Asian Journal of Andrology (2019) 21, 190–195
www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Germ Stem Cells

Direct modification of spermatogonial stem cells using lentivirus vectors *in vivo* leads to efficient generation of transgenic rats

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Spermatogonial stem cells (SSCs) transmit genetic information to the next progeny in males. Thus, SSCs are a potential target for germline modifications to generate transgenic animals. In this study, we report a technique for the generation of transgenic rats by *in vivo* manipulation of SSCs with a high success rate. SSCs in juvenile rats were transduced *in vivo* with high titers of lentivirus harboring enhanced green fluorescent protein and mated with wild-type females to create founder rats. These founder rats expressed the transgene and passed on the transgene with an overall success rate of 50.0%. Subsequent generations of progeny from the founder rats both expressed and passed on the transgene. Thus, direct modification of SSCs in juvenile rats is an effective means of generating transgenic rats through the male germline. This technology could be adapted to larger animals, in which existing methods for gene modification are inadequate or inapplicable, resulting in the generation of transgenic animals in a variety of species.
Asian Journal of Andrology (2019) **21**, 190–195; doi: 10.4103/aja.aja_80_18; published online: 12 October 2018

Keywords: germline modification; lentivirus; spermatogonial stem cell; testis; transgenic animal

INTRODUCTION

Spermatogenesis, a series of complex processes that produce millions of spermatozoa daily in the seminiferous tubule of the testis, occurs through serial divisions and differentiation of spermatogonial stem cells (SSCs).[1] SSCs in adult rats have high production capacity, and up to 4096 individual sperm cells are produced from a single SSC in the spermatogenic cycle.[1] As the foundation stem cells of spermatogenesis, SSCs are the mediators of male genetic information to the offspring. Therefore, when genetic modification is achieved at the SSC level, transgenic offspring are produced, and germline modification allows the stable delivery of the transduced characteristics to the descendants. Since spermatozoa produced from SSCs could be a vehicle by which male genes are delivered, transplantation is an essential technique for SSC-mediated transgenesis.[2,3]

SSC-mediated transgenesis has been optimized for use in SSCs through purification techniques and the development of efficient recipient animal production techniques.[4–8] These techniques have allowed the production of transgenic animals via genetic modifications and transplantation of highly purified SSCs. Several groups have already produced transgenic rats using SSC transduction.[9,10] Although these techniques allow male germline modification, there are several limitations that need to be overcome. To fully accomplish the SSC-mediated germline modification in rats, it is necessary to prepare the rat recipient animal model before transplantation. In mice, the best recipient that has been identified is the congenitally infertile W mutant mouse pup, which lacks endogenous spermatogenesis due to a mutation in the c-kit receptor tyrosine kinase. Several groups have reported the creation of transgenic mice by transplanting SSCs with modified genes into W recipients. However, congenitally germ cell-deficient breeds are not available for rats and other species. Instead, focal irradiation and chemotherapeutic drugs have been widely used to deplete endogenous male germ cells in nonmice recipients.[4,6,11,12] The major drawbacks of SSC transduction into such recipients are the low fertility rate and the lengthy time requirement (exceeding 6 weeks) to ensure successful depletion of endogenous germ cells. Alternatively, direct *in vivo* germline modification in donor SSCs would obviate the need for recipient animals. In mice, modification of SSCs using viral vectors directly injected into the seminiferous tubule or the interspace of the testes has been reported.[13–15] However, such direct modification of SSCs has not been performed in other animal models.

Among retroviruses, lentivirus causes chronic illnesses in the host organisms they infect. They are distinguished from other retroviruses by their ability to infect both dividing and nondividing cells, which has led to their utility as gene delivery vehicles,[16] especially in slowly dividing

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Received: 03 January 2018; Accepted: 05 August 2018

cells, such as SSCs. Several groups have reported the development of transgenic rats using the conventional lentivirus-mediated SSC transduction and transplantation technique.[9,10] To refine transduction conditions, we previously evaluated the transduction efficiency of mouse SSCs using various durations of lentiviral treatment, various multiplicities of infection and addition of different concentrations and types of polycationic agents.[17] As a model for human disease, rats have many advantages over mice and other animals. Due to their larger size and ease of surgical manipulation, rats are superior to mice in pharmacokinetic and transplantation studies. Furthermore, their greater intelligence allows better assessment of high-level learning.[18,19] However, the development of transgenic techniques in rats has been limited due to characteristics of the rat model that differs from the well-established mouse system. The main problem in using rats is the overall lack of experience in egg pronuclear injection techniques, compared to mice, which often restricts the possibility of experiments in rats.

