Lentiviral vector transduction of male germ line stem cells in mice

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Abstract Transgenesis can be achieved in mice by retroviral transduction of male germ line stem cells (GSCs). However, the transduction efficiency by a Moloney murine leukemia virus (MMLV)-based vector is low, probably due to the characteristically slow cell cycle of stem cells. Since lentiviral vectors can transduce non-dividing cells, they have the potential to efficiently transduce GSCs. Here we report that male GSCs of mice can be transduced in vitro by a lentiviral vector and generate complete spermatogenesis when transplanted into infertile host testes. Transduction efficiencies were comparable to those for MMLV transduction using similar experimental conditions. The results suggest that both lentiviral and MMLV vectors could be effective in transducing GSCs of other species. In addition, these and previous studies suggest that transduction of immature donor stem cells transplanted into immature recipient testes will provide the most efficient system for male germ line modification.

Key words: Male germ line; Spermatogonial stem cell; Lentivirus; Gene delivery; Transgenesis

1. Introduction

Spermatogonial stem cells, the germ line stem cells (GSCs) of the male in postnatal mammals, are the foundation of spermatogenesis [1,2]. The GSCs self-renew throughout life and produce committed progenitors that subsequently enter meiosis and form spermatozoa. This dual function of GSCs confers a lifelong reproductive potential to a male after puberty. In contrast, all female germ cells enter meiosis and lose their self-renewing potential prior to birth, resulting in the absence of GSCs in postnatal females. Thus, male GSCs are the only postnatal mammalian cell population that can self-renew, maintain the dependent tissue, and contribute genes to the next generation. Development of the spermatogonial transplantation technique allowed any testis cell population to be analyzed for the presence of this stem cell [3,4]. In this technique, testis cells obtained from one male are transferred to the testes of another infertile male, and the GSCs contained in the donor cell population colonize the seminiferous tubules of the recipient. Donor-derived spermatogenesis is established in the testes of the recipient, which subsequently produces offspring carrying the donor haplotype [3,4]. Thus this technique has provided unique opportunities to manipulate the genome of offspring through male GSCs.

The oncoretrovirus, Moloney murine leukemia virus (MMLV), has been one of the most frequently used vectors to deliver a foreign gene into target cells due to its intrinsic ability to integrate recombinant proviral DNA into the genome of a host cell [5,6]. We have previously demonstrated that gene delivery into the genome of male GSCs is feasible in mice using mouse-specific ecotropic MMLV vectors [7,8]. Following in vitro retroviral transduction of donor GSCs, transplantation of these cells into recipient seminiferous tubules resulted in generation of donor-derived spermatogenesis expressing a marker transgene [7]. The recipients subsequently produced transgenic offspring at a frequency (4.5%) comparable to that observed in conventional transgenic strategies using eggs and embryos [8]. The viral marker gene was expressed without silencing for at least three generations [8]. While these studies clearly indicate that MMLV-based vectors are valuable for transgenesis through the male germ line, they have a significant limitation for gene delivery to GSCs. Because the proviral DNA cannot translocate through the nuclear membrane, efficient transduction by MMLV vectors requires active host cell division, which is accompanied by nuclear membrane breakdown [5,9]. Since GSCs divide slowly [1], this requirement is a significant limiting factor in the genetic modification of the male germ line using MMLV vectors.

Lentiviruses are a subclass of retroviruses which have been developed as vectors in order to deliver target genes to the genome of non-dividing cells [5]. Lentiviral vectors, including those based on the human immunodeficiency virus, contain a matrix protein with a nuclear localization sequence that enables the viral pre-integration complex to be actively transported into the nucleus of a host cell [10]. Therefore, lentiviral vectors can transduce non-dividing cells. In addition, the envelope protein of lentiviral vectors is commonly replaced with the G glycoprotein of vesicular stomatitis virus (VSV-G), which uses an abundant membrane phospholipid as a receptor

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Abbreviations: GSC, germ line stem cell; MMLV, Moloney murine leukemia virus; EF, elongation factor; CMV, cytomegalovirus; VSV-G, vesicular stomatitis virus G glycoprotein; TE, transduction efficiency; HSC, hematopoietic stem cell; LTR, long terminal repeat

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and permits entry of these vectors into a wide range of cell types from a variety of species [11]. Therefore, the lentiviral vector could be an efficient vehicle to deliver foreign genes into male GSCs of many species.

In the present study, we used two types of lentiviral vectors, which encoded the *Escherichia coli lacZ* reporter gene driven by either a mammalian elongation factor (EF)-1α promoter or a viral enhancer/promoter derived from cytomegalovirus, to examine the transduction efficiency of male GSCs in mice.

