Retrovirus-mediated gene delivery into male germ line stem cells

Makoto Nagano, Takashi Shinohara, Mary R. Avarbock, Ralph L. Brinster*

Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104-6009, USA

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Abstract The male germ line stem cell is the only cell type in the adult that can contribute genes to the next generation and is characterized by postnatal proliferation. It has not been determined whether this cell population can be used to deliberately introduce genetic modification into the germ line to generate transgenic animals or whether human somatic cell gene therapy has the potential to accidentally introduce permanent genetic changes into a patient's germ line. Here we report that several techniques can be used to achieve both in vitro and in vivo gene transfer into mouse male germ line stem cells using a retroviral vector. Expression of a retrovirally delivered reporter *lacZ* transgene in male germ line stem cells and differentiated germ cells persisted in the testis for more than 6 months. At least one in 300 stem cells could be infected. The experiments demonstrate a system to introduce genes directly into the male germ line and also provide a method to address the potential of human somatic cell gene therapy DNA constructs to enter a patient's germ line.

Key words: Male germ line; Stem cell; Retrovirus; Gene delivery

1. Introduction

Spermatogonial stem cells, the male germ line stem cells in animals, divide throughout life. After puberty, they generate large numbers of differentiating spermatogonia that become spermatocytes, which undergo meiotic recombination to generate genetic diversity. Spermatids are then formed, which evolve into spermatozoa. Thus, stem cells are the foundation for spermatogenesis and male reproduction. Attempts to infect germ line stem cells with viral vectors or transfect them by other techniques have met with little success. Despite a long history of retroviral studies, there are no reports that show direct infection of postnatal male germ line stem cells. In mice, where retroviral infection of germ line stem cells is best characterized, both male and female stem cells can be infected before birth [1]. A series of classical studies in mice demonstrated that germ line transmission was high when preimplantation embryos were infected and very low ($<0.4\%$) when postimplantation embryos were infected [2–4]. If newborn mice were injected intraperitoneally with retroviruses, Leydig cells were infected but not germ cells [5]. In sheep, it was reported that no germ line transmission occurred when retrovirus supernatant or producing cells were injected into embryos in utero [6]. Use of other techniques to introduce genes into male germ line stem cells has been limited. Recently, in vivo whole-testis electroporation and lipofection by injecting DNA construct into mouse seminiferous tubules were described, but quantitative data on stable transfections into stem cells were not provided [7–10]. Taken together, the reports on infection by viral and transfection by non-viral techniques suggest that delivery of genes to postnatal male germ line stem cells is difficult. However, postnatal stem cells can be readily harvested compared to prenatal stem cells and continue to divide throughout life. Thus, they represent a unique and valuable resource to modify the germ line in any species. Recent development in the spermatogonial transplantation technique now allows an accurate determination of the presence of stem cells and assessment of the efficiency of various gene delivery attempts into stem cells [11–13]. In this transplantation system, a cell population containing putative spermatogonial stem cells is microinjected into the seminiferous tubules of an infertile recipient mouse, in which only a stem cell can generate a colony of spermatogenesis. Therefore, a stem cell marked by expression of a reporter gene can be unequivocally identified after transplantation. We used this assay method combined with a replication-defective ecotropic retroviral vector that contained the *Escherichia coli lacZ* structural gene, with the promoter of the phosphoglycerate kinase-1 (*Pgk*) gene (Gen$^-$PGKβgal) [14], to examine the potential of several infection systems to introduce a new gene into spermatogonial stem cells of postnatal mice.

2. Materials and methods

2.1. Animals

Donor cells were obtained from two sources. Neonatal testis cells were from C57BL/6×129/Sv (B6/129) F2 hybrid mice, 5–8 days of age. Adult cells were from C57BL/6 (B6) males made cryptorchid at 6–8 weeks of age, by securing the testes inside the abdominal cavity [15–17], and used 2 months later. Two types of recipient mice were prepared. B6 mice made cryptorchid were used for the in vivo infection system, and B6/129 F1 hybrid males treated with busulfan to destroy endogenous spermatogenesis were used for all other infection systems [12]. Cell collection methods were as previously described [18–20].

