Co-Culture of Spermatogonial Stem Cells with Sertoli Cells in the Presence of Testosterone and FSH Improved Differentiation via Up-Regulation of Post Meiotic Genes

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Abstract- Spermatogonial stem cells (SSCs) maintain spermatogenesis throughout life in the male. Maintenance of SSCs and induction of spermiogenesis *in vitro* may provide a therapeutic strategy to treat male infertility. This study investigated *in vitro* differentiation of mouse SSCs in presence or absence of Sertoli cells, hormones and vitamins. Spermatogonial populations were enriched from testes of 4-6 week old males by magnetic activated cell sorting and anti-Thy-1 antibody. Sertoli cells isolated from 6-8 week old testes were enriched using lectin-DSA-coated plates. Isolated SSCs were cultured in the presence of Leukemia inhibitory factor (LIF) for 7 days in gelatin-coated dishes, then dissociated and cultured for 7 days in media lacking LIF in the presence or absence of Sertoli cells, with or without FSH, testosterone and vitamins. After one week, the effects of Sertoli cells ± supplementary media on SSC differentiation was evaluated by microscopy and expression of meiotic and postmeiotic transcripts using RT-PCR. SSC colonies had limited development after LIF removal alone, exhibiting low expression of meiotic (Scp3, Th2b) but not postmeiotic transcript, and loss of Stra8 and Dazl expression. SSCs co-cultured with Sertoli cells, hormones and vitamins developed spermatid-like cells expressing postmeiotic markers (TP1, TP2, Prm1) at levels over 2-fold higher than Sertoli cells or hormone/vitamins alone. Our present SSC-Sertoli co-culture provides conditions that may allow efficient in vitro differentiation of SSCs for the treatment of male infertility.

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Introduction

Spermatogenesis originates from a small number of spermatogonial stem cells (SSCs) that reside on the basement membrane of seminiferous tubules and undergo self-renewal division throughout the life of adult animals (1). During the neonatal period, mouse gonocytes differentiate into spermatogonial stem cells, which give rise to all differentiating germ cells in the testis (2). Due to small number of spermatogonial stem cells in adult testis, identification of a specific SSCs marker and direct *in vivo* analysis of spermatogonial stem cells is difficult (3-5). An adult mouse testis contains 20000–35000 spermatogonial stem cells, representing 0.02%–0.03% of the total cells in the testis (3). Therefore, enrichment and *in vitro* culture of SSCs provides one practical approach for investigating early germ cell development. Furthermore, *in vitro* differentiation and maturation of male germ cells for assisted reproductive techniques (ART) may provide therapeutic strategies for treatment of male infertility (3, 6), such as testicular cancer, bilateral testicular damage or orchidectomy, and maturation arrest *in vitro* maturation techniques (following cryopreservation) could reduce risks of transplanting malignant cells back into patients after chemotherapy (6). Micromanipulation-ART with *in vitro* matured elongated spermatids from patient samples with spermatogenic arrest at the round spermatid or primary spermatocyte stage has resulted in healthy babies (7).

Testosterone and FSH actions provide additive and synergistic effects for meiotic and postmeiotic

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development *in vivo* (8). Somatic cells like Sertoli cells play a pivotal role in male germ line stem commitment to differentiation (9). *In vitro*, the presence of Sertoli cells and high concentrations of FSH and testosterone allowed meiotic progression and increased germ cell survival (decreased apoptosis) in cultured human testis tissue or cell samples (10,11). Testosterone and direct association of bull germ cells and Sertoli cells *in vitro* stimulated Sertoli cell function and germ cell differentiation to haploid stage (12). Different studies indicated Sertoli cells and hormone co-cultures induce postmeiotic development *in vitro* in maturated spermatocyte or spermatid (12-14).

Replacement and exchange of chromosomal proteins histone-protamine in spermatogenesis is crucial for postmeiotic development (15,16). So far there are few *in vitro* studies on SSCs derived spermatid dealing with expression of genes involved in this process (17). Expression of specific genes involved in chromosomal protein dynamics during spermatogenesis, such as H2A, Th2b and Ube1y, has already been reported in spermatid derived testis or *in vivo* or studies (13,17-19). However, there is no *in vitro* SSCs derived spermatid cells study in this regard in 2D medium culture. In this study, we co-cultured SSCs with Sertoli cells in the presence of FSH, testosterone, vitamins C, E and retinoic acid (RA) to replicate conditions somewhat similar to the natural niche. Efficient differentiation of SSCs to spermatid-like cells was characterized by histological staining and expression of the specific germ cell transcripts; in particular those involved in histone-protamine replacement. Our cell culture conditions produced spermatid-like cells *in vitro*, which provide insight into maturation conditions useful for treatment of male infertility.

Material and Methods

Experimental animals

Testis cells were obtained from 4–6 weeks old NMRI (National Medical Research Institute) male mouse. They were maintained under standard conditions with free access to food and water. Animal experiments were approved by the ethical committee of Tehran University of Medical sciences and were performed in accordance with University guidelines.

