In vitro culture and characterization of spermatogonial stem cells on Sertoli cell feeder layer in goat (*Capra hircus*)

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Abstract

Purpose To develop an efficient protocol for isolation, purification and long-term culture of spermatogonial stem cell (SSC) in goat.

Methods The isolation of SSC was performed by testicular disaggregation by enzymatic digestion using collagenase IV, trypsin and DNase I. Further SSCs were enriched using Percoll density gradient centrifugation. The purity of SSCs was assessed by immunocytochemistry (ICC) using $\alpha 6$ integrin. The SSCs were co-cultured on Sertoli cell feeder layer. The SSC colonies were characterized by studying the expression of SSC specific markers (viz., $\alpha 6$ integrin and PLZF) using ICC. The abundance of mRNAs encoding the markers of SSC (viz., $\beta 1$ integrin and Oct-4) and Sertoli cells (viz., vimentin) was also assayed using quantitative real-time PCR (qPCR).

Results The viability of isolated testicular cells was >90 % and the Percoll density gradient method resulted in 3.65 folds enrichment with a purity of 82.5 %. Co-culturing of SSCs with Sertoli cell feeder layer allowed the maintenance of stable SSC colonies even after one and half months of culture. The results of ICC analysis showed the expression of $\alpha 6$ integrin and PLZF in almost all the SSC colonies. qPCR analysis revealed a differential expression of mRNAs encoding $\beta 1$ integrin, Oct-4 and vimentin markers.

Conclusion Results of this study demonstrate a simple enzymatic digestion and Percoll density gradient method for isolation and enrichment of SSCs, and suitability of Sertoli cell feeder layer for long term in vitro culture of SSC in goats.

Capsule The results of this study demonstrated a simple three-step enzymatic digestion method for isolation, a Percoll density gradient method for enrichment and the use of Sertoli cell feeder layer for long term culture of SSC in goat.

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Results also suggest the possible application of non-caprine antibodies against SSC specific markers for the identification and subsequent assessment of SSCs in goats.

Keywords Discontinuous percoll density gradient · Enzymatic digestion · Immunocytochemistry · Integrin · Oct-4 · PLZF

Introduction

Spermatogonial stem cells (SSCs), a type of unipotent adult stem cells, are generated from the gonocytes in the postnatal testes. They are located in the basement membrane of the seminiferous tubules [1] and provide a continual supply of differentiating spermatogonia throughout life in males [2]. The SSCs can serve as a model for better understanding of adult stem cell biology and deciphering the mechanisms that control SSC functions. Recent studies advocate the implication of cryopreservation and transplantation of SSCs in transgenesis [1,3]. However, the exploitation of SSCs is limited due to the inherent problems of isolation, enrichment and establishment of long term culture [4–6]. Further, the SSCs that are being used in the production of transgenic animals require high purity [7,8].

Isolation of SSCs involves a multi-step enzymatic digestion of the testicular tissues by combinations of two or more enzymes like, hyaluronidase, collagenase, trypsin and DNase I [9,12]. The SSCs have been isolated using two enzymes in human [12,13], three enzymes in buffalo [14] and four enzymes in cattle [11] as well as goat [10]. Use of more enzymes ensures higher purity but it takes more time and may affect the viability of the cells. However, testicular disaggregation using less number of enzymes requires additional step of enrichment of isolated SSCs with high purity [13].

Several strategies are used for the enrichment of SSCs, including discontinuous Percoll density gradient [15],

differential plating [16], fluorescence activated cell sorting (FACS) [17] and magnetic cell sorting (MACS) [18]. The methods involving FACS and MACS need an expensive equipment set-up [19]. The differential plating is cost effective, but requires more time and compromises with the purity and concentration [20,21]. The density gradient protocol is considered to be a time saving and cost-effective method for the enrichment of isolated SSCs [13].

