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A simple method for isolation, culture, and in vitro maintenance of chicken spermatogonial stem cells

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Abstract Spermatogonial stem cells (SSCs) are expected to participate in male infertility therapy, endangered species preservation, and transgenic animal technology by their unique unipotency to differentiate into spermatozoa. The main challenges, however, remain to be addressed including the appropriate conditions to reach good number of these cells and how to derive, culture, and maintain them in vitro. In the present study, the testicular tissues were isolated from 1-d-old male chickens to establish primary cell cultures. This culture led to development of distinguished colonies which were further characterized by alkaline phosphatase (AP) activity assay and gene expression analysis. They were shown to be positive for AP activity and expressed two main transcription factors of *OCT4* and *STRA8* as indicated by reverse transcription-polymerase chain reaction. These were indications of carrying characteristics of SSCs by these colonies. The cultures were also exposed to different concentrations of glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF) growth factors to seek optimum colony-forming conditions. Colony-forming activity assay indicated that they were able to propagate in vitro with an increased self-renewal property when cultured in the presence of 15 ng/mL of GDNF, 20 ng/mL of bFGF, and 15 ng/mL of LIF. The present work provides an easy and practical method for isolation, culture, and in vitro maintenance of chicken spermatogonial stem cells and introduces appropriate cell culture conditions to improve and maintain their self-renewal property based on supplying the necessary growth factors.

Keywords Spermatogonial stem cell · Chicken SSC · Growth factors · GDNF · Colony-forming activity

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Introduction

In recent years, there has been an increasing interest in spermatogonial stem cells (SSCs) because of their unique properties in male fertility maintenance (Zohni et al. 2012). Spermatogenesis is a complex process to generate sperms from spermatogonial stem cells by meiosis (Golestaneh et al. 2009). SSCs are the only type of stem cells that can transmit genetic information to next generation of sexually inbred species (Dym et al. 2009). This unique property makes them a good candidate for very important researches in male infertility therapy (Vlajkovic et al. 2012; Zohni et al. 2012), endangered species preservation (Silva et al. 2012), and transgenic animal studies (Kanatsu-Shinohara et al. 2004; Ryu et al. 2007). For this, developing the proper techniques seems to be prerequisite for successful maintaining of the SSCs in vitro. During the past 15 yr, much more information has become available on mammalian SSC culture (Brinster and Nagano 1998; Nagano et al. 1998, 2001, 2002). Since the first report of SSC derivation from in vitro culture in 1998, using cultivation of the testis cells on STO feeder layer (Nagano et al. 1998), many researches on human, mouse, and rat SSC isolation, culture, and maintenance have been reported (Vlajkovic et al. 2012; von Kopylow et al. 2012; Wang et al. 2012). However, not many well-developed techniques about nonmammalian counterparts have been established by now, despite the fact that

many of these species play important roles in human life quality (Nobrega et al. 2010; Kawasaki et al. 2012).

Among nonmammalian vertebrates, poultries and specially chickens attract much more interests for transgenesis because of their high industrial and economic significance. Access to good numbers of SSCs from chicken and their successful culture would serve as a giant step in developmental biology and recombinant technology. It has been well confirmed that glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF) are three important molecular participants for maintaining self-renewal capacity of stem cells in vitro and for preparing chemically defined media, as these three factors have redundantly been exploited in SSC culture studies (Gritti et al. 1996; Berger and Sturm 1997; Meng et al. 2000).

In the present study, we tried to simplify the derivation and culture of chicken SSCs, followed by their characterization by exploiting the colony-forming assay (Yeh et al. 2007) and gene expression analysis. We also attempted to increase the efficiency of colony formation by planning combinatorial treatment of the cultures by growth factors.

Material and Methods

Testicular tissue preparation. Testes were obtained by surgery from 1-d-old male chickens (Hy-Line® variety W-36) provided by Morghak Company (Mashhad, Iran) in accordance with the codes of Bioethics Committee of Ferdowsi University of Mashhad.