SSCs enrichment

For obtaining testicular cells we used a modified method by Guan (2009). Briefly, decapsulated testis was cut into small pieces and seminiferous tubules were transferred to Collagenase type IV (1 mg/ml), DNase I (10 µg/ml) solution. Cells were incubated at 37 ˚C in a CO_2 incubator and dispersed by pipetting every 2–5 min until the tubules separated after about 20 min. Cells were washed twice with 10 ml of phosphate-buffered saline (PBS) as described (20). The dissociated testis cell suspension was overlaid on 30% (v/v) Percoll (Sigma) prepared in PBS containing 1% fetal bovine serum (FBS) (Gibco) and centrifuged at 600 x g for 8 min at 4˚C. For magnetic activated cell sorting (MACS), Miltenyi Biotec protocol was used. Sedimented cells (bottom fraction) of the Percoll gradient (3–8×10^6 cells in 90 µl of PBS) were incubated with 10 µl of anti-Thy-1 antibody (30-H12; Miltenyi Biotec) micro beads for 20 min at 4˚C. After rinsing with PBS containing 0.5% bovine serum albumin (BSA) (Sigma), Thy-1^+cells were selected by passing through an LS separation column (Miltenyi Biotec) placed in a magnetic field (21).

Flow cytometry

Flow cytometric analysis was performed on populations of testis cells before and after enrichment using a modified method of Shinohara (1999). Briefly, 10^6 cells were suspended in 0.1 ml of PBS/1%FBS. 10µl FITC Hamster Anti-Rat β1-integrin (CD29) antibody was added to the cells for 20 min at 4°C. The cells were then washed twice in 1 ml of PBS/FBS. Then 0.1 ml of PBS/1%FBS and 10µl PE rat anti-human α6-integrin (CD49f) (R&D) was added to the cells for 20 min at 4°C. The cells were washed twice with 1 ml of PBS/FBS. Control cells were not treated with any antibody (4).

Sertoli cells isolation and culture

Adult Sertoli cells were extracted from the testis of adult mice (6-8 weeks old) as previously described by Scarpino *et al.* (22). Briefly, culture dishes were coated with 5 µg/ml of Datura Stramonium agglutinin (DSA; Sigma) lectin in PBS at 37°C for 1 h. Then, the coated plastic dishes were washed three times with 0.5% BSA (Sigma). The mixed population of the cells obtained by enzymatic digestion was placed on lectin-coated dishes and incubated for 1 h at 32°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the non-adhering cells were collected by being washed twice with DMEM medium. Then DMEM and 10% FBS were added. After seven days the Sertoli cells formed a confluent layer (22).

Immunocytochemistry for characterization of adult sertoli cells

Vimentin was detected in cultured Sertoli cells by immunocytochemistry. After fixation with 4% paraformaldehyde, permeabilization by 0.4% Triton X100 (Sigma) and blocking with 10% goat serum (Sigma), the cells were incubated for 2 h at 37°C with mouse monoclonal anti-Vimentin antibody, diluted 1:100 (Sigma). After washing with PBS, the secondary antibody, goat anti-mouse labeled with fluorescent isothiocyanate (FITC) diluted 1:100 (Sigma) was applied for 3 hours. Control cells were treated under similar conditions except for the removal of the first antibody. Nuclei were stained with 5µg/ml propidium iodide (Sigma) (23).

SSCs culture

Enriched SSCs were cultured (6–10 $\times 10^4$ cells/cm^2) in minimum essential medium-alpha (MEMα)(Gibco) containing 10% FBS, 1 x nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 10^3U/ml human recombinant leukemia inhibitory factor (LIF) (B&D), 0.4mM Pyruvate (Sigma), 1x Glutamine (Gibco), 100 u/ml Penicillin and 100 µg/ml Streptomycin (Sigma), on gelatin-coated dishes for 7 days. Culture medium was changed every other day. All culture media were maintained at 32˚C in an atmosphere humidified with 5% CO_2 (20). SSCs colonies were stained with alkaline phosphatase after 7 days (24). SSCs number was determined with a hemocytometer. Trypan blue (0.4%) exclusion assays were used to determine the percentage of surviving cells following isolation and culture (25).

SSCs differentiation

The cells was dissociated with trypsin/EDTA solution and 5×10^5 cells transferred to four types of medium without LIF :1) MEMα medium containing 10% FBS, 100 u/ml Penicillin, 100 µg/ml Streptomycin ,10^{-4} M vitamin C (Sigma), 10 mg/ml vitamin E (Sigma), 3.3×10^{-7} M RA (Sigma), 2.5×10^{-5} U FSH (sigma), 10^{-7} M testosterone (sigma) co-culturing with Sertoli cells; 2) MEMα medium containing 10% FBS, 100 u/ml Penicillin, 100 µg/ml Streptomycin co-culturing with Sertoli cells; and 3) MEMα medium containing above supplements (FSH, testosterone and vitamins E, C, RA) without Sertoli cell co-culturing ; 4) MEMα medium without LIF, Sertoli cells and any supplements.