Current methods of long term culture of SSCs primarily involve either of the two strategies: first, by adding growth factors (viz., bFGF, GFR α -1 and GDNF) in the media [21,22]; second, by growing SSCs on a feeder layer [23]. Bovine SSCs were successfully cultured on a monolayer of STO (SIM 6-thioguanine resistant ouabain resistant) feeder cells [24], bovine embryonic fibroblast cells (BEF) [25] and Sertoli cells [26]. Bovine SSCs were also cultured for short term on laminin coated plates in vitro [11]. However, when co-cultured with Sertoli cells the numbers and diameter of murine SSC colonies increased significantly [27].

The SSCs are invaluable tool in modifying the male germ line to generate transgenic animals [28]. Among the livestock species, goats are often considered as unique for transgenesis because of their small body size, shorter gestation period, prolificacy and relatively higher protein content in their milk [29]. There are several reports of SSC culture in different species including mice [22,30], rat [31,32], cattle [11,33], buffalo [34,35] and human [13,36]. But the isolation and enrichment as well as establishment of long term culture system for SSCs have not yet been exploited substantially in goats except a very recent report [10]. Hence, in the present study, we demonstrated a three enzymatic digestion for the isolation of SSCs which was further enriched by using Percoll density gradient centrifugation technique for the first time in goats. The enriched SSCs subsequently used for co-culturing with Sertoli feeder. Besides, information pertaining to the germ cell markers is also limited in this species. Therefore, we assessed the expression of different markers using immunocytochemistry and quantitative real-time PCR (qPCR) analysis.

Materials and methods

Unless otherwise specified, all chemicals, buffers and media used in the present study were obtained from Sigma (USA) and antibodies except vimentin were procured from Santacruz Biotechnology (Germany).

Animals and collection of testis

The testes from prepubertal (aged 3–4 months) bucks ($n=5$) were collected from the local abattoir, Bareilly, Uttar Pradesh, India. Within 30 min of sacrifice of the animal, the testes were

transported to the laboratory in phosphate buffer saline (PBS) containing 50 μ g/ml Gentamicin (Sigma, USA).

Enzymatic isolation of testicular cells

Extra fat surrounding the testes was removed using sterile scissors. The testes were washed several times with PBS containing 50 μ g/ml Gentamicin. After removing the tunica albuginea, the testis was minced into small pieces on a petri dish containing Dulbecco's Minimum Essential Media (DMEM; Gibco, USA) supplemented with 1 $\times$ antibiotic-antimycotic solution (Invitrogen, USA). The minced pieces were sequentially digested with 1 mg/ml collagenase IV (Gibco, USA) in DMEM for 30 min, 7 mg/ml DNase I in DMEM (Roche, Switzerland) for 5 min and 0.25 % (v/v) trypsin-EDTA (Invitrogen, USA) in PBS for 20 min. The activity of trypsin was stopped by addition of 5 ml heat-inactivated fetal bovine serum (FBS). All enzymatic digestions were carried out at 37 °C with intermittent agitation, and the cell suspension resulting from each stage was washed by centrifugation (@800 rpm, 5 min) with pre-warmed (37 °C) PBS. The final cell suspension was filtered through cell strainers (100 μ m and 70 μ m pore size, BD Falcon, USA) and washed 3–4 times. The cell pellet was finally resuspended in DMEM containing 5 % (v/v) FBS. Numbers and viability of the cells were determined by Trypan blue staining. The isolated cells were used for either for preparation of Sertoli cell feeder layer or enrichment of SSCs.

Preparation of Sertoli cell feeder layer

Testicular cells in DMEM were added on datura stramonium agglutinin (DSA-lectin, 10 μ g/ml)-coated 6-well plate containing equal amount of DMEM and Ham's F-12 (Gibco, USA) supplemented with 10 % (v/v) FBS, essential amino acids (EAA), non essential amino acids (NEAA) and antibiotic-antimycotic solution (Invitrogen, USA) and incubated for 2 h at 37 °C in a humidified atmosphere of 5 % CO₂ in air. After discarding the non-adherent cells, the adherent Sertoli cells were cultured for 7–9 days. When it reached 90 % confluency, the Sertoli cells were treated with 10 μ g/ml mitomycin C for 3 h. The cells were washed 3–4 times with DMEM-F12 and used subsequently as feeder layer.