Primary cell culture. The dissected tissues were washed three times, with phosphate-buffered saline (PBS), supplemented with 1,000 units/mL penicillin and 1 mg/mL streptomycin to avoid microbial contamination. They were then cut into small pieces using a sterile surgical blade and transferred into culture flasks containing Dulbecco's modified Eagle's medium (Gibco, Washington DC), supplemented with 10% fetal bovine serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 1,000 units/mL penicillin, and 1 mg/mL streptomycin. These primary cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and passaged every 2–3 d enzymatically with trypsin-ethylenediaminetetraacetic acid (EDTA). The first passage was performed using a 70 μm cell strainer (BD Biosciences, San Jose, CA) to remove tissue debris from the cultured cells.

Alkaline phosphatase assay. The media were removed from the culture flasks and the cells, including the colonies, were fixed with 4% buffered paraformaldehyde for 10 min. They were then rinsed with fresh PBS for three times and subjected to BCIP-T/NBT substrate solution and alkaline phosphatase (AP) buffer (pH 9.5; Thermo Scientific, Bonn, Germany). After about 10 min, the plates were analyzed microscopically.

Colony-forming activity assay. The primary cell cultures at passage two were trypsinized and counted. The singled cells were then seeded in 96-well culture plates with a seeding density of 1×10^4 cells/well. Every day, the wells were examined microscopically and the numbers of colonies were recorded. This experiment was repeated separately for different treatments of GDNF (Sigma-Aldrich, Germany; 0, 10, 15, 20, and 25 ng/mL), bFGF (Sigma-Aldrich; 0, 10, 15, 20, 25, and 30 ng/mL bFGF plus basic concentration of 15 ng/mL of GDNF in each treatment), and LIF (Sigma-Aldrich; 0, 10, 15, 20, 25, and 30 ng/mL LIF plus basic concentration of 15 ng/mL of GDNF in each treatment).

RNA extraction and reverse transcription-polymerase chain reaction. Total RNA was extracted from the testis tissues and cultured cells using RNX-plus RNA extraction kit (CinnaGen, Tehran, Iran). Briefly, 1 mL of RNA extraction solution was added to 1×10^6 cells or 50–100 mg of the homogenized testis tissues in a 1.5 mL microfuge tube. Extraction of the RNA was performed by conventional way using the protocol supplied by the kit. The extracted RNA was dissolved in 20 μL of RNase-free water and was immediately subjected to DNase I treatment (GeNet Bio, Daejeon, Korea). To do so, about 1 μg of the RNA, 1 μL of 10× reaction buffer, 1 μL (1 unit) of DNase I and up to 10 μL DEPC-treated water were added to a microtube (0.2 mL) and incubated for 30 min at 50°C. One microliter of 50 mM EDTA was then added and incubated for 10 min at 65°C. First strand cDNAs were synthesized using SuPrimeScript RTase Kit (GeNet Bio, Korea) and used as templates for PCR amplification of *GAPDH* as a house-keeping gene and *STRA8* as a germ cell marker.

The primers used in this experiment are listed in Table 1. PCR was done in a final volume of 20 μL with the following cycling conditions: an initial cycle at 94°C (3 min); followed by 35 cycles of 94°C (30 s), 60°C (20 s), and 72°C (30 s); and a final extension cycle at 72°C for 5 min.

All real-time PCR runs were performed in duplicates for β-*actin* as a house-keeping gene and *OCT4* as a stem cell marker gene and each reaction mixture was prepared using the SYBRGreen PCR Master Mix (GeNet Bio, Korea) in a total volume of 20 μL with the following volumes of each component: 10 μL of Master Mix, 2 μL of each sample cDNA (1 ng/μL), 1 μL of primers mix (10 pmol/μL), and 7 μL of DNase-free water. The primers used in this experiment are listed in Table 1. PCR was performed in a 0.2 mL microtube with the following cycling conditions: an initial cycle of 95°C (10 min); followed by 35 cycles of 95°C (30 s), 63°C (20 s), and 72°C (20 s); and an added cycle of 10 s at 95°C. The

