Absence of Germline Infection in Male Mice Following Intraventricular Injection of Adenovirus

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The possibility of inadvertent exposure of gonadal tissue to gene therapy vectors has raised safety concerns about germline infection. We show here that the receptor for coxsackie B viruses and adenoviruses 2 and 5 (CXADR) is expressed in mouse germ cells, suggesting the possibility that these viruses could infect germ cells. To directly assess the risk of germline infection *in vivo*, we injected an adenovirus carrying the germ-cell-specific protamine promoter fused to the bacterial *lacZ* reporter gene into the left ventricular cavity of mice and then monitored expression of the reporter gene in germ cells. To differentiate between infection of stem cells and differentiating spermatogenic cells, we analyzed expression of the reporter cassette at different times after viral delivery. Under all conditions tested, mice did not express the *Escherichia coli* β-galactosidase protein in developing spermatids or in mature epididymal spermatozoa. Primary germ cells cultured *in vitro* were also refractory to adenoviral infection. Our data suggest that the chance of vertical germline transmission and insertional mutagenesis is highly unlikely following intracoronary adenoviral delivery.

Key words: gene therapy, adenovirus, germline infection, testis, sperm, protamine

INTRODUCTION

Until recently, somatic gene therapy clinical trials have been restricted to patients with serious or life-threatening conditions. Clinical trials are now in progress that are investigating treatments for chronic, non-life-threatening diseases, and patients of child-bearing potential will receive somatic gene therapies. The possibility of exposure of gonadal tissue to viral vectors has raised safety concerns about vertical germline transmission and insertional mutagenesis with gene therapy products [1].

Adenoviruses have been widely used as vectors for gene transfer and can infect both dividing and nondividing cells. The efficiency of adenoviral infection depends on the presence of appropriate binding and entry mechanisms on target cells [2–5]. Adenovirus serotype 5 (Ad5) infection requires the attachment of the viral capsid protein to a cellular receptor and viral penton base binding to certain cellular integrins, followed by cell entry by means of receptor-mediated endocytosis [2]. The mouse and human homologues for a high-affinity receptor of coxsackie B viruses and adenoviruses 2 and 5 (CXADR) have been identified [6–8]. Further, HLA class I molecules may also contribute to Ad5 fiber binding to the cell surface [9]. Adenovirus internalization is mediated by the integrins $a_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ [2,10,11]. After cell entry, the adenovirus life cycle does not normally involve integration into the host genome, rather replication occurs as episomal (extrachromosomal) elements in the nucleus of the host cell. However, in *in vitro* studies, adenovirus vectors have been found to integrate at a very low frequency (10^{-3} to 10^{-5} per cell) [12].

To gain access to the male germ line, a viral vector has to surmount several barriers. A virus would initially need to escape from the bloodstream and enter the testicular lymph, cross several myoid cell layers, and traverse the

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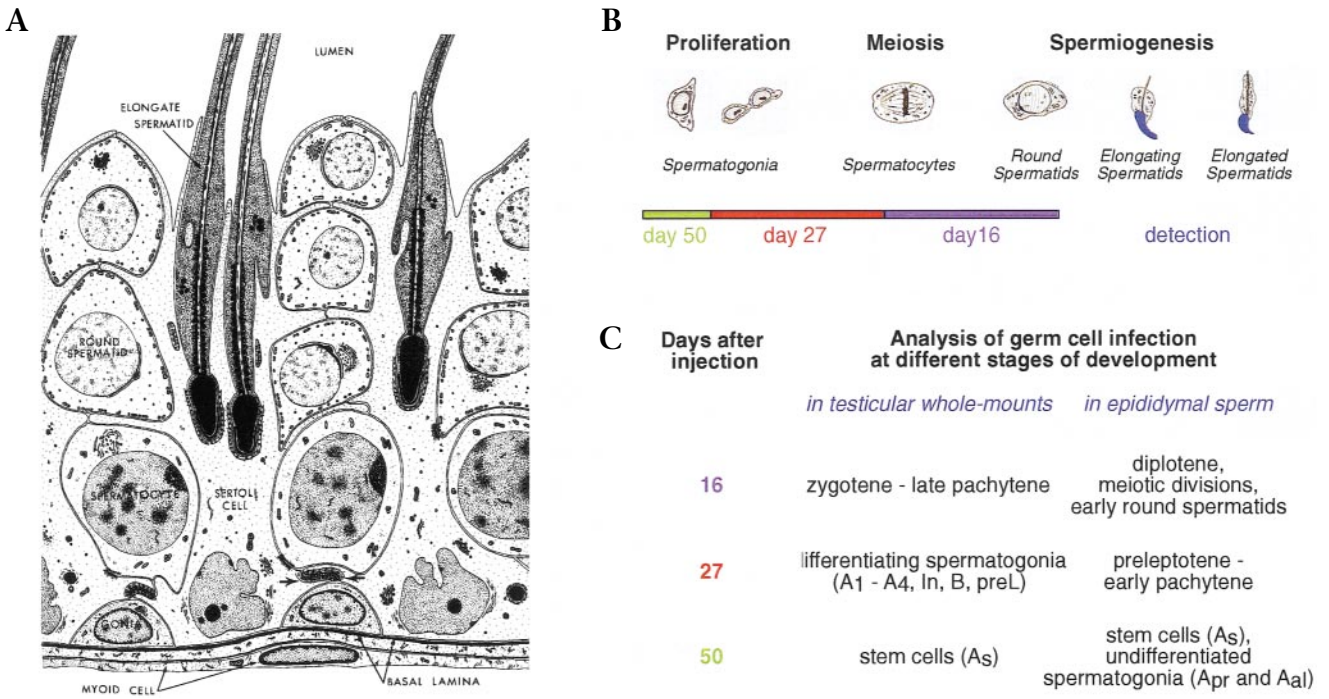

