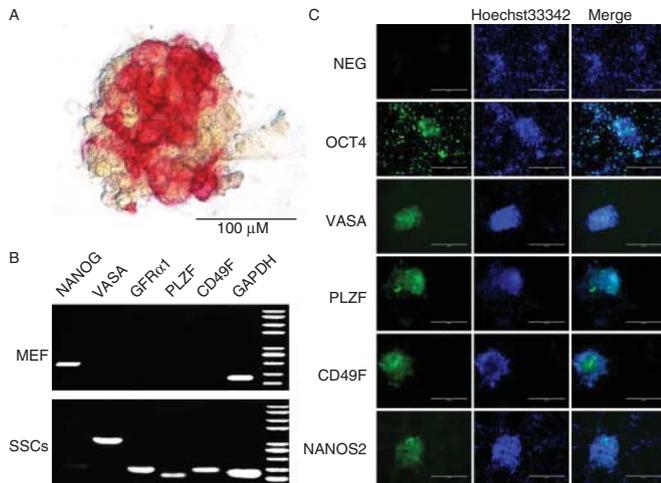


Figure 1 Analysis and identification of the isolated SSCs. (A) The alkaline phosphatase staining of the isolated SSCs (scale bar = 100 μ M). (B) The semi-quantitative RT-PCR analysis of pluripotent gene marker (*NANOG*), germ cell marker (*VASA*) and SSC markers (*GFRA1*, *PLZF*, and *CD49F*) in the isolated SSCs. (C) Immunofluorescence staining of pluripotent gene marker (*OCT4*), germ cell marker (*VASA*) and SSC markers (*PLZF*, *CD49F*, and *NANOS2*) in the isolated SSCs (scale bar = 100 μ M).

10–30 min at room temperature, and washed again with PBS to terminate staining.

Immunofluorescence staining

Dairy goat's testicular tissues were collected from 30 days postnatal (dpp), 50 dpp, 90 dpp, and adult. Then the tissues were dissected, fixed in 4% PFA respectively. They were then paraffinized, deparaffinized and rehydrated following the standard methods. The slides were dipped in three changes of xylene for 6 min each, two changes of 100%, 95%, 75% alcohol for 3 min respectively, afterwards rinsed twice in deionized water for 5 min. The slides were soaked in the boiling citrate buffer for 15–25 min, followed by three washes in cold PBS, each for 5 min. The washed slides were blocked with 1% BSA for at least 30 min and incubated with primary antibodies against ERK1/2 (1:1000, C.S.T. Consultants, Inc., ON, Canada), pERK1/2 (1:1000, C.S.T) overnight at 4 $^{\circ}$ C. The tissues were washed in PBS for three times, and then incubated with secondary antibody (1:500, Chemicon International, Inc., Temecula, CA, USA) following the manufacturer's manual. The nuclei of cells were stained by Hoechst 33342 (Harichandan *et al.* 2013).

The cell samples were fixed in 4% PFA, and 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 *OCT4* (1:200, C.S.T), *NANOS2* (1:200, Abcam, Cambridge, MA, USA), *VASA* (1:200, Abcam), *PLZF* (1:200, Bioss, Beijing, China), and *CD49F* (1:200, Bioss) respectively for overnight at 4 $^{\circ}$ C. After washing three times in PBS, appropriate secondary antibodies were incubated and stained by Hoechst 33342. The untreated cells were used as the negative control (Niu *et al.* 2014).

Semi-RT-PCR analysis

Total RNAs for semi-RT-PCR analysis were extracted from adult dairy goat SSCs and dairy goat embryonic fibroblasts (GEFs) using TRIzol (Tiangen Biotech Co. Ltd, Beijing, China). CDNA were synthesized from 500 ng RNA using a commercially available reverse transcription kit (TaKaRa, Biotech. Co. Ltd, Dalian, China). The PCR steps were as follows: initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 35 cycles at 94 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s, and a final extension at 72 $^{\circ}$ C for an additional 10 min. The primers were listed in Table 1. The primers were synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd (Beijing, China). The PCR products were analyzed in 2% agarose (Invitrogen) gel electrophoresis, stained with ethidium bromide (Invitrogen), and visualized under u.v. illumination (Cao *et al.* 2011).