Recently, germline competent embryonic stem cells were created from rat blastocysts, allowing the production of transgenic rats by injecting modified embryonic stem cells into blastocysts.[20,21] However, the technique is expensive, time-consuming, and labor intensive, and requires several female donors. Moreover, mosaic/chimeric mutant progeny is often created during the developmental process after blastocyst injection. On the other hand, using rat SSCs to produce transgenic rats may be more efficient than those alternative techniques. In this study, we report the efficient production of transgenic rats by spermatogonial gene modification using lentiviral vectors.

MATERIALS AND METHODS

Animals and transplantation

All procedures were performed according to the approved guidelines for the ethical treatment of animals as established in the regulations of Chung-Ang University (Anseong, Korea; IACUC No. 2015-00016) and in compliance with standard international regulations. Sprague–Dawley (SD) rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). Recipient rats were intraperitoneally anesthetized with both ketamine (75 mg kg^{-1}; Yuhan, Seoul, Korea) and medetomidine (0.5 mg kg^{-1}; Orion, Orionintie, Espoo, Finland) for transplantation. To generate transgenic offspring, immature recipient SD pups (5, 8, and 12 days old) were injected with approximately 4 μl of lentivirus into the seminiferous tubules via the efferent duct (**Supplementary Figure 1**). To assess the toxicity of polycationic agents on the testis, 4 μg ml^{-1} of polybrene (Sigma-Aldrich, St. Louis, MO, USA) and 2 μg ml^{-1} of dioctadecylamidoglycyl spermine (DOGS; Promega, Madison, WI, USA) were dissolved in Dulbecco's phosphate-buffered saline (PBS) and approximately 20 μl was injected into the seminiferous tubule of the testes of 3-week-old rats. The concentrations of polybrene and DOGS were chosen based on our previous study.[17]

Lentiviral vector

Frozen lentivirus conjugated with enhanced green fluorescent protein (*EF1-eGFP*) was purchased from Cellexium Biomedica Inc. (Taipei, Taiwan, China). The final titer of the lentivirus concentrate was 3.9×10^9 IU ml^{-1} as measured by quantitative PCR (titer was provided by Cellexium Biomedica Inc.). DOGS (2 μg ml^{-1}) and 10% Trypan blue dye (Thermo Fisher Scientific, Waltham, MA, USA) were added to the virus cocktail before microinjection into the testis tubule via the efferent duct. All procedures for the lentiviral preparation were performed on ice.

Analysis of pups and testes

To produce transgenic offspring from transduced rat SSCs, recipient SD rats transplanted with *EF1-eGFP* lentivirus were mated with SD females beginning 2 months after transplantation. Breeding was allowed to continue for 11–21 months, after which the recipient males were sacrificed and the testes were weighed. Pups positive for GFP expression were identified from litters by exposure to ultraviolet (UV) light. Using this method, both male and female founders were identified and used to generate GFP transgenic lines. Offspring from these founder animals were confirmed to be GFP-positive by UV light examination. Some pups were sacrificed and the organs were also examined for GFP expression by UV light.

At the end of each experiment, lentivirus-injected testes were collected and examined using fluorescent microscopy (TE2000-U; Nikon, Tokyo, Japan) to detect GFP-positive colonies of transduced SSC-derived spermatogenesis. Two months after the injection of the polycationic agent, testes were collected and prepared sections were stained with hematoxylin and eosin (H&E; Vector Labs, Burlingame, CA, USA) and examined by light microscopy (TE2000-U; Nikon) to assess the number of tubules filled with germ cells.