Cytochemistry staining

After inducing differentiation for one week (26), cultured germ cells were stained with PAS and Shorr staining to assay acrosome granules in spermatid-like cells cytoplasm (27).

Reverse transcriptase polymerase chain reaction

After 7 days, the expression of specific genes such as Histone 2A (H2A), synaptonemal complex protein (Scp3) and testis-specific histone protein 2B (TH2B) expressed in spermatocytes, and Ubiquinin 1 y (Ube1y), transition proteins (TP1, TP2) and Protamine (Prm1) expressed in spermatids were studied by RT-PCR. Primer sequences are given in Table1. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RT-PCR was performed by cDNA synthesis kit (Bioneer, South Korea) using 1 µl of total RNA according to the manufacturer's protocol. PCR was performed using Tag DNA polymerase (Cinagene, Tehran, Iran) in a Gene Amp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After an initial denaturation at 94 °C for 5 min, cDNA was subjected to 33 cycles of PCR. Testicular cells from adult testis were used as positive control and liver cells used as negative control. To normalize RT-PCR samples, expression of β-actin was examined. PCR products were observed on 2% agarose gels, and the intensity of bands assayed by UVI doc gel documentation system (Avebury house 36 a union lane Cambrige CB4 1QB-UK).

Statistical analysis

The results were expressed as mean ± SD. The statistical significance between the mean values was determined by one-way ANOVA analysis of variance, Tukey and Duncan post-test. $P\leq0.05$ was considered significant.

Results

Spermatogonial stem cells enrichment

The surface antigenic phenotype of SSCs in the mouse testis were reported to be α6-integrin, β1-integrin, Thy-1(CD90), CD24, GFRα1, Ep-CAM, GPR125 (5). In the current study, SSCs were isolated by using anti-Thy-1 antibody and MACS. The purity of the isolated cells was determined by flow cytometry using α6 and β1-integrin antibodies. The flow cytometric analysis revealed that of the enriched Thy-1 purified cells, 67.2±2.2% (n=6) expressed β1 integrin, 37.7±1.8% (n=6) expressed α6-integrin and 30.8±1.9% (n=6) of Thy-1-enriched cells expressed both integrins (Figure 1), consistent with previous data (51.3±11.9 for β1-integrin) (4).

Table 1. Primer sets used for amplification of specific genes.

Genes	Primers sequences (5' to 3')	Product length (bp)
Dazl	Forward: AAGGCAAAATCATGCCAAAC	186 bp
	Reverse: TTCTGCACATCCACGTCATT	
Stra8	Forward: CTGTTGGACCAGATGCTGAA	165 bp
	Reverse: GCAACAGAGTGGAGGAGGAG	
H2A	Forward: GCTGTCACGAAAGTGCAGAAG	206 bp
	Reverse: GGTCGAGCGCTTGTTGTAATG	
Th2b	Forward: GATGCCGCGAAGAGAGTTACTC	173 bp
	Reverse: GGTCGAGCGCTTGTTGTAATGCG	
Scp3	Forward: CCGCTGAGCAAACATCTAAAGATG	169 bp
	Reverse: GGAGCCTTTTCATCAGCAACAT	
Ube1y	Forward :T TTACTCCCGCCAGCTGTAT	223 bp
	Reverse: AATGCCCTGATCATGGAGAG	
TP1	Forward: GCCGCAAGCTAAAGACTCATGG	189 bp
	Reverse: GGGATCGGTAATTGCGACTTG	
TP2	Forward :GGCCTCAAAGTCACACCAGTAAC	205 bp
	Reverse :TTGCAGGTGAGTGTCGAGAGTGC	
PRM1	Forward: ATGGCCAGATACCGATGCTGC	113 bp
	Reverse:CAGCATCTTCGCCTCCTCCG	
β-actin	Forward: TTCTACAATGAGCTGCGTGTGG	115 bp
	Reverse: GTGTTGAAGGTCTCAAACATGAT	

