

## Retroviral Gene Expression in Spermatogonial Stem Cells during Long-term Culture

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**ABSTRACT :** The spermatogonial stem cell (SSCs) is unique in that it is the only cell in the adult male that can contribute genes to a subsequent generation. Permanent modification of the germ cell line may be realized if stem cells could be cultured, transfected with unique genes, and then transplanted into recipient testes. We developed a culture system that supported long-term viability of SSCs. We used a retrovirus vector (pMSCV including  $\beta$ -galactosidase) to stably transfect spermatogonia following long-term culture using the system developed. Expression of the reporter gene  $\beta$ -galactosidase controlled by the retroviral vector was stable in long-term cultured SSCs. We confirmed the retroviral-mediated  $\beta$ -galactosidase gene could be expressed in germ cells in recipient mice following SSCs transplantation. (**Key Words :** Retrovirus, Gene Transfer, Electroporation, Stem Cell Medium)

### INTRODUCTION

The spermatogonial stem cell (SSCs) is unique, since it is the only cell in the male adult body that undergoes a dramatic morphological change to generate a sperm that can contribute genes to subsequent generations (McLaren and Durcova-Hills, 2001). Following fusion of the male and female gametes and the formation of an embryo, the fate of germ cells in gonads depends on the sex of the embryo (Donovan, 1998). In the female, the PGCs undergo meiosis and become oocytes, thereby ending their stem cell potential. In the male, gonocytes found in the neonatal testis migrate to the seminiferous tubule basement membrane and differentiate into testicular germ cells or initiate spermatogenesis (Brinster, 2002). The genetic manipulation of germ line stem cells will allow for direct germ-line transfer of genes to subsequent generations.

Transplantation techniques in both hematopoietic (Harrison, 1980; Till and McCulloch, 1980) and spermatogenic (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994) systems could be utilized for cell therapy (Kanatsu-Shinohara, 2002). Transplantation and

genetic manipulation of SSCs could be used to answer fundamental questions about spermatogenesis and SSCs renewal and determine the testicular cell responsible for infertility in animal models (McLean et al., 2002).

In general, germ cell cultures using mammalian species have been only moderately successful over long time-periods. The efficiency of germ cell development in the mammalian testis is enhanced by the presence of Sertoli cells; however, the co-culture of mammalian germ cells and Sertoli cells has not improved germ cell survival *in vitro*. Mouse SSCs have been maintained in culture with STO feeder layers for approximately 4 months. These cultured stem cells were transplanted into recipient testes and successfully generated mouse spermatogenesis (Nagano et al., 1998). Permanent modification of the germ cell line would be possible only if stem cells could be cultured, transfected with unique genes, and transplanted (Cooker et al., 1993). Recently, an alternative means to generate genetically modified sperm was reported through the use of *in vitro* generation of spermatocytes and spermatids with telomerase-immortalized mouse type A spermatogonial cells in the presence of stem cell factors (Feng et al., 2002).

Retroviral vectors provide an efficient gene transfer system for many gene therapy applications. However, these vectors have significant limitations because retroviral

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integration into the host cell DNA requires cells to go through M-phase of the cell cycle. The techniques for culturing testicular germ cells need to be improved to provide efficient division of SSCs. In the testis, these cells divide at a slow rate and the factors necessary to stimulate stem cell activity are unknown. Cell culture of spermatogonial stem cells followed by SSCs transplantation provides a means to elucidate and evaluate factors needed for stem cell division and differentiation (Risley, 1990; Cooker et al., 1993; Rassoulzadegan et al., 1993).

The establishment of culture conditions for SSCs could promote high levels of cell survival and stimulate proliferation that will enhance subsequent efforts to carry out retroviral transduction. In addition, direct introduction of DNA into male germ cells could provide a new alternative strategy for gene therapy or transgenic animal production (Avarbock et al., 1996; Nagano et al., 2000). The *in vivo* gene transfer technique for mouse testes was previously used to develop a novel transient expression assay system for transcriptional regulatory elements of spermatogenic specific genes (Muramatsu et al., 1997). We used this technique combined with a retroviral infection system to transfect SSCs. The purposes of the present study were to establish and refine long-term culture of SSCs, identify the effects of retroviral infection of these SSCs and determine the expression of reporter genes following transplantation into recipient testes.

