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Identification of Niche Conditions Supporting Short-term Culture of Spermatogonial Stem Cells Derived from Porcine Neonatal Testis

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ABSTRACTS

Despite that porcine spermatogonial stem cells (pSSCs) have been regarded as a practical tool for preserving eternally genetic backgrounds derived from pigs with high performance in the economic traits or phenotypes of specific human diseases, there were no reports about precise definition of niche conditions promoting proliferation and maintenance of pSSCs. Accordingly, we tried to determine niche conditions supporting proliferation and maintenance of undifferentiated pSSCs for short-term. For these, undifferentiated pSSCs were progressively cultured in different composition of culture medium, seeding density of pSSCs, type of feeder cells and concentration of growth factors, and then total number of and alkaline phosphatase (AP) activity of pSSCs were investigated at post-6 day culture. As the results, the culture of 4×10^5 pSSCs on mitotically in activated 2×10^5 STO cells in the mouse embryonic stem cell culture medium (mESCCM) supplemented with 30 ng/ml glial cell line-derived neurotrophic factor (GDNF) was identified as the best niche condition supporting effectively the short-term maintenance of undifferentiated pSSCs. Moreover, the optimized short-term culture system will be a basis for developing long-term culture system of pSSCs in the following researches.

(Key words : pig, niche, short-term culture, spermatogonial stem cells)

INTRODUCTION

To date, pigs showing high performance in the economic traits have played an important role in developing pig-related livestock industry. Simultaneously, due to the outstanding physiological and genomic similarities between pigs and humans (Aigner *et al.*, 2010; Meurens *et al.*, 2012; Fan and Lai, 2013), pigs have also provided a uniquely relevant animal model for human diseases related with heart (Hasenfuss, 1998), skin (Swindle *et al.*, 2012), brain (Deacon *et al.*, 1998), respiratory (Elahi, 2005; Khatri, 2010) and reproductive organs (Prieto and Castro, 2005). Accordingly, the continuous supply of valuable pigs possessing high performance in the economic traits and phenotypes of specific human diseases has been of great interest and porcine spermatogonial stem cells (pSSCs), which can self-renewal and differentiate in functional spermatozoa via spermatogenesis during lifetime (Aponte *et al.*, 2005; Oatley and Brinster, 2008), has been regarded as a practical tool making it possible to preserve valuable pigs by transferring eternally precious genetic information into the next generation, regardless of the death of useful pigs.

Recently, many trials to isolate, culture and maintain SSCs *in-vitro* have been conducted in all model animals (Izadyar *et al.*, 2002; Guan *et al.*, 2006; Goel *et al.*, 2007; Kossack *et al.*, 2009; Heidari *et al.*, 2012). In case of pigs, post-the first isolation of pSSCs from minipig testis by Cheng and Feng (Cheng and Feng, 2006), conditions of niche promoting proliferation and maintenance of pSSCs has also not been defined precisely (Goel *et al.*, 2007; Han *et al.*, 2009; Lee *et al.*, 2013). Accordingly, before establishing long-term culture system of pSSCs, we tried to identify systematically niche conditions supporting short-term maintenance of pSSC characteristics and investigated alteration of pSSC self-renewal by manipulating composition of culture medium, seeding density of pSSCs, type of feeder cells

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and concentration of growth factors.

MATERIALS AND METHODS

1. Animal

From 1- to 4-day-old crossbred (Landrace × Yorkshire) or purebred (Yorkshire × Yorkshire) male piglets were kindly provided from Gumbo Inc. (Wonju, Korea) and collection of testes from them were conducted through routine castration surgery. Moreover, pregnant female ICR mouse mated with male ICR mouse and C57BL/6 mouse mated with male DBA/2 mouse were purchase from DBL (Eumseong, Korea). The Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-131106-1) approved all animal experimental procedures, which were conducted according to the Animal Care and Use Guideline of Kangwon National University.