2. Materials and methods

2.1. Animals

Donor cells were obtained from adult and pup mouse testes. Adult testis cells were collected from C57BL/6 (B6) males made cryptorchid at 6–8 weeks of age, by securing the testes inside the abdominal cavity to allow elimination of differentiated germ cells [12]. Cells were collected 2–3 months after surgery. This procedure results in a 20–25-fold enrichment of stem cells [12,13]. Pup testis cells were collected from B6×129/SvCP (B6/129) F_1 hybrid mice at 5–8 days of age. Adult B6/129 males were used as recipients; they were pretreated with 50 mg/kg body weight of busulfan at 4–6 weeks of age to destroy endogenous spermatogenesis, and used 4 weeks or more after treatment. All animal experimentation procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.2. Virus production

Viral vectors were produced as described [14]. HEK 293T cells were plated on poly-D-lysine-coated 10-cm plates. One day later, cells were transfected with 5 μg of pMD.G (encoding the VSV-G envelope glycoprotein) [15], 15 μg of pCMVΔR8.2 (encoding viral structural, enzyme, and accessory genes) [15], and 20 μg of the transfer plasmid, either HIV-CMV-*LacZ* [15] or SIN-EF-n*LacZ* [16], using the CalPhos transfection kit (Clontech). The cells were rinsed 12–16 h later, and the medium was changed to the germ cell culture medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 6 mM lactate, 0.5 mM pyruvate, 6 mM L-glutamine, 0.1 mM β-mercaptoethanol, 30 μg/ml penicillin, and 50 μg/ml streptomycin) [7]. Viral supernatants were collected ~48 h post-transfection, and centrifuged at 200×*g* for 5 min at 4°C and passed through a 0.45-μm filter to remove any contaminating virus producer cells. Unconcentrated vector stocks were used for transduction and titering immediately after collection. Titering was performed on canine Cf2TH cells in the presence of 8 μg/ml polybrene (Sigma) using serial dilutions of viral supernatants, and titers were determined by reacting the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) 48 h after transduction [15,16]. The titer was ~10^5 transduction U/ml for both vectors (Table 1), which was similar to that of MMLV-based vectors reported in previous studies [7,8].

2.3. In vitro manipulation of male GSCs by lentiviral vectors and transplantation

In vitro culture and infection procedures were based on our previous studies with slight modifications [7,8]. Testis cells were prepared using a two-step digestion of donor testes, and 2×10^6 cells were placed in a 9.6-cm^2 tissue culture well [7]. Mitomycin C-treated SIM mouse embryo-derived thioguanine- and ouabain-resistant (STO) fibroblast cell line served as feeder cells (5×10^5 cells/well) [7]. Four wells for each type of vector were prepared per experiment. The following day, donor testis cells were treated twice with freshly prepared lentiviral supernatant (1 ml with 4 μg/ml polybrene); first for 6–8 h followed by a second treatment for 12 h. On the second day, cells were washed with 3 ml of Dulbecco's phosphate-buffered saline three times, harvested by trypsinization, resuspended in the germ cell culture medium, and transplanted into seminiferous tubules of recipient testes [7,17]. Each recipient testis received approximately 10 μl of cell suspension. Recipient testes were stained with X-gal to detect donor-derived spermatogenesis expressing the viral *lacZ* marker gene 2–3 months or 6 months following transplantation [17]. Recipient seminiferous tubules were paraffin-sectioned and counter-stained with eosin to examine the morphology of donor-derived spermatogenesis.

3. Results and discussion

We used lentiviral vectors with two different promoters, each carrying the *lacZ* marker gene, to transduce male GSCs. One vector (CMV-*lacZ*) contained an internal enhancer/promoter of cytomegalovirus (CMV) [15], and the other (EF-n*lacZ*) contained an internal promoter from EF-1α and a nuclear translocation sequence [16]. Adult and pup donor testis cells were transduced in vitro using each vector, and transplanted into recipient testes to evaluate viral transduction of GSCs. The transplantation assay is necessary because, based on current knowledge, GSCs can be identified only by their ability to generate complete spermatogenesis.