## MATERIALS AND METHODS

### Experimental animal

Protocols for the use of animals in these experiments were approved by the Washington State University and Cheju National University Animal Care and Use Committee and in accord with National Institutes of Health Standards established by the Guidelines for the Care and Use of Experimental Animals. Donor testis cells and recipient mice were obtained from B6/129 mouse testes of males at 4 weeks of age. Animals were housed in a standard animal facility with free access to food and water.

### Cell collection and culture

Donor testes were enzymatically digested as described by O'Brien (1993) with modifications as described by McLean et al. (2002). Briefly, testes were removed, immersed in Hank's buffer, and the tunica removed. Testes were transferred to tubes containing digestion medium, consisting of 0.5 mg/ml of collagenase type IV (Sigma, St Louis, MO), 0.25 mg/ml of trypsin (Gibco BRL, Bethesda, MD) and 0.05 mg/ml of DNase (Sigma) in Ca- and Mg-free Hanks buffer (pH 7.4). Testes were digested for 15 min by shaking at 33°C to dissociate tubules. The sample containing the tubule suspension was transferred to ice and

the tubules were allowed to sediment for 5 min. The supernatant was removed, fresh digestion medium added and the tubules digested for 15 min by shaking at 32°C. Following the second digestion, the cell concentration was determined using a hemacytometer.

Testicular germ cells, from 4-week old mice, were cultured in 24 well plates (Falcon plastics) or in 6 well plates with stem cell culture medium consisting of DMEM supplemented with 10% FBS (Hyclone), 12 ng/ml of murine leukemia inhibitory factor (mLIF; Sigma), 20 ng/ml of bovine basic fibroblast growth factor (bFGF; Sigma), 0.04 ng/ml of human interleukin-11 (h-IL-11; Sigma), 10 ng/ml of human stem cell factor (hSCF; Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine,  $5.5 \times 10^{-5}$  M 2-mercaptoethanol, 100 fig/ml of streptomycin, 100 units/ml of penicillin, 10 ng/ml of oncostatin M (OSM; Sigma), 1 ng/ml of platelet derived growth factor (PDGF; Sigma) and 15 ng/ml of human insulin-like growth factor-I (IGF-I; Sigma). The isolated germ cells from one testis were seeded into wells of the 6 well culture plates and incubated in a CO<sub>2</sub> incubator at 32°C until the stem cells had colonized as a primary culture (approximately 7-12 days). Mitomycin C-treated STO cells were use as feeder cells in culture. The feeder cells were seeded on the culture surface at a concentration of  $5 \times 10^6$  cell/cm<sup>2</sup> for 1-2 days before testis cells were added. Medium was changed twice a week.

### *In vivo* DNA transfection

The mice were lightly anesthetized by intraperitoneal administration of ketamine (0.1 mg/g) and xylazine (0.05 mg/g). A midline incision was made and the testis exposed. Plasmid pMSCVB-gal DNA construct was used and it was linearized by BamHI restriction endonucleases, ethanol precipitated and resuspended in sterile TE buffer (10 mM Tris, and 1 mM EDTA, pH 7.5) and 0.04% trypan blue to be injected into the rete testis through the efferent bundle with a glass needle. A total of 2 µg of plasmid was injected into the testis. Immediately after the injection, square electric pulses were applied with an electro-square porator T820 (BTX, San Diego, CA, USA) at 40 voltages with a loading period of 40 ms and 1 pulse. After the procedure, the testes were replaced and the animals were maintained for 48 h upon which they were sacrificed for preparation of germ cells and identification of transient expression.