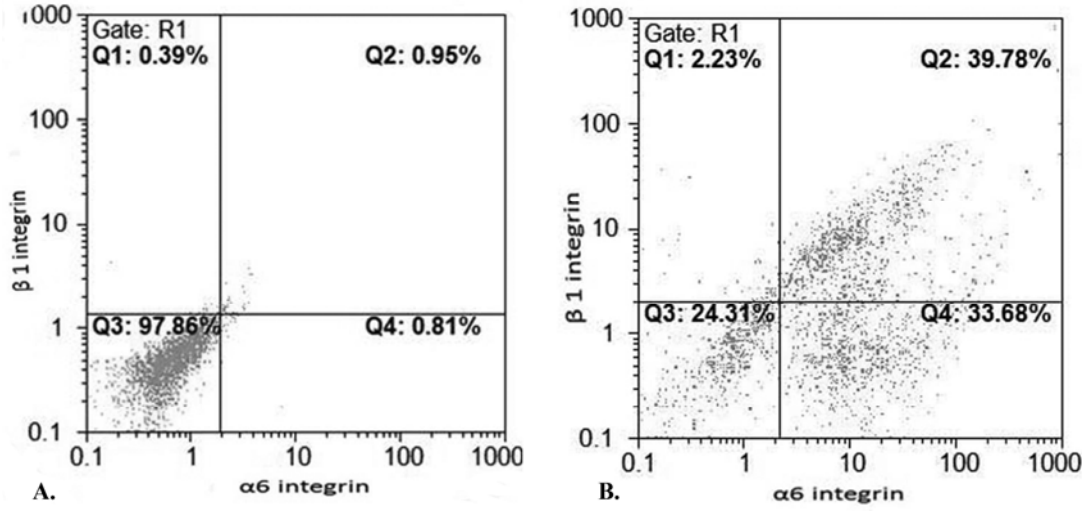


Figure 1. Flow cytometric analysis for detection of α6 and β1-integrin in SSCs. While there is no cells with expression of β1 and α6-integrins before MACS (A), there is considerable cells with positive β1 and α6-integrins following MACS purification (B).

SSCs culture

Isolated Thy1+ SSCs were cultured in the presence of LIF. In the first day of culture, SSCs were single and attached to gelatin coated dishes (Figure 2A). After two days the majority of round cells aggregated into clumps in which no cytoplasmic bridges could be discerned (Figure 2B), and then colonies were formed on 2-3th day (Figure 2C). These cell colonies were positive for alkaline phosphatase activity (Figure 2D), indicating the colonies were SSCs (21,24).

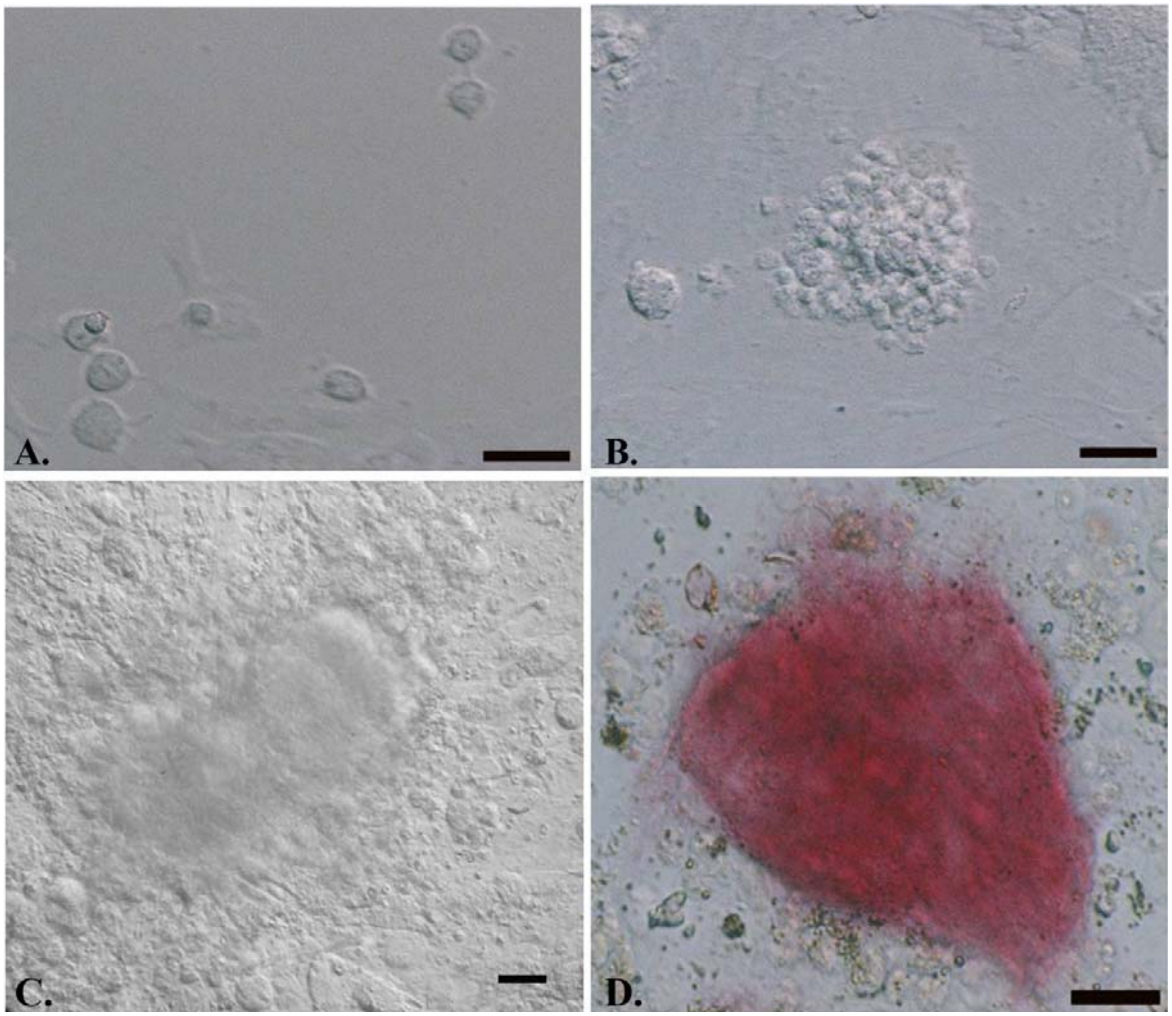


Figure 2: Microscopy morphology of SSCs derived from 4-6 week-old male mice. SSCs were single on 1st day of cell culture (A). After 2-3th days of cell culture Small SSCs colonies were formed (B), on 7th day of cell culture the size of colonies was increased(C) and positive for alkaline phosphatase activity (D). Bar: 100µm