FIG. 1. Experimental design. (A) Schematic representation of a seminiferous tubule illustrating the close association of Sertoli cells with germ cells at successive stages of development (taken from [13] with permission of the publisher). The "blood–testis barrier" is composed of tight junctions (arrowheads) between Sertoli cells secluding spermatocytes, spermatids, and spermatozoa from direct contact with blood-born factors. (B, C) Spermatogenesis initiates when a stem cell (As) divides to produce two type A paired spermatogonia (Apr) that are joined together by intercellular bridges. One or several subsequent rounds of division produce chains of aligned spermatogonia (Aal), which then differentiate by means of six mitotic divisions into pre-leptotene spermatocytes before entering meiotic prophase. In the mouse, this process takes approximately 2.5 to 3.5 weeks depending on the number of divisions of the Aal spermatogonia [45,46]. The subsequent meiotic prophase and divisions and the differentiation of haploid rounds spermatids, via elongating and elongated spermatids into testicular sperm (spermiogenesis), occurs in about 26 days [47]. By monitoring β-galactosidase activity (represented by blue color in (B) in elongating and elongated spermatids and in testicular and epididymal sperm at different days after virus administration), infection of germ cells at different stages of development (colored bars in (B)) was investigated. At day 16, infection of cells in the zygotene substage of the meiotic prophase up to early rounds spermatids is analyzed. At day 27, infection of differentiating spermatogonia up to cells in early pachytene of meiotic prophase is determined. Finally, at day 50, infection of stem cells and undifferentiated spermatogonia is investigated. A_1–A_4, In, and B correspond to different types of differentiating spermatogonia [28]. Pre-L denotes pre-leptotene spermatocytes.

basal lamina of the seminiferous tubules where spermatogenesis takes place [13]. All of these barriers could severely restrict access to the germ line. Within the seminiferous epithelium the somatic Sertoli cells provide structural and nutritional support to the germ cells. Numerous tight junctions between Sertoli cells (Fig. 1A) create an adluminal compartment, modulated by Sertoli cells, and a basal compartment, influenced by vascular products and paracrine factors produced by other testicular somatic cells [14]. The blood–testis barrier created by the tight junctions between Sertoli cells is likely to be a major barrier for viral infection of the meiotic spermatocytes and the post-meiotic spermatids, which are localized in the adluminal compartment. However, infection of the spermatogonial stem cells, or the differentiating diploid spermatogonial cells, would not require virus to cross the blood–testis barrier as these cells lie in the basal compartment of the seminiferous epithelium.

Several studies have suggested that, when virus is injected intravenously or into nongonadal tissue, it is capable of reaching either testis or ovary [15–19]. However, the use of highly sensitive, PCR-based methods of viral detection may not give an accurate estimate of the number of viral particles that reach the gonad, or determine the viral particles that reach the gonadal tissue and infect germ cells. In studies in which investigators have assayed for transmission of virus to progeny [17,18], or assayed expression of reporter gene cassettes that have been cloned into adenovirus backbones [19–21], evidence of germline infection has not been reported. However, in these *in vivo* studies either the number of potential infection events analyzed was low, or the germ cell types that were assayed were limited. Another major limitation of these studies has been the use of non-germ-cell promoters to drive the expression of reporter genes. Without prior knowledge that a

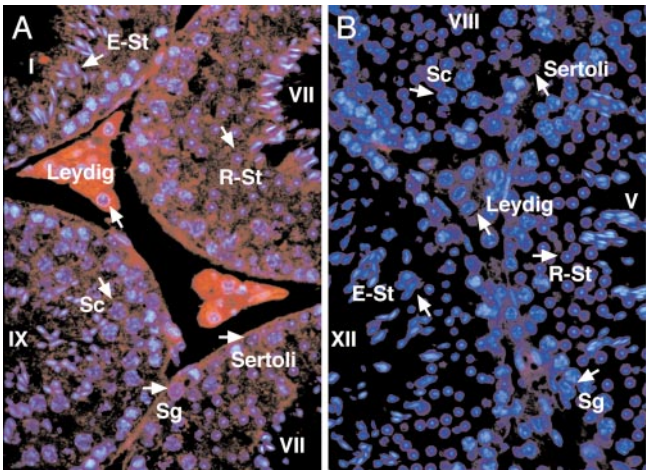

FIG. 2. CXADR expression in mouse germ cells. (A) Immunofluorescence staining of mouse testis sections with anti-CXADR monoclonal antibody (RmcB clone). CXADR expression (red stain) was detected in all germ-cell types and in the somatic Sertoli and Leydig cells. (B) The CXADR signal is absent from the no-primary controls. Sg, spermatogonial cells; Sc, spermatocyte in meiotic prophase; St, spermatid; R-St, round spermatid; E-St, elongating spermatid. Stages of mouse seminiferous tubule epithelium are represented in roman numerals [13].

reporter gene cassette will be expressed in germ cells it is not possible to make definitive conclusions about the lack of infectibility of the germ line. Perhaps most importantly, it has yet to be determined if the adenoviral vectors used in gene therapy are capable of infecting the stem cell population in the testis.

In this study we aimed to estimate the risk of germline infection by human recombinant Ad5 following administration into the left ventricular cavity of the heart, using the mouse as a model system. Our goal was to design a simple and robust method for detecting and quantifying viral infection and integration of the germ line at different stages of differentiation. For this purpose, we generated an adenovirus vector carrying the gene *lacZ* encoding a nuclear-localized β-galactosidase gene product under the transcriptional control of the protamine-1 (*Prm1*) gene. *Prm1* is a testis-specific gene that is transcribed exclusively in haploid round spermatids [22]. Previous transgenic studies using the *Prm1* promoter to drive the expression of *lacZ* have demonstrated the power of this system to detect the products of rare meiotic or spermatogonial recombination events in individual differentiating spermatids and in mature spermatozoa [23–26]. To differentiate between infection of stem cells and differentiating spermatogenic cells, we analyzed expression of the reporter cassette at different times after viral delivery. In addition to these *in vivo* experiments, we estimated the frequency of germ-cell infection in *in vitro* germ cell cultures and determined the expression profile of CXADR in mouse testis.

RESULTS

Immunolocalization of CXADR in Mouse Testis

To determine the expression profile of CXADR in germ cells, we carried out immunolocalization studies on sections of mouse testis using the RmcB monoclonal antibody raised against human CXADR [27]. In the presence of the CXADR primary antibody, we saw ubiquitous expression in somatic Leydig and Sertoli cells as well as in germ cells of all stages of differentiation (Fig. 2A). We observed CXADR expression in testes fixed in Bouin's and Carnoy's fluids, and in acetone-fixed cryosections. Positive controls for antibody staining included HeLa and 3T3 cells (data not shown). Both of these cell types were readily infected by an adenovirus containing an RSV-*lacZ* expression cassette (Ad-RSV-*lacZ*). In contrast, mouse L cells were very hard to infect with the Ad-RSV-*lacZ* virus and showed very weak CXADR immunostaining. We therefore believe that the observed staining pattern in mouse testes represents endogenous CXADR expression.