Cell cycle analysis

For cell cycle analysis, dairy goat SSCs were cultured in control medium or media containing MEK1/2 inhibitor-PD0325901 (0, 2.5 and 10 μ M, Sigma) for 48 h, then resuspended into single cells, and washed in precooling PBS. After that, the cells were resuspended and incubated (Cell Cycle Kit, LianKe Biology, Hangzhou, China) with 1 ml A liquid and 10 μ l B liquid for 30 min, cell cycle analysis was determined by a Beckman flow cytometry (Cao *et al.* 2012).

BrdU incorporation

SSCs proliferation was determined by BrdU incorporation assay as described previously (Zhu *et al.* 2014). BrdU-positive cells

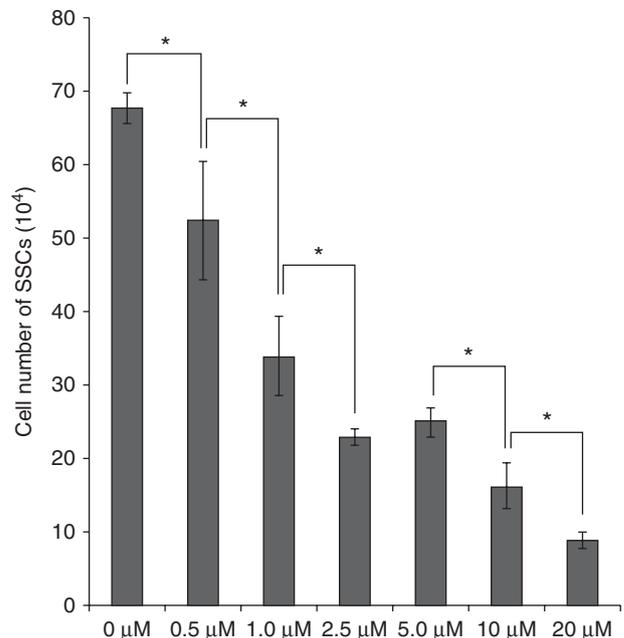


Figure 2 SSCs proliferation and viability dynamic with specific MAP2K/MEK1/2 inhibitor treatment. The number of SSCs cultured by the different PD0325901 concentrations (0, 0.5, 1, 2.5, 5, 10, and 20 μ M), * P < 0.05.

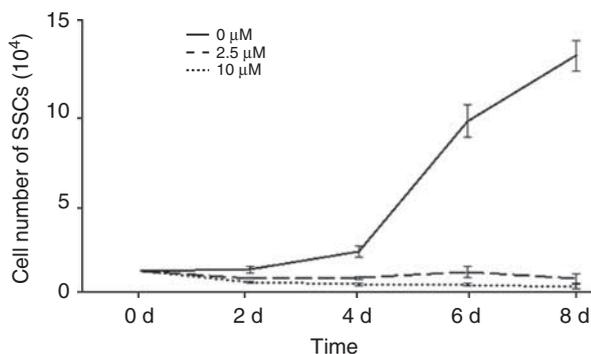


Figure 3 The growth curve of SSCs with specific MAP2K/MEK1/2 inhibitor treatment. The cell count of SSCs cultured by the different concentrations of PD0325901 (0, 2.5, and 10 μ M) for 8 days (scale bar = 100 μ M).

were detected by incubating them in FITC-conjugated secondary antibody (1:500, Millipore) for 1 h at room temperature. After three washes in PBS, cells were visualized by fluorescence microscopy and analyzed for Brdu incorporation (Cao *et al.* 2012).

TUNEL assay

The cells cultured with different concentration of PD0325901 (0, 2.5, and 10 μ M) were fixed with 4% PFA for 30 min at room temperature, washed twice with PBS, and permeated with 0.1% Triton X-100 for 10 min. The cells were then washed with PBS and incubated with the TUNEL reaction mix (Roche, 454 Life Sciences) for 60 min in the dark, and analyzed using a Leica fluorescent microscope. The rate of TUNEL-positive cells in the absence or presence of PD0325901 was made by manual counting under fluorescent microscope (Cao *et al.* 2012).