Following transplantation of donor cells transduced by the CMV-*lacZ* vector, no colonies of spermatogenesis expressing

Table 1
Colonization of recipient testes by male GSCs transduced by a lentiviral vector in vitro

Donor age	Experiment number	Viral titer[a] (U/ml)	Number of cells/testis ($\times10^5$)[b]	Number of testes analyzed[c]	Number of testes colonized[c]	Number of colonies observed	Testis cell TE[d]
Adult	1	1.3×10^5	6.7	8	2	5	0.9
	2	6.7×10^3	6.3	11 (4)	4 (0)	10	1.4
	3	2.4×10^5	6.7	9 (2)	2 (0)	4	0.7
	1–3[e,f]	$1.3\pm0.7\times10^5$[e]	6.6 ± 0.1[e]	28 (6)[f]	8 (0)[f]	19[f]	1.0
Pup	4	3.5×10^5	5.3	6	6	15	4.7
	5	4.6×10^3	6.7	8 (1)	8 (1)	11	2.1
	6	4.8×10^4	5.7	9 (4)	9 (4)	36	7.0
	4–6[e,f]	$1.3\pm1.1\times10^5$[e]	5.9 ± 0.4[e]	23 (5)[f]	23 (5)[f]	62[f]	4.6

The lentiviral vector contained a reporter gene, EF-n*lacZ*. No donor cell colonization was observed in recipients of CMV-*lacZ*-transduced cells using the same experimental conditions (44 testes analyzed; data not shown).
[a]The viral titer was determined using canine Cf2TH cells and expressed as a mean of two titrations per experiment.
[b]Number of cells/testis was calculated based on the number of testis cells originally placed in culture (8×10^6 cells in all experiments, except in experiment 2, in which 7.6×10^6 cells were used). Cultured cells were harvested after 2 days and suspended in 120 μl (experiments 1–3 and 5), or 150 and 140 μl (experiments 4 and 6), respectively. Approximately 10 μl of each cell preparation was injected into a recipient testis. Thus, in experiment 1, 6.7×10^5 cultured testis cells (8×10^6 cells ×10 μl/120 μl) were injected per recipient testis.
[c]Values in parentheses indicate the number of recipient testes analyzed 6 months following transplantation. Other recipient testes were analyzed 2 or 3 months following transplantation. Thus, in experiment 2, four out of 11 recipient testes were analyzed at 6 months and no colonies were found in these four testes.
[d]Testis cell TE represents the number of colonies generated from 10^6 testis cells originally placed in culture.
[e]Mean ± S.E.M. Average of three experiments.
[f]Total of three experiments.

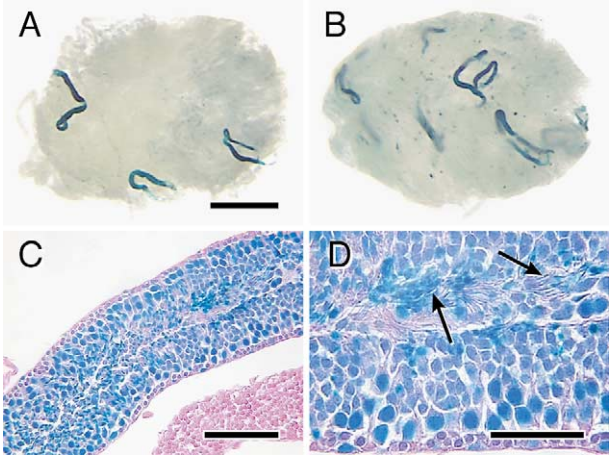

Fig. 1. Spermatogenesis arising from GSCs transduced in vitro by EF-n*lacZ* vector following transplantation into recipient testes. Recipient testes were stained for *lacZ* activity to detect colonies of spermatogenesis expressing the reporter gene. A: Spermatogenic colonies derived from adult donor cells. Three months after transplantation. B: Spermatogenic colonies derived from pup donor cells. Three months after transplantation. In A and B, blue segments of seminiferous tubules represent colonies of donor-derived spermatogenesis arising from transduced GSCs. C: Histology of spermatogenic colony derived from transduced pup GSCs. Two months following transplantation. Counter-stained with eosin. D: Higher magnification of C. Note qualitatively complete spermatogenesis with spermatozoa (arrows). Scale bars: A and B = 2 mm, C = 100 μm, D = 40 μm.

the *lacZ* marker gene were established in a total of 44 recipient testes analyzed from three experiments with both donor ages. Either this vector did not transduce GSCs or expression of the reporter gene was suppressed in GSCs and differentiated germ cells arising from transduced stem cells. Suppression of reporter gene expression appears a more likely cause because expression of the marker gene was seen in transduced testis cells in vitro (not shown), and the CMV-*lacZ* vector used in this study also transduced neural cells but its expression was silenced (D.J. Watson and J.H. Wolfe, unpublished data).