2. Harvest of Spermatogonial Stem Cells from Porcine Testes

Testes were transferred from a local farm (Gumbo Inc.) to our laboratory in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) including 1% (v/v) antibiotic-antimycotic solution (Welgene) within 1 hour. Firstly, in order to isolate testicular cells from testes, removal of the tunica albuginea and epididymis from testis were conducted and digestion of the seminiferous tubules utilizing 0.1% (w/v) type IV collagenase (Worthington Biochemical, Lakewood, CA) in high glucose Dulbecco's modified Eagle's medium (DMEM; Welgene) was performed at 37℃ for 15 minutes. Subsequently, fragments of seminiferous tubules were dissociated singly by incubation at 37℃ for 10 minutes in a mixture of high glucose DMEM supplemented with 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and 0.25% trypsin-EDTA (Welgene), respectively. The dissociated cells were filtered using a 70-μm nylon mesh (SPL, Pocheon, Korea) in order to eliminate myofibroblasts and Sertoli cells, and removal of erythrocytes were conducted by incubating the dissociated and filtered cells in red blood cell lysis buffer (Sigma-Aldrich) for 15 minutes at room temperature. Then, in order to harvest spermatogonial stem cells from testicular cell populations, 5×10^6 of isolated testi culcells were plated on 0.1% (w/v) gelatin (Sigma-Aldrich) -coated 100-mm Petri dishes (SPL) and incubated in high glucose DMEM supplemented with 10% (v/v) heat-in activated fetal bovine serum (FBS; Welgene) and 1% (v/v) antibiotic-antimycotic solution at 37℃. After 16 hours, the suspended spermatogonial stem cells were collected and counted using a hemocytometer.

3. Preparation of Feeder Cell-based Cellular Niches

For obtaining mouse embryonic fibroblasts (MEFs), head, legs, tail and embryonic internal organs were removed from fetuses retrieved from uteri of 13.5-day pregnant female mouse. Then, the remainders of the fetuses were minced by razor blade, and the minced tissues were dissociated singly with 0.25 % trypsin-EDTA and filtered using a 70-μm nylon mesh for eliminating non-digested tissue pieces. Culture of the dissociated cells were conducted in high glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic solution at 37℃ under 5% CO_2 in a humidified air atmosphere. For collecting testicular stromal cells (TSCs) from porcine testes, testicular cells retrieved enzymatically from testes were seeded on 0.1% (w/v) gelatin-coated 100-mm Petri dishes and incubated for 16 hours in high glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic solution at 37℃. Subsequently, the floating cells were eliminated and the attached TSCs to the bottom of the culture dishes kept on being cultured in high glucose DMEM supplemented with 20% (v/v) heat-inactivated FBS, 1% (v/v) non-essential amino acids (NEAA; Gibco Invitrogen, Grand Island, NY, USA), 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Inc., Rocky Hill, NJ, USA) and 1% (v/v) antibiotic-antimycotic solution at 37℃ under 5% CO_2 in a humidified air atmosphere. STO cells purchased from ATCC (Manassas, VA, USA) were maintained in high glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic solution at 37℃ under 5% CO_2 in a humidified air atmosphere. Subsequently, confluent MEFs, TSCs and STO cells were inactivated mitotically by the treatment of 10 μg/ml mitomycin C (Sigma-Aldrich) for 3 hours at 37℃ and cellular niches based on inactivated 2×10^5 MEFs, TSCs and STO cells was prepared on 0.1% (w/v) gelatin-coated 4-well culture dish (SPL), respectively.

4. Preparation of Pocine Spermatogonial Stem Cell Culture Media

According to experimental design, the sorted SSCs were cultured in the following medium: GDNF-supplemented mouse embryonic stem cell culture medium (mESCCM) (GDNF-