Genotyping and western blot

At 4 weeks postcoitum, F2- and F3-generation pups were genotyped by PCR. Tail samples were collected from the pups for DNA extraction. The specific primers used for genotyping were as follows: *eGFP*: 5'-ATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3' (forward primer), 5'-CTTGTACAGCTCGTCCATGCCGAG AGTGAT-3' (reverse primer); and glyceraldehyde-3-phosphate dehydrogenase: 5'-AGGCCGGTGCTGAGTATGTC-3' (forward primer), 5'-TGCCTGCTTCACCACCTTCT-3' (reverse primer). For western blot analysis, tail tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer and the protein quantified by the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Twenty-five micrograms of protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal anti-GFP antibody (1:500; Abcam, Cambridge, UK) and detected by enhanced chemiluminescence (ECL). Blots were stripped and re-probed with alpha-tubulin (1:1000; Abcam) as a loading control.

Statistical analysis

Statistical significance in the evaluation of toxicity between the polybrene and DOGS was evaluated using the Student's *t*-test. All statistical analyses were conducted using GraphPad Prism software (GraphPad, La Jolla, CA, USA). Differences resulting in P value < 0.01 were considered significant.

RESULTS

Effect of polycationic agents on testis development

The polycationic agents polybrene and DOGS are often used to increase the transduction efficiency in SSCs *in vitro*.[17] However, they are cytotoxic following virus infection. Two months after polycationic agent injection, the testes were harvested and sections were prepared and stained using H&E. The seminiferous tubules were classified as damaged when there was no evidence of regeneration of seminiferous epithelium or normal when complete seminiferous epithelium with spermatids and mature spermatozoa was evident. In the DOGS-treated rats, most of the seminiferous tubules had thick walls with narrow lumens 2 months following injection. Walls of the normal seminiferous tubules consisted of several layers of seminiferous epithelial cells, with the spermatogonia located near the basement membrane, spermatocytes in the middle, and the spermatozoa and spermatids protruding toward the lumen (**Figure 1a** and **1b**). In the polybrene-treated rats, many of the seminiferous tubules were damaged, with

even thinner walls and only one layer of spermatogonia apparent (**Figure 1c** and **1d**). The mean proportion of damaged tubules in the DOGS and polybrene groups (both $n = 3$) was 3.82% (standard error of the mean [s.e.m.]: 1.19%) and 64.23% (s.e.m.: 2.49%), respectively. The difference was statistically significant ($P < 0.0001$; **Figure 1e**). Based on these findings, lentiviruses were injected with 2 μg ml^{-1} DOGS in subsequent experiments.

Transduction of SSCs by lentivirus microinjection into seminiferous tubules

Mature seminiferous tubules comprise multiple layers of cells that contain differentiating germ cells and Sertoli cells, as well as tight junctions between the Sertoli cells.[1] In contrast, the immature testis comprises a single layer of spermatogonia lacking tight junctions. Based on these histological differences, we expected that lentivirus microinjected into immature seminiferous tubules would have a higher probability of successfully targeting the stem cell population, than lentivirus injected into mature seminiferous tubules. To assess the timeline of testis maturation, we collected the testes of 5-, 8-, and 12-day-old rats and microscopically analyzed the H&E-stained sections. Only the testes obtained at day 5 showed tubules with a single layer of spermatogonia without differentiating germ cells that would be expected in immature testes (**Figure 2**). In another experiment, lentivirus with 2 μg ml^{-1} DOGS was microinjected into the seminiferous tubules of 5-, 8-, and 12-day-old rats, and the transduction efficiency was analyzed by GFP fluorescence microscopy. In the testes injected on day 5 with lentiviral vector, strong GFP expression was observed in all cell types in the seminiferous tubule (**Figure 3**). However, GFP expression in testes injected on day 8 or 12 was apparent only as solitary dots in the seminiferous tubule, indicating that the lentivirus could not successfully transduce the SSCs (**Figure 3**).

