J Assist Reprod Genet (2012) 29:39–46
DOI 10.1007/s10815-011-9687-5

STEM CELL BIOLOGY

Short-term in-vitro culture of goat enriched spermatogonial stem cells using different serum concentrations

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Received: 30 August 2011 / Accepted: 21 November 2011 / Published online: 11 December 2011
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Abstract
Purpose To investigate the effect of serum supplementing on short-term culture, fate determination and gene expression of goat spermatogonial stem cells (SSCs).
Methods Crude testicular cells were plated over Datura-Stramonium Agglutinin (DSA) for 1 h, and non-adhering cells were cultured in the presence of different serum concentrations (1, 5, 10, and 15%) for 7 days in a highly enriched medium initially developed in mice. Colonies developed in each group were used for the assessment of morphology, immunocytochemistry, and gene expression.
Results Brief incubation of testicular cells with DSA resulted in a significant increase in the number of cells that expressed the germ cell marker (VASA). The expression of THY1, a specific marker of undifferentiated spermatogonia, was significantly higher in colonies developed in the presence of 1% rather than 5, 10 and 15% serum.
Conclusion Goat SSCs could proliferate and maintain in SSC culture media for 1 week at serum concentrations as low as 1%, while higher concentrations had detrimental effects on SSC culture/expansion.

Summary Serum concentration adversely affects in-vitro culture of goat spermatogonial stem cells.

Capsule The result of this study showed maintenance and proliferation of goat SSC are adversely affected by high serum concentration.

Keywords Goat · Spermatogonial stem cell · Serum · THY1

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Introduction

Spermatogenesis is supported by rare testis stem cells called spermatogonial stem cells (SSCs). 0.02–0.03% of total germ cells, 1.25% of all spermatogonia, and 10.6% of undifferentiated spermatogonia in the rodent testis are SSCs, thereby amounting to 35,000 SSCs per mouse testis [1, 2]. Therefore, the development of efficient methods for in-vitro culture and expansion of SSCs is imperative to attaining the great potentials of SSCs in medicine and agriculture.

The first report of SSC culture dates back to 1998 when Brinster et al. [3] cultured entire cells of mouse seminiferous tubule in medium supplemented with fetal bovine serum (FBS) over an embryonic fibroblasts feeder layer. Since then, despite great advances achieved in this field, almost invariably all culture systems have been supplemented with serum and/or cultured over feeder cells or layers. However, a considerable volume of evidence indicates that, like other types of stem cells cultured in-vitro, feeder and

serum may complicate SSC self-renewal analyses [4]. Therefore, development of defined culture systems may be imperative for the assessment of the signaling pathways controlling self-renewal and differentiation of SSCs [5]. However, serum is a complex composition including compounds both useful [6–8] and harmful to SSC maintenance, cultivation and propagation [9]. Therefore, the effect of serum on SSCs needs to be further investigated. For example, Kanatsu-Shinohara et al. (2011) [4] observed that deletion of serum prevented germ cell stem cells (GS) from attaching to laminin-coated plates and stopped their proliferation.

The goat (Capra hircus) is one of the most suitable farm animals for the transgenic production of recombinant proteins [10]. Therefore, there is an interest in the application of SSCs as an alternative approach to create transgenic animals. However, there are no reports on goat SSC culture and only direct transplantation of crude testicular cells to the recipient goat testis has been investigated in previous studies [11–13].

The present study investigated the short-term culture of caprine spermatogonial stem cells without feeders in a highly enriched culture system developed for in-vitro proliferation of mouse SSCs [14]. Moreover, the effects of different concentrations of serum on caprine spermatogonial stem cell propagation and fate were determined.

Materials and methods

Unless otherwise specified, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively.