Table 1. The sequence of primers

Primer	Forward sequence	Reverse sequence	Reference
GADPH	CCTTCATCGATCTGAACTACATGG	GGAGCTGAGATGATAACACGCTTA	Ref.NM204305
STRA8	GATGTGAGGGACAGTGGAGGTAA	CAGAAATGCCGCTTGTAAATGA	Ref. XM416179
β-actin	GCTCTGACTGACCGCGTTACTC	CGACCCACGATAGATGGGAACAC	Ref. NM_205518
OCT4	AATGAGGCAGAGAACACGGACAAC	GGGACTGGGCTTCACACATTTGC	Ref. NM_001110178.1

melting curve analysis was performed at temperature range of 65–95°C by 0.5°C increment for 5 s.

Statistical analysis. The SPSS v.21 statistical program was used to analyze the data. All data were expressed as mean± SD. Significance of the data were examined in the level of $p < 0.05$ using the one-way analysis of variance and Tukey's test.

Results

The primary testicular cell cultures generated colonigenic cell populations. The cultured dissected tissues of the 1-d-old chicken testes gave rise to primary cultures with mixed cellular morphologies. By the time and after 2 d, some cells appeared showing defined rounded shape morphology. They were either single, paired, aligned, or even forming small colonies (Fig. 1). After day 3, the colonies grew larger in size, while the number of them remained unchanged (Fig. 2). These colonies were considered as possible SSC populations and left for further characterizations.

Characterization of the cultured cells. The cultured cells developed into distinct morphologies of spindle and round shapes, presumably representing the fibroblasts and SSCs, respectively. This assumption was tested by examining the alkaline phosphatase activity of the mixed culture. Both rounded single cells and those forming the colony structures were confirmed as AP^+, while the fibroblast-like cells of the same plates were negative for AP activity (Fig. 3).

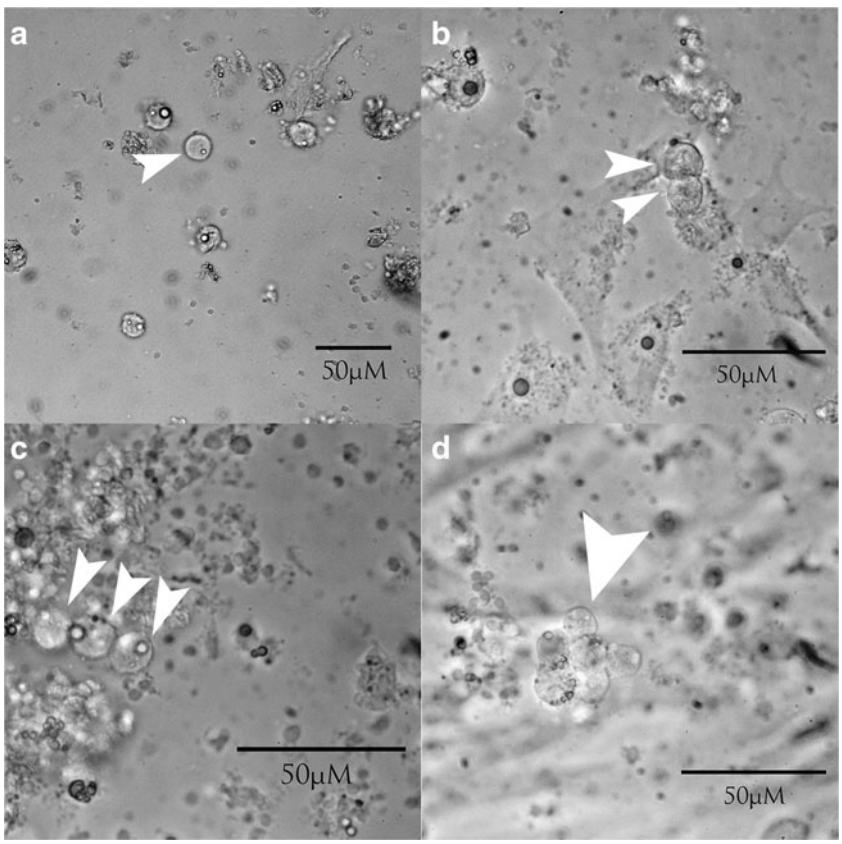

Figure 1. Primary cell culture from testicular tissues of 1-d-old male chickens. The cell populations with morphological characteristics of SSCs emerged 2 d after culture. These structures as indicated by arrows include (*a*) round single cells, (*b*) paired cells, (*c*) aligned cells, and (*d*) typical cellular colonies.