In contrast, following transduction by the EF-n*lacZ* vector, transplantation of adult and pup donor cells resulted in establishment of spermatogenic colonies arising from transduced GSCs in 28.6% (8/28) and 100% (23/23) of recipient testes, respectively (Fig. 1 and Table 1). The transduced stem cells generated complete spermatogenesis following transplantation, as indicated by the production of spermatozoa in these colonies (Fig. 1D). Although all recipient testes contained donor-derived colonies at all time points when pup donor cells were used (Table 1, line 8), no colonies were observed in recipient testes analyzed 6 months following transplantation, when adult donor cells were used (Table 1, line 4). However, when the data were examined by chi-square analysis, the difference between adult testis cells at 2–3 months vs. 6 months was not significant ($P = 0.368$), suggesting that the absence of adult donor-derived colonies at 6 months resulted from the small number of recipient testes analyzed, rather than attenuation of marker gene expression.

To evaluate the regeneration efficiency of donor-derived spermatogenesis arising from GSCs transduced by the EF-n*lacZ* lentivirus, we determined the testis cell transduction efficiency (TE) for adult and pup donor cells. The TE is calculated from the number of spermatogenic colonies expressing the marker gene per 10^6 donor testis cells originally placed in culture (Table 1), and each colony is considered to arise from a single stem cell [7,8]. Thus, the value indicates the number of transduced donor stem cells that colonized recipient testes as a fraction of all donor testis cells cultured. When adult testis cells were used, 19 colonies were obtained in 28 recipient testes, which were transplanted with 6.6×10^5 cells/testis (Table 1, line 4), resulting in a transduction efficiency of 1.0 [(19 colonies) $\times 10^6$/(6.6×10^5 cells/testis $\times$ 28 testes)]. Calculated in the same manner, the value for pup testis cells was 4.6 (Table 1, line 8). Therefore, transduction efficiency was 4.6-fold greater for pup than adult donor testis cells. This difference was not due to variation in lentivirus titer among experiments because no correlation was observed between testis cell TE and virus titer (Table 1, columns 3 and 8).

These results, which suggest that pup GSCs are more efficiently transduced than adult GSCs, are more compelling when one considers that pup testes contain a lower stem cell concentration than adult cryptorchid testes [18]. We previously demonstrated that when donor cells with a reporter transgene were transplanted without culture, 358 and 140 colonies were established per 10^6 donor cells derived from adult cryptorchid and pup testes, respectively [8]. Using these values as denominators, we next calculated the stem cell TE, which represents the percentage of GSCs that were transduced in vitro. We found that 0.3% (1.0/358) of adult GSCs and 3.3% (4.6/140) of pup GSCs originally placed in culture were transduced by lentiviral vectors, indicating that stem cell TE was 11-fold (3.3/0.3) higher for donor pup than adult GSCs (Table 2). A similar age-related difference in testis cell TE and stem cell TE was observed in our previous studies of GSC transduction by an MMLV-based vector [7,8]. When a MMLV vector was used, testis cell TE and stem cell TE were

Table 2
Comparison of male GSC transduction by lentiviral and MMLV-based vectors

Vector type	TE[a]	Adult donor	Pup donor	Pup/adult[b]
Lentivirus[c]	Testis cell TE[e]	1.0	4.6	4.6
	Stem cell TE[f]	0.3	3.3	11
MMLV[d]	Testis cell TE[e]	0.7	2.5	3.6
	Stem cell TE[f]	0.2	1.8	9

[a]Transduction efficiency.
[b]Ratio of TE values for pup and adult donor cells.
[c]Male GSC transduction by a lentiviral vector (this study).
[d]Male GSC transduction by a MMLV-based vector [7].
[e]Testis cell TE represents the number of colonies generated from 10^6 testis cells originally placed in culture.
[f]Stem cell TE represents percentage of GSC transduced in vitro by viral vectors. Stem cell TE for MMLV was calculated in the same manner as in this study; 0.7/358 = 0.2% for adults and 2.5/140 = 1.8% for pups.

approximately 3.6-fold and nine-fold higher for pup than adult GSCs, respectively (Table 2).