Enzymatic digestion of the testicular tissue

The process of testicular cell extraction was performed according to Honaramooz et al. (2003a) [11]. In brief, six neonatal (20–40 days old) goats were castrated and the encapsulated testes (around 1.75 g each testis) were washed and thoroughly minced with the razors. The minced fragments were digested by three-step enzymatic treatment with i) collagenase IV [2 mg/ml Dulbecco's modified eagle medium supplemented with Glutamax® (DMEM)] for 30 min, ii) a mixture of collagenase IV (1 mg/ml DMEM), hyalorunidase (1 mg/ml DMEM), and DNase I (7 mg/ml DMEM, Roche) for 30 min, and finally iii) 0.25% trypsin-EDTA for 10 min, followed by addition of 5 ml FBS to stop trypsin. All enzymatic digestions were carried out at 37°C with intermittent agitation, and the cell suspension resulting in each stage was rinsed by centrifugation (700 g, 10 min) with pre-warmed phosphate buffered saline free of calcium and magnesium (PBS, 37°C). The final cell suspension was passed through nylon mesh (100 μm pore-size, BD, Falcon®) to remove undigested tissue and cell clamps before being used for the experiments.

Experimental design

The viability of cell suspensions resulting from enzymatic digestion was assessed using the trypan blue exclusion test of cell viability [15]. The mixed population of cells at a concentration of $1.5\times10^5/cm^2$ was plated over Datura-Stramonium agglutinin (DSA, 5 μg/ml PBS) at 37°C, 5% CO_2 for 1 h to enrich SSCs [16]. After that, non-adhering cells that were floating over DSA were recovered and used for i) cell viability assessment, ii) immunocytochemistry analyses of germ- and somatic- cell specific markers using flow cytometry, and iii) in-vitro culture in the presence of different concentrations of FBS (1, 5, 10, and 15%) for 7 days. The colonies developed in each group were then used for i) morphologic assessments using phase contrast microscopy, ii) immunocytochemistry (ICC) analyses of germ- and somatic- cell specific markers, and iii) real-time assessment of gene expression.

Flow cytometry

The process of cell staining for flow cytometry was as described by Kanatsu et al. (1996) [17]. Accordingly, the cells were fixed in paraformaldehyde (PF, 4% in PBS) for 30 min at RT (room temperature). Fixed cells were then washed two times with PBS for 5 min each, permeated by treatment with 0.1% Triton-X100 in PBS for 30 min, before being blocked with the blocking buffer [BF: 10% goat serum and 1 mg/ml bovine serum albumin (BSA) in PBS for 30 min at 37°C]. The cells were then incubated with the primary antibodies including i) rabbit anti-DDX4 (VASA: 1:400), ii) mouse anti-vimentin (1:50 in BF) at 4°C overnight. After thorough washing with PBS plus 0.05% Tween 20 (PBST), the cells were incubated with the secondary antibodies [FITC-conjugated goat anti-rabbit IgG (1:80 in PBS) for anti- VASA] and FITC-conjugated anti-mouse IgG (1:50 in PBS) for anti-vimentin for 1 h at 37°C in the dark. After washing twice with PBST for 5 min each, the cells were collected on a Becton Dickinson fluorescent activated cell sorter (FACS Calibur, Becton Dickinson) and were analyzed using Modfit® software. Appropriate controls were considered to test the system and protocol fidelity.

Cell culture and colony assessment

The SSC medium used in this study was based on a highly efficient medium designed for SSC culture in rodents [14] and contained DMEM supplemented with antibiotic (100 IU/ml penicillin, 100 μg/ml streptomycin), L-glutamine (6 mM), B27-Vit A (1X), non-essential amino

Table 1 Please check if data in table 1 are presented correctly. Primer sequences for quantitative reverse transcription-PCR analyses

