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Full Length Research Paper

Effects of growth-promoting factors on proliferation of mouse spermatogonial stem cells (SSCs) *in vitro*

Jian-Hong Hu[1]*[#], Wen-Chang Zhang[2#], Peng Wang[1], Zhi-Guo Fan[1], Zhong-Liang Jiang[1], Qing-Wang Li[1], Huai-Ping Shi[1], Yu Liu[1], Jin-Xin Mo[1] and Zhe Qiang[1]

[1]College of Animal Science and Technology, Northwest A and F University, Yangling, Shaanxi 712100, P. R. China.
[2]College of Animal Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, P. R. China.

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The proliferation, cultivation and identification of spermatogonial stem cells (SSCs) *in vitro* are critical to our understanding of male infertility, genetic resources and endangered species conservation. To investigate the effects of growth-promoting factors, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) on the proliferation of mouse (SSCs) *in vitro*, three culture schemes (single factor, double factor combinations and triple factor combinations) were designed to culture mouse SSCs *in vitro*. The optimal proliferation rate of SSCs cultivation *in vitro* among single factor groups, double factor combinations and triple factor combinations was 41.60, 61.00 and 56.60%, respectively. The results of the proliferation rates of SSCs cultivation *in vitro* indicated that 10 ng/ml EGF based on pre-treatment with 30 ng/ml 10% follicle stimulating hormone (FSH) was able to greatly enhance the proliferation of mouse SSCs *in vitro*. The 20 ng/ml EGF and 20 ng/ml IGF-1 combination based on post-treatment with 30 ng/ml 10% FSH, and the 20 ng/ml EGF, 20 ng/ml IGF-1 and 10 ng/ml bFGF combination based on post-treatment with 30 ng/ml 10% FSH were a preferable combination to the proliferation of SSCs cultivation, respectively. In conclusion, it was apparent that 20 ng/ml EGF and 20 ng/ml IGF-1 was the optimal combination which could stimulate the proliferation of mouse SSCs *in vitro*.

Key words: Growth-promoting factors, mouse spermatogonial stem cells (SSCs), proliferation.

INTRODUCTION

Due to the increasing incidence of male infertility, researches on spermatogonial stem cells (SSCs) proliferation and culture *in vitro* have become one of the important issues in reproductive medicine. The self-renewal and differentiation of the testis stem cells is mediated by growth factors produced by the Sertoli cells (Hofmann et al., 2005). Li et al. (2008) demonstrated that the growth factors can indirectly stimulate spermatogonial proliferation via Sertoli or pericystic cells. The basic fibroblast growth factor (bFGF) can promote endogenous progenitor cell proliferation (Wada et al., 2003), while the insulin-like growth factor-1 (IGF-1) can stimulate mitotic DNA synthesis of male germ cells (Soder et al., 1992; Loir and Le, 1994). The IGF-1 is necessary for the initiation of spermatogenesis, which can induce the proliferation of SSCs (Takeshi and Chiemi, 2001). The IGF-1 also increases NO activity (Michell et al., 1999; Schini-Kerth et al., 1999; Kaplan et al., 2005) which is an endothelial survival factor enhancing endothelial cell proliferation (Cooke and Losordo, 2002). The epidermal growth factor (EGF) is beneficial to the DNA synthesis (Wahab-Wahlgren et al., 2003) and the formation of the cloning clusters of cultivation mouse SSCs *in vitro* (Anjamrooz et al., 2006). It is reported that the stem-cells-like cells of male germ isolated from the testicular tissues of non-obstructive azoospermic patients can be

*Corresponding author. E-mail: hjh19732008@yahoo.com.cn. Tel: +86 29 87092102. Fax: +86 29 87092164.

Abbreviations: SSCs, Spermatogonial stem cells; **EGF,** epidermal growth factor; **IGF-1,** insulin-like growth factor-1; **bFGF,** basic fibroblast growth factor; **FSH,** follicle stimulating hormone.