Production of transgenic rats using in vivo modification of SSCs

We next examined whether the SSCs that were infected with the lentivirus could successfully produce transgenic animals. We microinjected *eGFP*-lentivirus into the seminiferous tubules of 5-, 8-, and 12-day-old rats with 7, 6, and 10 recipient rats in each age group, respectively (**Table 1**). After 8 weeks, the animals were each bred to 2 SD female rats to produce offspring. Although some of the injected animals could not sire offspring due to postoperative adhesions associated with the operation, 83.7% of the 5-day-old rats, 83.3% of the 8-day-old rats, and 100% of the 12-day-old rats injected with lentivirus remained fertile (**Table 1** and **Supplementary Table 1**). All resulting offspring were examined for the presence of GFP under UV illumination (**Figure 4a**). Of the 5-day-old rats, 50.0% (3/6) of the fertile animals had at least one GFP-expressing offspring during the analysis period, demonstrating that transgenic offspring can be derived from lentiviral-transduced SSCs. Conversely, the 8- and 12-day-old rats injected with lentivirus did not generate any transgenic offspring expressing GFP (**Table 1**). In the group that received the lentivirus on day 5, the percentage of transgenic animals produced from the founder males ranged from 1.2% (1/84) to 2.9% (2/69). Furthermore, the first evidence of transgene expression appeared in the progeny from a founder rat as early as 81 days after lentivirus injection, with an average of 86 days.

In a subsequent experiment, a female transgenic *eGFP* founder animal was produced by mating a lentivirus-recipient male (no. 1200) to wild-type SD females. The *eGFP* transgene was inherited and expressed by the founder animal and two subsequent generations of progeny from the founder, indicating stable integration of the transgene into the original donor SSC genome. The pedigree for the offspring produced from this recipient demonstrated that the *eGFP* gene was transmitted and expressed by both sexes, and inheritance followed a Mendelian pattern (**Figure 5**). Furthermore, the progeny generated from mating of the founder with a wild-type male expressed the GFP transgene in all tissues and organs (**Figure 4b**). Thus, stable

Table 1: Efficiency of transgenic rat offspring of different ages

Age (day)	*Injected animals (n)*	*Fertile animals, n (%)*	*Animals with transgenic offspring, n (%)*
5	7	6 (85.7)	3 (50.0)
8	6	5 (83.3)	0
12	10	10 (100.0)	0

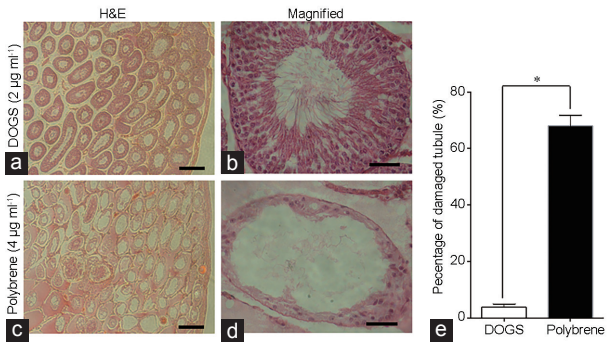

Figure 1: Toxicity of the polycationic agents on rat testes. Histology of rat testes after injection of polycationic agent. DOGS (2 μg ml^{-1}) and polybrene (4 μg ml^{-1}) were introduced into the seminiferous tubule of 3-week-old male rat testes. The testes were harvested 8 weeks later, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). (**a**) Testis harvested from a rat injected with 2 μg ml^{-1} DOGS. (**b**) Magnified image of **a**. (**c**) Testis harvested from a rat injected with 4 μg ml^{-1} polybrene. (**d**) Magnified image of **c**. (**e**) Quantification of spermatogenic seminiferous tubules in DOGS- and polybrene-injected testes. All data are presented as the mean ± standard error of the mean. Scale bars = 0.5 mm in **a** and **c**; scale bars = 50 μm in **b** and **d**. $^*P < 0.0001$, the percentage of damaged tubules after DOGS group is compared to polybrene-injected group. DOGS: dioctadecylamidoglycyl spermine.