Sertoli cells isolation and characterization

Cells cultured on lectin DSA coated plates were initially round and adhered firmly to the bottom of the dish. After 1 day, cells began to flatten, spread out and take on an epithelioid appearance (Figure 3A). The presence of Vimentin protein on Sertoli cells (23) was confirmed by immunocytochemistry, demonstrating that the majority (up 85%) (data not shown) of freshly isolated cells attached to lectin were Vimentin positive (Figure 3B). All these results indicated the Sertoli cells properly isolated (Figure 3B and C).

SSCs differentiation

Isolated Thy1+ SSCs were cultured in the presence of LIF for 7 days as described above. Then SSC cells were dissociated and cultured for 7 days in the absence of LIF (denoted SSC$^-$LIF group) to investigation developmental potential without Sertoli cells and supplements (hormones/vitamins). SSC colonies had limited development after LIF removal alone (data not shown), and cells exhibited low expression levels of meiotic (Scp3, Th2b) but no detectable postmeiotic transcripts, and lost detectable Stra8 and Dazl expression (Figure 4B).

In order to provide the optimum cell culture condition for differentiation of SSCs to spermatid like cells, we then compared three different conditions; i) SSCs cultured with Sertoli cells alone, denoted S group ii) SSCs cultured with Sertoli cells in presence of vitamins (C, E and RA) and hormones (FSH and testosterone), denoted the SHV group iii) and SSCs cultured with only hormones and vitamin (no Sertoli cells), denoted the HV group.

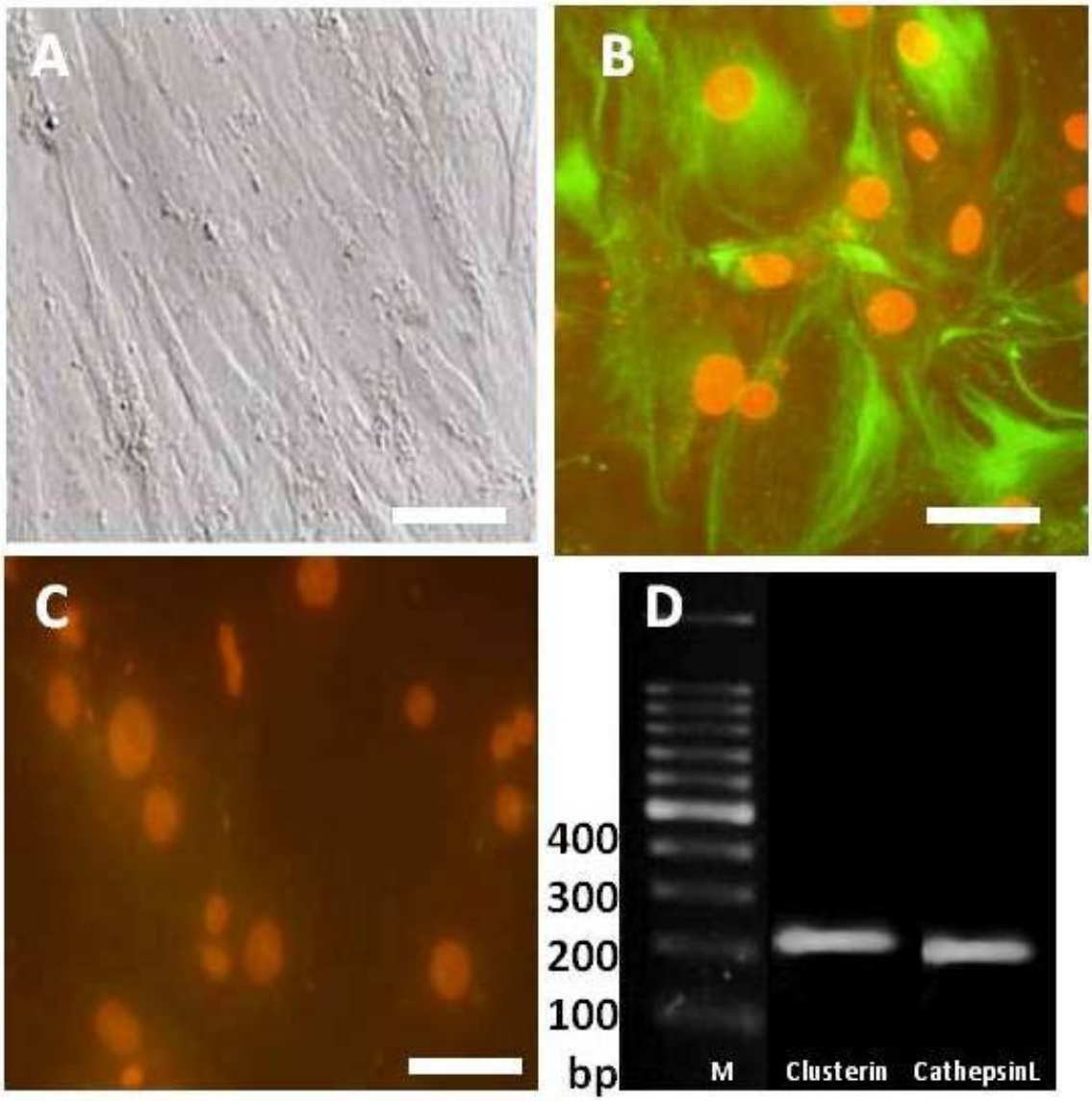
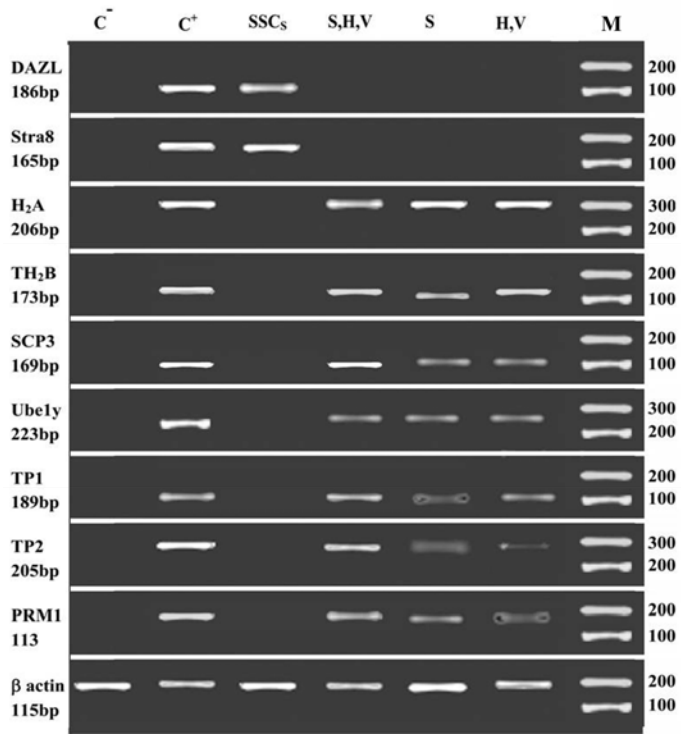