#These authors contributed equally to this work.

proliferated by EGF *in vitro* (Lee et al., 2006). Wang et al. (2008) reported that EGF could promote the formation of cloning clusters of mouse SSCs *in vitro*. Although, numerous studies reported that single growth factor could increase the proliferation of SSCs and other cells *in vitro*, the combination of different growth factors on the proliferation of SSCs was rarely reported.

The proliferation and cultivation of SSCs *in vitro* are critical to our understanding of the origin of certain testicular neoplasias, and the causes of male infertility. In order to improve the proliferation rate of SSCs *in vitro*, the objective of the present study is to determine the compatibility effect of bFGF, IGF-1 and EGF on it. Therefore, the single factor groups, double factor combinations and triple factor combinations of these growth-promoting factors were involved in the culture medium of SSCs. The culture schemes were designed to obtain comfortable environmental conditions and increase the information obtained on the proliferation of SSCs *in vitro* by the combination of bFGF, IGF-1 and EGF.

MATERIALS AND METHODS

Mice, aged 7 days, were taken from the Experimental Animal Center of Medical College of Xi'an Jiaotong University, and their testes were removed and used in this study. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Northwest Agricultural and Forestry University.

Chemical agents

All chemical agents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of orchilytic cell suspension

Sixteen testes were removed from 7-day-old mice and placed in a culture dish containing phosphate buffer solution (PBS) supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin after wiping out albuginea and visible blood vessels. The testicular tissue was cut into small fragments, and then transferred into a centrifugal tube. As reported before, the two-step enzymatic digestion was utilized to prepare single cell suspensions from testis tissues (Herrid et al., 2007). Finally, the cell was cultured with DMEM containing 10% FBS, and cell suspension was inoculated into a 0.1% (w/v) gelatin-coated 24-well cell culture plate and cultivated with culture medium I in an incubated room at 37°C with 5% CO_2 and 95% humidity.

Isolation of sertoli cells

When the cell suspension was cultured for 10 to 15 h in 24-well cells culture plates with culture medium I in an incubated room at 37°C with 5% CO_2 and 95% humidity, the medium was gently drawn off and the Sertoli cells stuck to the gelatin-coated plates. Then, the cell suspension was sucked out and the Sertoli cells were cultured continually. When the Sertoli cells almost covered the bottom of the culture plates, they were disposed by 10 µg/ml mitomycin C for 3 to 4 h and the DMEM containing 10% FBS was added to terminate the reaction. Then, the Sertoli cells were cultivated unceasingly under similar conditions. The medium was updated after the first 12 h, then changed every 24 h on a regular basis.

Purification of SSCs

When the cell suspension was cultivated for 10 to 15 h in 24-well cells culture plates with culture medium I in an incubated room at 37°C with 5% CO_2 and 95% humidity, the medium I was gently drawn off with Sertoli cells attached and polarized. However, majority of SSCs did not stick to it, as such, they were transferred to new cell culture plates to continue proliferation under same conditions. The procedure was repeated twice to improve the purity of SSCs. The cell suspension was transferred into a centrifugal tube and centrifuged at 201 ×g (1000 rpm) for 5 min at 16°C, and the supernatant was discarded. The remaining cells were cultivated with culture mediums I and II in an incubated room at 37°C with 5% CO_2 and 95% humidity. Mediums I and II were updated after the first 24 h together, then changed every 48 h on a regular basis. It was not until the sixteenth day that the purity of SSCs reached above 75%.

Morphology observation of SSCs

Numerous researchers had demonstrated that the 8 cell-chains and 16 cell-chains were often considered as the typical trait of SSCs (Huckins, 1971; Oakberg, 1971; Dym and Fawcett, 1971; De Rooij and Russell, 2000; Yoshida et al., 2004). Thus, the morphology, number, formation and maintenance of cell colonies of SSCs were observed daily by an inverted phase contrast microscope (Nikon Imaging Sales Co Ltd., Tokyo, Japan).