Prm1-nlacZ Expression in Transgenic Control Mice

To mimic adenovirus driven β-galactosidase expression in germ cells as a positive control, we generated transgenic mice that carried a *Prm1-nlacZ* reporter cassette flanked by adenoviral sequences, Tg(Ad5-*Prm1-nlacZ*) (Fig. 3A). We stained whole mount seminiferous tubules of these transgenic mice with X-gal and saw intense blue staining in the adluminal region of the tubules corresponding to the localization of spermatids (Fig. 3B). More detailed analysis showed β-galactosidase expression in almost all tubules of transgenic testes (Fig. 3D), which is in agreement with the expected expression in all stages of the seminiferous epithelium. We saw weak expression in step-8 round spermatids and strong staining in later developing spermatids (Figs. 3E–3G). In contrast, in testes of non-transgenic littermates, we found only extratubular staining, which we attribute to low levels of an endogenous β-galactosidase-like activity (Figs. 3C and 3H).

In transgenic animals we observed blue X-gal staining of epididymal sperm heads (Fig. 3M). In some spermatozoa, the midpiece was stained as well. We easily detected blue sperm in 1:1000 mixtures of transgenic with non-transgenic sperm samples (Fig. 3O). Comparing two transgenic lines with either two or eight copies of the same transgene, we did not observe any differences in the intensity of X-gal staining of epididymal sperm (data not shown), suggesting that the presence of a single copy in an adenoviral vector is most likely sufficient for detection.

In Vivo Virus Exposure

We initially determined whether Ad5-*FGF4* virus, when injected in the left ventricular cavity of the heart at high titer (1.85×10^8 vp/g body weight), could be detected by PCR at 4 and 14 days post-injection. At day 4 post-injection we detected viral DNA in eight of eight heart samples

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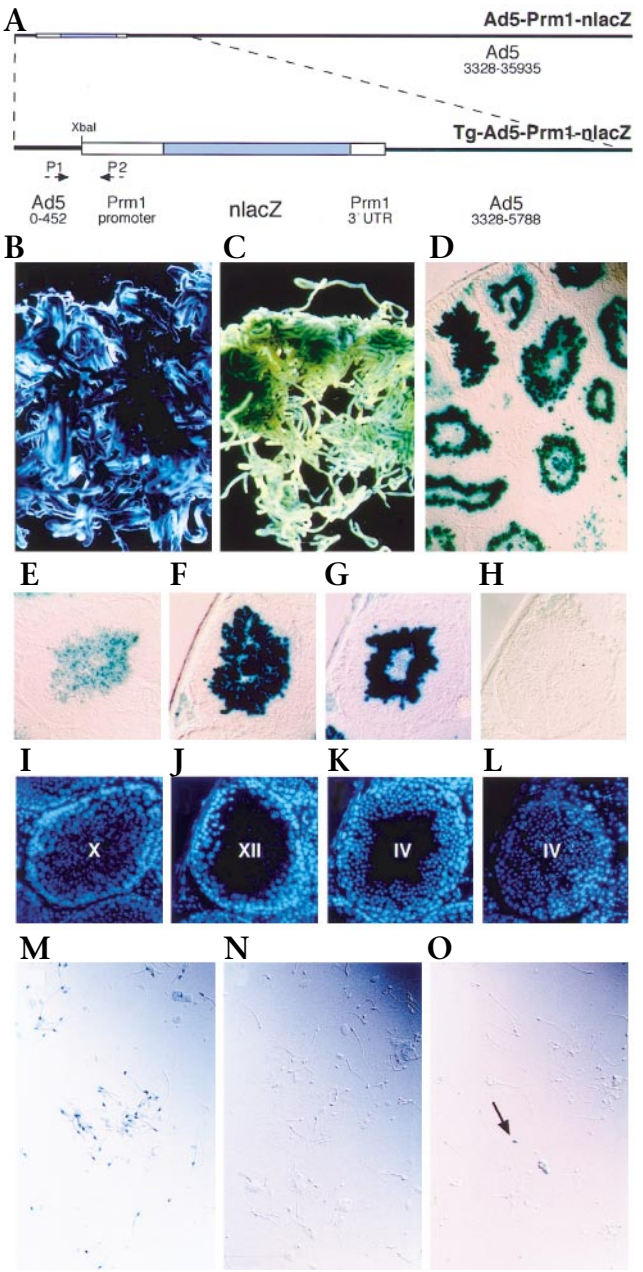

FIG. 3. Vector design and transgenic control. (A) Schematic structure of the *Prm1-nlacZ* reporter cassette in the Ad5 virus (top) and as a transgene (bottom). Primers P1 and P2 and the *Xba*I restriction site were used for genotyping of transgenic mice. (B–L) X-gal staining of seminiferous tubules in whole mount (B, C) or cryosections (D–L) of transgenic (B, D–G, I–K) and non-transgenic (C, H, L) mice. (D–H) X-gal staining photographed using differential interference contrast illumination. (I–L) Corresponding DAPI staining used for staging of the seminiferous epithelium. Stages are represented by roman numerals. (M–O) Staining of isolated transgenic epididymal sperm (M) resulted in sperm with blue stained heads and sometimes blue stained midpieces, whereas non-transgenic sperm (N) did not show any staining. (O) The 1:1000 dilution mixtures of transgenic with non-transgenic sperm samples before X-gal staining revealed single blue sperm heads surrounded by many unstained sperm heads.

(≥ 10 copies/μg genomic DNA) and two of eight testes (Table 1). At day 14 post-injection we continued to detect Ad5-*FGF4* in six of eight heart samples, but failed to detect virus (< 10 copies/μg genomic DNA) in the testes of all animals injected (n = 8). These data suggest that when virus is injected in the left ventricular cavity of the heart, systemic circulation is capable of disseminating virus to the testis but it is not retained.

To determine if adenovirus can infect the germ line *in vivo*, we injected mice with an adenovirus carrying the *Prm1-nlacZ* expression cassette. We assayed infection of germ cells at different stages of differentiation by analyzing β-galactosidase expression in seminiferous tubules dissected from whole testes and in isolated epididymal sperm at different times after administration of the virus (Figs. 1B and 1C). Assaying viral-mediated transfection in whole seminiferous tubules makes it possible to identify the cell type in which the reporter is expressed. It also allows one to assay large numbers of cells and to quantitate the frequency of infection should it occur. If adenovirus can cross the tight junctions connecting Sertoli cells and infect post-meiotic spermatids, we expected to detect β-galactosidase activity in spermatids within a few days of viral delivery. Similarly, if adenovirus can cross the blood–testis barrier and infect meiotic spermatocytes, we expected to be able to detect β-galactosidase activity in spermatids approximately 2 weeks post-injection—the time required for the differentiation of spermatocytes into late spermatids. Infection of the differentiating spermatogonia would be assessed by detection of β-galactosidase activity approximately 4 weeks post-injection. Last, if adenovirus can infect spermatogonial stem cells, we expected to continue to detect spermatids with β-galactosidase activity beyond 6–7 weeks of viral injection—the time required for differentiation of stem cells into mature spermatozoa.