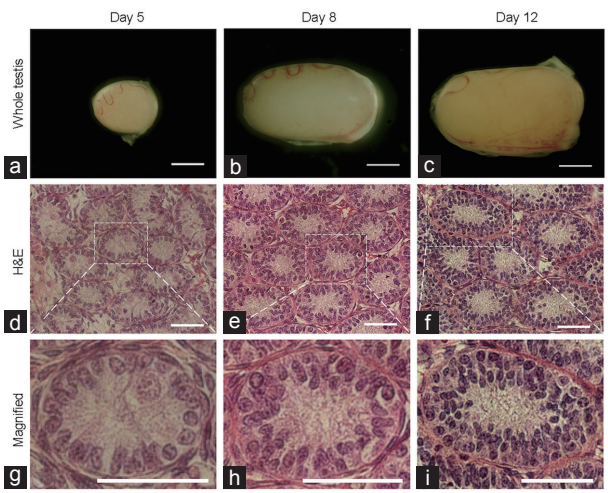

Figure 2: Macroscopic and histological analyses of testes from rats of different ages. Macroscopic appearance of (**a**) day 5, (**b**) day 8, and (**c**) day 12 testes. Histological appearance of (**d**) day 5, (**e**) day 8, and (**f**) day 12 testes. Each testis was stained with hematoxylin and eosin. (**g**–**i**) Magnified images of **d**–**f**, respectively. Scale bars = 2 mm in **a**–**c**; 60 μm in **d**–**i**. H&E: hematoxylin and eosin.

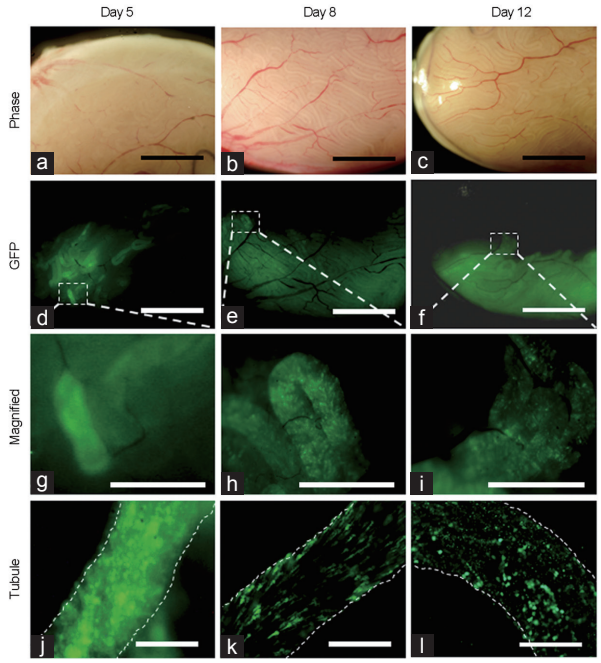

Figure 3: Lentiviral transduction of rat spermatogonial stem cells. Lentivirus expressing the *eGFP* gene was microinjected into the seminiferous tubules of rats of different ages. GFP expression was visualized by fluorescence microscopy at the end of the mating period. Green fluorescent tubules represent colonies from transduced spermatogonial stem cells. To isolate a single tubule, the testes were treated with 1 mg ml^{-1} collagenase for 3 min at room temperature and then with 1 mg ml^{-1} of DNase I for 5 min at room temperature in a shaker. Macroscopic appearance of (**a**) day 5, (**b**) day 8, and (**c**) day 12 testes transduced using lentivirus. GFP expression on transduced (**d**) day 5, (**e**) day 8, and (**f**) day 12 testes. The colonies are asymmetrically distributed toward the ends. (**g**–**i**) Magnified images of **d**–**f**, respectively. GFP expression pattern in single tubules collected from the transduced (**j**) day 5, (**k**) day 8, and (**l**) day 12 testes. Scale bars = 5 mm in **a**–**f**; 1.25 mm in **g**–**i**; 300 μm in **j**–**l**. GFP: green fluorescent protein; eGFP: enhanced green fluorescent protein.

integration and expression of a transgene can be achieved through the male germline using a lentiviral vector to modify the SSC genome *in vivo*.