Primer	Sequence (5′–3′)	Product size (bp)	Annealing Tm (°C)
GAPDH	F: TGCCGCCTGGAGAAACC R: TGAAGTCGCAGGAGACAACC	121	62
THY1	F: CGTCTCCAATAAGGATGTC R: GTCACAAGGAGATGAAGTC	146	60
c-Kit	F: TACCAACCAAGGCAGACAA R: CTTTGAGGCAAGGAACGC	164	62
Vimentin	F: CTTTCTTCCCTGAACCTGAG R: AAGTTTCGTTGATAACCTGTCC	125	62
FGFR3	F: CGTGATGAAAATCGCCGACT R: CAGGACCCCGAAGGACCA	163	58
Bcl2	F: AGCATCACGGAGGAGGTAGAC R: CTGGATGAGGGGGTGTCTTC	161	61

acids (NEAA, 1X), 2-mercaptoethanol (5×10^{-5} mM), human glial cell line-derived neurotrophic factor (GDNF, 40 ng/ml, R & D Systems), human basic fibroblast growth factor (bFGF, 20 ng/ml, Royan Institute), human epidermal growth factor (EGF, 20 ng/ml, R & D Systems) in twelve-well culture dishes (Falcon®) at 37°C, 5% CO_2 and maximum humidity for 7 days. The culture dishes were assessed daily for colony formation and the developed colonies were counted and imaged using an inverted phase contrast microscope (CKX 41, Olympus, Japan) equipped with a high sensitivity camera (DP-72, Olympus, Japan) operated on DP2-BSW software. On day 7, the total number of colonies developed in each group was determined.

Immunocytochemistry (ICC)

The primary antibodies used for ICC included mouse monoclonal anti-OCT3/4 (1:250 in BF, Santacruz Biotechnology, INC), mouse anti-inhibin α (1:100), mouse anti- αSMA (1:100), rat monoclonal anti-human α-6 Integrin (1:100, R&D system), rat monoclonal anti mouse β-1 Integrin (1:100, R&D system), rabbit anti VASA (1:400) and goat anti PLZF (1:100). The alkaline phosphatase (AP) activity was assessed using Alkaline Phosphatase Kit. For ICC, the colonies over cover slips were fixed as described for flow cytometry, except for the permeabilization with Triton-X100 which was not performed for surface-specific antibodies (anti -α-6 integrin, and -β1 integrin). The samples were incubated with the primary antibodies overnight at 4°C. The samples were washed three times with PBST, 15 min each, and then incubated with the secondary antibodies: FITC conjugated goat anti-mouse IgG (1:50, for anti-OCT3/4, anti-inhibin α, and anti-αSMA), anti-rat FITC IgG (1:80, for anti-α-6 Integrin, and anti-mouse β-1 Integrin), anti-rabbit FITC IgG (1:80, for anti-VASA), and anti-goat FITC IgG (1:50, for anti-PLZF) at 37°C for 1 h in the dark. The samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI: 1 μg/ml) to detect the nuclei. The prepared samples were observed using a fluorescent microscope (BX51, Olympus, Japan) equipped with a stabilized HBO100 mercury vapor lamp and 490 nm excitation and 525 nm emission filters. Digital images of colonies were taken with a high sensitivity camera (DP-72, Olympus, Japan) operated on DP2-BSW software at 400X magnification. Appropriate positive and negative controls were considered.

It is worth noting that the negative controls of each staining in immunocytochemistry and flow cytometry were samples that were stained with secondary antibody without primary antibody. However these figures are not shown.

Quantitative Real-Time PCR (qRT-PCR)

The total RNA of the colony cells was extracted using RNeasy Mini Kit (Qiagen®), according to the manufacturers' protocol. Concentration of the extracted total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer and DNase treatment was performed using DNase I (Fermentase®). First-strand cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas®). First-strand complementary DNA was