Western blotting

Total cell extracts were prepared from SSCs cultured in control medium or media containing different concentration of PD0325901. Total proteins were resolved by PhosphoSafe (Millipore), and western blotting analysis was performed according to the protocol we described previously (Cao *et al.* 2011, Yu *et al.* 2014). Briefly, proteins were transferred to PVDF membrane and incubated with antibodies including β -actin (1:1000, Beyotime, Haimen, Jiangsu, China), PCNA (1:2000, Millipore), c-MYC (1:2500, Chemicon), ERK1/2 (1:1000, C.S.T), pERK1/2 (1:1000, C.S.T) at 4 $^{\circ}$ C for overnight. The next day, the secondary antibody were added, and incubated. Then, the detection was performed using the BM-chemiluminescence blotting substrate (Roche) (Yu *et al.* 2014, Zhang *et al.* 2011).

Statistical analyses

The data were presented as mean \pm S.E.M. and the S.E.M. in this study were calculated for at least three replicates in each

of the 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 *P* value of <0.01 was considered to be highly significant difference.

Results

Isolation, cultivation, and identification of the dairy goat SSCs

The dairy goat SSCs were isolated, purified, cultured, and identified as described in previous studies (Wu *et al.* 2013, Zhu *et al.* 2013). The cells were identified by AP staining, semi-quantitative RT-PCR and immunofluorescence (Fig. 1). The AP staining showed positive for a part of dairy goat SSCs (Fig. 1A). The semi-RT-PCR showed that pluripotent marker *NANOG* was at low expression level, the germ cell marker *VASA*, and SSC markers *GFRA1*, *PLZF*, and *CD49F* were at high expression levels in goat SSCs (Fig. 1B). They indicated that the isolated SSCs were purified and maintained as putative SSCs. Also the immunofluorescence staining of pluripotent marker *OCT4*; germ cell markers *VASA*; and SSC markers *PLZF*, *CD49F*, and *NANOS2* was positive (Fig. 1C), which indicated purified and well-maintained the isolated SSCs.

Ras/ERK1/2 pathway is critical for the proliferation of dairy goat SSCs

Previous study showed that Ras/ERK1/2 pathway is critical for SSCs' self-renewal (He *et al.* 2008, Ishii *et al.* 2012). First, the SSCs were cultured in the different concentrations of specific MEK1/2 inhibitor PD0325901 (0, 0.5, 1, 2.5, 5, 10, and 20 μ M), and the proliferation of SSCs was analyzed by cell count, cell growth curve, Brdu staining, and TUNEL assay. The cell counting showed that the number of cells decreased as the PD0325901 concentration increased (Fig. 2), which indicated Ras/ERK1/2 pathway is critical for the proliferation of dairy goat SSCs. Besides, AP staining showed that the rate of AP-positive cells was decreased as the

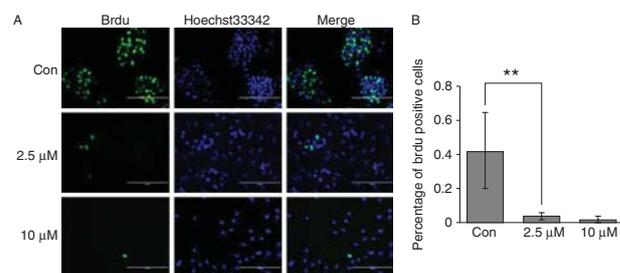


Figure 4 Brdu assay of SSCs with specific MAP2K/MEK1/2 inhibitor treatment. (A) Brdu-positive SSCs in different concentrations of PD0325901 (0, 2.5, and 10 μ M) were detected by immunofluorescent staining (scale bar = 200 μ M). (B) The statistical analysis of the rate of Brdu-positive SSCs in the three different concentrations of PD0325901, ***P* < 0.01.