### Virus production and *in vitro* assay

The Murine Stem Cell Virus (MSCV) vectors (Clontech, CA, USA) were derived from the Murine Embryonic Stem Cell Virus (MESV) and the LN retroviral vectors. The vectors achieve stable, high-level gene expression in hematopoietic and embryonic stem cells through a specifically designed 5' long terminal repeat (LTR). This LTR is from the murine stem cell PCMV virus, and it

differs from the MoMuLV LTR used in other retroviral vectors by several point mutations and a deletion (Grez et al., 1990). The pMSCV- $\beta$ -gal vector was produced in HEK 293T retrovirus packaging cells maintained in DMEM. HEK 293T cells ( $3-4 \times 10^6$  per 100 mm plate) were transfected with 20  $\mu$ g of pMSCV- $\beta$ -gal using the electroporator (BTX T820). The conditions for electroporation were modified from Hong et al. (1998). Briefly,  $3-4 \times 10^6$  HEK 293T cells were mixed with 20  $\mu$ g pMSCV- $\beta$ -gal in 400  $\mu$ l of Opti-MEM (Gibco) containing 1.25% DMSO (v/v) for 1 min at room temperature. Electroporation conditions were optimized in HEK 293T cells. Cells were electroporated at 250 V with a capacitance of 10 msec and one pulse time. In addition, to transfect in HEK 293T using the lipofectamine 2000 (Invitrogen, CA, USA) one day before transfection, HEK 293T cells were incubated in growth medium without antibiotics so that they would be 85-95% confluent at the time of transfection. pMSCV-p-gal plasmid DNA was diluted in 50  $\mu$ l of Opti-MEM I medium and mixed gently. Lipofectamine 2000 was mixed gently before use, then diluted in 50  $\mu$ l of Opti-MEM I medium and incubated for 5 min at room temperature. After the 5 min incubation, the diluted DNA was combined with the diluted Lipofectamine 2000. The combined complex was incubated for 20 min and 100  $\mu$ l of transfection complex added to each plated well with HEK 293T cells. The cells were incubated at 37°C in a CO<sub>2</sub> incubator for 24 h until ready to assay for X-gal screening. Stably transfected cells were selected by treatment with G418 (1 mg/ml) and G418-resistant colonies were passaged for virus production and virus titer assay. Retroviral vector transfected HEK 293 T cells were stained with X-gal to detect  $\beta$ -galactosidase activity and the efficiency of transfection. In order to stain the cell, medium was removed, the cell layer washed once with PBS, fixed with 0.5% glutaraldehyde for 20 min in ice, washed twice with PBS and then incubated with X-gal until blue color developed.

### ***In vitro* infection and transplantation**

Virus-conditioned medium was obtained from the confluent retrovirus-producing cells that were selected by G418 selection method for 25 days and filtered (0.4  $\mu$ m) to remove debris and floating cells. Testicular germ cell cultures were treated with the virus-conditioned medium with polybrene (8  $\mu$ g/ml). Four-gene transduction protocols were evaluated (described in detail below): single infection protocol, two infection protocol, single infection protocol after *in vivo* gene transfection, and two infection protocol after *in vivo* gene transfection. All cells treated with retrovirus were cultured for 2 months.

Retroviral infection protocols were as follows: Single infection protocol (hereafter referred to as Protocol 1):