Mice were injected with 1.85×10^7 (low dose, LD) or 1.85×10^8 (high dose, HD) of Ad5-*Prm1-nlacZ* vp/g body weight. As a negative control, we injected Ad5-*FGF4* at 1.85×10^8 vp/g body weight, the same dose used in the virus exposure study above. In all cases virus was injected in the left ventricular cavity of the heart. At days 16, 27, and 50 after virus delivery, we prepared seminiferous tubules from whole testes and stained them with the X-gal substrate to localize β-galactosidase activity in germline cells. Had the virus infected the germ line we expected to be able to deduce the germ-cell type that had been infected from the timing of detection of β-galactosidase activity in spermatids. Additionally, integration of the viral DNA into the genome of pre-meiotic cells would lead to clones of β-galactosidase-positive spermatids. The size of the clone would reflect the cell type in which the virus had integrated.

Expression of β-galactosidase in spermatids at 50 days post-injection would have detected integrations that had occurred in spermatogonial stem cells; integration into

a single stem cell would generate a clone of up to 2048 β-galactosidase positive spermatids [13]. In visual inspection of seminiferous tubules dissected from 33 testes at 50 days post-injection, we never observed clones with the expected pattern of β-galactosidase expression. Assuming there are approximately 35,000 stem cells per testis [28], we assayed a total of 1.2×10^6 stem cells (Table 2).

TABLE 1: Number of mice with PCR-positive tissues[a]

Days post-injection	Heart		Testis	
	Saline	Ad5-*FGF4*	Saline	Ad5-*FGF4*
4	0/8	8/8	0/8	2/8
14	0/8	6/8	0/8	0/8

[a]Positive results/number animals tested. Sensitivity of PCR approximately 10 copies of Ad5-*FGF4* per μg genomic DNA. The two animals that tested positive by PCR contained 59 and 126 copies of Ad5-*FGF4* DNA/μg genomic DNA.

At 27 days post-injection we would have detected integrations that had occurred in differentiating spermatogonia or preleptotene spermatocytes; clones would have varied in size between 4 and 256 cells [28]. In careful visual inspection of seminiferous tubules from animals injected with the two titers of Ad5-*Prm1-nlacZ* virus, we did not detect clones of spermatids with the expected pattern of β-galactosidase expression. We scored approximately 5.4×10^6 differentiating spermatogonia and preleptotene spermatocytes per testis (Table 2). In total, approximately 1.8×10^8 cells were analyzed in 34 testes from 20 animals exposed to the Ad5-*Prm1-nlacZ* virus 27 days after injection.

In most cases the seminiferous tubules lacked any indication of X-gal staining. However, on occasion, and irrespective of the condition tested, we detected a low number of lightly stained regions of nonspecific β-galactosidase activity within tubule fragments. Figure 4A shows an example of a collection of β-galactosidase positive seminiferous tubule fragments of one Ad5-*Prm1-nlacZ* HD mouse at day 27 after delivery. Figure 5 summarizes the number of stained patches at different virus conditions and different days after administration. To determine whether blue staining colocalized with late-differentiating spermatids, we made cryosections of the blue-stained seminiferous tubules. In most samples of virus-treated mice, X-gal staining colocalized with elongating or elongated spermatids (Figs. 4B and 4C). However, we made the same observation in tubule fragments of wild-type mice unexposed to any virus (Figs. 4D and 4E). We never detected expression similar to that seen in control transgenic animals (Figs. 4F and 4G). Closer analyses showed that the morphology and configuration of these nuclei within the seminiferous epithelium were abnormal (Fig. 4C, arrow). Using an immunofluorescence approach, we failed to detect the β-galactosidase protein in blue tubules of virus-treated and untreated mice, whereas the protein was detectable in Tg(Ad5-*Prm1-nlacZ*) transgenic control testes (data not shown). These data indicate that X-gal staining was not due to presence of Ad5-*Prm1-nlacZ* derived β-galactosidase activity, but was caused by an endogenous β-galactosidase-like enzyme active in degenerating spermatids. These results are consistent with others in which β-galactosidase activity was observed in damaged or dying human or rodent cells [29,30].

At day 16 post-injection, integration would have occurred in primary spermatocytes. Completion of the two meiotic divisions would have generated single spermatids with the gene cassette. However, transfer of the β-galactosidase protein through the intercellular bridges that connect post-meiotic cells may lead to adjacent cells sharing β-galactosidase activity [31]. As discussed above, we occasionally observed small groups of spermatids that contained weak β-galactosidase activity (Fig. 5). However, as similar numbers of small clusters of cells were also observed in animals injected with the Ad5-*FGF4* virus, and none of the clusters stained as darkly as the control transgenic tubules, we attribute the observation to degenerating spermatids expressing an endogenous β-galactosidase-like activity.

We carefully inspected X-gal stained epididymal sperm from a random subset of the injected animals and found the rare blue sperm. However, similar to what we had observed in the seminiferous tubules, we detected

TABLE 2: Number of germ cells analyzed in seminiferous tubules

Day post-injection	Cell type assayed	No. cells/testis[a]	No. testes[b]	Total cells assayed
16	zygotene/pachytene spermatocytes	1.6×10^7	34	5.4×10^8
27	differentiating spermatogonia (A1-A4, In, B) and preleptotene spermatocytes	5.4×10^6	34	1.8×10^8
50	spermatogonial stem	3.5×10^4	33	1.2×10^6

[a]Taken from [13,28,45].
[b]Sum of testes analyzed at both concentrations of virus containing the *Prm1-nlacZ* cassette.