Alkaline phosphatase (AP) staining of SSCs

The SSCs were fixed on the slide for 20 min with 4% paraformaldehyde. Then, 500 µl Tris-HCl solution, 5 µl NBT (P-Nitro-Blue tetrazolium chloride) and 5 µl BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) were appended on the slide to treat SSCs for 30 min at 20 to 25°C (without light). The morphology was inspected by an inverted phase contrast microscope (Nikon Imaging Sales Co Ltd., Tokyo, Japan).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA of SSCs and Sertoli cells was extracted by Trizol reagent (Amercian Invitrogen Life Technologies Ltd., Camarillo, America) according to the manufacturer's instructions (Amercian Invitrogen Life Technologies Ltd., Camarillo, America) and was transcribed using an oligo dT (20) Primer by M-MLV Reverse Transcriptase (Promega, Madison, WI) following the manufacturer's instructions (Amercian Invitrogen Life Technologies Ltd., Camarillo, America). PCR was subsequently performed to identify the presence of transcripts Beta-actin and Ngn3. The primers were as follows: Beta-actin (GenBank No.NM_007393.3) UpperPrimer: 5'-GTTA CCAACTGGGACGACA-3'; LowerPrimer: 5'-CATGGATGCCACA-GGATT-3', 601bp. Ngn3 (GenBank No. NM_009719.6) UpperPrimer: 5'-GCAAACAGCGAAGAAGCC-3'; LowerPrimer: 5'-ACTACCTCCCACTCCAGACG-3', 489bp. PCR amplification was performed using the following programs: 94°C for 6 min, 30 to 38 cycles at 95°C for 35 s; 56°C for 35 s; 94°C for 35 s; 55°C for the addition of poly-A overcharges and cooled to 4°C. PCR products

were then separated on a 1.2% agarose gel and visualized with ethidium bromide using a Fluor-S Multi-Imager densitometer (ET9970616AA, Bio-Rad laboratories, New York, America).

Treatment of the primary SSCs

The SSCs was pre-treated using two kinds of methods in our study. Firstly, the SSCs were incubated into a medium with 30 ng/ml 10% FSH for 15 min before being inoculated on the feeder layer cells (Sertoli cells), which was called pre-treatment. Secondly, when SSCs were cultured for 20 h on the feeder layer cells, one third of the solution was sucked out. Afterwards, culture medium I supplemented with 30 ng/ml 10% FSH was utilized to incubate SSCs for 30 min, and then SSCs were cultured with culture medium I without FSH, which was called post-treatment.

Culture medium I and II of SSCs

The primary component of culture medium I is DMEM, supplemented with 10% FBS, 4 mM L-Glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 µM vitamin E, and 0.1 mM non-essential amino acids (NEAA). The culture medium II involves growth-promoting factors, including single factor groups(10ng/ml of EGF, IGF-1, bFGF, respectively), three double factors combinations groups (20 ng/ml of EGF and IGF-1, IGF-1 and bFGF, EGF and bFGF, respectively) and triple factors combinations group(20 ng/ml of EGF, IGF-1 and bFGF plus 10 ng/ml bFGF), respectively.

Culture methods of SSCs

Firstly, the cell suspension was cultured for 10 to 15 h in 24-well cell culture plates with culture medium I in an incubated room at 37°C with 5% CO_2 and 95% humidity. The SSCs and Sertoli cells could be obtained through the repeated isolation and purification described in the section of isolation of Sertoli cells and purification of SSC, respectively. While the quantity of primary SSCs, including single cell, 4 cell-chains, 8 cell-chains and 16 cell-chains cloning clusters surpassed 45% in possessory cells, the pre-treatment method was carried out and then culture mediums I and II were jointly used to culture SSCs unceasingly. In treatment groups, medium I and II were updated after the first 24 h together, then changed every 48 h on a regular basis. However, the control groups were only cultured with medium I. While the first passage of SSCs was cultured for 30 min in culture medium I, the post-treatment method was performed and then culture mediums I and II were jointly used to culture SSCs unceasingly. Mediums I and II were updated after the first 24 h together, then changed every 48 h on a regular basis. Cells were normally grown for 16 days in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Culture medium of the Sertoli cells

The culture medium of the Sertoli cells is DMEM, supplemented with 10% FBS, 5 µM vitamin E, 100 IU/ml penicillin and 100 mg/ml streptomycin.