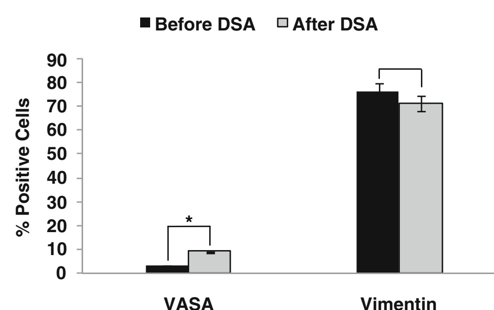

Fig. 1 Comparison of immunostaining percentages of cells before and after DSA incubation by anti-VASA $P\leq0.01$, -vimentin ± SEM, $P\geq$ 0.05

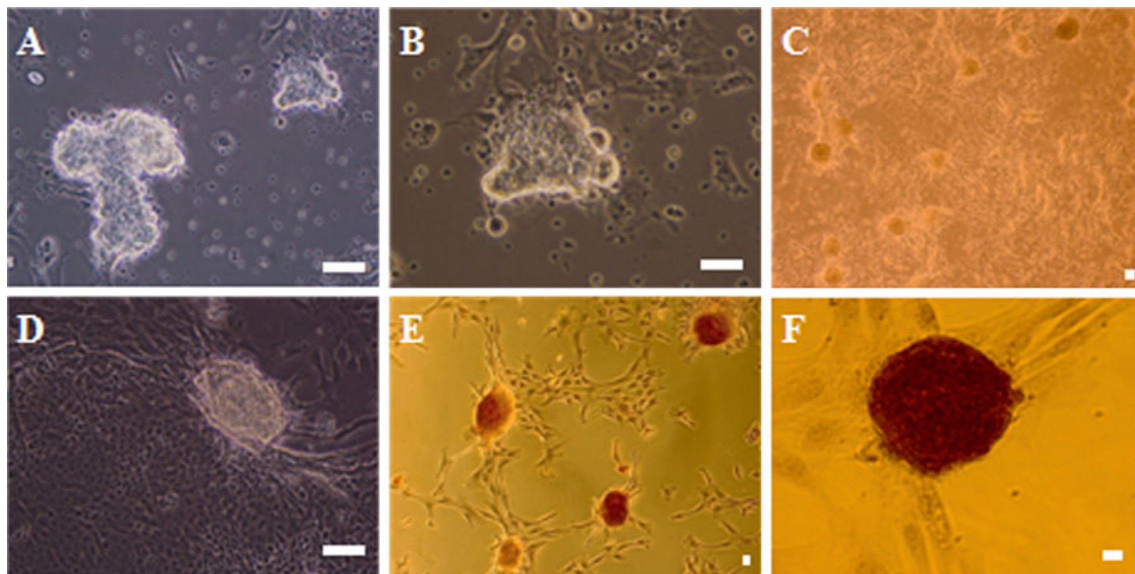

Fig. 2 Different morphology of colonies in day 4 (**A**–**B**) and day 7 (**C**–**D**). **E** and **F** represent the alkaline phosphatase activity of colonies in culture. All *bars* represent 50 μm except in part F where it represents 10 μm

synthesized by 1 μl of random hexamere, adjusted to a volume of 12 μl using DEPC-treated water and incubated at 65°C for 5 min. Then, the following components were added in the incubate order: 4 μl 5X reaction buffer, 1 μl of Ribolock RNaseTM inhibitor (20 U/μl), 2 μl 10 mM dNTP and 1 μl Revert Aid M-MuLV reverse transcriptase (200 U/μl). The resulting mixture was incubated at 25°C for 5 min followed by 60 min at 42°C and 70°C for 5 min. The qPCR primers were designed using the similar regions of sequence of targeted genes including THY1, vimentin, c-Kit, Bcl2 and FGFR3 by Primer premier 5 (Table 1). GAPDH gene was used as a reference gene in the qPCR analyses. The qPCR was performed with Quantitect SYBR Green PCR kit (fermentase®) using 20 μl of reaction solution containing 10 μl of 2X Quantitect SYBR Green PCR master mix, 25 ng of cDNA, 10 pM primers and reaching a final volume of 20 μl by addition of sterile distilled water. Relative gene expression for each gene was calculated by ratio of target gene expression to that of reference by ΔΔCT (threshold cycle) analysis [18].