Calculation of transduction efficiency for both stem cell and testis cell populations from adults and pups allowed identification of factors contributing to successful regeneration of spermatogenesis from transduced GSCs. First, stem cell TE values were higher (~10-fold) for pup than adult GSCs regardless of viral vector types (Table 2), suggesting that either the level of viral receptor expression or the cell cycle activity is higher in pup than adult GSCs, since these are two important limiting factors in viral transduction [5,6]. However, it is unlikely that the difference in expression levels of viral receptors was a significant cause, since this lentivirus does not require specific proteins on the target cell surface for viral entry [11]. Rather, it is more likely that pup GSCs are more active in cell cycle progression than adult GSCs, which results in a better transduction efficiency. This would apply particularly to MMLV retroviral vectors, since the rate of host cell division is a significant limiting factor for these viruses [5,6]. Although the lentivirus is capable of transducing non-dividing cells, transduction of quiescent cells arrested at the G_0 stage in the cell cycle is less efficient due to a requirement for deoxynucleoside triphosphates that can lead to a block at the reverse transcription step [19–23]. Therefore, our results using lentiviral and MMLV-based vectors suggest that ~10-fold more pup GSCs are actively progressing through the cell cycle in vitro than adult GSCs. Second, regardless of vector types, the age-related difference of testis cell TE was less than that of stem cell TE (~4-fold vs. ~10-fold, Table 2), probably because of the lower concentration of donor stem cells in pup vs. adult cryptorchid testes [18]. In addition, regeneration efficiency of spermatogenesis arising from transduced GSCs is also influenced by recipient environment. By using infertile pups rather than infertile adults as recipients, regeneration of spermatogenesis following transplantation can be increased for both pup and adult donor cells [7,8]. Combined with our finding that pup donor cells are more efficiently transduced, this shows that the best system for viral vector transduction of male GSCs and transgenesis is provided by using donor cells from pups for transduction followed by transplantation into infertile recipient pup testes.

It has been suggested that self-inactivating lentivirus-based vectors have several potential advantages relative to ecotropic MMLV-based vectors [5]. Lentiviral vectors can: (1) be pseudotyped with VSV-G to achieve high titers ($>10^8$ U/ml) and to allow transduction of a broad range of host cell types and species; (2) deliver a vector gene that is expressed for long periods without transgene silencing because negative regulatory elements in the long terminal repeat (LTR) are absent; and (3) transduce both dividing and non-dividing cells. However, the results of our present and previous studies indicate that transduction efficiency of male GSCs by a lentiviral- and MMLV-based vector is similar (Table 2). Several relevant observations are consistent with our findings. First, Barrette et al. [24] demonstrated that a mouse-specific ecotropic MMLV vector transduced mouse hematopoietic stem cells (HSCs) as efficiently as a lentiviral vector, indicating that an envelope protein appropriate for the host cell species allows efficient transduction of HSCs by MMLV. Likewise, transduction efficiency by ecotropic MMLV and lentivirus was similar in our studies with mouse GSCs. Both lentiviral and MMLV vectors pseudotyped with VSV-G can be produced at high titers, and both vectors when pseudotyped will have a wide range of host species and cell types [25,26]. Thus, while our studies were done with mice, they suggest that VSV-G pseudotyped lentiviral and MMLV vectors could have similar efficiencies in transducing male GSCs derived from other animal species. Second, recent observations indicate that vector gene silencing can be a problem with both lentivirus and MMLV [5,27]. In the present study, the CMV enhancer/promoter was not effective in achieving lentiviral transduction and expression in GSCs. In contrast, MMLV [7,8] and lentiviral (present study) vectors with other promoters were demonstrated to successfully transduce GSCs without transgene silencing. Therefore, transgene expression can be affected by vector construction (e.g. LTR, internal promoter, etc.) for both MMLV and lentiviral vectors. Third, it has been reported that lentiviral transduction requires host cell cycle progression to at least the G_{1b} stage in HSCs [23]. Since at least 75% of HSCs are resting at the G_0 stage in the cell cycle [28], this high percentage of quiescent cells may preclude efficient transduction by lentiviral vectors [22,23]. The majority of GSCs also is believed to be quiescent [1], which may be the cause of similar transduction efficiencies for lentiviral and MMLV vectors in GSCs. Thus, it appears that the advantages of pseudotyping and the problems of vector construction (e.g. LTR- and promoter-type) are applicable to both lentiviral and MMLV vectors, and cell cycle kinetics of the target cell may be the major factor in determining transduction in these GSC populations. A similar situation may exist for other stem cells.

The transduction efficiency for GSCs found in the present study for lentiviral vectors was similar to those found previously for MMLV vectors using the same experimental conditions. Thus, if appropriate modifications were made to each vector (e.g. pseudotyping, internal promoter, etc.), both viral vectors should prove useful in generating transgenic animals in a wide range of species. In addition to optimizing vector characteristics, our studies suggest that improvement of male GSC transduction by either vector will require development of techniques to activate the cell cycle in GSCs (e.g. culture conditions, growth factors, etc.). Finally, based on our results in this and previous studies, the use of donor stem cells from immature animals transplanted to immature recipients will provide the best system to generate transgenic animals using spermatogonial stem cell transplantation in mice and possibly other species.

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