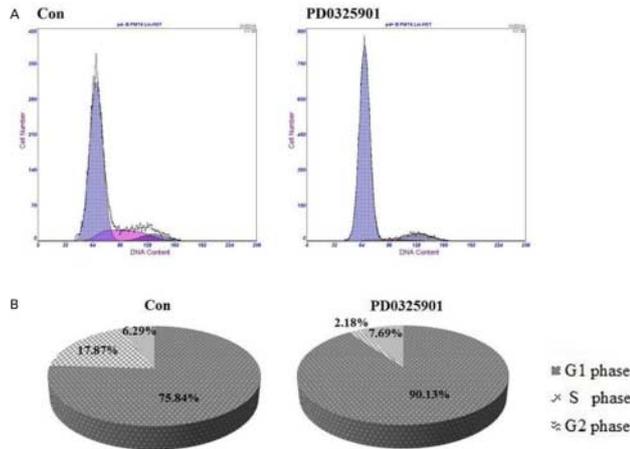


Figure 5 S phase dynamic of SSCs with specific MAP2K/MEK1/2 inhibitor treatment. (A) Flow cytometer analysis of SSCs cultured with 2.5 μ M PD0325901, Con is for SSCs without PD0325901 treatment. (B) Proportion of S phase dynamic SSCs cultured with 2.5 μ M PD0325901, Con is for SSCs without PD0325901 treatment.

concentration of PD0325901 increased, but there were not significant differences among SSCs with concentration of 2.5, 5, 10, and 20 μ M group (Supplementary Figure 1, see section on supplementary data given at the end of this article). Then, the cells were cultured in 0, 2.5, and 10 μ M PD0325901. Compared with the control (0 μ M), the growth of SSCs cultured in 2.5 and 10 μ M PD0325901 became significantly slower. However, no obvious differences have been detected between the two groups (Fig. 3).

Parallel results were provided in proliferation by Brdu incorporation assay. The cells were labeled with Brdu and immunofluorescence staining detection showed that the rate of Brdu-positive SSCs cultured in 2.5 and 10 μ M PD0325901 was significantly downregulated compared with the control (0 μ M) (with 38.48% in 0 μ M, 3.09% in 2.5 μ M, and 1.35% in 10 μ M) (Fig. 4). Moreover, 2.5 μ M was identified as a critical concentration of specific MEK1/2 inhibitor PD0325901 (Fig. 2). The cell cycle of SSCs was performed to analyze by flow cytometer, and the proportion of S phase SSCs was extremely decreased in 2.5 μ M PD0325901 group compared with the control (0 μ M) (with 17.871% in 0 μ M, and 2.184% in 2.5 μ M) (Fig. 5). These results indicated that the decrease in S phase cells and increase in G1 phase might be the reason for the decline of SSCs proliferation and viability.

The effect of Ras/ERK1/2 pathway on dairy goat SSCs' apoptosis

The cellular apoptosis of SSCs cultured with various concentrations of PD0325901 (0, 2.5, and 10 μ M) was analyzed by the TUNEL method. In contrast to the control group (0 μ M PD0325901), the rate of TUNEL-positive cells in 2.5 and 10 μ M PD0325901 showed a

significant rise. The positive rate in control group was 7.02%, while 2.5 and 10 μ M groups were 24.16 and 25.40%, respectively. However, there was no significant difference between 2.5 and 10 μ M groups (Fig. 6).

Ras/ERK1/2 pathway regulate the expression of self-renewal markers

In order to detect the level of MAPK phosphorylation in different periods of dairy goat testis, we choose dairy goat testicular tissues at three different ages (newborn, adolescence, adult) for ERK and pERK immunofluorescence staining. The rate of pERK and ERK-positive cells in adolescence is significantly higher than newborn but not different from adulthood (Fig. 7). Western blotting showed that pERK was decreased depending on the concentrations of PD0325901 (Supplementary Figure 2, see section on supplementary data given at the end of this article). However, when the concentration of