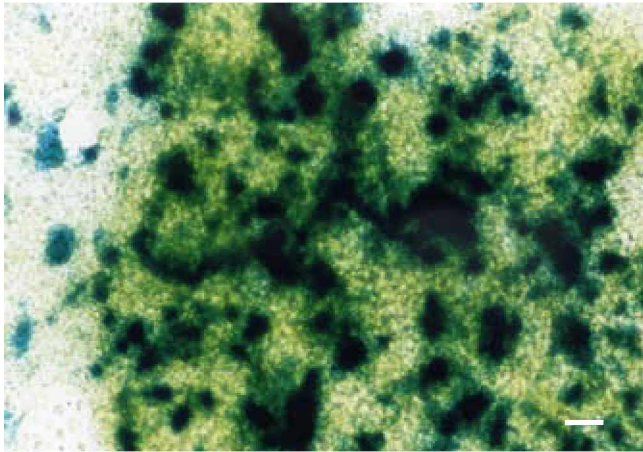
Testes cells isolated from 4-week old 129/B6 mice were cultured for 1 day, and were infected with virus-producing medium on the 2nd day for 24 h. These cells were then cultured for 2 months after washing with DMEM media. Two infection protocol (hereafter referred to as Protocol 2): Single infected cells were incubated for 3 days after the initial infection and were then infected with virus-producing medium on the 5th day for 24 h and cultured for 2 months after washing with DMEM media. Single infection protocol after *in vivo* gene transfection (hereafter referred to as Protocol 3): Four-week old mice testes were injected with pMSCV- $\beta$ -gal retroviral vector DNA. After injection, testes cells were isolated and infected with virus-producing medium for 24 h and cultured for 2 months after washing with DMEM media. Two infection protocol after *in vivo* gene transfection (hereafter referred to as Protocol 4): Four-week old mice testes were injected with pMSCV- $\beta$ -gal retroviral vector DNA. Forty eight hours after injection, testes cells were isolated and infected with virus-producing medium and exposed to retroviral medium for 24 h, incubated for 3 days, followed by a second retroviral exposure on the 5th day for 24 h and then cultured for 2 months after washing with DMEM media.

Cultures of retrovirally infected cells were treated with trypsin to obtain a single cell suspension and the cell suspension was centrifuged at 500 $\times$  g for 4 min. Cells were resuspended in EKRB medium containing 0.03% trypan blue (Gibco BRL) at a concentration of 10<sup>7</sup> cell/ml. Cultured donor germ cells were transplanted into testes of B6/129 mice, 4 weeks following treatment with busulfan (40 mg/kg). A midline incision was made in the recipient mouse abdomen and the testis exposed. A small hole was made in the efferent bundle and a glass needle containing 7  $\mu$ l of the germ cell suspension inserted into the rete testis and the cells were injected into the seminiferous tubules via the rete testis. The testis was replaced and the animal allowed for recover. Recipient mice were sacrificed after 1 week or 2 months by cervical dislocation and the testes removed. Testes were fixed in 4% paraformaldehyde in PBS, pH. 7.4, for 1 h on ice and washed twice in LacZ buffer (0.2 M sodium phosphate, pH 7.3, 2 mM MgCl<sub>2</sub>, 0.02% NP-40 and 0.01% sodium deoxycholate) for 30 min.  $\beta$ -galactosidase positive cells were stained by incubating testes in LacZ stain solution (LacZ buffer containing 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide and 1 mg/ml X-gal).

## **RESULTS**

### **Retroviral transduction of short-term cultured mouse spermatogonial stem cells**

The first step to transfect spermatogonial stem cells was to generate a high expression packaging cell line/HEK 293T.

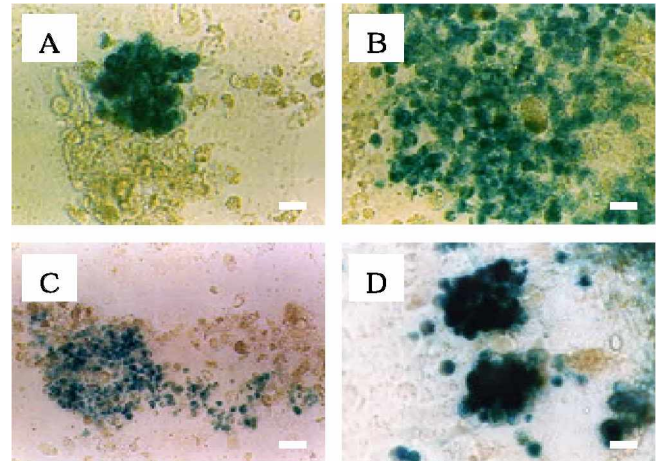


**Figure 1.** Appearance of p-galactosidase (pMSCV-β-gal; retroviral vector) expression in HEK 293 T cells following electroporation. Bar = 33 μm. The experiments were repeated three times, each experiment comprised triplicate cultures.