Data collection and statistical analysis

All samples were replicated 3 times for the control and treatment groups and their results were expressed as mean values ± SEM. The mean values of the percentages of the proliferation rates of SSCs were compared using Duncan's multiple range tests by ANOVA procedure, when the F-value was significant (P<0.05). All statistical analyses were performed using Statistical Product and Service Solutions (SPSS 11.5 for Windows; SPSS, Chicago, IL, U.S.A).

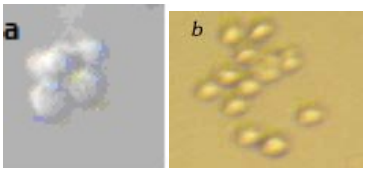

Figure 1. The typical 4-cell chains and 16-cell chains (200× magnification).

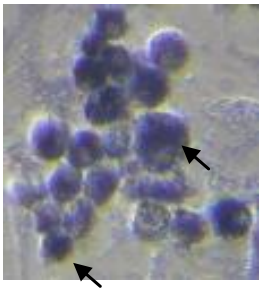

Figure 2. The identification of SSCs with AP staining (400× magnifications).

RESULTS

Culture of the feeder layer cells (Sertoli cells)

During the process of cultivation, the Sertoli cells began to stick to the layer after 5 to 7 h and were completely stuck to it within 48 h. The favorable Sertoli cells mainly stuck to the layer and became a monolayer; it showed that the first, second and third passages of Sertoli cells were suitable in this study. The monolayer of Sertoli cells was maintained for 2 to 5 days and the cells began to detach from the layer.

Morphology observation of SSCs

About 90% SSCs attached to the feeder layer 24 h after inoculation turned into cloning clusters, which were regularly globular and could be observed clearly through a microscope. A few double-cell cloning clusters emerged and triple-cell appeared 48 h later after the double-cell cloning clusters were outnumbered. After 72 h, some multi-cell cloning clusters that were higher than 8-cell cloning clusters (8 cell-chains) were detected and then they initiated aggregation. After 5 days interval, 16-cell cloning clusters (16 cell-chains) that had a resemblance with grapes were found under high power field (Figure 1).

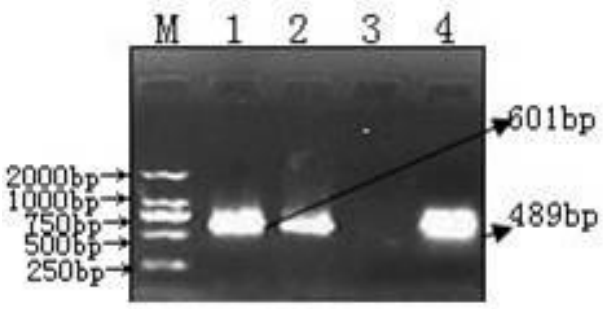

Figure 3. The identification of SSCs with Beta-actin gene and Ngn3 genes. Lane 1 shows the cDNA of Beta-actin gene in Sertoli cells. Lane 2 shows the cDNA of Beta-actin gene in SSCs. Lane 3 shows that the cells were Sertoli cells. Lane 4 shows the cDNA of Ngn3 gene in the SSCs.

Identification of SSCs with alkaline phosphatase (AP) staining

Alkaline phosphatase could be highly expressed in SSCs, so the cells became blue-violet or dark-brown through NBT/BCIP Kit in Figure 2.