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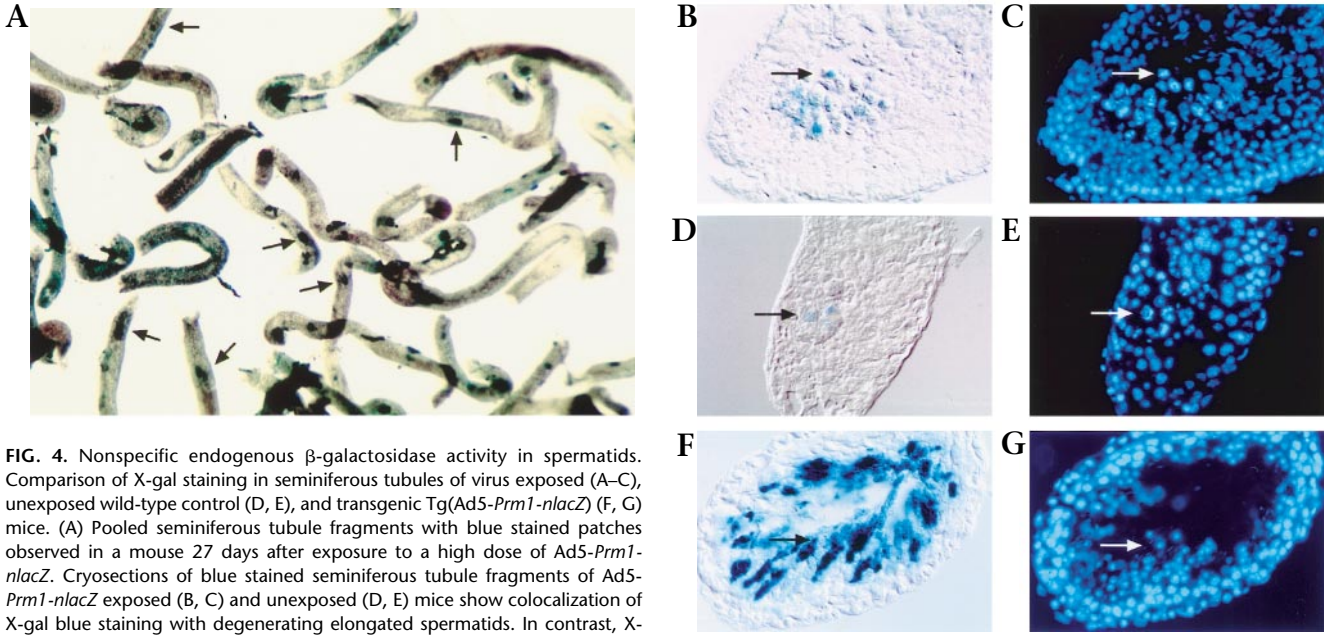

FIG. 4. Nonspecific endogenous β-galactosidase activity in spermatids. Comparison of X-gal staining in seminiferous tubules of virus exposed (A–C), unexposed wild-type control (D, E), and transgenic Tg(Ad5-*Prm1-nlacZ*) (F, G) mice. (A) Pooled seminiferous tubule fragments with blue stained patches observed in a mouse 27 days after exposure to a high dose of Ad5-*Prm1-nlacZ*. Cryosections of blue stained seminiferous tubule fragments of Ad5-*Prm1-nlacZ* exposed (B, C) and unexposed (D, E) mice show colocalization of X-gal blue staining with degenerating elongated spermatids. In contrast, X-gal staining in Tg(Ad5-*Prm1-nlacZ*) testis (F) is localized to elongating spermatids with normal nuclear morphology (G).

blue sperm in all samples irrespective of the condition analyzed. Semi-quantitative analysis of epididymal sperm from six animals that had been injected with virus at the day 50 time point is shown (Table 3). The intensity of the staining was weaker in these sperm samples than in sperm of transgenic Tg(Ad5-*Prm1-nlacZ*) mice (Fig. 6), suggesting that similar to what we observed in the seminiferous tubules, a low percentage of wild-type cells contain an endogenous β-galactosidase-like activity.

In Vitro Germ-Cell Infection

To determine whether mouse germ cells can be infected by adenovirus *in vitro*, we exposed primary germ-cell cultures to Ad5-CMV-GFP. At day 1 after virus exposure we observed GFP expression in approximately 0.2 to 1% of testicular cells using a multiplicity of infection (MOI) of either 500 or 5000 (approximately 4×10^6 cells per MOI analyzed). The number of GFP-positive cells remained grossly constant at subsequent days of culture. Using DAPI staining and differential interference contrast microscopy, we were able to identify different cell types among the GFP-positive cells. The majority consisted of Sertoli cells as these cells were large in size and contained a uniformly DAPI stained nucleus with prominent nucleoli characteristic for this cell type (Figs. 7A–7C). The remaining cells were intermediate in size and displayed an oval-shaped nucleus that was never localized in the center of the cell and had several prominent blocks of heterochromatin within the nuclear periphery (Figs. 7D–7F). These cells are most likely Leydig cells (Fig. 2D). Occasionally, we observed cells attached to the culture dish, which were most likely myoid cells (Figs. 7G–7I).

To specifically detect infection in differentiating spermatids, we exposed testicular cells to Ad5-*Prm1-nlacZ* (Fig. 3A) at an MOI of 500 or 5000 (~ 0.9×10^6 cells per MOI analyzed). We never observed X-gal staining in any of these cultures. In addition, we exposed testicular cells isolated from transgenic Tg(Ad5-*Prm1-nlacZ*) mice (Fig. 3A) to the Ad5-CMV-GFP vector at an MOI of 500 or 5000 (~ 2.2×10^6 cells per MOI analyzed). Similarly, we never observed colocalization of GFP expression and X-gal staining (Figs. 7J–7L).

Finally, to determine whether transgene expression in germ cells depends on the physiological state of the cell or on the interaction of germ cells with Sertoli cells, we co-cultured mouse testicular cells after Ad5-CMV-GFP exposure with 15P-1 cells. The 15P-1 cell line exhibits Sertoli cell characteristics and supports meiotic and postmeiotic differentiation of diploid premeiotic germ cells into haploid spermatids [32,33]. Co-culturing after adenovirus exposure resulted in the characteristic aggregation of germ cells on top of the 15P-1 cells (Fig. 7M), indicating that the cell isolation procedure and the virus exposure had not affected the physiological state of the germ cells. Most of the 15P-1 cells were infected by the Ad5-CMV-GFP virus and showed GFP expression (Fig. 7N), but the frequency of transgene expression in the various testicular cells described above was not increased (4×10^6 cells per MOI analyzed) and we did not detect GFP expression in germ cells.