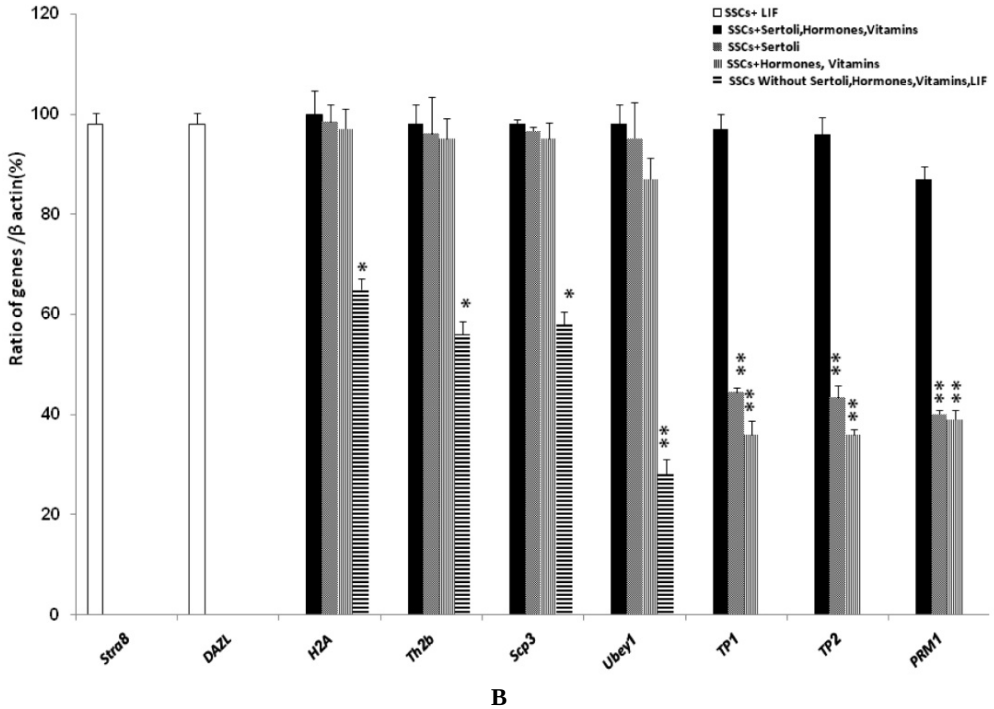
Figure 3. Microscopy morphology of Sertoli cells derived from 6-8 week-old male mice. Sertoli cells were isolated by lectin DSA and characterized by immunocytochemistry. Monolayer Sertoli cells began to flatten and spread out following one day plating on lectin DSA (A). Sertoli cells were positive for Vimentin in cytoplasm (green color) and nuclei were stained with propidium iodide (B)(orange color); Negative control (C). Bar: A and C: 100μm; B: 50μm

In order to evaluate the differentiation of germ cells, levels of meiotic spermatocytes and postmeiotic specific spermatid transcripts were studied by RT-PCR. All genes were expressed in testis as positive control but absent in liver as the negative control.