Identification of SSCs with Beta-actin gene and Ngn3 gene

To identify SSCs and Sertoli cells, the total RNA expressions were examined by RT-PCR. The SSCs were identified by the Beta-actin and Ngn3 genes in thisstudy. The Beta-actin gene which could be used to identify the normality of culture system is considered the congenerous gene of SSCs and Sertoli cells. The Ngn3 gene is the typical gene of the SSCs and does not exist in Sertoli cells (Yoshida et al., 2004). In Figure 3, Lanes 1 and 2 showed the cDNA of Beta-actin gene in Sertoli cells and SSCs, respectively. Lane 3 showed the cDNA of Ngn3 gene in Sertoli cells. Total RNA was extracted from the Sertoli cells and the primer sequences based on the Ngn3 gene were used for the amplification. The band was not present in Lane 3, attesting that Ngn3 gene was expressed only in SSCs. Lane 4 showed the cDNA of Ngn3 gene in SSCs. Total RNA was extracted from SSCs and the primer sequences based on the Ngn3 gene were used for amplification. The band existed in Lane 4 and the expression of Ngn3 gene cDNA was detected, indicating that it was expressed in SSCs. These results indicated that Beta-actin gene was expressed in SSCs and Sertoli cells, but Ngn3 gene was expressed only in SSCs. Thus, we can identify SSCs and Sertoli cells by estimating the expression of Beta-actin and Ngn3 genes.

Effects of single factor on proliferation of mouse SSCs *in vitro*

The effects of single factor on the proliferation of SSCs are shown in Table 1. The total cloning clusters of mouse SSCs in IGF-1 group based on post-treatment with 10% FSH (30 ng/ml) had a slight difference when compared with the bFGF group without treatment. The cloning clusters in the EGF group based on pre-treatment with 10% FSH (30 ng/ml) were bigger than those of the EGF group based on post-treatment with 10% FSH (30 ng/ml). The results indicated that the EGF group based on pre-treatment with 10% FSH (30 ng/ml) could greatly promote SSCs proliferation (Figure 4b). The effect of EGF (10 ng/ml) based on pre-treatment with 10% FSH (30 ng/ml) was the best (41.60%) in comparison with others, yet the lowest level was only 11.87% ($P<0.05$). It also indicated that EGF (10 ng/ml) based on pre-treatment with 10% FSH (30 ng/ml) could easily stimulate the proliferation of SSCs *in vitro*.

Effects of double factors on proliferation of mouse SSCs *in vitro*

The effects of double factors on the proliferation of SSCs are shown in Table 1. The proliferation of SSCs had no significant difference between EGF and bFGF combination based on pre-treatment with 10% FSH (30 ng/ml) and IGF-1 and bFGF combination based on pre-treatment with 10% FSH (30 ng/ml) ($P>0.05$). The EGF and IGF-1 combination based on post-treatment with 10% FSH (30 ng/ml) was beneficial to forming bigger SSCs cloning clusters (Figure 4c) than that of IGF-1 and bFGF combination. The interaction effects of EGF (20 ng/ml) and IGF-1(20 ng/ml) combination based on post-treatment with 10% FSH (30 ng/ml) resulted in the highest proliferation rate (61.00%) of SSCs than that of other groups, while the lowest proliferative rate was 36.87% ($P<0.05$). It indicated that there was synergetic effect between EGF (20 ng/ml) and IGF-1 (20 ng/ml) based on post-treatment with 10% FSH (30 ng/ml) on the proliferative rate of SSCs *in vitro*.

Effects of triple factors on proliferation of mouse SSCs *in vitro*

The effects of triple factors on the proliferation of SSCs are also shown in Table 1. There are bigger SSCs cloning clusters in the combination of 20 ng/ml EGF, 20 ng/m IGF-1 and 10 ng/ml bFGF based on post-treatment with 10% FSH (30 ng/ml) than that of the control group (Figure 4d) ($P<0.05$). However, there was no significant difference in the amount of cloning clusters between the combination of 20 ng/ml EGF, 20 ng/m IGF-1 and 10 ng/ml bFGF based on pre-treatment with 10% FSH (30 ng/ml) and the control group.

DISCUSSION

In mammals, the blood-testis barrier consists of tight

Table 1. The proliferation-promoting rates (%) of the SSCs cultivation *in vitro*.