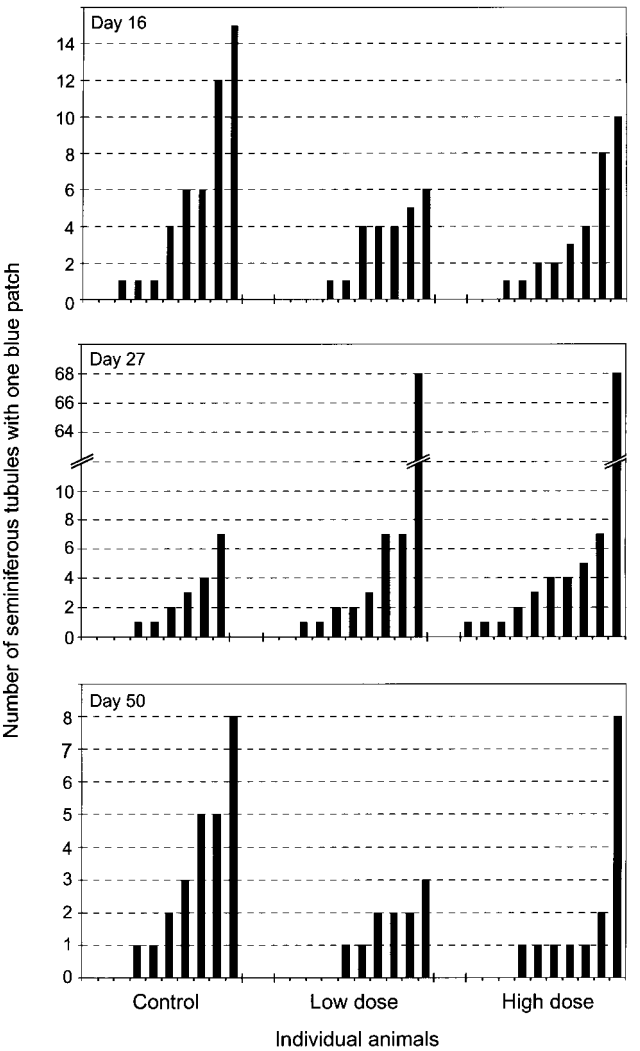

FIG. 5. X-gal staining in seminiferous tubule fragments at days 16, 27, and 50 after virus exposure. The total number of seminiferous tubule fragments each containing one blue stained patch is given for each animal. Control, 1.85×10^8 Ad5-*FGF4* vp/g body weight. Low dose and high dose, 1.85×10^7 and 1.85×10^8 Ad5-*Prm1-nlacZ* vp/g body weight, respectively.

DISCUSSION

The application of gene therapy to non-life-threatening conditions for patients of child-bearing age introduces the potential risk of gonadal dissemination and infection of the germ line. In this study we wanted to determine the frequency of testicular germ line infection after systemic administration of adenovirus in a mouse model. Our intent was to also mimic the worst case of systemic distribution by injection of adenovirus injection of high doses of virus in the left ventricular cavity of the heart. Attempts to target specific organs like the heart by techniques such as delivery into the lumens of coronary arteries would limit systemic distribution. The high dose used in this study corresponds to a total dose of 1.3×10^{13} vp for a 70 kg human on a body weight adjusted basis. This dose is more than 2 log units greater than the highest dose currently used in clinical trials testing the angiogenic effect of a replication-deficient human adenovirus (serotype 5) containing the human gene *FGF4* on ischemic coronary artery disease.

We obtained PCR evidence for the presence of virus in the testis 4 days after injection in the left ventricular cavity of the heart, but it did not persist at 2 weeks. Others have also shown that virus injected into nongonadal tissue is capable of reaching either testis or ovary [15–19], suggesting that germline infection could occur. However, in our analysis of testicular spermatids and epididymal sperm we found no evidence for infection of mouse germ cells. By analyzing *lacZ* expression in seminiferous tubules at 16, 27, and 50 days post-injection, we scored a total of 5.4×10^8 spermatocytes, 1.8×10^8 differentiating spermatogonia and pre-leptotene spermatocytes (equivalent to having assayed 7.2×10^9 to 4.6×10^{10} sperm), and 1.2×10^6 stem cells (equivalent to having assayed 2.4×10^9 sperm), respectively (Table 2). Had Ad5-*Prm1-nlacZ* infected a spermatogonial stem cell and integrated into the genome we would have expected a clone of 2048 spermatids expressing β-galactosidase. However, we never observed such a pattern, suggesting that the germline and, most importantly, the stem cell populations are refractory to adenovirus infection. Our *in vivo* seminiferous tubules and epididymal sperm data are in agreement with our *in vitro* studies. We were not able to detect germ-cell transfection by adenovirus using CMV-GFP or *Prm1-nlacZ* expression cassettes, despite using extremely high MOI and co-culture of primary germ cells with a Sertoli cell line.

Our data are consistent with other studies designed to investigate infection of the germ line by adenovirus [16–21]. The major differences between our studies and those of others was the method of viral delivery; we used an expression cassette driven by a germ-cell-specific promoter and assessed infection of the stem cell population. However, a previous study [34] showed that *in vitro*, and under highly unusual conditions *in vivo*, spermatogenic stem cells are capable of infection by retroviruses. Infection *in vivo* required removal of testis cells from an animal and reintroduction of the cells, along with virus-producing cells, into lumens of seminiferous tubules of mice that had been previously made cryptorchid. Surgical cryptorchidism is known to reduce the cell types within the seminiferous tubules to primitive spermatogonial cells and somatic Sertoli cells, a condition thought to be favorable for stem cell infection [34]. However, with direct testicular injection of virus into cryptorchid males the investigators did not detect infection of the germ line.

Why does the male germ line seem to be refractory to infection by adenovirus? Our immunohistochemistry

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TABLE 3: Number of X-gal blue epididymal sperm per million sperm at day 50 after viral exposure

Virus construct	Dose (vp/g[a] body weight)	Animal 1 (*n*)	Animal 2 (*n*)
Ad5-*FGF4*	1.85×10^8	0.7 (1.4×10^6)	23 (1.4×10^6)
Ad5-*Prm1-nlacZ*	1.85×10^7	25 (0.7×10^6)	0.9 (1.2×10^6)
Ad5-*Prm1-nlacZ*	1.85×10^8	13.3 (0.9×10^6)	73 (1.8×10^6)

[a]vp/g, viral particles per gram. *n*, total number of sperm analyzed.

data indicate that the high-affinity receptor responsible for Ad5 binding is expressed in germ cells of mouse testis, suggesting that adenovirus should be capable of binding germ cells. Shinohara and co-workers [35,36] have reported that antibodies against β_1 and α_6 can be used to affinity-enrich for the presence of stem cells used in germ cell transplantation experiments in mice, suggesting lack of integrins may not be responsible for the absence of infection of the stem cells. In human testis, Schaller and colleagues [37] have shown that β_1, and its α_3, α_5, and α_6 subunits are expressed in spermatocytes, spermatids, and testicular spermatozoa, whereas the α_1, α_2, and α_4 subunits of β_1 could not be detected. *In vivo* it is likely that the blood vessel wall, the peritubular myoid cell layer (which is several cells thick in humans), and the basal lamina of the seminiferous epithelium all contribute to providing a barrier to adenoviral entry. In addition, the tight junctions between Sertoli cells that form the blood–testis barrier provide further protection to the meiotic and postmeiotic cells.