Co-culture of SSCs with Sertoli cells in presence of vitamins and hormones (SHV) improved the detection of haploid transcripts compared to the other groups (Figures 4A and B). There was no significant difference in expression of primary spermatocyte-specific genes, H2A and Scp3 ,and spermatocyte expressed Th2b (13), between the three different SHV, S and HV groups. By comparison, the SSC^{-}LIF cells had significantly reduced H2A, Scp3 and Th2b expression compared to all other groups ($P \leq 0.05$) (Figure 4B). There was also significant increase in detection of spermatid-specific transcripts (Ube1y, TP1, TP2, Prm1) in SHV relative to the S and HV groups (>2-fold, $P<0.001$) (Figure 4B). Interestingly, there was no significant difference between postmeiotic transcripts detected in S versus HV groups, except a modest increase in Tp1 and Tp2 expression in S relative to HV condition.



A



B

Figure 4. Electrophoresis of PCR products on 2 percent agarose gels. After 7 days cultivating of SSCs in different conditions, the expression of some specific genes was studied by RT-PCR. C^-: Negative Control group; C^+: Testes cells for positive control group; SSCs: spermatogonial stem cells before induction of differentiation; S, H, V: *In vitro* differentiated cells co-cultured with Sertoli cells in presence of hormones (FSH, testosterone) and vitamins (C, E, RA); S: *In vitro* differentiated cells only co-cultured with Sertoli cells; HV: *In vitro* differentiated cells co-cultured only in presence of hormones and vitamins; M: 100 bp ladder (A). Ratio of Genes/β-actin has been shown by a histogram (B).* $P \leq 0/05$, ** $P \leq 0/001$

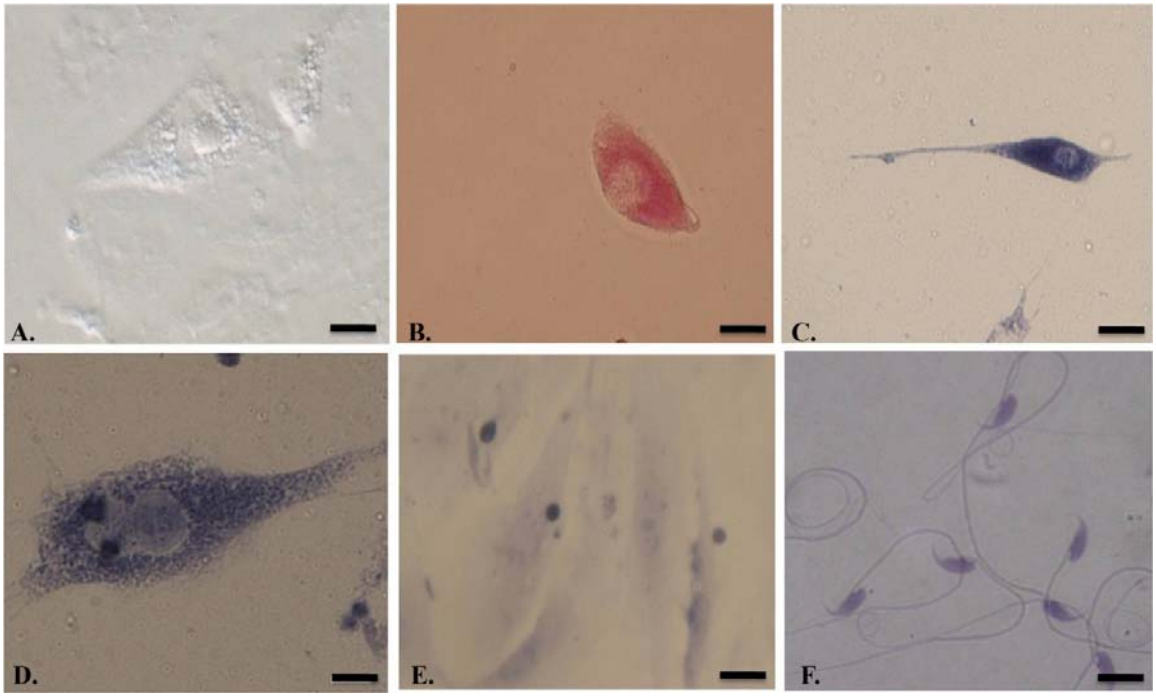


Figure 5. Specific staining of SSCs derived-spermatid like cells. Phase contrast microscopy photograph of SSCs derived-spermatid like cell (A). Acrosome granules were detected by PAS (B) and Shorr staining (C, D) No acrosome granules were observed in SSCs (E). Normal sperm was used as a positive control for acrosome granules (F). Bar: 50µm (A, C, D); 100µm (B, E, F)

The differentiation of spermatid-like cells was further evaluated using specific staining (Shorr and PAS staining). As shown in figure 5, the existence of acrosome granules in SHV, S, HV groups suggests that spermatid-like cells were successfully differentiated under these conditions.