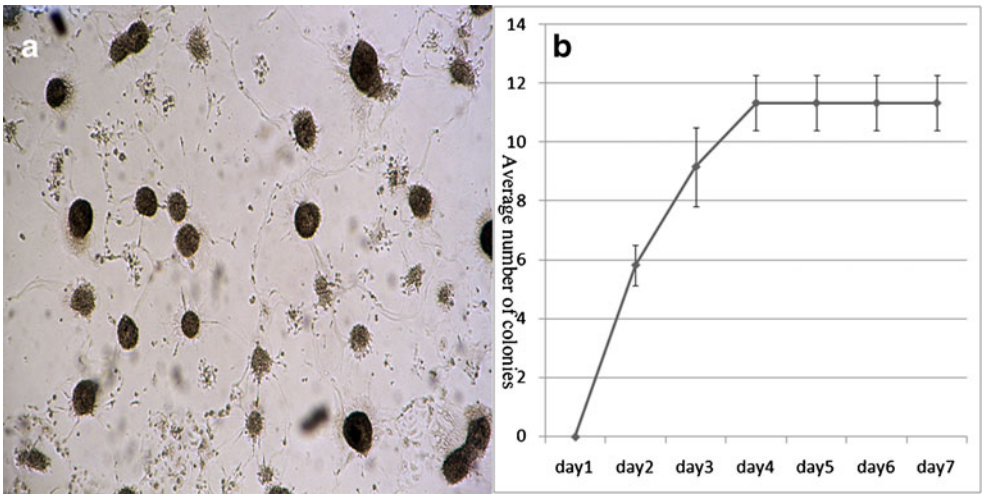

Figure 2. Development of cellular colonies, representing the growth of chicken spermatogonial stem cells, derived from in vitro culture of 1-d-old chicken testicular tissues. While size of the colonies were increasing by the time (*a*), their number remained unchanged after day 6 (*b*). Results are represented as means ± SD of six repeats.

Improvement of colony-forming efficiency by growth factors. With the knowledge of GDNF, bFGF, and LIF positive effects on self-renewal property of stem cells, the cultures were treated with variable concentrations of the mentioned growth factors. Overall these treatments increased the number of colonies, reaching to its highest efficiency at 15 ng/mL of GDNF, 20 ng/mL of bFGF, and 15 ng/mL of LIF (Fig. 4). Using higher concentrations the frequencies of the colony formation remained unchanged in cases of the GDNF and bFGF treatments, but in the case of the LIF the efficiency was even decreased.

Gene expression analysis. STRA8, as a well-known marker for germ cells and spermatocytes, was shown to be expressed in 1-d-old chicken testes as examined by reverse transcription-polymerase chain reaction (RT-PCR). In the same experiments, the cultured cells, from these testes, also proved to express this gene 1 wk after derivation and culture (Fig. 5). The impact of growth factors (15 ng/mL of GDNF, 20 ng/mL of bFGF, and 15 ng/mL of LIF) was also tested at molecular level on expression of *OCT4* gene as a stemness-specific marker by real-time RT-PCR. Its expression in the untreated cells tended to decrease by time, up to 2 wk after the initial culture. This trend was, however, reversed in the treated cells as indicated by increasing the *OCT4* mRNA expression level. As shown in Fig. 6, 1 and 2 wk after the treatment with growth factors, the rate of *OCT4* expression experienced 2.2- and 21.95-fold increases, respectively, as compared to their untreated counterparts at the same time points.