Enrichment of SSCs using Percoll density gradient centrifugation

The SSCs were enriched following the protocol described earlier [37] with some modifications. Briefly, iso-osmotic Percoll was prepared by adding 82.2 % (v/v) Percoll in to the diluting media (DM) containing 0.6 % (w/v) BSA, 45 μ g/ml DNase in DMEM. Then discontinuous density gradients (65, 50, 40, 36, 32, 30 and 28 %) of Percoll were

prepared in DM. The cells suspended in DM was layered on the top of the Percoll gradients and centrifuged at 800 g for 30 min at 18°C. The fractions at 32, 36 and 40 % Percoll density were collected and washed two times with DMEM-F12. The cell pellet was suspended in DMEM-F12 and was seeded on Sertoli cell feeder layer. A fraction of this cell suspension was used for immunocytochemistry for assessing the purity of SSCs.

Immunocytochemistry (ICC) analysis of SSC population

In order to assess the enrichment, both the Percoll enriched and non-enriched SSCs were subjected to ICC analysis. The cells were fixed in 4 % (w/v) paraformaldehyde (PFA) in PBS and a smear was made on a clean poly-L-lysine coated glass slide. The slides were washed in PBS and blocked with 1 % (w/v) BSA in PBS for 30 min at RT. Then incubated with rabbit polyclonal anti- $\alpha 6$ integrin (1:50) primary antibody for 2 h in a moist chamber at 37 °C. After washing with PBS, FITC conjugated mouse anti-rabbit IgG (1:200) secondary antibody was added for 1 h at 37 °C (in the dark). The slides were again washed, mounted with fluoroshield and examined under fluorescent microscope (Leica Microsystems, Germany). The fluorescent cells were counted in 20 fields and the number was recorded.

Culture of SSC

The enriched SSCs (3×10^6 cells) in DMEM-F12 supplemented with 10 % (v/v) FBS, $1 \times$ essential amino acids (EAA) and $1 \times$ Non-essential amino acids (NEAA) were added on to each well of 6-well plate containing Sertoli cell feeder layer. Then cell culture plates were incubated at 37 °C in a humidified CO₂-incubator with a 5 % CO₂. The culture was maintained for 2 months by replacing the medium on every alternate day. After 3rd day, plates were assessed for colony formation on every alternate day. The numbers of colonies were counted and images were recorded using an inverted phase contrast microscope (Leica Microsystems, Germany).

ICC analysis of SSC colonies

The primary antibodies used for ICC included rabbit polyclonal anti- $\alpha 6$ integrin (1:50), mouse monoclonal anti-PLZF (1:50) and mouse monoclonal anti-vimentin (1:40; Sigma, USA). After 14 days of culture, cells in the 6-well plates were fixed with 4 % (w/v) PFA in PBS for 10 min at RT. After fixing, the wells were washed twice with $1 \times$ PBS/0.1 % (v/v) Tween-20 (PBST) for 5 min each. Then cells were permeated by treating with 0.3 % (v/v) Triton- $\times 100$ for 10 min (only for PLZF and vimentin). Cells were treated with blocking buffer (1 % (w/v) BSA in PBST) for 30 min at RT and then incubated with the primary antibodies for overnight at 4 °C. After three

washing with 0.1 % PBST, the cells were incubated at 37 °C for 1 h in the dark in a moist chamber with the secondary antibodies: FITC conjugated mouse anti-rabbit IgG (1:200 for anti- $\alpha 6$ integrin) and FITC conjugated chicken anti-mouse IgG (1:100 for anti-PLZF and anti-vimentin). The samples were counterstained with propidium iodide (PI; 1 μ g/ml) and examined under fluorescence microscope.