Statistical analysis

All experiments in this study were repeated three times. Data were analyzed by paired samples *t*-test, general linear models, and two-way repeated measuring using the ANOVA model of SPSS 18. Differences were compared by Tukey multiple comparison post-hoc test. All data were presented as means ± S.E.M. and differences considered as significant at $P<0.05$.

Results

Flow cytometry

The initial viability of the extracted cell suspension was 91.7% and did not change after incubation with DSA. As shown in Fig. 1, flow cytometry assessment of somatic and germ cell markers indicated that incubation with DSA insignificantly reduced vimentin$^+$ cells compared to unsorted cells (76.1 vs. 71.2%). Importantly, this reduction in the percentage of vimentin$^+$ cells was concomitant with significant 2.8-fold increases in VASA$^+$ (9.2 vs. 3.3%) cells compared to unsorted cells. After culture for 7 days, the colonies could barely be dissociated to single cells to be used for flow cytometry.

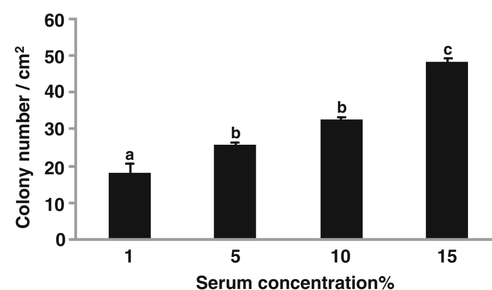

Fig. 3 Comparison of the effect of serum concentration on the formation of colonies in caprine spermatogonial cultures (Number of colonies per cm^2 after 7 days of culture $P<0.05$). The results are presented as the mean ± S.E.M. of three independent experiments