DISCUSSION

In this study, donor rat testes were transduced with an *eGFP* transgene using direct injection of a lentiviral vector, which demonstrated efficient generation of transgenic offspring in 5-day-old recipient rats and natural mating. Transgenic rats have previously been produced by lentiviral transduction of SSCs *in vitro* followed by transplantation into busulfan-treated recipient rats.[9,10] In these studies, busulfan was used to deplete the endogenous stem cells before transplantation; the treatment damages the germ cell environment and affects fertility restoration.[22–24] In mice, techniques for the direct modification of SSCs following injection into the seminiferous tubule using concentrated retroviral vectors without specific preparation of recipients have been reported.[13] The authors of that study reported that 26% of the fertile animals sired offspring expressing the transgene during the analysis period. The success rate might be improved using lentivirus, since lentivirus can transduce both dividing and nondividing cells. Although multiple studies regarding genetic modification of the SSC genome have been conducted, a direct modification of rat SSCs has not been examined. When we used 5-day-old rats as recipients for direct

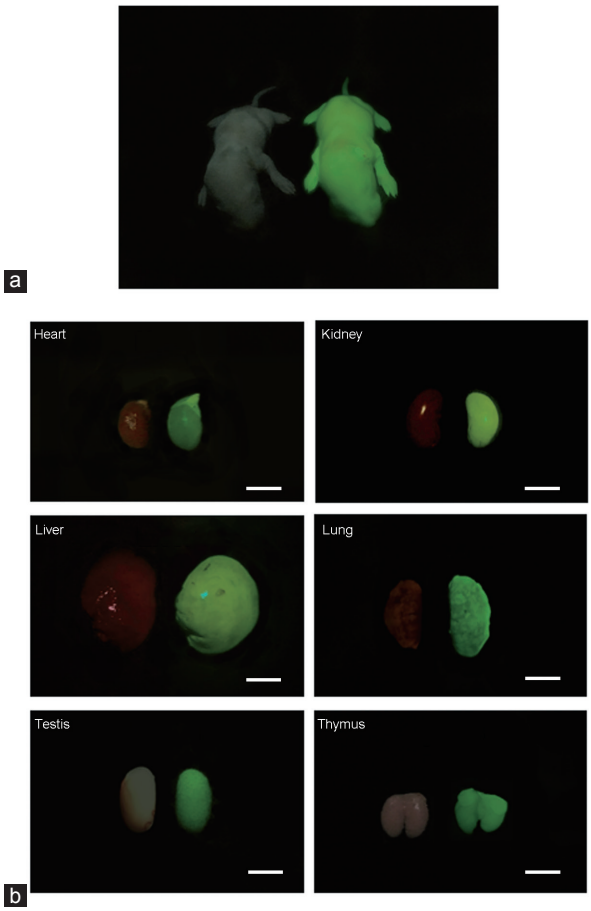

Figure 4: Production of transgenic rats by direct lentiviral transduction of rat spermatogonial stem cells followed by natural mating. (**a**) Green fluorescent protein (GFP) was expressed in newborn pups, which allowed the identification of transgenic pups and ultimately the establishment of founders. (**b**) Expression of the GFP transgene in various organs from rats generated from founder animals. Nonfluorescent wild-type SD organs are shown on the left. Scale bars = 0.5 mm. SD: Sprague–Dawley.

modification of SSCs *in vivo*, 50.0% (3/6) of the fertile animals sired offspring expressing GFP during the analysis period (**Table 1**). Because lentivirus can infect both dividing and nondividing cells, including germ cells,[9,10] it has a clear advantage over retrovirus, which can only infect dividing cells. Therefore, our technique overcomes the difficulties associated with both *in vitro* infection and *in vivo* transduction and enhances the capability to manipulate SSCs.

Two groups recently reported less time-consuming and relatively inexpensive techniques for the generation of transgenic mice using electroporation and lentiviral transduction *in vivo*.[15,25] In these studies, both the intratubular injection of DNA followed by electroporation and the intertubular injection of lentivirus generated ubiquitously expressing transgenic mice. Based on these reports, we also injected high titers of the lentiviral cocktail into the intratubular space of SD rat testes. However, no transgenic pups were produced, and we could not find any GFP-positive germ cells in the recipients' testes (data not shown). The difference may be due to the thickness of the basement membrane of the rat seminiferous tubules as well as other subtle differences between the two rodent species.