2.2. Cell infection and transplantation

Three different procedures were used for in vitro infection: feeder infection, insert infection and periodic infection systems. For all in vitro systems, donor cells were cultured at 37°C at $2.2\pm0.1\times10^5$ cells/cm^2 with 4 μg/ml polybrene.

In the feeder infection system, donor testis cells were cultured on Gen$^-$PGKβgal virus-producing cells (10^5 cells/cm^2 in 25 cm^2 flasks), which were mitotically arrested with mitomycin C. Medium was changed and new feeder cells added on day 3. Cells were washed with Dulbecco's phosphate-buffered saline (D-PBS), harvested and transplanted into recipient testes on day 7.

In the insert infection system, donor testis cells were cultured on mitomycin C-treated STO mouse embryonic fibroblasts (5.2×10^4 cells/cm^2 in 6-well plates). Gen$^-$PGKβgal cells (8.6×10^4 cells/cm^2) were seeded on a permeable membrane in cell culture inserts (3 μm pore size); then the inserts were placed over the germ cell culture to

*Corresponding author. Fax: (1)-215-898 0667.

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separate virus-producing cells from germ cells. Medium and inserts with virus-producing cells were changed on days 3 and 5. Inserts were removed on day 7, cultures washed three times with D-PBS and incubated for 20 h. On day 8, cultures were washed with D-PBS, harvested and transplanted into recipient testes.

In the periodic infection system, donor testis cells were cultured on mitomycin C-treated STO cells (5.2×10^4 cells/cm^2 in 25 cm^2 flasks). Two days later, the cells were fed with virus-containing medium for 2 h, then incubated with regular medium for 1 h. This cycle was repeated 3–4 times a day for 6 days. Then the cells were washed and harvested as in the insert system.

For in vivo luminal infection, a mixture of Gen$^-$PGKβgal cells and freshly collected testis cells (1:1) was injected into recipient testes. Methods for cell culture and transplantation were as previously described [18,20,21]. The retroviral vector (Gen$^-$PGKβgal), based on the Moloney murine leukemia virus, and its producer cell line (derived from GP+E-86 packaging cells) were a generous gift of P. Soriano and were maintained as described [14]. The original titer of Gen$^-$PGKβgal virus on Swiss 3T3 cells was $2.5\pm0.4\times10^5$ colony forming units (cfu)/ml.

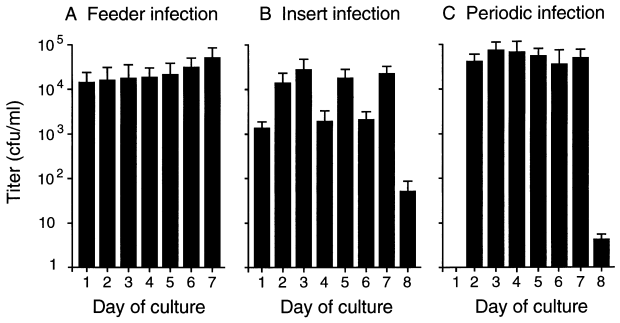

Fig. 1. Retroviral titers in mouse testis cell culture supernatants. A: Feeder infection system. B: Insert infection system. C: Periodic infection system. Viral titers in medium supernatant are indicated by bars, and means during culture periods with viruses were: $A=2.9\pm0.5\times10^4$; $B=1.4\pm0.3\times10^4$; $C=5.6\pm0.6\times10^4$ cfu/ml. Titers on day 8 were 55.1 ± 29.7 and 4.3 ± 0.9 cfu/ml for B and C, respectively. All values are means ± S.E.M. for three or more determinations.

3. Results and discussion

In preliminary experiments, we compared different techniques to deliver a foreign gene to testis cells. For in vitro gene delivery, we used lipofection and retrovirus infection, and for in vivo gene delivery, whole-testis electroporation [7–10]. Since only retrovirus infection resulted in unequivocal introduction of a marker gene into spermatogonial stem cells, we focused on this approach.