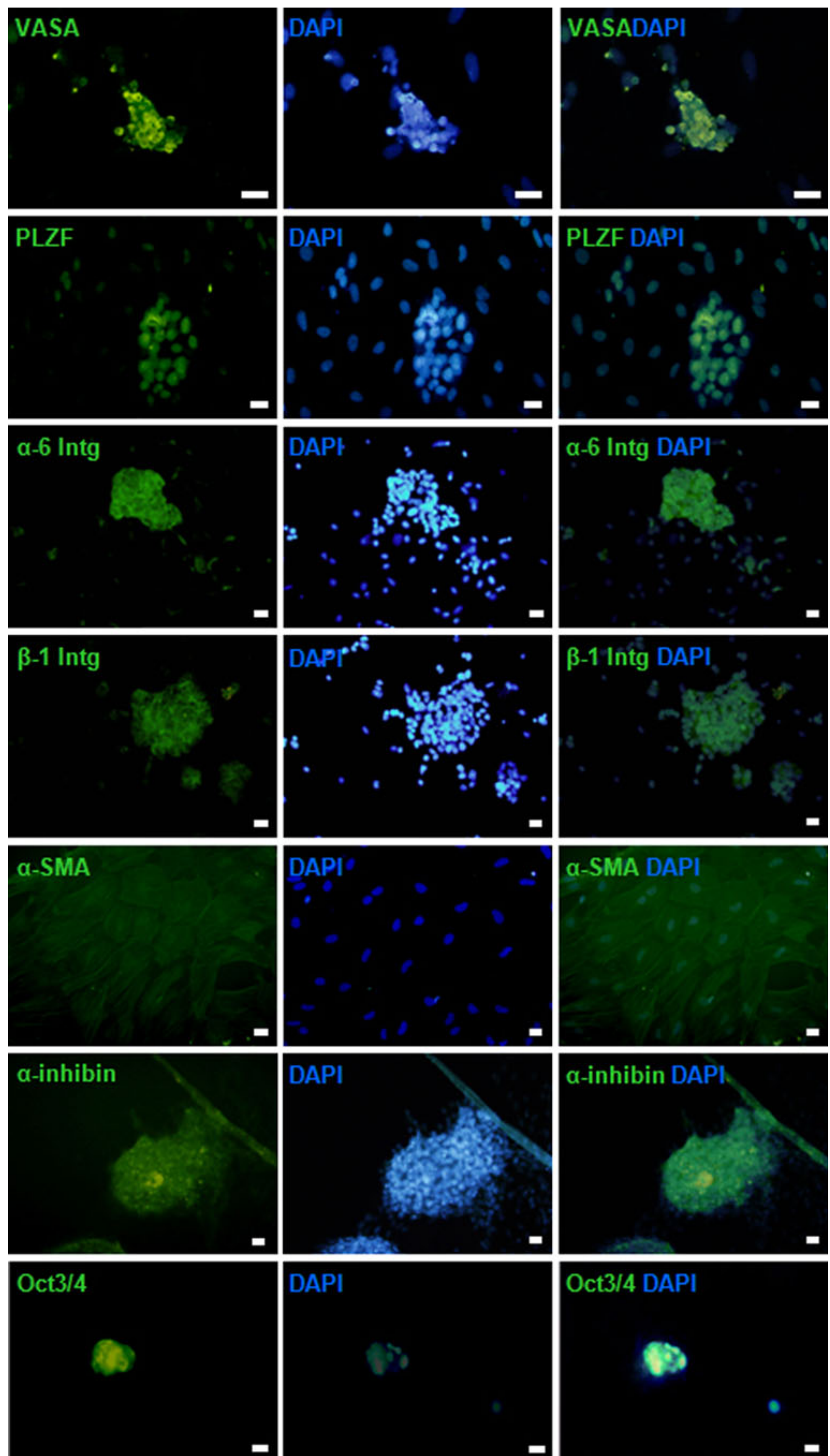

Fig. 4 Immunostaining of germ cell and somatic cell markers. Expression of VASA, PLZF, α-6 integrin, β-1 integrin, α-SMA, α-inhibin and Oct3/4 in the colony in the first column, DAPI in the second column and a merge of FITC and DAPI in the third column. Bar=40 μm

Colony formation

The first evidence of colony formation was observed at day 4 of culture in all treatment groups (Fig. 2). There was a marked direct influence of serum concentration on the number of colonies developed at day 7 of culture. Accordingly, the number of colonies developed in the presence of 1% FBS was significantly lower than in the other group (Fig. 3).

Immunocytochemistry

Figure 4 shows the results of immunocytochemistry performed on the colonies developed in different FBS treatment groups. As shown, irrespective of FBS concentration, the expressions of OCT3/4, α-6 Integrin, and β-1 Integrin, PLZF, α-inhibin and VASA were observed in the colony cells developed in all treatment groups, suggesting that the colony cells contained somatic and germ cells. The α-SMA$^+$ staining (the marker of myoid cells) was observed only in background cells, suggesting that myoid cells did not participate in the colony formation. Moreover, the colony cells showed AP-activity in all groups (Fig. 2E,F). However, none of the colony cells in different groups contained absolutely positive cells for a certain germ cell marker, suggesting that the colony contained a mixed population of germ cells and somatic cells other than myoid. The colonies could barely be dissociated to single cells to be used for quantitative assessment by flow cytometry.

Real-time assessment of gene expression

As shown in Fig. 5, the concentration of FBS and cryopreservation markedly influenced the pattern of gene expression. Accordingly, the minimum and maximum expressions of vimentin were observed in colonies developed in the presence of 1 and 15% serum, respectively, which were significantly different with 5 and 10% serum. The expressions of THY1 and FGFR3 in the colonies developed using 1% was significantly higher than 5, 10 and 15% serum. C-Kit expression was significantly higher in 1% serum than in 10% and 15% serum. Bcl2 was highest in colonies grown in 15% serum.