Quantitative real-time PCR (qPCR) analysis

Total RNA from the cells were extracted using TRI-reagent after 14 days of culture. The RNA samples were treated with DNA-free DNase kit (Ambion, USA). The concentration and purity of RNA were determined spectrophotometrically and the integrity of the RNA samples was ascertained using agarose gel electrophoresis. One microgram of DNA-free RNA sample was used for reverse transcription in a final volume of 20 μ l according to the manufacturer's protocol (RevertAid H Minus First Strand cDNA Synthesis Kit, Fermentas, USA). Synthesized single-stranded cDNA was stored at - 20 °C till further use.

The gene-specific primer pairs for $\beta 1$ integrin, Oct-4 and vimentin and β -actin (Table 1) were designed using Primer Express (Ver 3.0). β -actin was used as a reference gene. The qPCR was carried out using Applied Biosystems 7500 real-time PCR system. All PCR reactions were performed in triplicates in a reaction volume of 20 μ l containing $1 \times$ Power SYBR® Green Master Mix (Applied Biosystems, USA), 50 nM of each gene-specific primer and 40 ng of cDNA template. PCR cycling conditions were as follows: 50 °C for 2 min, initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s 60 °C for 60 s. The specificity of PCR and the efficiency (E) of primer pairs were determined as described elsewhere [38]. The 'E' of $\beta 1$ integrin, Oct-4, vimentin and β -actin was 107, 102, 96 and 106 %, respectively.

Statistical analysis

The mRNA expression levels of different markers were analyzed using $2^{-\Delta\Delta CT}$ method [39]. Differences between groups were statistically analyzed by either one-way ANOVA or Student's *t* test. A *p*-value < 0.05 was defined as statistical significance.

Results

Isolation and enrichment of goat SSCs

We isolated the goat SSCs using a protocol involving sequential enzymatic digestion with three enzymes viz., collagenase IV, trypsin and DNase I. Trypan blue staining of the isolated

Table 1 Gene-specific primers used for qPCR analysis

SI No.	Gene	Primer sequence (5'–3')	Source	Product size (bp)
1	$\beta 1$ integrin	F: ATCGTTTTACCGAATGATGGA R: AGATAATGTTCTACCGCTGACT	JQ965819	207
2	Oct-4	F: GGTTCTCTTTGGAAAGGTGTTC R: ACACTCGGACCACGTCTTTC	NM_001285569	314
3	vimentin	F: CTTTCTCCCTGAACCTGAG R: ACACTCGGACCACGTCTTTC	XM_004014247	124
4	β -actin	F: AGCTCGCCATGGATGATGA R: TGCCGGAGCCGTTGT	JX046106	54

cells indicated viability of vast majority (>90 %) of the cells. The ICC results showed the presence of $\alpha 6$ integrin positive SSCs in both the enriched and non-enriched fractions. However, following Percoll density gradient centrifugation, the enriched fractions contained 3.65 fold ($p < 0.001$) more $\alpha 6$ integrin positive cells than those (22.62 ± 2.03 %) in the non-enriched fraction (Fig. 1).

Sertoli cell feeder layer

After 4–5 days of culture, the testicular cells, which were seeded on to DSA lectin coated plates, showed a slow-down in proliferation. The adherent Sertoli cells showed a typical morphology with enlarged body and dramatically spread cytoplasm with 3–4 enations like irregular

polygon. After 7–9 days, the Sertoli cells formed a confluent layer which was used subsequently as feeder layer for SSC culture.

Morphological analysis of SSC colonies on Sertoli cell feeder layer

SSCs were observed as round or oval bodies, with a large nucleus and little cytoplasm. SSCs began to form cell clusters at 48 h and colonies were visible at 4th day (Fig. 2a). The size of the colonies increased till 10–12 days. Co-culturing with the Sertoli cells yielded more numbers of SSC colonies of larger size which could be maintained till one and half months (Fig. 2b).