mESCCM), porcine embryonic stem cell culture medium based on FBS (pESCCM-FBS) (GDNF-pESCCM-FBS) and porcine embryonic stem cell culture medium based on knock-out serum replacement (pESCCM-KSR) (GDNF-pESCCM-KSR), and porcine spermatogonial stem cell culture medium (pSSCCM), respectively. GDNF-mESCCM consisted of high glucose DMEM supplemented with 15% (v/v) heat-inactivated FBS, 0.1 mM β-mercaptoethanol, 1% (v/v) NEAA, 2 mM L-glutamine (Gibco Invitrogen), 1% (v/v) antibiotic-antimycotic solution, 1,000 units/ml mouse leukemia inhibitory factor (mLIF; Chemicon International, Inc., Temecula, CA, USA), and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; R&D Systems, Inc., Minneapolis, MN, USA). GDNF-pESCCM-FBS was composed of 1:1 mixture of low glucose DMEM (Welgene) and Ham's F-10 (Gibco Invitrogen) medium supplemented with 0.2 mM β-mercaptoethanol, 1% (v/v) NEAA and 1% (v/v) antibiotic-antimycotic solution and 20 ng/ml bFGF and 10 ng/ml GDNF. GDNF-pESCCM-KSR consisted of Minimum essential medium (MEM) alpha medium (Gibco Invitrogen) supplemented with 10% (v/v) KSR (Gibco Invitrogen), insulin-transferrin-selenium (ITS; Gibco Invitrogen), 10 ng/ml bFGF, 20 ng/ml epidermal growth factor (EGF; PeproTech), 1,000 units/ml mLIF and 10 ng/ml GDNF. MEM alpha medium supplemented with 1% (v/v) antibiotic-antimycotic solution, 1% (v/v) NEAA, 0.1 mM β-mercaptoethanol, N2-1 supplement (Merck Millipore; Darmstadt, Germany), DL-lactic acid (Sigma-Aldrich), 1% (v/v) MEM vitamin solution (Sigma-Aldrich), 30 ng/ml β-estradiol (Sigma-Aldrich), 1% (v/v) heat-inactivated FBS, 10 ng/ml bFGF, 20 ng/ml EGF, 1,000 units/ml mLIF and 10 ng/ml GDNF was used as pSSCCM.

5. Experimental Design

In order to establish culture system maintaining self-renewal of porcine spermatogonial stem cells (pSSCs) for short-term, culture of pSSCs for 6 days at 37℃ under 5% CO_2 in a humidified air atmosphere were conducted under different niche conditions organized by a variety of culture media, cell seeding density, feeder cells and growth factors and each niche component was optimized by measuring number and alkaline phosphatase (AP) activity of pSSC-derived colonies in each experiment. Firstly, optimization of culture medium was conducted by culturing 2×10^5 SSCs on STO cell feeder layer in GDNF-mESCCM, -pESCCM-FBS and -pESCCM-KSR and pSSCCM, and seeding density of pSSCs was determined by culturing 2, 4, 6 and 8×10^5 pSSCs on STO cell feeder layer in the optimized culture medium. Moreover, type of feeder cells was optimized by culturing the optimized number of pSSCs on STO cell, F1 MEF, ICR MEF and porcine TSC (pTSC) feeder layer in the optimized culture medium, and optimization of each growth factor concentration was performed by culturing the optimized number of pSSCs on the optimized feeder layer in the optimized culture medium containing different concentration of GDNF, EGF and bFGF.

6. Alkaline Phosphatase Staining

Cells fixed with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan) were washed twice with DPBS and they were incubated in solution consisting of 0.1 M Tris buffer (pH 8.2) supplemented with 0.2 mg/ml naphthol AS-MX phosphate (Sigma-Aldrich), 2% (v/v) dimethyl formamide (Sigma-Aldrich), and 1 mg/ml Fast Red TR salt (Sigma-Aldrich). After 90 minutes, washing of stained cells with DPBS were conducted twice and the yield of AP-positive cells was calculated using a hemocytometer.

7. Statistical Analysis

All the numerical data shown in each experiment were analyzed statistically using the Statistical Analysis System (SAS) program. When a significance of the main effects through variance (ANOVA) analysis in the SAS package were detected, the least-square or DUNCAN method were used for comparing among each treatment, and p values less than 0.05 were regarded as indicative of significant differences.

RESULTS

1. Identification of Culture Medium Stimulating Self-renewal of pSSCs

Regardless of culture medium type, pSSCs isolated from neonatal testes formed colonies with grape-like morphology at 6 day of culture (Fig. 1C) and maintenance of AP activity in these colonies could be identified by observing pSSCs stained positively by AP staining (Fig. 1D). However, number of colonies derived from pSSCs was significantly different according to different type of culture medium and significantly the highest number of colonies was detected in the culture of pSSCs in GDNF-mESCCM (Fig. 1A). Whereas, no significant increase or decrease in the yield of pSSCs maintaining AP activity were detected among culture medium composed differently (Fig.