**Figure 2.** pMSCV-β-gal (retroviral vector), produced by HEK 293T cells, gene expression in SSCs using several infection methods including single transfection *in vitro* (protocol 1), two *in vitro* transfections (protocol 2), *in vivo* electroporation followed by *in vitro* transfection (protocol 3) and *in vivo* electroporation followed by two *in vitro* transfections (protocol 4). The data are expressed as the mean percent of transduced cells of all pooled experiments. A significant increase in the efficiency of retroviral gene transfection was observed in protocol 2 (\*  $p < 0.05$ ). The experiments were repeated three times, each experiment comprised triplicate cultures. Data represent the mean  $\pm$  SEM of one representative experiment

To accomplish this goal, we optimized a method to obtain high expression of β-gal in the HEK 293T cells with the use of electroporation at multiple voltages (Jeong, 2005). To increase the efficiency of retroviral productive gene expression, HEK 293T cells electroporated with MSCV-p-gal were grown in DMEM with G418 (1 mg/ml) for 25 days (Figure 1). Retroviral containing medium from stably transfected HEK 293 cells was subsequently used to transfect germ cells in culture. We first examined whether

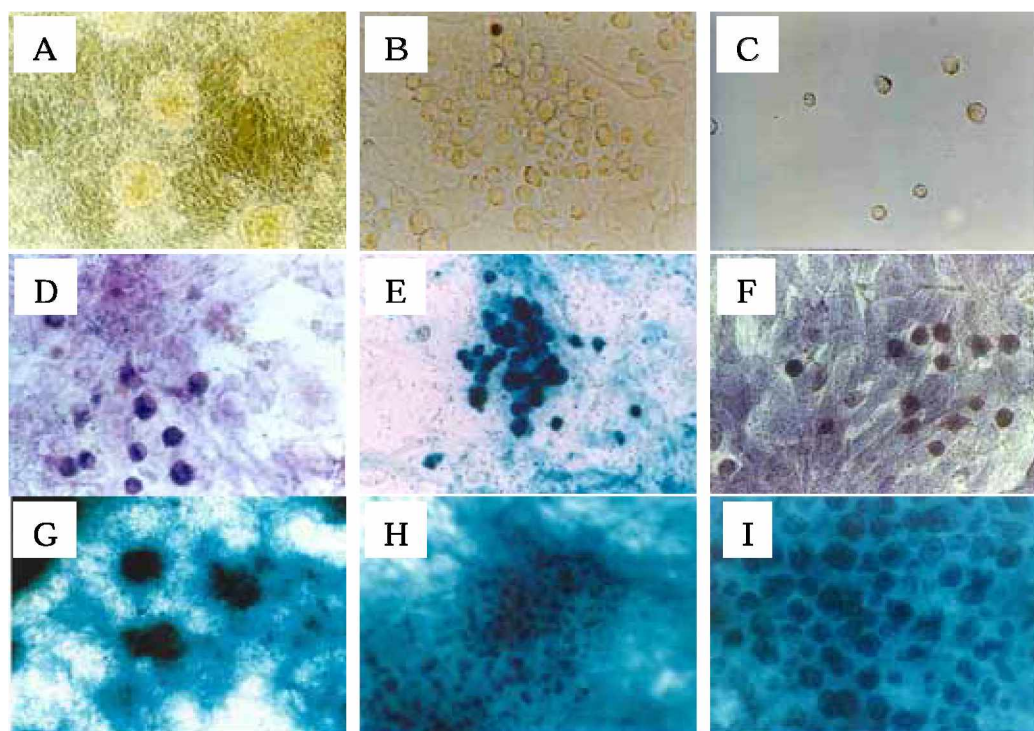


**Figure 3.** Retrovirus infection into SSCs using the high titer virus (pMSCV-β-gal retroviral vector). (A) One infection (B) Two infections (C) One infection after *in vivo* transfection into the testis. (D) Control (ROSA26 male germ cells). Bar = 33 μm (C), 66 μm (A, B, D). The experiments were repeated three times, each experiment comprised triplicate cultures.