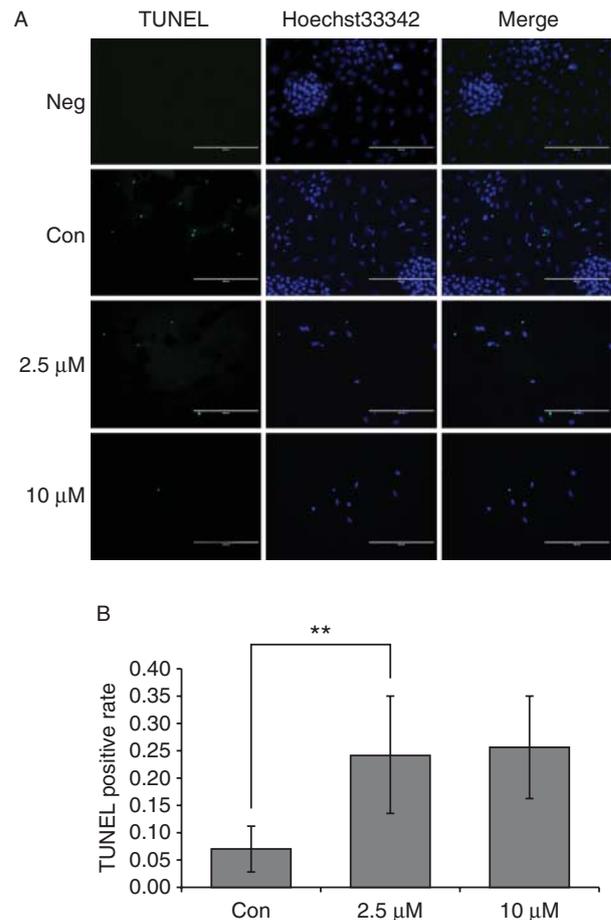


Figure 6 TUNEL assay of SSCs cultured with specific MAP2K/MEK1/2 inhibitor treatment. (A) TUNEL-positive SSCs in different concentrations of PD0325901 (0, 2.5, and 10 μ M) were detected by immunofluorescent staining, NEG is for SSCs without TUNEL incubation (scale bar=200 μ m). (B) The statistical analysis of the rate of TUNEL-positive SSCs in the three different concentrations of PD0325901, ** P <0.01.

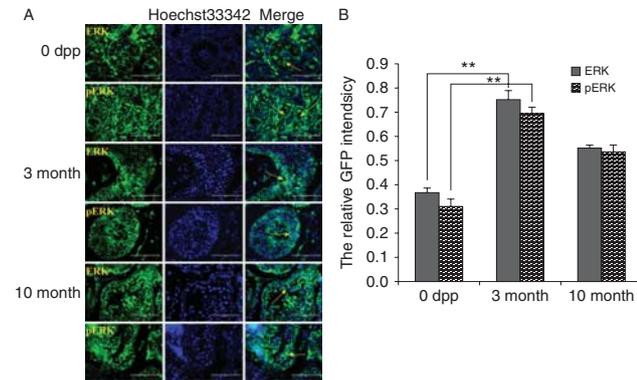


Figure 7 Protein expression of ERK and pERK in dairy goat testis at different ages. (A) Immunofluorescent staining of ERK and pERK in dairy goat testis at different ages (0 dpp, 3 month, and 10 month). (B) The statistical analysis of the immunofluorescent staining level of ERK and pERK in dairy goat testis at three ages, $^{***}P < 0.01$.

PD0325901 was increased up to $2.5 \mu\text{M}$, the pERK cannot be detected any more.

We detected the expression of SSCs' self-renewal markers *Etv5* and *Bcl6b*, which is the downstream gene of Ras/ERK1/2 pathway by immunofluorescence staining. The results showed that the proportion of *ETV5* and *BCL6B*-positive cells was significantly downregulated in $2.5 \mu\text{M}$ PD0325901 group compared with control (Fig. 8). PCNA and C-MYC indicate proliferation as detected by western blotting, and the results showed that PCNA and C-MYC were significantly downregulated in $2.5 \mu\text{M}$ PD0325901 treatment compared with control (Fig. 8).