Polycationic agents are often used to increase the transduction efficiency in mammalian cells.[26] In the absence of polycationic agents, viruses and

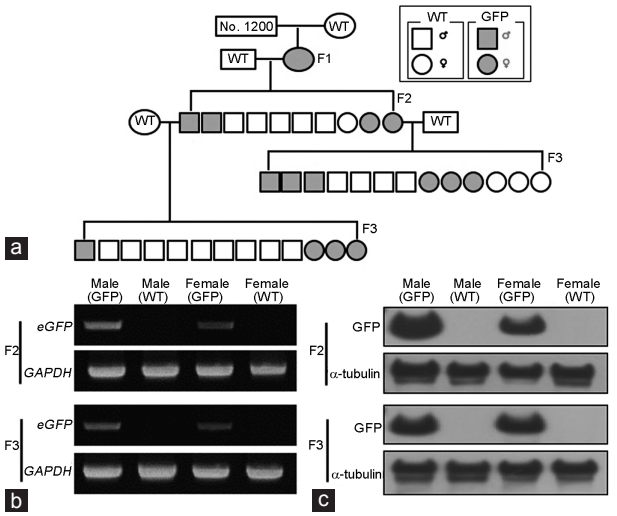

Figure 5: Generation of transgenic rats via injection of lentivirus to drive GFP expression. (**a**) Pedigree of recipient rat no. 1200 that had been directly injected with lentiviral vector at day 5 after birth. This pedigree demonstrates the transmission of the transgene for 3 generations. Green symbols indicate progeny expressing the GFP. (**b**) Expression of *eGFP* transgene was detected by PCR. (**c**) Western blot analysis using anti-GFP antibody. GFP: green fluorescent protein; eGFP: enhanced green fluorescent protein; WT: wild type; PCR: polymerase chain reaction. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

cells tend to repel each other as they both have negatively charged surface membranes. Polycationic agents mask the negative charges on the virus to facilitate their passage through the negatively charged cell membrane. Thus, these agents need to be biocompatible, nontoxic, and nonimmunogenic and must have a high vector-carrying capacity. We examined the *in vivo* cytotoxicity of polycations for potential use in lentiviral gene delivery systems, especially for SSCs in the rat testes. When we compared the tubules after injecting both polycations, testes injected with 4 μg ml^{-1} of polybrene demonstrated a 17.8-fold increased rate of tubule damage compared to those with 2 μg ml^{-1} DOGS, and the damaged tubules did not display differentiating germ cells or spermatozoa in the lumen (**Figure 1**). We have previously shown that lentiviral vectors can be used to efficiently modify the mouse SSC *in vitro*. In that study, we demonstrated lower cytotoxicity for the DOGS-treated group compared to the polybrene-treated group.[17] Similarly, a lower cytotoxicity was observed in the present study for the DOGS-treated group compared to the polybrene-treated group (**Figure 1**), thus highlighting the usefulness of DOGS as a polycationic agent in the transduction of SSCs *in vitro* and *in vivo*.

A potentially important application of our findings concerns the transgenesis of large animals. There are several current limitations for transgenesis in large animals. The culture system is not available for the majority of species and this limits the number of cell populations. Furthermore, SSCs in large animals are less well characterized, and methods to enrich stem cells are not available. Since the testicular microinjection technique has been established for many animal species, the microinjection of lentivirus vectors into immature animals can be applied to other animals.[11,12,27–30] Therefore, our *in vivo* transduction of rat SSCs provides a novel strategy for manipulating SSCs and warrants new efforts geared toward understanding and using this valuable population of cells from different species.

AUTHOR CONTRIBUTIONS

BJK and YHK designed and performed the experiments, collected and analyzed the data, and wrote the manuscript. MGO, KJK, SEJ, and JHJ carried out the animal experiments and molecular analysis. SUK and KSM participated in the design of the study and data analysis. BYR conceived the experiments, analyzed the results, and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

We would like to thank Steve S. Cho (Perelman School of Medicine, University of Pennsylvania) for critical evaluation of the manuscript and helpful comments. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2017R1A2B4003798), the Bio & Medical Technology Development Program of the NRF funded by the Korean government (MSIT) (2018M3A9H1023139), and KRIBB Research Initiative Program (KGM4251824).