gene expression occurs in cultured spermatogonial stem cells after infection with retrovirus obtained from HEK 293T cells, since stable gene integration by retrovirus occurs only in cells that are actively replicating (Figure 2). Two approaches were used: Infection of cultured testis cells by retrovirus *in vitro* (protocols 1 and 2) and *in vivo* electroporation of germ cells with β-gal plasmid in the seminiferous tubules followed by culture of these cells and *in vitro* infection with retrovirus produced by HEK 293T cells (protocols 3 and 4). In addition, cultured testis cells were exposed to retrovirus from HEK 293T cells either once or twice during the culture period (see Materials and Methods for details). The efficiency of gene expression by these approaches and protocols was compared. Protocol 1 (single infection) infected short-term cultured spermatogonial stem cells with retrovirus from HEK 293T cells. Gene expression was observed in germ cells at a level of 28%. Using protocol 2 (two infections), gene expression in germ cells was significantly increased to 50% ( $p < 0.05$ ) when compared to protocol 1. These experiments in cultured germ cells showed that the highest gene expression that occurred with the use of protocol 2 was 50%.

Additional protocols were developed to get higher gene expression in SSCs. Protocols 3 and 4 were developed to include *in vivo* electroporation of SSCs and *in vitro* infection with HEK 293T produced retrovirus. In protocol 3, the efficiency of gene expression in SSCs was 38%, which was slightly higher than protocol 1. In addition, the efficiency of gene expression in SSCs using protocol 4 was 39.87%. The SSCs, which were stained by X-Gal, were typically a dark blue color indicating high reporter gene expression.

The gene expression slightly increased in germ cells



**Figure 4.** Identification of spermatogonial cells after retrovirus transduction. (A) 2 month cultured SSCs derived colonies. (B) Dispersion of germ cell derived colony (Single germ cells were derived from cell colonies during long-term culture). (C) Primary single cell suspension from mouse testis cells (4-week old male) (D) Alkaline phosphatase expression of single cell suspension (B). (E)  $\beta$ -Galactosidase expression of retrovirus transduced single germ cells. (F) Alkaline phosphatase expression of retrovirus transduced single germ cells. (G)  $\beta$ -Galactosidase expression of 3 month cultured cell colony (100 $\times$ ). (H) Higher magnification of G (200 $\times$ ). (I) Higher magnification of G (400 $\times$ ). Bar = 17  $\mu$ m (A, G), 33  $\mu$ m (H), 66  $\mu$ m (B, C, D, E, F, I). The experiments were repeated three times, each experiment comprised triplicate cultures.

between protocol 1 and protocol 2. In protocol 1,  $\beta$ -galactosidase gene expression was observed in a small area as a cell colony (Figure 3A). As shown in Figure 3B, the gene expression levels increased significantly using protocol 2 when compared to protocol 1, 3 and 4. These data indicated that the optimal conditions were protocol 2 by transfecting SSCs twice *in vitro* under the experimental conditions described.

#### Identification of long-term cultured spermatogonial cells with retrovirus mediated gene expression

The cells infected by the retrovirus required consistent cell division. Recently, we developed a long-term culture method using several growth factors and STO feeders for mice SSCs (Jeong et al., 2003). To improve the gene transduction efficiency of mouse SSCs during long-term culture by retroviral-mediated gene transfer, the virus that was previously produced with protocol 2 was used to infect mice testicular germ cells from 4-week old mice and was cultured for 2 months. The culture cell proliferation ratio was the same as the control. Colonies of  $\beta$ -gal positive cells appeared from the 7th day of culture and continued through 2 months of culture. The size and number of colonies also

increased over the course of the 2 months of culture (Figure 4A). Cells from some of the colonies separated into single cells (Figure 4B). The morphology of these cells was similar to SSCs separated from primary testes (Figure 4C).