Discussion

In this study, we isolated and cultured the dairy goat SSCs according to previous study, and the cells exhibited the typical characteristics of goat SSCs detected by AP staining, RT-PCR, and immunofluorescence. Our previous study have shown that overexpressed *DAZL* in the isolated SSCs lead to the meiosis *in vitro* (Niu *et al.* 2014). These results demonstrated that these cells shared the typical SSCs characteristics. Then, PD0325901, a MEK1/2-specific inhibitor was used to evaluate its effects on the self-renewal of dairy goat SSCs. Cells counting, AP staining, growth curve and Brdu incorporation, and TUNEL assay were used to evaluate the effects of PD0325901 on the self-renewal of dairy goat SSCs. Results showed that between 1 and $10 \mu\text{M}$ PD0325901, and upon $2.5 \mu\text{M}$, the suppression of self-renewal of SSCs is significant. These results showed that PD0325901 clearly inhibited the proliferation of goat SSCs, and the effects are dependent on PD0325901 concentrations. TUNEL, a common method for detecting DNA fragmentation that results from apoptotic signaling cascades, has become one of the main methods for

detecting apoptotic programmed cell death apoptosis (Cao *et al.* 2012).

In total, blocking of MEK1/2 pathway also resulted in the arrest of SSCs in G1 stage and cannot replicate, percentage of S stage cells and the AP-positive rate is declined, and the apoptosis rate was increased. These results showed that the Ras/ERK1/2 signaling pathway plays an important role in the maintenance of dairy goats SSCs' self-renewal.

Many transcription factors show their expression and function from embryonic stem cells (ESCs) to adult stem cells, their perplexing and coordinating operation with pathways make stem cells self-renewal and development (Hobbs *et al.* 2010, 2012). A group of transcription factors are regulated by the GDNF, including *BCL6B*, *ETV5*, and *LHX1* (Oatley *et al.* 2006). Furthermore, *ETV5*, *BCL6B*, and *LHX1* are also regulated by FGF2 (Ishii *et al.* 2012). These genes are the common targets of GDNF and FGF2, which are regulated by Ras/ERK1/2 signaling pathway to maintain the self-renewal of SSCs. *ETV5* plays the critical role in maintaining the self-renewal of SSCs (Morrow *et al.* 2007). *Etv5*-knockout mice showed serious spermatogenesis dysfunction (Morrow *et al.* 2007). *BCL6B* is the downstream gene of *ETV5*, which is regulated by *ETV5* (Ishii *et al.* 2012). *ETV5* can also regulate miR-201 and Brachyury, and Brachyury may promote SSCs proliferation and survival (Wu *et al.* 2011).

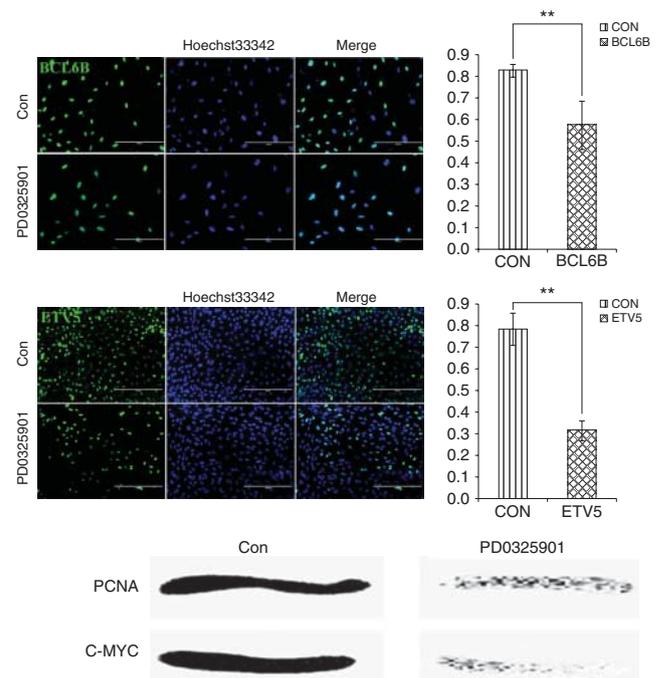


Figure 8 The expression of SSCs' self-renewal markers: *BCL6B*, *ETV5* (immunofluorescence), PCNA, and C-MYC (western blot) was significantly downregulated in $2.5 \mu\text{M}$ PD0325901 treatment compared with control. $^{***}P < 0.01$.