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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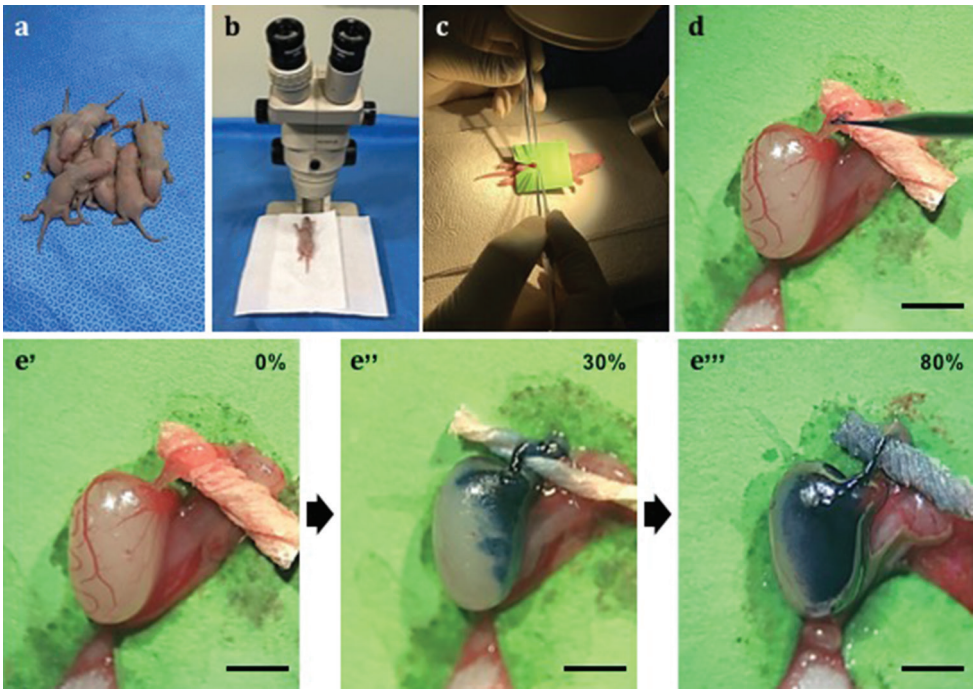

Supplementary Figure 1: Injection of recombinant lentiviruses into rat testis. (**a**) Donor male rats (day 5 after birth). (**b** and **c**) Equipment used to inject virus suspension. An anesthetized rat with the testis exposed has been placed on the central platform. (**d**) The fatty tissue around the ducts has been clearly removed and a small piece of paper towel has been placed under the efferent duct. A micropipette has been inserted into a large duct and threaded almost into the rete. Pressure in the pipette has been increased and the rete has been filled with dye solution. (**e**) Testis filled with lentivirus suspension. (**e'**) Testis without injection. (**e''**) Testis filled with 30% portion of the recipient testis. (**e'''**) Testis filled with 80% portion of the recipient testis.

Supplementary Table 1: Transgenic rats from spermatogonial stem cells infected *in vivo*[a]

Founder animal	*Time to analysis (day)*	*Time to first transgenic (day)*[b]	*Colonies/testis*[c]		*Transgenic/total offspring (%)*[d]
			R	*L*	
898	422	84	1	-	1/84 (1.1)
899	422	NA	-	-	0/132 (0)
900	422	NA	-	-	0/84 (0)
1214	476	92	13	16	2/69 (2.9)
1215	476	NA	-	-	0/96 (0)
1216	476	81	7	12	2/76 (2.6)
1217	476	NA	-	-	0/103 (0)

[a]R: right testis; L: left testis; [b]Days from transplantation to birth of first transgenic progeny sired by the animal; [c]Number of green colonies in each testis; [d]The numerator is the number of transgenic progeny. NA: not available