The infected SSCs were stained with X-Gal to determine the gene expression (Figure 4E and G). As shown in Figure 6E and G strong gene expression in SSCs was identified. In addition, all  $\beta$ -galactosidase positive cells had AP activity (Figure 4D and F). We identified the formation of colonies of SSCs through long-term culture but it was difficult to identify characteristics of the single cells that formed the colonies. Before scattering into single cells, the colonies were characterized by microscopy. The morphology of the single cells was similar to spermatogonial cells, and they grew in very compact colonies.

#### Transplantation of cultured germ cells

To examine whether cultured SSCs were stably transfected and would initiate donor-derived spermatogenesis these cells were transplanted into the testes of azoospermic busulfan treated mice. Germ cells cultured and transfected with protocols 1, 2, 3 and 4 were all

**Table 1.** Colonization of retroviral mediated lacZ gene transduced spermatogonial stem cell in recipient mouse testes

Protocol <sup>a</sup>	Months after transplantation	Colonized testes <sup>b</sup>	Average no. of colonies <sup>c</sup>
1	2	1/4	11±2
2	2	5/10*	76±8*
3	2	2/8	53±6
4	2	0/8	ND <sup>d</sup>

<sup>a</sup> Protocol from four separate experiments.

<sup>b</sup> Number of testes colonized per number of testes analyzed.

<sup>c</sup> Mean±SEM of all testes for each age group.

<sup>d</sup> ND = Not determined.

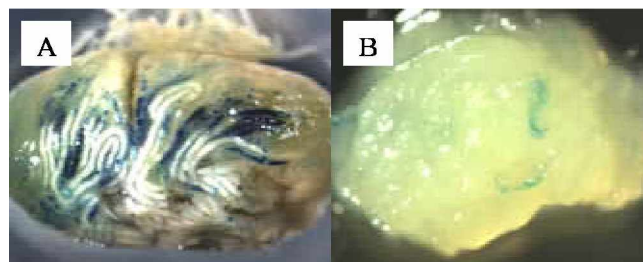
\* Superscript is significantly different at  $p < 0.05$ .

transplanted to evaluate the effect treatments had on colonization efficiency and gene expression. Using protocol 3, donor-derived spermatogenesis of  $\beta$ -galactosidase expressing SSCs was detected in 2 out of 8 mice transplanted. Spermatogenesis of  $\beta$ -galactosidase expressing SSCs using protocols 1 and 2 was detected in one out of 4 mice transplanted and 5 out of 10 mice transplanted, respectively (Table 1). Under these conditions, donor-derived spermatogenesis of stably transfected SSCs was increased slightly by the number of infections. When donor SSCs were transplanted into the recipient mouse seminiferous tubules, they settled, proliferated, and interacted with recipient mouse SSCs (Figure 5). Therefore, the foreign gene transduced SSCs, cultured for a long-term period by the retroviral infection system, were sufficient to generate donor-derived spermatogenesis following transplantation. However, the ratio of spermatogenesis was not increased by protocol 3 and 4.

## DISCUSSION

The production of cell lines stably transduced with retroviral vectors is an important issue in retroviral mediated gene transfer. Currently, retroviral-mediated gene transfer is widely utilized to obtain efficient transduction of mammalian cells *in vitro* (Greig et al., 1990). To date, while standard retroviral vectors are well suited for use in such *ex vivo* application, the vectors have found only limited use in strategies involving direct *in vivo* gene transfer (Yamazaki et al., 2000). The defects of the common retroviral vector include the difficulty to easily purify and concentrate large amounts of virus often needed for direct *in vivo* gene transfer application and integration in quiescent cells of most murine-based vectors (Ory et al., 1996; Mulligan, 1993; Roe et al., 1993).