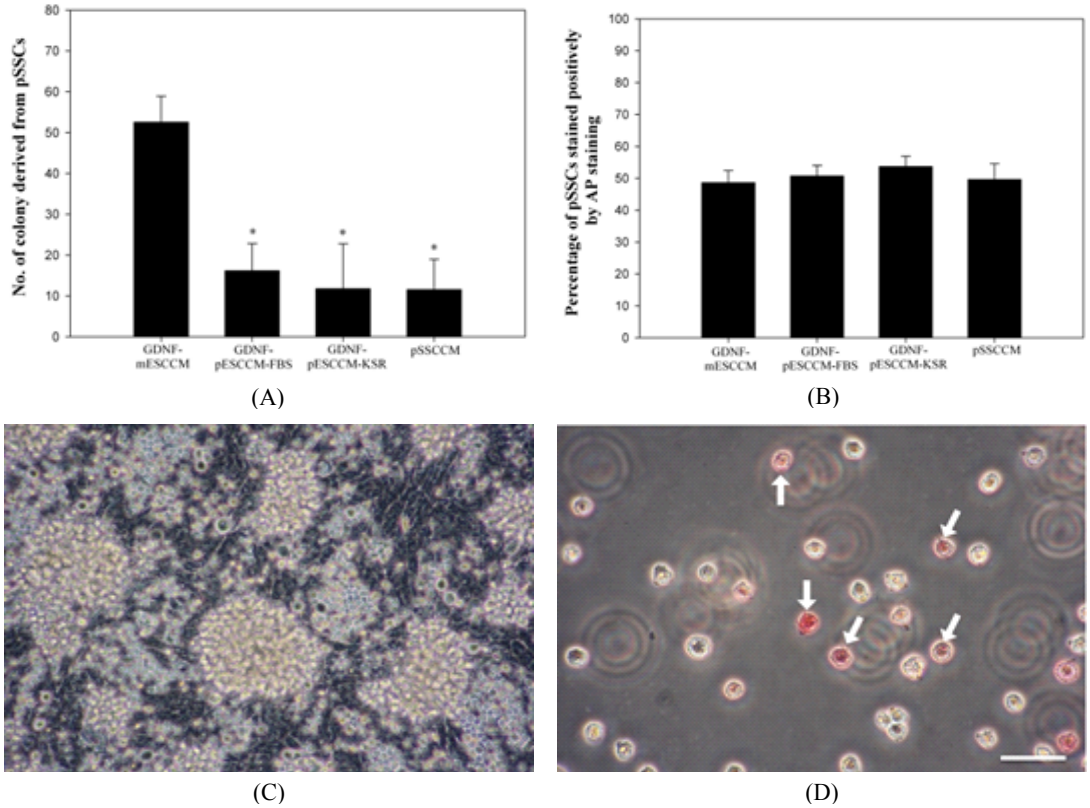


Fig. 1. Determination of culture medium stimulating formation of colonies and maintenance of alkaline phosphatase (AP) activity in porcine spermatogonial stem cells (pSSCs). Two times 10^5 pSSCs retrieved from testis were plated onto mitotically in activated STO cells and cultured for 6 days in GDNF-supplemented mESCCM (GDNF-mESCCM), pESCCM-FBS (GDNF-pESCCM-FBS) and pESCCM-KSR (GDNF-pESCCM-KSR), and pSSCCM, respectively. Subsequently, the number of pSSC-derived colonies (C) was enumerated under inverted microscopeand the yield of pSSCs with AP activity (D, arrow) was calculated by dividing the number of pSSCs stained positively by AP staining by the number of total pSSCs. The culture in GDNF-mESCCM showed significantly the highest number of colonies derived from pSSCs (A). However, no significant difference in the proportion of pSSCs stained positively by AP staining was observed among culture medium (B). Error bars represent S.D. n=3. * $p<0.05$. Scale bars represent 50 μm.