Discussion

Brief incubation with DSA partially enriched SSCs in the goat

When unsorted cell suspension was plated over DSA for 1 h, flow cytometry analyses of the non-adherent fraction indicated insignificant reduction (0.9-fold) in the percentage of vimentin$^+$ that was concomitant with

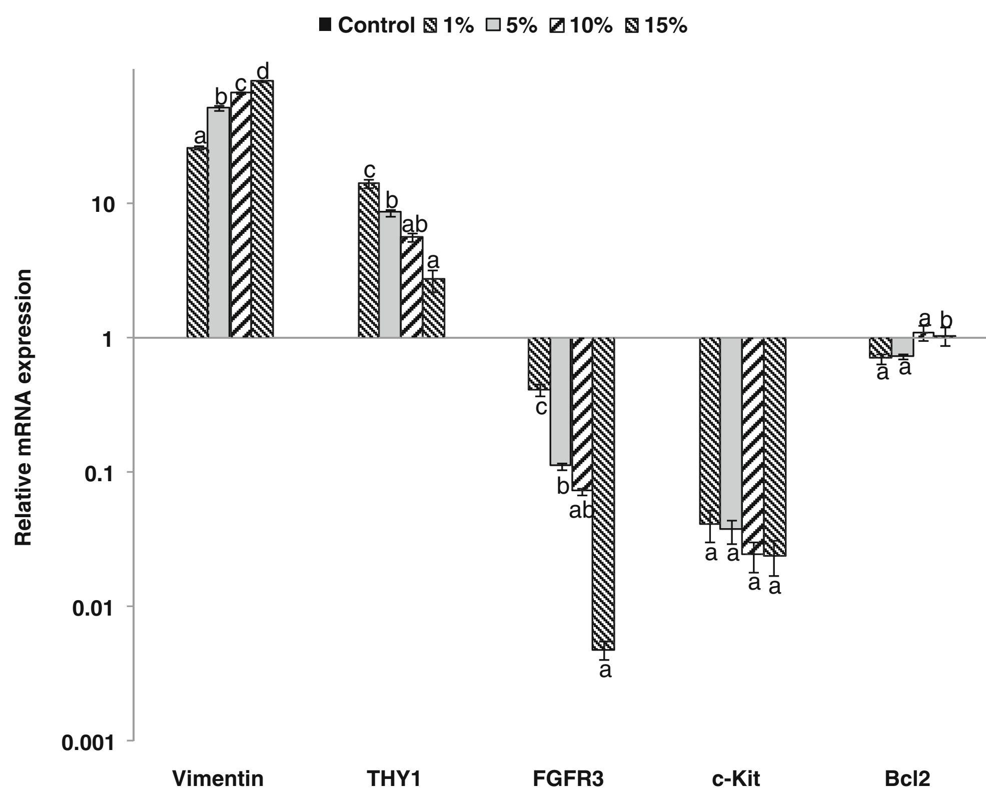

Fig. 5 Effects of different concentration of FBS on relative mRNA expression of vimentin, THY1, FGFR3, c-Kit and Bcl2 in logarithmic base 10. Relative mRNA expressions were log transformed (in base 10). The results are presented as the mean ± S.E.M. of three independent experiments ($P<0.05$)

significant increases (2.8-fold) in the percentages of VASA^{+} cells. DSA is a lectin that specially binds to β-(1——4)-linked oligomers of N-acetyl-D-glucosamine, and therefore, considering the large amount of terminal sugars existing in sertoli cells, Scarpino et al. (1998) [16] successfully used DSA-coated dishes as a rapid (1 h) method for the isolation and purification of sertoli cells in rats. Moreover, Herrid et al. (2009) [19] demonstrated that overnight incubation in flasks coated with 20 μg/ml DSA produced a 3.6-fold increase in bovine type A spermatogonia in the non-adherent fraction. Therefore, it seems that DSA-coated dishes can be used as an approach to enrich the initial cell population of goat SSCs. However, further studies are needed to optimize the concentration and duration of DSA incubation which profoundly affects the final outcome of SSC enrichment in goats.