In this study the pMSCV- $\beta$ -gal retroviral vector was modified from the MoMuLV LTR used in other retroviral vectors by several point mutations and a deletion that would provide a high level of gene expression in EC or ES cells (Greig et al., 1990; Lewis and Emerman, 1994). By the gene expression assay, we demonstrated that male germ cells



**Figure 5.** Spermatogonial transplantation of retroviral mediated lacZ gene transduced cells. (A) A recipient testis after transplantation of lacZ gene transduced cells. Number counted for testes with blue stretches of seminiferous tubules represent colonies from donor cells. (B) X-gal stained seminiferous tubules hard to find in recipient testes for protocol 4.

were efficiently transduced in long-term culture by a retrovirus produced from retroviral vector transfer into HEK 293T cell using electroporation. In general, retroviral vectors are transferred into host cells mainly by calcium phosphate coprecipitation (Danno et al., 1999). However, we used the method that had previously recorded levels of reporter gene expression in germ cells at 80% using electroporation (Yamazaki et al., 1998). The application of the retroviral vector gene transfection method by electroporation was first attempted to produce highly efficient virus. We identified that the electroporation method is a simpler and a more straightforward gene transfection method than other methods because it showed high efficiency of gene expression in HEK 293 T cells. To compare the efficiency of gene expression and generally used amount of virus, we infected virus derived from the most efficient cells (89.02%) with NIH 3T3 cells. After counting all the colonies ( $3.75 \times 10^5$ , data not shown), our results were consistent with previous reports (Nagano et al., 2000). Through these results, we were able to confirm that the titer of virus could be determined by a simple X-Gal stain method. The retrovirus infection can only be used for fast proliferating and cultured cells. Applying the foreign gene transfection method into SSCs by retrovirus infection, a low efficiency of transfection was repeatedly observed (data not shown). We developed a 3-month long-term SSCs culture method (Jeong et al., 2003). By using this method, protocol 2 had the most efficient results. Unlike this method, Nagano et al. (2003), performed SSCs culture over a shorter time period. They reported that the efficiency of gene expression gradually decreased in a time-dependent manner. We increased the gene expression in short-term culture by protocols 1 and 2 by including a second exposure to retrovirus. Specifically, the efficiency of gene expression was increased to 22% by the number of infections of retrovirus produced by HEK 293T cells. The addition of the *in vivo* gene transfection method by the one infection system did not affect the efficiency of gene expression. In addition, a negative effect showed that there was no

spermatogenesis using protocol 4.

$\beta$ -Galactosidase expression by germ cells could have been affected by heat shock from the *in vivo* electroporation. There was low reporter gene expression by electroporation transiently in germ cells and many somatic cells. Possibly, the efficiency was decreased due to the competition from endogenous stem cells that were not transfected.

Recently, there was a report that the efficiency of retroviral infection depends on the kind of vector used (Miguel and Donovan, 2002). Interestingly, MSCV had the lowest efficiency when compared to ALV and MLV during *in vitro* culture. But this study showed that the efficiency of MSCV expression increased by long-term culture as confirmed by transplantation. Also, these authors confirmed that the efficiency of gene expression was increased by the culture period, which is consistent with our results. They suggested that the most efficient culture condition for spermatogonia includes several growth factors including forskolin, LIF, EOF and OSM. We also observed that these growth factors combined with bFGF, mLIF, hIL-11, hSCF, mOSM, PDGF, and IGF-1 were very important in long-term culture of spermatogonia through our previous study (Jeong et al., 2003). Therefore, the efficient culture method of SSCs will be established by the combination of appropriate growth factors.

In summary, the retrovirus transfection method developed by this study can select a cell line that produces highly efficient virus straightforwardly and transduces it into SSCs efficiently. Second, protocol 2 was more effective than protocol 4. In addition, spermatogenesis did not appear after SSCs transplantation in protocol 4. These results showed that the efficiency of spermatogenesis was different depending on the quality of spermatogonial cells transfected with foreign gene, although transplanted spermatogenesis was normal. Third, the efficiency of gene transduction was affected by the long-term culture. Through this result, we could identify that the establishment of an efficient culture method is essential to foreign gene transduction. The efficiency of gene transduction could be increased by the long-term culture.

Therefore, the retrovirus gene infection method combined with the long-term culture method is very useful in SSCs gene transduction. These methods may be used as an efficient method for production of transgenic animals and gene therapy.

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