Discussion

Finding optimized conditions for stable in vitro chicken SSC culture is regarded as a promising prerequisite to study the events of spermatogenesis in vitro and exploit the potentials of these cells for generation of transgenic chickens to enjoy their great industrial significance. Despite reports on successful derivation of SSCs from different mammals including human

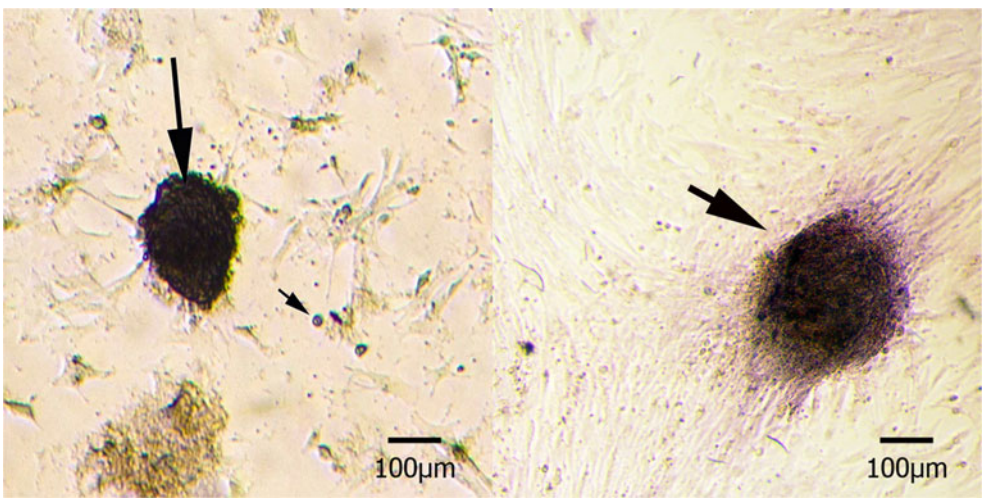

Figure 3. Characterization of cellular colonies, derived from 1-d-old chicken testicular tissues by alkaline phosphatase activity assay. Two distinguished colonies and some individual round-shaped cells, indicated by arrows, are shown to be AP^{+}, while the surrounding cells do not seem to show the activity.