Serum concentration adversely affected in-vitro culture of SSCs in the goat

The present study for the first time demonstrated that short-term in-vitro culture of goat SSCs maintained their activity and allowed them to propagate and to form colony cells. Morphologic and ICC analyses indicated that the developed colonies were highly organized structures containing SSCs that intermingled with somatic cells. Accordingly, irrespective of FBS concentration, the colonies developed in different treatment groups contained OCT3/4^{+}, α-6 integrin^{+}, β-1 integrin^{+}, PLZF^{+}, and VASA^{+} cells that were embraced by α-inhibin $^{+}$ cells. Importantly, differential ICC analyses indicated that the somatic cells in the background were mainly myoid cells (α-inhibin^{-}, αSMA^{+}), whereas the somatic cells in colonies were mainly sertoli cells (α-inhibin^{+}, αSMA^{-}), thus suggesting that the increase in serum concentration resulted in the proliferation of sertoli cells in the colonies. However, the majority of the immunostained colonies have cells that were positive for SSC markers, indicating that the presence of somatic cells in the colonies does not interfere with the maintenance and/or proliferation of SSCs in the goat. This observation is in agreement with the study of Aponte et al. (2008) [20], who observed that bovine SSC colonies included densely packed somatic cell areas, inside which spermatogonia were always located.

Quantitative real-time assessment of gene expression in isolated colonies indicated that serum concentration had a marked adverse effect on the maintenance and proliferation of SSCs in culture. Accordingly, the highest expressions of THY1 and vimentin were observed in the colonies developed in the presence of 1 and 15% FBS, respectively. Hypothetically, this contrasting effect of FBS concentration on the expression of SSC- and somatic- cell markers may result from either SSC-differentiation, or somatic cell over-proliferation in high (>1%) FBS concentrations. However, since there was no significant difference between the expression of c-Kit, the marker of SSC differentiation, it seems the increase in FBS concentration resulted in somatic cell proliferation rather than SSC-differentiation.

FGFR3 (fibroblast growth factor receptor 3) is a SSC surface marker with a vital role in SSC self-renewal. In this study, the expression of FGFR3 was significantly diminished by the increase in FBS concentration, thus suggesting that high FBS concentration may hamper SSC self-renewal in culture. This finding is in agreement with the study of Kanatsu-Shinohara et al. (2005) [21] in mice, that showed enhancement of stem cell renewal using 1% serum, though high concentrations of serum stimulated proliferation of testicular somatic cells and interfered with the germ cell growth. In contrast, Kubota et al. (2004) [9] described that the expansion of spermatogonial stem cells could not be achieved at concentrations of serum as low as 0.1 and 1%, and they observed SSC expansion even with the use of 10% serum. In their natural niche, sertoli cells control SSC apoptosis and balance SSC self-renewal. Notably, the expression of Bcl2, the anti-apoptotic marker, in the colonies developed in the presence of 15% FBS was significantly higher than in the other groups. Although the exact reason for this phenomenon was not understood, it seems that serum may contain factors that act as anti-apoptotic. Also, one may consider that low serum concentration may improve stem cell renewal, as verified by the high expression of Bcl2, while simultaneously inducing apoptosis in somatic cells.

In conclusion, the results of this study provide a background for further studies on goat SSC culture through presenting the methods for partial enrichment and culture. Overall, the results indicated the adverse effect of FBS on SSC culture and highlighted the need to design serum-free cultures that may provide significant practical advantages for investigating the signaling pathways controlling the self-renewal and differentiation of SSCs.

Acknowledgement This study was funded by a grant from the Royan Institute (P83P88213). Authors declare that there is no conflict of interest in this study.

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