In our initial experiments, we microinjected Gen$^-$PGKβgal virus-producing cells into the seminiferous tubules of recipient mice that were made cryptorchid by securing the testes inside the abdominal cavity. After injection, the testes were returned to the scrotum, anticipating that the in situ microenvironment of seminiferous epithelium surrounding stem cells should facilitate continuing stem cell mitosis, a prerequisite for retroviral integration and expression. Cryptorchid testes are devoid of the multiple layers of differentiating germ cells, which are characteristic of the normal tubular epithelium, and contain only dividing primitive spermatogonia and Sertoli cells on the basement membrane of the tubules [15–17]. Despite these supposedly favorable characteristics, no colonies of spermatogenesis arising from transduced stem cells were found in the 14 testes injected (Table 1, line 1), apart from one recipient testis containing a very small cluster of transduced spermatogonial-type cells. However, this small cluster did not generate a blue colony indicative of developing spermatogenesis. A potential explanation for the inability of virus to infect spermatogonial stem cells and generate colonies expressing the transgene is that supporting somatic Sertoli cells cover and shield stem cells from the virus present in the tubule lumen.

To determine if spermatogonial stem cells could be infected when displaced from their protected niche in the tubule, we employed three different in vitro systems: feeder infection, insert infection and periodic infection (Fig. 1). These systems were selected to maximize the titer of virus to which donor cells were exposed and to vary conditions of infection. In the feeder infection system, donor testis cells were cultured on virus-producing feeder cells, and at the end of culture, donor and feeder cells were transplanted together into recipient testes. In the insert and periodic systems, donor testis cells were cultured on STO feeder cells, and virus-producing cells

Table 1
Colonization of recipient testes by male germ line stem cells infected in vitro or in vivo by a retroviral vector

Infection system	Type of testis cell	Number of experiments	Number of testis cells cultured ($\times10^6$)[a]	Number of cells injected per testis ($\times10^5$)[b]	Number of testes analyzed[c]	Number of testes colonized	Number of colonies	TCE[d]
In vivo	–	7	–	12.9 ± 1.7	14	0	0	0
Feeder	Neonate	11	8.7 ± 0.9	8.4 ± 0.9	28	13	20	8.5
	Adult	10	6.5 ± 1.2	6.2 ± 1.0	36	6	9	4.0
Insert	Neonate	9	10.9 ± 1.3	10.9 ± 1.0	43	3	3	0.6
	Adult	5	9.6 ± 1.2	12.3 ± 1.5	35	5	6	1.4
Periodic	Neonate	3	7.3 ± 1.1	7.8 ± 1.0	14	10	27	24.7
	Adult	3	5.7 ± 0.3	5.8 ± 0.7	15	4	6	6.9
Control (insert)	Adult	4	8.2 ± 1.2	4.4 ± 0.3	62	60	1068	391.5
Luminal	Neonate	4	–	4.7 ± 0.3	23	16	42	38.9
	Adult	4	–	5.0 ± 0.0	23	5	8	7.0

[a]Mean ± S.E.M. Number of testis cells originally placed in culture per experiment.
[b]Mean ± S.E.M. Number of cells injected was calculated based on the percent of recovered cells actually injected into a recipient testis times the number of testis cells placed in culture (in vitro infections). All cells recovered or collected cannot be injected into a recipient testis because the seminiferous tubule volume is limited. For in vivo infection, only Gen$^-$PGKβgal virus-producing cells were injected into recipient seminiferous tubules. For luminal infection, only the number of testis cells is shown.
[c]Testes were analyzed 2–3 months after cell transplantation. Differentiation from primitive type A_1 spermatogonia to spermatozoa takes 35 days in mice [24].
[d]TCE = (number of colonies)(10^7)/(number of cells injected per testis)(number of testes analyzed).

were cultured separately from the testis cells (see Section 2). In these two systems, the titer of virus particles associated with testis cells at the time of transplantation was minimized by washing the culture 1 day before transplantation and incubating the culture with fresh medium without the virus (Fig. 1). Donor testis cells for infection were obtained from two sources. The first was neonatal testes, 5–8 days of age, at which stage the stem cells are proliferating. In addition, neonatal testes at this stage should be enriched for stem cells because formation of differentiating germ cells is just beginning. The second source was adult experimental cryptorchid testes, in which spermatogonia are actively dividing [15,17], and the testis cell population is enriched for stem cells because differentiating germ cells are destroyed by the high core body temperature [16,22].