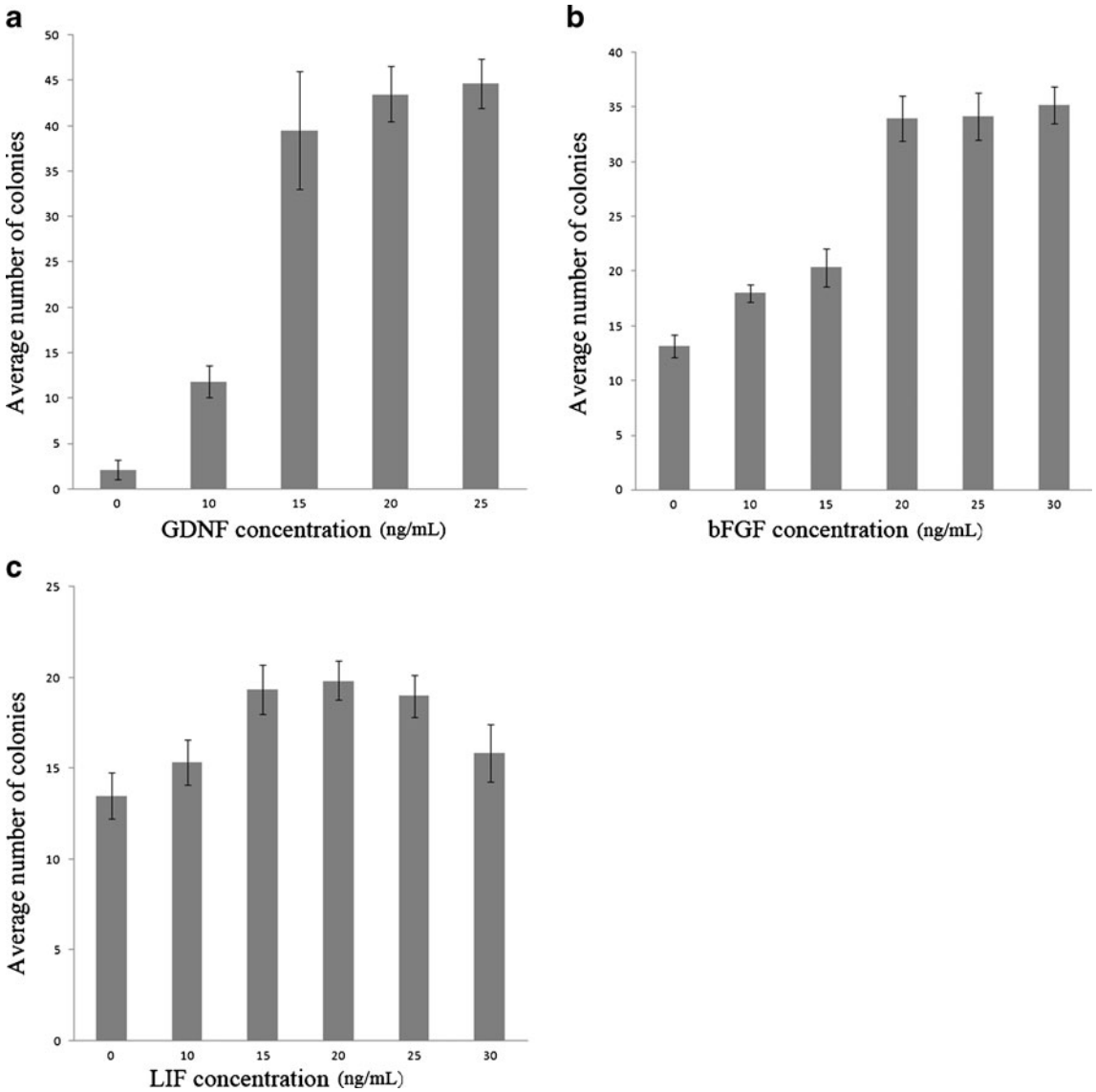

Figure 4. Growth rate and maintenance of the chicken spermatogonial stem cells are affected positively by application of different growth factors of GDNF (*a*), bFGF (*b*), and LIF (*c*) in primary cultures of 1-d-old chicken testicular tissues. The effect of each growth factor was assayed by comparison of the colony forming efficiency in different concentrations. Results are represented as means ± SD of six repeats.

(Liu et al. 2011; He et al. 2012) and some laboratory animals like mouse and rat and a number of farm animals (Guan et al. 2009; Honaramooz and Yang 2010; Oatley 2010; Liu et al. 2011), only a few studies have been dedicated to establish SSC cultures from poultries (Li et al. 2010; Liu et al. 2010; Yu et al. 2010). These reports have applied the same culture conditions as mammals to grow SSCs from chickens. Therefore, our study questioned this principle and tried to seek optimum conditions applicable to chicken SSC derivation and culture. We tried not to use enzymatic detachment of the testis tissues with anticipation of potential damage to the cells by application of the enzymes. Instead, we smashed the tissues mechanically and passed them through a 70-μm mesh. The procedure used in this study was rapid compared to the previously reported methods, which apply a two-step enzymatic digestion by type I collagenase and trypsin (Liu et al.

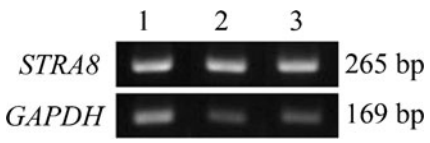

Figure 5. Expression analysis of *STRA8* at mRNA level by conventional RT-PCR in testes of 1-d-old chickens and primary cultured cells. *1*: One week untreated cell cultures; *2*: 1-wk cultures treated with GDNF, bFGF, and LIF; and *3*: testicular tissues from 1-d-old chickens. The *lower panel* represents mRNA expression in the same tissues to serve as the internal control for equal loading of the cDNAs in PCR experiment.