Infection of the female germ line is also of concern in somatic gene therapy. The absence of an equivalent blood–ovary barrier would seem to enhance the possibility of germline infection in females. On the other hand, the fewer number of total germ cells in the ovary compared with the testis and the lack of a stem cell population might balance the sex-specific anatomical differences. Recent attempts to infect the female germ line with a marked adenovirus suggest that, like in males, germ cells in females are highly refractory to infection [38].

We developed and applied a relatively simple quantitative method of estimating the risk of germline infection at different stages of spermatogenic differentiation. Its main advantage lies in the ability to assay a large number of events and to deduce the cell type that had been infected. The evolutionary conservation of the protamine genes should make it possible to easily adapt this assay to other mammals and to nonhuman primates.

MATERIALS AND METHODS.

Construction of plasmids and transgenic mouse lines. Plasmid *Prm1-nlacZ* consists of a 3.1-kb fragment of the *Escherichia coli lacZ* gene containing the simian virus 40 (SV40) nuclear localization signal [39] inserted into the mouse protamine-1 gene (*Prm1*). Upstream of *lacZ* there is ~ 1 kb of *Prm1* promoter sequences and 0.1 kb of *Prm1* 5′ untranslated sequences (UTR). Downstream of *lacZ* is the remainder of the *Prm1* gene including the intron and the 3′ UTR.

DNA from Ad5 was cloned both 5′ and 3′ of *Prm1-nlacZ* to generate a transgene for use as a positive control. Nucleotides 0–452 of Ad5 (GenBank M73260) were transferred as an *Eco*R1-*Xba*I fragment from the pXCJL.1 plasmid (gift from Andre Lieber, University of Washington, Seattle, WA) into the *Sma*I and *Xba*I sites of *Prm-nlacZ*. Subsequently, a *Bam*H1-*Stu*I fragment of pXCJL.1 containing Ad5 nucleotides 3328–5779 was cloned downstream of the *Prm1-nlacZ* cassette into the *Bgl*II and *Pst*I sites. DNA from Ad5-*Prm1-nlacZ* was used to generate transgenic mouse lines Tg(Ad5-*Prm1-nlacZ*) for use as a positive control.

A 7.5-kb *Kpn*I–*Hin*dIII fragment of Ad5-*Prm1-nlacZ* was injected into pronuclei of fertilized FVB/J mouse eggs as described [40]. Genotyping was done by PCR and confirmed by Southern blot analysis. Amplification of genomic tail DNA with the forward primer (5′-CGCGTAATATTTGTCTAGGGC-3′) positioned in the Ad5 upstream region (Ad5 333–354 nt), and the reverse primer (5′-GCAGTGTTTGGACTGAGGGAGG-3′) located in the *Prm1* promoter region, resulted in a 556-bp fragment containing a unique diagnostic *Xba*I site. Two lines of transgenic mice were analyzed. The copy number of the transgene, approximately 2 and 8, was determined relative to the endogenous *Prm1* gene by quantitative Southern blot analysis after *Xba*I and *Eco*R1 digestions using an 862-bp *Xba*I-*Nco*I fragment of *Prm1-nlacZ*, encompassing the *Prm1* promoter, as probe.

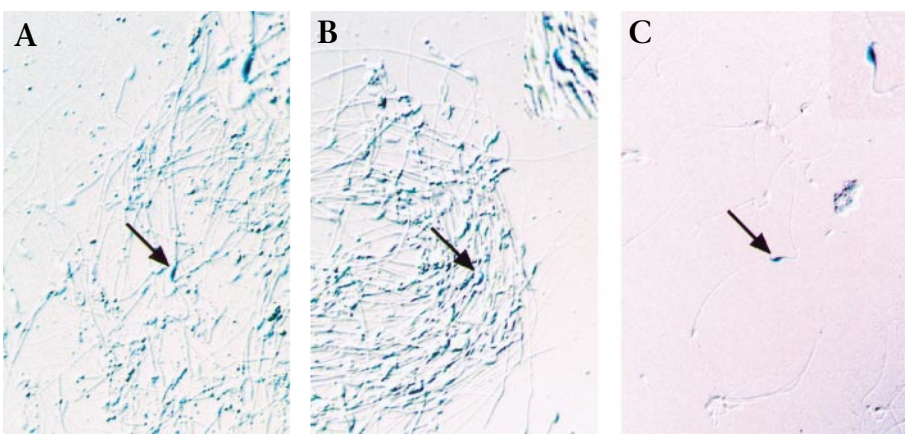

FIG. 6. Nonspecific endogenous β-galactosidase activity in sperm. X-gal staining in epididymal sperm of Ad5-*Prm1-nlacZ* virus exposed (A; high dose), unexposed wild-type control (B), and transgenic Tg(Ad5-*Prm1-nlacZ*) (C) mice.

Virus construction. To produce Ad5-*Prm1-nlacZ* virus, the *Xba*I-*Bgl*II fragment of *Prm1-nlacZ* containing the reporter cassette was first cloned into the *Xba*I and *Bam*H1 sites of pXCJL.1. Subsequently, human HEK-293 cells were transformed with pXCJL.1-*Prm1-nlacZ* plasmid and pJM17 (Microbix) as described [41]. Virus was isolated and amplified as described [42]. Virus production using HEK293 cells in serum-free suspension culture was performed by Berlex Biosciences (Richmond, CA). Following freeze-thaw disruption, virus was purified by ion-exchange chromatography, formulated in PBS-sucrose, and stored frozen until use. The purified virus was free of measurable endotoxin and had an infectivity/particle count ratio of 1–3%. The negative control Ad5-*FGF4* virus has been described [43] and was also produced and tested by Berlex Biosciences. Ad5-CMV-GFP [44] was provided by André Lieber (University of Washington, Seattle, WA).