To determine the efficiency of stem cell transduction by retrovirus, both neonatal and cryptorchid testis cells were exposed to virus using each of the three infection systems and then transplanted into recipient mouse testes (Table 1). Two to three months after transplantation, 171 recipient testes were analyzed, and 71 blue areas representing colonies of spermatogenesis originating from retrovirally transduced stem cells were found (Table 1; Fig. 2). Spermatogenesis from donor stem cells that are not infected with the retroviral vector or in which the vector is not expressed will not produce blue-stained cells. In addition, it is believed that each area of spermatogenesis generally represents colonization by the progeny of one stem cell [12,13]. Therefore, at least 71 stem cells integrated and expressed the retroviral vector (Table 1, lines 2–7). The transduction/colonization efficiency (TCE) for the two types of testis cells in the three infection systems varied, but two patterns were observed (Table 1). First, periodic and insert infections produced the highest and lowest TCE, respectively. This could be due to differences in viral titers in each infection system (Fig. 1) or differences in the ability of the systems to support stem cells. Second, higher transduction efficiency was achieved with neonatal cells than with adult cryptorchid cells in the periodic and feeder systems, but this pattern was opposite in the insert system. Cells derived from neonatal testes divide more rapidly than those from adult cryptorchid testes. In addition, the insert system contains more cells per cm^2 culture surface than the periodic or feeder systems. Therefore, there could be more rapid depletion of nutrients in the neonatal insert compared to the adult cryptorchid insert culture environment, and poor stem cell survival result in the former. The better results with neonatal cells in periodic and feeder systems may reflect a greater number of stem cells in the original neonatal cell population, better stem cell proliferation, or both.

Establishment of complete spermatogenesis by donor cells (Fig. 2) can be achieved only from spermatogonial stem cells. Therefore, these results clearly demonstrate that retroviral vectors can be used to deliver genes into male germ line stem cells of two different developmental ages (neonatal and adult) with three separate systems. Since successful retroviral infection with resultant gene expression requires cell division for chromosomal integration of the viral genome, the present results also indicate that the stem cells must be replicating in vitro. Normal spermatogenesis with characteristic structural morphology and mature spermatozoa were found in the blue areas colonized by transduced stem cells (Fig. 2B–D). Expression of the *Pgk-lacZ* transgene in areas of spermatogenesis was present at least 6 months after transplantation, and previous studies indicate that colonies arising from transplanted stem cells are permanent and produce spermatozoa that transmit the donor haplotype to progeny [11,21,23].