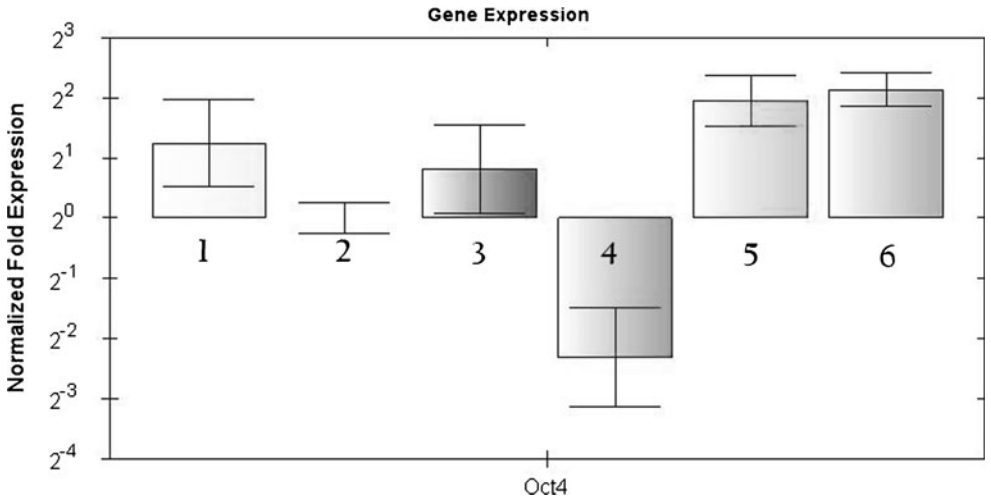

Figure 6. *OCT4* expression analysis of the cultured cells from testicular tissues of 1-d-old chickens by real-time RT-PCR before and after treatment with three growth factors of GDNF, bFGF, and LIF. *Column 1* represents the mRNA expression level from 1-d-old testes as positive control and *columns 2–6* represent the primary cultured cells: without growth factors 2 d after culture; without growth factors 1 wk after culture; without growth factors 2 wk after culture; cells treated with GDNF, bFGF, and LIF 1 wk after culture including GDNF, bFGF, and LIF 2 wk after culture respectively.

2011; Panda et al. 2011). Moreover, the enzymatic treatments often have negative effects on cell viability because they destroy the extracellular matrix and cellular damage could be induced by such enzymes (Yoon et al. 2013). Omitting this step in our study resulted in increased viability and higher yield of SSCs. This protocol is also reproducible and could be practiced without using extra enzymatic digestion steps which are usually tedious and expensive.

We chose to test the impact of GDNF, bFGF, and LIF growth factors on the efficiency of the colony generation from the cultured cells because these growth factors have been shown to be required at various concentrations throughout development of different species (Nagano et al. 2003; Kubota et al. 2004). Therefore, a range of different concentrations of the growth factors was tried in the culture to find the optimal conditions for proliferation and maintenance of the SSCs. This notion was assessed by colony-forming assay and also analyzing the mRNA expression level of *OCT4* as a very well-known marker of self-renewing stem cells. Our data showed that the cultured cells of the chicken testicular tissues in the presence of the three growth factors expressed *OCT4* mRNA at a higher level when compared to untreated cells showing the presence of more SSCs in these cultures.

The quantitative gene expression analysis demonstrated that without the aid of the three growth factors, the mRNA level of *OCT4* was gradually decreased throughout the first and second weeks of culture. On the contrary, in the treated cell cultures, the *OCT4* expression experienced an opposite pattern by increasing its level by time, up to 2 wk of examination. This was in line with the results obtained from colony-forming assay experiments, where it was shown that the number of colonies, originated from SSCs, increased significantly after treatment of the cells by these growth factors (Fig. 4).

Conclusion

To reach an optimal condition for successful SSC derivation, culture, and maintenance, the primary cell cultures from chickens were exposed to different concentrations of three growth factors of GDNF, bFGF, and LIF. The rate of success for the SSC derivation and their maintenance in the presence of these factors was improved greatly as examined by colony-forming assay followed by molecular characterization for the stemness markers. The experimental conditions proposed here could be utilized in future experiments aiming to provide in vitro sources of SSCs for use in developmental biology studies or producing transgenic birds.

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