Fig. 1 **a** Effect of enrichment using Percoll density gradient centrifugation on the numbers of $\alpha 6$ integrin positive SSCs. More numbers of green fluorescent cells were observed after the purification; **b** Graphical representation of comparison of immunostained SSCs before and after gradient centrifugation ($***p < 0.001$). Scale bar: 100 μ m

Fig. 2 Phenotypic characteristics of SSC colonies obtained by co-culturing of Spermatogonial cells on Sertoli cell feeder layer after **a** 4 days and **b** one and half months of culture. Black arrows indicate SSC colonies. Scale bar: 50 μ m

ICC of SSC colonies

The results of ICC analysis showed the expression of $\alpha 6$ integrin (Fig. 3a & b) and PLZF (Fig. 3c & d) in almost all the SSC colonies. Immunoreactivity of vimentin was detected in the Sertoli cells (back ground cells) surrounding the SSC colony (Fig. 3e & f) and in some of the colonies (Fig. 3g & h).

Expression of SSC and Sertoli cell specific markers using qPCR

Quantitative real-time PCR (qPCR) analysis revealed the expression of mRNAs encoding the markers for SSC (viz., $\beta 1$ integrin and Oct-4) and Sertoli cells (viz., vimentin) (Fig. 4). The expression of $\beta 1$ integrin was higher than that of Oct-4. However, there was no significant difference ($p > 0.05$) in the expression between both the genes. A significantly ($p < 0.001$) higher abundance of vimentin was detected in the culture.

Discussion

Mammalian testes possess a limited population of SSCs constituting only 0.03 % of total germ cells [1]. The scarce availability of SSCs in the testes necessitates developing efficient and newer methods for isolation and subsequent enrichment of SSCs. In addition, disaggregation of the testicular tissues is a crucial step for obtaining sufficient numbers of

viable cells that determines the efficiency of isolation of SSCs from mammalian testis. Broadly, disaggregation is carried out either by mechanical method or by multi-step enzymatic digestion [11,40]. Both the methods yield equal numbers of cells, but the viability of the isolated cells is compromised in mechanical method [41]. On the other hand, the enzymatic digestion protocols are relatively complex and time-consuming. Further, the use of more numbers of enzymes might cause cell injury while less numbers increase the possibility of cell contamination [13,42]. Previously, in farm animals, attempts were made to isolate the SSCs using either four [10,11], three [14] or two [43] enzymes with variable success. In the present study, we performed testicular disaggregation by enzymatic digestion using three enzymes without any reduction in the total cell count. Most importantly, we obtained cell viability as high as 90 % as previously demonstrated in human [13]. Following testicular disaggregation, Percoll gradients (32, 36 and 40 %) centrifugation yielded 3.65 folds more abundance of $\alpha 6$ integrin positive cells in the enriched fractions suggesting a level of purity up to 82.75 %. Although, similar level of purity was obtained by Percoll density gradient (30–32 %) centrifugation in rat and cattle, but it required four enzymes for testicular disaggregation [15,16]. On the other hand, when numbers of enzyme were reduced to two, it required an additional enrichment step of differential surface-attachment after Percoll density gradient to obtain a purity nearing to 86.7 % [13].

Normally, the establishment of SSC culture is challenging primarily because of the decrease in the cell viability,