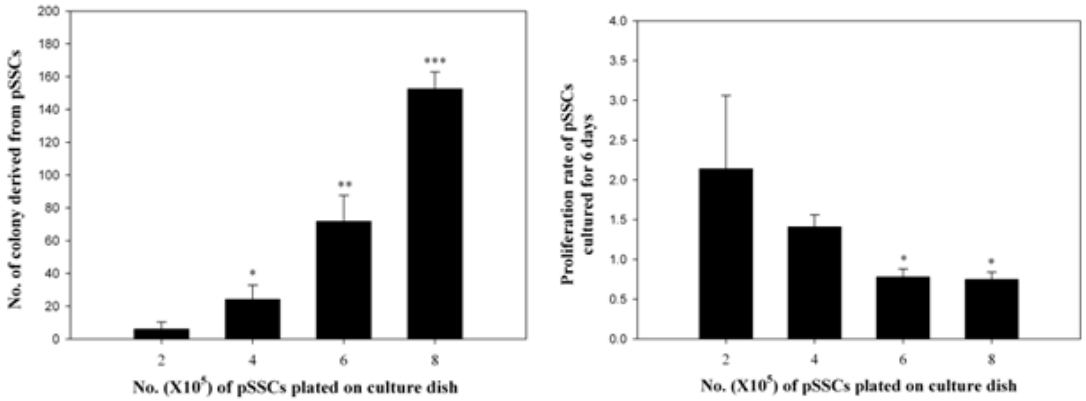
1B). These results demonstrated that GDNF-mESCCM can be used as a basic medium supporting *in-vitro* maintenance of pSSCs characteristics for short-term.

2. Determination of Seeding Density Supporting Proliferation of pSSCs

Subsequently, under GDNF-mESCCM, the optimal seeding density simulating colony formation and proliferation rate of pSSCs was determined according to experimental design. As the results, the number of colonies formed was significantly increased according to increase of pSSC seeding density (Fig. 2A). However, as shown in Fig. 2B, high seeding density groups such as 6 and 8 × 10^5 pSSCs showed significantly lower proliferation rate than low seedling density groups such as 2 and 4× 10^5 pSSCs. Moreover, seeding densities of 6 and 8 × 10^5 pSSCs inhibited proliferation of pSSCs and stimulation of pSSC proliferation was observed in 2 and 4 × 10^5 pSSCs seeding density. From these results, we could identify that the application of 4 × 10^5 seeding density to the *in-vitro* culture of pSSCs was effective in proliferating pSSCs in the GDNF-mESCCM.

3. Identification of Feeder Cells Supporting Maintenance of Undifferentiated pSSCs

Next, by culturing 4 × 10^5 pSSCs for 6 days on avariety of feeder cells in GDNF-mESCCM and measuring AP activity, cellular niche inhibiting differentiation of pSSCs into spermatozoa were determined. As the results, STO cell-derived cellular niche showed significantly higher percentage of AP-positive



(A) (B)

Fig. 2. Optimization of seeding density stimulating colony formation and proliferation rate of porcine spermatogonial stem cells (pSSCs). Two, 4, 6 and 8 × 10^5 pSSCs seeded onto mitotically in activated STO cells were cultured for 6 days in GDNF-supplemented mESCCM. Inverted microscope was used for counting the number of pSSC-derived colonies and proliferation rate of pSSCs was calculated by dividing the number of pSSCs cultured for 6 days by the number of pSSCs initially plated. According to increase of seeding density, significant increase in the number of colonies derived from pSSCs was detected (A), where as proliferation rate of pSSCs was significantly decreased and significantly increased in high (6 and 8×10^5) and low (2 and 4×10^5) seeding density groups, respectively (B). Error bars represent S.D. n=3. $^{*\sim***}$ p<0.05.

pSSCs than F1 and ICRMEF and pTSC-derived cellular niche and no significant difference in the yield of PSSCs stained positively against AP activity was detected among cellular niches derived from F1 and ICRMEFs and pTSCs (Fig. 3). In addition, the formation of pSSC colonies with grape-like morphology was justly detected in the culture on STO cells and no pSSC colonies were formed on the other feeder cells (not shown data). Accordingly, these results demonstrated that cellular niche derived from STO cells was appropriate to maintain effectively undifferentiation of pSSCs.