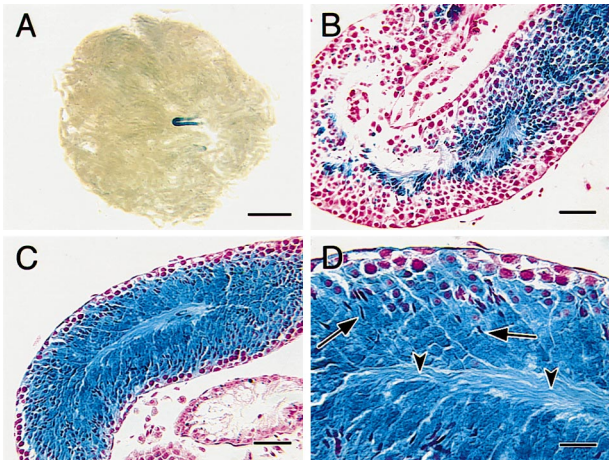

Fig. 2. Spermatogenesis produced from stem cells into which a transgene was introduced by retroviral infection during in vitro culture. Blue areas represent colonies of spermatogenesis arising from donor stem cells transplanted to recipient testes, which were stained to demonstrate expression of the viral transgene 2–6 months after cell transplantation. A: Spermatogenic colony (blue) derived from donor neonatal stem cells transduced in the periodic infection system. Whole mount of testis was stained 6 months after cell transplantation. B: Histological section of spermatogenic colony derived from adult cryptorchid stem cells transduced with the insert infection system, analyzed 3 months after cell transplantation. Expression of transgene indicated by blue staining is seen primarily in differentiated, late stages of spermatogenesis in this section. C: Histological section of spermatogenic colony derived from neonatal stem cells transduced by the periodic infection system, analyzed 3 months after cell transplantation. Expression of transgene is present in germ cells at all stages of differentiation. Differences in staining intensity and pattern between B and C may reflect the effect on gene expression of the integration site of virus and transgene in the stem cell chromosome [2,25]. Similar differences were observed among colonies produced using each system. D: Higher magnification of C. Presence of spermatozoa heads (arrows) and spermatozoa tails (arrowheads) indicates that complete spermatogenesis has been established in the colony. Non-transduced transplanted stem cells also generate complete spermatogenesis in recipient testes beginning at approximately 2 months after donor cell transplantation [12]. Stain: 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), A–D; nuclear fast red, B–D. Scale bars: A = 2 mm, B and C = 50 μm, D = 20 μm. Testes of recipient mice were analyzed for transgene expression of β-galactosidase by incubation with X-gal to produce blue staining [11,12].

The TCE represents the number of blue colonies produced from 10^7 testis cells originally placed in culture and is a measure of the ability of each infection system to introduce a foreign gene into stem cells (Table 1). However, the number of stem cells actually present at any time in culture cannot be determined because there are no morphological or biochemical markers to identify the stem cells in vitro. Therefore, a functional assay, such as testis cell transplantation, must be used to identify stem cell activity. To estimate the number of stem cells present at the end of culture, a control population of cryptorchid testis cells from a transgenic mouse expressing *lacZ* in male germ cells (C57BL/6J-*Gtrosa26*) was cultured using the insert system with GP+E-86 packaging cells that produced no virus. Then the total number of colonies generated by these cultures after transplantation was determined

(Table 1, line 8). This value is a measure of the number of stem cells that survived conditions similar to those experienced by cryptorchid cells infected using the insert system (Table 1, line 5), and therefore is equivalent to 100% stem cell TCE. Comparison of the TCE of adult cryptorchid cells exposed to virus (1.4; Table 1, line 5) with the control TCE (391.5; Table 1, line 8) indicates that about one in 280 stem cells was infected and expressed the transgene using the insert system.

On the basis of the above results, the ability to infect stem cells in vivo was reexamined using a system in which freshly collected testis cells and Gen$^-$PGKβgal virus-producing cells were coinjected into the seminiferous tubules of recipient mice (luminal infection). As shown in Table 1, the luminal infection resulted in a high TCE (Table 1; lines 9 and 10). However, a major factor likely contributing to this transduction efficiency is the much greater number of stem cells present in freshly collected donor cell populations without in vitro manipulation. When 10^5 cryptorchid cells were injected into a recipient testis immediately after collection, about 50 colonies were formed [22], which translates into a control TCE of 5000. This is a value more than 10 times greater than we found for control adult cryptorchid testis cells after 1 week in culture (391.5). Therefore, only about one in 714 (7.0/5000; Table 1, line 10/control) stem cells appear to be infected with the viral vector in the luminal system. Thus, while the absolute number of stem cells infected is greater with the in vivo luminal system, the efficiency of infection of cryptorchid stem cells is about 2.5 times (714/280) better with the in vitro insert system.

Previous studies suggested that postnatal male germ line stem cells were difficult or impossible to transduce using viral agents. However, the absence of a functional assay to directly measure stem cell activity interfered with a conclusive determination. Although our initial studies with an in vivo infection approach agreed with published reports, the protection of the stem cell in a niche surrounded by Sertoli and other somatic cells could explain the inability to detect infection. This niche may offer a degree of protection for patients undergoing somatic cell gene therapy. However, our studies indicate that the potential exists for infection of the male germ line by viral vectors. In addition, it is estimated that at least 4% of these insertion sites will disrupt endogenous gene activity [4]. Using several infection systems, which provided viral access to the stem cell, we clearly established that viral transduction of neonatal and adult spermatogonial stem cells can be achieved at quite high levels in vitro and in vivo. Moreover, the use of higher titer virus preparations available from other vector-producing systems might allow greater levels of infection. The present studies lay the groundwork for introduction of new genes directly into the male germ line of many animal species, and suggest that other transfection approaches should be feasible when used with spermatogonial stem cell transplantation.

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