Fig. 3 Immunostaining of SSC colonies on Sertoli cell feeder layer after 14 days of culture. Expression of $\alpha 6$ integrin (a & b) and PLZF (c & d) in SSC colonies; Expression of vimentin in individual Sertoli cells (e&f) and in SSC colony (g&h). The positive cells produced green fluorescence. In the first column, without nuclear stain and in the second column, the nuclei (red) of cells were stained by propidium iodide (PI). Scale bar: 50 μ m

reduction in cellular proliferation and also the changes in the original cell characteristics [44]. Initially, attempts were made to improve the viability of SSCs by adding growth factors in the culture medium [45], but it failed to yield much improvement [44]. The requirement of glial cell line-derived neurotrophic factor (GDNF) was also reported for long-term in vitro culture of self-renewing SSCs [25,31]. Subsequently, co-culturing of SSC on feeder layers (viz., Sertoli cells, STO cells and embryonic fibroblast cells) was exploited as an alternative [46–48]. Among the co-culturing system, Sertoli cells have been emerged as the most commonly used feeder layers [48]. Many laboratories have established Sertoli cell lines to support SSCs culture [23]. In mice, a short-term co-

culture with Sertoli cells resulted in a significant increase in both number and diameter of SSC colonies compared with culture containing growth factors including GDNF only [27]. However, these cell lines tend to transform and lose some of their typical characteristics, making them unsuitable for co-culturing [49]. In the present study, while culturing the SSCs on Sertoli cells feeder layer, we recorded an appearance of SSC colonies as early as on 4th day of culture. However, we observed that SSC colonies appeared after 5th day on laminin coated plates (unpublished data). The previous report in goat demonstrated a short term culture of SSC in feeder-free condition for only 1 week [10]. In contrast, even after one and half month of culture, we and others [13] noticed the presence of

Fig. 4 The relative expression of markers for SSC (viz., Oct-4 and $\beta 1$ integrin) and Sertoli cell (viz., vimentin) after 14 days of SSC culture ($***p < 0.001$). The culture showed a significantly higher expression of vimentin than SSC markers

stable colonies without any differentiation. This indicates a better suitability of Sertoli cell feeder for long term in vitro propagation of goat SSCs.

The limited availability of SSC-specific markers poses challenge in isolation and characterization of SSCs [4,23]. Literature suggests a high degree of conservation in germ cells and SSC markers between goat and rodents despite having a long evolutionary distance between those species [50]. Integrins, a marker for SSC, act as laminin receptor and mediate cell attachment [51] while PLZF, one of the transcription factors, is essential for self renewal of SSCs [52]. In the present study, using anti-rabbit $\alpha 6$ integrin and anti-mouse PLZF antibodies, we demonstrated the expression of SSC specific markers by almost all the SSC colonies. Morphological and immunocytochemistry analysis revealed that the SSC colonies are intermingled with the somatic cells as some colonies also expressed vimentin. Earlier studies also reported densely packed somatic cell areas in SSC colonies [11,26]. Vimentin, an intermediate filament of Sertoli cell cytoskeleton, plays an important role in maintenance of spermatogenesis in the testes [53]. Using qPCR, we further confirmed the expression of mRNA encoding other SSC specific markers (viz., $\beta 1$ integrin and Oct-4) required for colonization and self renewal of SSCs [54,55]. A relatively higher abundance of vimentin mRNA observed in the study might be due to the fact that most SSC cultures contain a mixture of testicular cells constituting as less as 1.33 % SSCs and other testicular cells mainly comprising of Sertoli cells [26].

Although the SSCs are unipotent stem cells, under different culture conditions they may acquire pluripotency. SSCs derived from neonatal and adult mice testis generated embryonic stem (ES)-like cells in vitro [56,57]. Similarly in human, the pluripotent cells produced from SSCs showed cellular and molecular characteristics of ESCs and differentiated into various cell types of the three germ layers [58,59]. However, in the present study, as we didn't change the culture conditions, the colonies were maintained their normal state till the end of culture.

In conclusion, we demonstrated a simple enzymatic digestion method for isolation and a Percoll density gradient method for enrichment of SSCs in goat. In addition, Sertoli cells appear as suitable feeder layer for long term culture of SSC. Results also suggest the possible application of non-caprine antibodies against SSC specific markers for the identification and subsequent assessment of SSCs in goats. The characterization and understanding of SSCs will have immense practical implications considering the major genetic gain comes through male.

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