Combination	Group	Control group	Pre-treatment group	Post-treatment group
Single factor (no combination)	EGF(10 ng/ml)	11.87±0.57^{b}	41.60±0.81^{a}	40.93±0.74^{a}
	IGF-1(10 ng/ml)	18.23±0.96^{c}	26.67±0.18^{b}	36.67±0.87^{a}
	bFGF (10 ng/ml)	37.20±0.50^{a}	30.67±0.24^{b}	15.29±0.44^{c}
Double factors combination	20 ng/ml EGF and 20 ng/ml IGF-1	36.87±0.24^{c}	53.33±0.55^{b}	61.00±0.81^{a}
	20 ng/ml EGF and 20 ng/ml bFGF	40.73±0.55^{a}	40.40±1.06^{a}	38.80±0.53^{a}
Triple factors combination	20 ng/m IGF-1 and 20 ng/ml bFGF	41.27±0.59^{b}	41.40±0.53^{b}	55.23±0.61^{a}
	20 ng/ml EGF, 20 ng/m IGF-1 and 10 ng/ml bFGF	39.40±0.46^{b}	39.40±0.46^{b}	56.60±0.69^{a}

Values are mean ± S.E of the proliferation-promoting rate in different factors groups/combinations. Values in the same row with different letters mean significant difference (P<0.05).

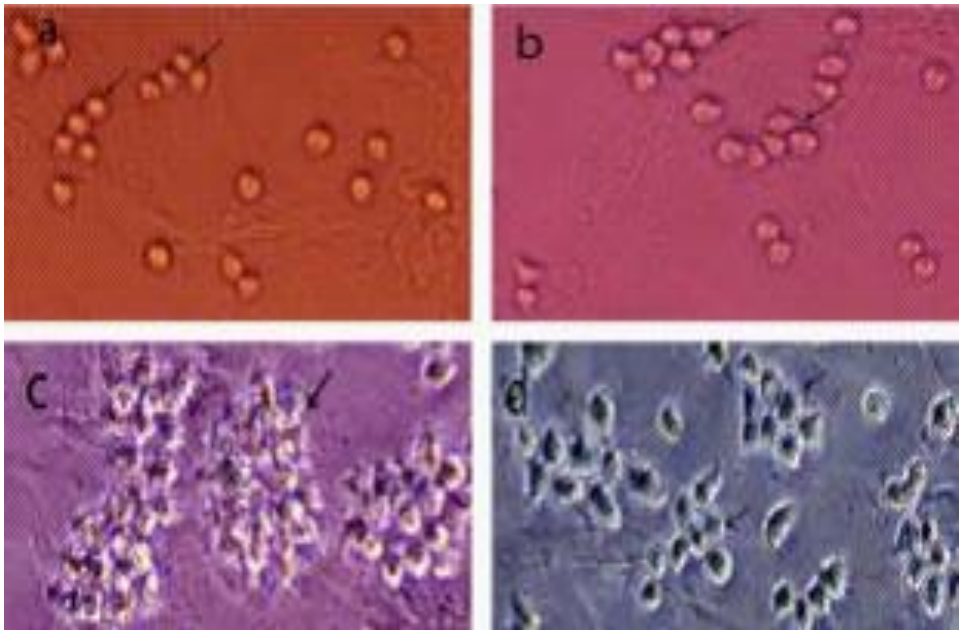

Figure 4. Morphology observation of SSCs from the first passage (200× magnification). a. Control group; b. EGF (10 ng/ml) group based on pre-treatment with 10% FSH (30 ng/ml); c. EGF (20 ng/ml) and IGF-1 (20 ng/ml) combination based on post-treatment with 10% FSH (30 ng/ml); d. EGF (20 ng/ml), IGF-1(20 ng/ml) and bFGF (10 ng/ml) combination based on post-treatment with 10% FSH (30 ng/ml.