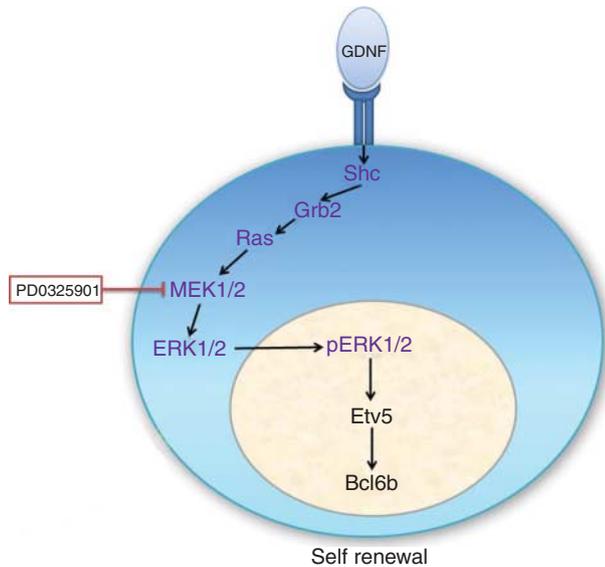


Figure 9 Schematic representation of the role of MAPK signal in regulation of SSCs.

In this study, we found that both ERK and pERK signals exist in dairy goat SSCs by immunofluorescence detection, and suggests that ERK and pERK control the self-renewal of dairy goat SSCs, and the MAPK phosphorylation was involved in the proliferation of dairy goats' SSCs (Fig. 9). In adolescence, the expression of ERK and pERK level is higher, indicates that SSCs initiate meiosis and enter the spermatogenesis wave, and promote the ability of self-renewal and maintain the SSCs in an active state upon puberty. The balance between the self-renewal and differentiation in SSCs maintain the normal development, meiosis, and produce spermatocytes, and also keeps a large number of SSCs in the testis, to ensure its potentiality to generate subsequent spermatocytes. Different cells need different levels of MEK signal to maintain their function. In mice and rats ESCs, generally 1 μ M PD0325901 clearly blocked the Ras/ERK1/2 signaling pathway (Buehr *et al.* 2008, Ying *et al.* 2008). In mice SSCs, 4 μ M PD0325901 was used to inhibit MEK1/2 signal (Ishii *et al.* 2012). In our study, western blotting analysis proved that 2.5 μ M PD0325901 can completely block the Ras/ERK1/2 signaling pathway in dairy goat SSCs. Additionally, for different cell types, different species and inhibitor itself are likely to influence the blocking effects for specific signals.

Blocking of the Ras/ERK1/2 signal pathway can lead to the decrease in *ETV5* and *BCL6B* expression. These results demonstrated that *ETV5* and *BCL6B* might be the downstream target of Ras/ERK1/2 pathway in dairy goat SSCs. The activation of Ras/ERK1/2 signals caused the downstream gene *CREB1* and *CREM*, *ATF*-like-1 phosphorylation, and promote *c-FOS* transcription, and then promote *CDK2* and cyclinA expression (He *et al.* 2008). Under the stimulus of GDNF and FGF2, reactive oxygen

species via PI3K-AKT and Ras/ERK1/2 pathway regulate the self-renewal of mice SSCs (Morimoto *et al.* 2013), and active oxygen is necessary for SSCs' self-renewal. Blocking of the Ras/ERK1/2 signaling pathways in dairy goat SSCs caused the inhibition of their self-renewal related to this pathway. Previous study showed histone deacetylase displayed different transcriptional regulation patterns, which may affect the self-renewal of SSCs (Kofman *et al.* 2013). However, more studies are needed to explore the real mechanisms.

Taken together, this study showed that the Ras/ERK1/2 pathway play a key role in maintaining the self-renewal of dairy goat SSCs via regulation of *ETV5* and *BCL6B*. This study laid an efficient platform for studying the mechanism of goat SSCs' self-renewal.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0506>.

Declaration of interest

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

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