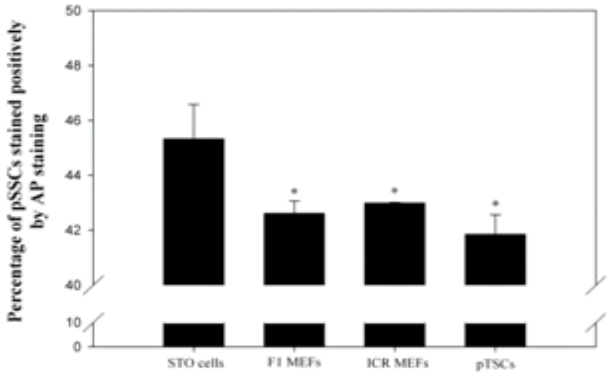


Fig. 3. Effects of different feeder cells on maintenance of alkaline phosphatase (AP) activity in porcine spermatogonial stem cells (pSSCs). Four times 10^5 pSSCs retrieved from testis were respectively seeded onto mitotically inactivated STO cells, MEFs derived from B6D2F1 strain mouse (F1MEFs) and ICR strain mouse (ICRMEFs), and porcine testicular stromal cells (pTSCs) and then cultured in GDNF-supplemented mESCCM. After 6 days culture, AP activity was identified by AP staining and the percentage of AP acitivity-possessing pSSCs was represented as dividing the number of pSSCs stained positively by AP staining by the number of total pSSCs. The pSSCs cultured on STO cells showed significantly the best yield of AP-positive pSSCs, compared to the other groups. Error bars represent S.D. n=3. * p<0.05.

4. Optimization of Growth Factor Concentration Supporting Maintenance of Undifferentiated pSSCs

Finally, the optimization of concentration of growth factors, known to support self-renewal of SSCs, was conducted by culturing 4 × 10^5 pSSCs on STO feeder cells in the mESCCM containing different concentration of GDNF and in the optimized GDNF-mESCCM supplemented with different concentration of EGF or bFGF, and analyzing AP activity after 6 days of culture. As the results, significantly the highest percentage of pSSCs stained positively against AP activity was observed in mESCCM containing 30 ng/ml GDNF (Fig. 4). However, supplementation of EGF or bFGF into the 30 ng/ml GDNF-containing mESCCM didn't induceany significant alteration in the yield of pSSCs stained positively by AP staining (Fig. 5). Accordingly, we could identify that maintenance of undifferentiated pSSCs was supported strongly by 30 ng/ml GDNF and both EGF and bFGF didn't be requested necessarily for

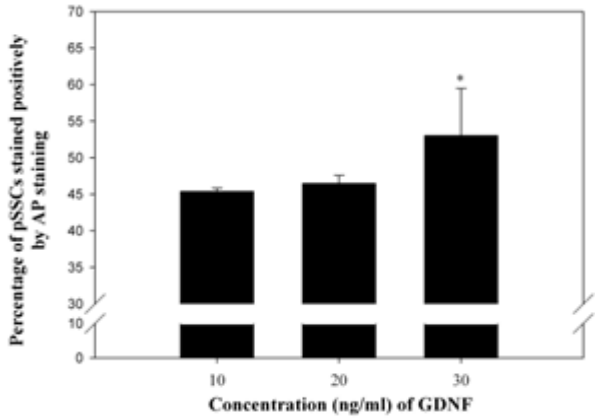


Fig. 4. Determination of GDNF concentration supporting maintenance of alkaline phosphatase (AP) activity in porcine spermatogonial stem cells (pSSCs). Four times 10^5 pSSCs plated on mitotically in activated STO cells were cultured for 6 days in 10, 20 and 30 ng/ml GDNF-containing mESCCM, respectively. Then, identification of AP activity was conducted by AP staining and the percentage of AP-positive pSSCs was represented as dividing the number of pSSCs stained positively by AP staining by the number of total pSSCs. As the results, 30 ng/ml GDNF-containing mESCCM showed significantly higher percentage of AP-positive pSSCs than 10 and 20 ng/ml GDNF-containing mESCCM. Error bars represent S.D. n=3. * $p<0.05$.

maintaining undifferentiation of pSSCs. Simultaneously, we could optimize culture system supporting short-termmaintenance of undifferentiated pSSCs by culturing 4×10^5 pSSCs on mitotically in activated 2×10^5 STO cells in the mESCCM supplemented with 30 ng/ml GDNF.

DISCUSSION

In this study, we tried to determine progressively type of basic culture medium and feeder cells, seeding density of pSSCs and feeder cells, and concentration of growth factors for the purpose of establishing a niche maintaining effectively self-renewal of undifferentiated pSSCs. Therefore, effect of each niche component on proliferation or AP activity of pSSCs was evaluated and specific niche conditions stimulating proliferation and AP activity of pSSCs could be decided. As the results, we could identify that pSSC self-renewal was maintained for a short term by culturing 4×10^5 pSSCs on mitotically in activated 2×10^5 STO cells in the mESCCM supplemented with 30 ng/ml GDNF.