Discussion

The isolation and proliferation of SSCs may provide a valuable tool for the treatment of male infertility. In this study we have employed a simple but versatile method to efficiently produce spermatid-like cells *in vitro*, using Sertoli cell co-culture and supplements comprising known important factors for germ cell survival and development.

Mouse SSCs were enriched using anti-Thy-1 antibody and MACS technology. Our results revealed that the isolated SSCs had high purity and expressed two specific surface markers for mouse spermatogonial stem cells i.e. α6 and β1-integrin (4). Similar to our findings, Kubota *et al*. used Thy-1 microbeads to isolate SSCs by MACS (21). However, as source for SSCs they used cryptorchid adult, pup and neonate testis, in which SSCs are present at a considerably higher % of total testis cells relative the % of SSCs found in adult testes (21). In contrast, in our present study, adult mouse testes were was used as a source of SSCs, and approximately same purity of SSCs was obtained as determined by the expression of β1- and α6-integrins (75% compared to 51%)(4) by flow cytometry, indicating efficiency of our enrichment method.

In vitro differentiation of SSCs may provide a valuable therapeutic treatment of some diseases (6). This study investigated the differentiation of SSCs to spermatid-like cells in a co-culture with Sertoli cells in the presence or absence of vitamins and hormones, deliberately designed to mimic the natural niche of SSCs. Our *in vitro* results strongly showed that Sertoli cells combined with hormones/vitamins can improve differentiation of mouse SSCs to spermatid-like cells.

Tesarik *et al*. demonstrated that germ cells cultured in association with Sertoli cells can progress spermatogenesis during short-term culture, including initiation or completion (28). Also intercellular junctions, endocrine and paracrine regulation of cells seems to be critical to *in vitro* spermatogenesis process (12). Sertoli cells provide essential factors necessary for successful differentiation of spermatogonia into spermatozoa and their endocrine or paracrine factors play an important role in maintaining the viability of spermatogonia cells and stimulating animal meiosis *in vitro* (29). Our results also revealed that Sertoli cells can induce meiosis *in vitro*. FSH and testosterone are

reported to be a prerequisite for the completion of meiosis and spermiogenesis *in vitro* and they are necessary for alterations in spermatid morphology in an *in vitro* culture system (30). Sertoli cells express receptors for androgen (31) and are instrumental to stem spermatogonia differentiation, a process that critically depends on RA (32), consistent with our findings of improved *in vitro* differentiation in the presence of Sertoli cells combined with hormones/vitamins. Similar findings have been reported by Sousa *et al*. (14). However, they co-cultured spermatocytes or early spermatids with Sertoli cells in the presence of FSH and testosterone. They showed that these three factors, Sertoli cells in the presence of FSH and testosterone, act as survival factor and induce meiosis entry and full spermatid differentiation (14). *In vivo*, FSH and testosterone combined for positive effects on mouse germ cell development (33). It is noteworthy that FSH and testosterone regulate germ cell development and play an important supportive role for this process (34).

We demonstrated that enriched SSCs cultured in the absence of LIF developed meiotic markers (H2A, TH2B and Scp3). Differentiation of embryonic stem cells to early stage germ cells without LIF was reported (35). In our study, SSCs failed to develop markers of postmeiotic development, although classic mitotic markers (Stra8, Dazl) were down regulated, consistent with meiotic progression (Figure 5B). Stra8 and Dazl are expressed in premeiotic germ cells that do not undergo further differentiation (36). Our study also demonstrated that cultured SSCs without LIF developed to cells expressing genes involved in the initiation of meiosis, such as Scp3 and H2A, again indicating a degree of SSC differentiation occurred in the absence of known somatic cell (Sertoli) support or important hormones. However, our results demonstrated the beneficial role of Sertoli cells, FSH, testosterone and vitamins in up regulating the expression of these genes, suggesting SSC meiotic differentiation was more efficient with somatic and trophic support.

Correct histone-protamine exchange is essential for completion of differentiation of spermatids and production of fertile sperm (16). For male germ cell development and differentiation ubiquitination is also necessary and plays a key role in histone-protamine transition stage (18). We showed that the SSCs derived spermatid-like cells expressed key genes involved in histone-protamine exchange, such as Ube1y, Tp1, Tp2 and Prm1.

The combination of Sertoli cells, FSH, testosterone and vitamins improved the efficiency of differentiation, as indicated by higher expression of post meiotic genes (Ube1y, Tp1, Tp2 and Prm1). In conclusion, our findings indicated that Sertoli cells, FSH, testosterone and vitamins (E, C and RA) improved SSCs differentiation. Our simple co-culture system may be applicable for treatment of male infertility, although the efficiently of these cells for ART remains to be answered and warrant further complementary studies.

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