junction that is critically selective to small molecules that can enter the abdominal compartment (Wong and Cheng, 2005). Therefore, growth factors are able to act directly on spermatogonia as well as on somatic cells when added in tissue culture *in vitro*. The current study clearly indicated that three culture schemes have significant effects on the proliferation of mouse SSCs and all of the three growth factors (EGF, bFGF and IGF-1) could stimulate SSCs proliferation *in vitro*. The current findings are in agreement with the report which states that growth factors such as bFGF and EGF are necessary for SSC proliferation *in vitro* (Kubota et al., 2004). The preferable culture schemes of the single factor, double factors combination and triple factors combination were 10 ng/ml EGF through pre- treatment, 20 ng/ml EGF and 20 ng/ml IGF-1 combinations through post-treatment, 20 ng/ml EGF, 20 ng/ml IGF-1 and 10 ng/ml bFGF combinations through post-treatment, respectively. The optimal culture scheme was 20 ng/ml EGF and 20 ng/ml IGF-1 combinations through post-treatment (30 ng/ml 10% FSH), and its proliferative rate of mouse SSCs was up to 61.0%. We suggested that there might be synergy between 10% FSH (30 ng/ml) and growth-promoting factors. Thus, the cooperation among EGF, IGF-1, bFGF and FSH was likely to maintain SSCs proliferation *in vitro*.

The growth-promoting factors have a very important function in the culture of SSCs *in vitro*. bFGF is a neurotrophic and vasoactive factor, and has therapeutic potential for some central nervous system (CNS) disorders (Ma et al., 2008). bFGF can stimulate proliferation and differentiation of neural precursors during development (Temple and Qian, 1995), as well as adulthood (Wagner et al., 1999). bFGF increases germ cell proliferation in co-cultures of gonocytes and Sertoli cells (Van Dissel-Emiliani et al., 1996). bFGFs may play a key role during early development when SSCs differentiate from gonocytes and proliferate mitotically to give rise to the full complement of SSCs that subsequently support spermatogenesis. Nugent and Iozzo (2002) confirmed that bFGF can promote cells in the stage of the G0 and G1 to come into the stage of S and finish the proliferation of the fibroblast cells, epithelial cells and vascular endothelial cells via analysis of cells' cycles. bFGF participates in regulating the cells proliferation by activating the Ras-Raf-MEK-MAPK-c-myc pathway. Skaletz-Rorowski et al. (2005) found that bFGF takes part in the proliferation of coronary artery smooth muscle cells by activating the Ras-Raf-MEK- MAPK-c-myc pathway. In addition, PI3K and PLC pathways are involved in cells proliferation by activating FGFR1. bFGF and IGF-1 promote the proliferation of bovine coccygeal cells by phosphorylation of ERKs and Akt, but the function of bFGF was weaker than that of IGF-1 in AKt pathway (Pratsinis and Kletsas, 2007).

IGF-1 is a polypeptide protein hormone, similar in molecular structure to insulin, which plays an important role in cell migration, cell cycle progression, cell survival and proliferation (Ma et al., 2009). IGF-1 can promote the microglia/macrophage proliferation in the ischemic mouse brain and then promote proliferation of the 3T3-L1 preadipocytes. In addition, IGFBP-5 [IGF (insulin- like proliferation factor)-binding protein-5] has been shown to be associated with calcification in plaques (Kim et al., 2007). The insulin-like growth factors (IGFs) can stimulate DNA synthesis of trout male germ cells by interacting directly with these cells via IGF receptor *in vitro* (Loir and Le, 1994). The proliferation of SSCs can be induced by 11-ketotestosterone, which is the main androgen in teleost. IGF-1 is necessary for the action of 11-ketotestosterone in the initiation of spermatogenesis (Takeshi and Chiemi, 2001). IGF-1 can inhibit the enzyme of cyclin protein D to enhance the accumulative amount of cyclin protein D in the nucleus, which can promote the cells proliferation by activating P13k/AKt pathway (Frederick et al., 2007). In this study, IGF-1 (10 ng/ml) was beneficial to form smaller cloning clusters based on pre-treatment with 10% FSH (30 ng/ml) and the effects were possibly related with P13k/AKt pathway.