Unfortunately, long-term culture of pSSCs didn't be successfully conducted in the optimized short-term pSSC culture system. Decrease of proliferation rate and AP activity were observed in pSSCs cultured for 12 days in the established niche (not shown data), suggesting that the optimized pSSC culture system is inappropriate to maintain pSSC self-renewal for a long time. Accordingly, studies on developing a novel niche showing improved maintenance of pSSCs self-renewal should

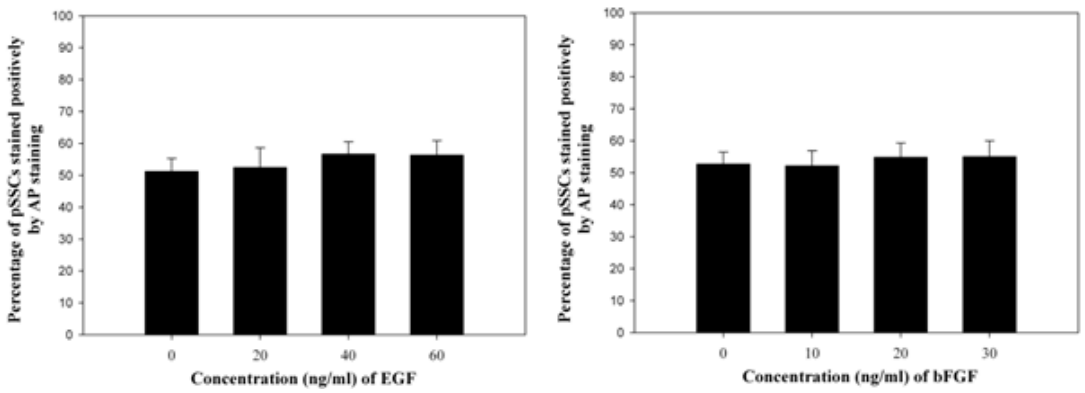


Fig. 5. Effects of EGF or bFGF supplementation to 30 ng/ml GDNF-containing mESCCM on maintenance of alkaline phosphatase (AP) activity in porcine spermatogonial stem cells (pSSCs). The culture of 4×10^5 pSSCs plated on mitotically in activated STO cells were conducted in 30 ng/ml GDNF-containing mESCCM. Subsequently, at 6 days of culture, AP staining was performed and the calculation of the yield of AP-positive pSSCs was conducted by dividing the number of pSSCs stained positively by AP staining by the number of total pSSCs. No significant differences in the increase or decrease of the percentage of AP-positive pSSCs were induced by EGF or bFGF treatments. Error bars represent S.D. n=3.

be conducted necessarily in the future.

Interestingly, pSSCCM used universally for pSSC culture (Kanatsu-Shinohara *et al.*, 2003; Kubota *et al.*, 2004) didn't show any significant increase of colony number and AP activity in the culture of the retrieved pSSCs on STO cells, which have already been reported as a helper providing niche supporting maintenance of embryonic stem cell and primordial germ cell self-renewal (Shim *et al.*, 1997; Shamblott *et al.*, 1998). Rather, in the STO cell-based niche, mESCCM containing GDNF could stimulate significantly colony formation (Fig. 1A) and inhibit significantly decrease of AP activity (Fig. 3). In other words, a niche combining STO cells with GDNF-mESCCM showed the highest efficacy in the proliferation and AP activity of pSSCs. Moreover, positive effect of EGF or bFGF on pSSC self-renewal (Kuijk *et al.*, 2009) wasn't detected under this niche condition (Fig. 5). These results demonstrate that responsibility of the same cells can be altered according to different niches and difference of one of factors constituting niche can induce alteration of entire niche system. Moreover, these are supported by the fact that composition of stem cell culture medium can be differed according to the presence or absence of feeder cells (Kanatsu-Shinohara *et al.*, 2011).

In conclusion, niche condition supporting maintenance of pSSC self-renewal for a short time was established by culturing 4×10^5 pSSCs on mitotically in activated 2×10^5 STO cells in the mESCCM supplemented with 30 ng/ml GDNF. This niche will be abletogreatly contribute to conducting researches requiring maintenance of pSSCs self-renewal for a short time. Moreover, this will be a basis for developing long-term culture system of pSSCs in the following researches.

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