EGF superfamily of peptide growth factors, including EGF and transforming growth factor-α (TGF-α) have been implicated as regulators of germ cells development (Niederberger et al., 1993). The receptor of the EGF with intrinsic tyrosine kinase activity is widely expressed in SSCs (Wells, 1999). Four members of these receptors are known: erbB1 (Ullrich et al., 1984), erbB2 (Coussens et al., 1985), erbB3 (Kraus et al., 1989) and erbB4 (Plowman et al., 1993), respectively. The EGF and EGFR (EGF receptor) can promote the over-expression of cycloxygenase-2, which can improve the activity of mitosis of SSCs (Robert et al., 1997). The effects of EGF on the proliferation of SSCs can be mediated by activating JAK-STAT pathway of SSCs *in vitro* (Yu and Xu, 2010). Wang et al. (2008) reported that EGF (10^{-7} to 10^{-6} mol/ml) could significantly promote the formation of the cloning clusters of SSCs. EGF (0.1 ng/ml) associated with FSH (10 ng/ml) improves the proliferation of SSCs because of the additive effects observed (Zhang et al., 2007), which are similar to those of this study. EGF can also stimulate Sertoli cells to synthesize lactate (Boussouar and Benahmed, 1999) and promote the proliferation of SSCs (Wahab-Wahlgren et al., 2003). In this study, it was indicated that Sertoli cells were almost stuck after the culture of 48 h and the degree of the convergence rose from 80 to 90%. EGF (10 ng/ml) based on pre-treatment with 10% FSH (30 ng/ml) immensely facilitated the proliferation of SSCs *in vitro* and the proliferation-promoting rate was 41.60% among three kinds of single factors. The effects of EGF on the proliferation of SSCs can be mediated by activating JAK-STAT pathway (Yu and Xu, 2010), which regulated the transcription of mRNA and promoted cells proliferation.

It is well known that FSH activates Sertoli cells which then produce some paracrine factors to stimulate germ cells as well as other somatic cell types (Jegou, 1993;

Jegou and Pineau, 1995). Nakayama et al. (1999) reported that both FSH and IGF-I stimulated a differentiation of spermatogonia into primary spermatocytes in organ culture, indicating that IGF-I as well as FSH activated the differentiation through Sertoli cells. Toebosch et al. (1988) demonstrated that FSH acted indirectly on the gonocytes by inducing Sertoli cell expression of follistatin and inhibin which enable germ cell maturation. Mather et al. (1990) and Boitani et al. (1995) evidenced that FSH stimulate Sertoli cells proliferation during early post-natal testis development in *in vitro* culture systems. Tadokoro et al. (2002) established that the proliferation of SSCs was dependent on FSH, because FSH possibly combined with the receptor on SSCs to activate the SSCs. In this study, SSCs were pre-treated with two methods of pre- and post-treatment. It could probably be that the growth factors of EGF, IGF-1 and bFGF which can activate SSCs proliferation stimulate Sertoli cells afterward. Collectively, FSH activates Sertoli cells to produce EGF, IGF-1 and bFGF factors which later stimulate SSCs proliferation during the process of cultivation *in vitro*.

Conclusion

Based on the results of this study, it could be concluded that single factor of EGF (10 ng/ml) based on pre-treatment with 10% FSH (30 ng/ml), double factors of EGF (20 ng/ml) and IGF-1 (20 ng/ml) based on post-treatment with 10% FSH (30 ng/ml), triple factors of EGF (20 ng/ml), IGF-1 (20 ng/ml) and bFGF (10 ng/ml) based on post-treatment with 10% FSH (30 ng/ml) were the preferable methods in treating and culturing the mouse SSCs which were beneficial to the proliferation of mouse SSCs *in vitro*. However, the optimal culture scheme was 20 ng/ml EGF and 20 ng/ml IGF-1 combinations through post-treatment (30 ng/ml FSH). The growth factors supplemented in the medium up-regulated activation by FSH and acted on Sertoli cells to produce some growth factors; their combined action activates SSCs proliferation during the process of cultivation afterward. The culture schemes of multiple factor combination on the proliferation of mouse SSCs have not been reported so far; as such, more researches would be needed to evaluate and understand the synergetic mechanisms.

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