Immunohistochemistry and immunofluorescence. For paraffin sections, testes of FVB/J mice (The Jackson Laboratory, Bar Harbor, Maine) were fixed in either Bouin's fluid (0.9% picric acid, 10% formaldehyde, 5% glacial acetic acid), Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), or 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4°C and embedded in paraffin according to standard procedures. For cryosections, testis tissue was embedded in OCT (Tissue-Tek) and snap-frozen in isopentane submerged in liquid N_2. Cryosections were postfixed for 1 h in acetone at –20°C. The CXADR receptor was detected using a mouse monoclonal antibody raised against human CXADR (RmcB clone, provided by Jeffrey Bergelson, Children's Hospital of Philadelphia, Philadelphia, PA). The CXADR receptor was visualized in 5 μm paraffin sections or 10 μm cryosections using either the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) and the Zymed AEC detection kit (Zymed, San Francisco, CA) according to the manufacturer's protocols, or immunofluorescence. In the later case, PBS containing 5% non-fat milk and 10% goat serum was used as blocking and antibody dilution solution (BS). Primary antibodies were applied overnight at 4°C and were subsequently detected using either the appropriate Alexa-488 or Alexa-568 conjugated secondary antibody (Molecular Probes, Eugene, OR) or a biotinylated secondary antibody (Zymed, San Francisco, CA) followed by Streptavidine-Alexa-568 (Molecular Probes, Eugene, OR). Immunofluorescence sections were counterstained with 4′,6′-diamino-2-phenylindole (DAPI, Boehringer) and mounted in Vecta Shield (Vector Laboratories) antifade solution. We analyzed the preparations using a Nikon microscope equipped with epifluorescence illumination and photographed images directly on 400 ISO Kodak Elite color slide film using single or triple band-pass filter sets. Immunohistochemistry sections were counterstained with hematoxylin (Sigma).

In vitro infection. Primary testis cultures were isolated from adult wild-type or Tg(Ad5-*Prm1-nlacZ*) transgenic FVB/J testes as follows. After removal of the tunica, we extensively washed the seminiferous tubules in PBS to remove as many interstitial cells as possible. Tubules were minced in a drop of medium (Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F12 medium containing penicillin, streptomycin, and 2 mM L-glutamine; all from Gibco BRL) into small fragments using two razor blades. A single-cell suspension was generated by repeated pipetting. After settlement of the larger tubule fragments, cells were centrifuged for 5 min at 1000 rpm in a new tube and resuspended in fresh medium. After counting, 10^6 cells per 1 ml medium were exposed to either Ad5-Prm1-nlacZ or Ad5-CMV-GFP virus at a MOI of 0, 500, or 5000 for 2 h at 25°C under continuous gentle shaking. Subsequently, cells were washed once with PBS and 4.5×10^5 cells in 0.8 ml medium containing 10% fetal bovine serum were seeded in a 1.8 cm^2 well of a 4-well Coverglass Chamber Slide System (LabTec) and cultured for one or several days at 32°C and 5% CO_2. For each MOI, nine cultures were analyzed, each containing 4.5×10^5 cells. In some experiments, 15P-1 cells were added to the testicular cells in a 1:50 ratio after virus exposure and PBS washing. 15P-1 cells were cultured as described [32]. GFP expression, DAPI staining, and differential interference contrast (DIC) images were analyzed and recorded using a Zeiss inverted microscope, a Quantix CCD camera and the siftWoRx Version 2.5 software package (Issaquah, WA). For the GFP-X-gal double labeling experiment we used normal glass chamber slides (LabTec) and analyzed the expression with a Nikon microscope equipped with epifluorescence illu-

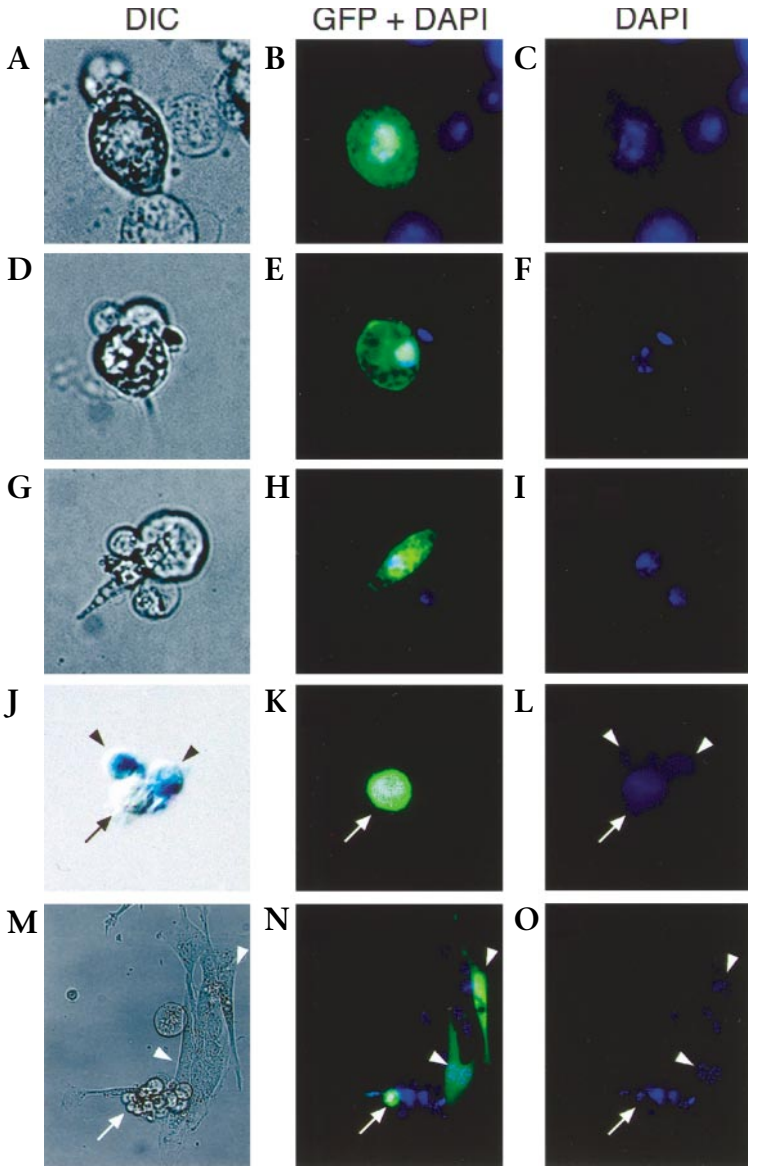

FIG. 7. Expression of GFP in different testicular cell types after *in vitro* infection of testicular cell suspensions with Ad5-CMV-GFP. Cell suspensions were obtained from wild-type (A–I, M–O) or transgenic Tg(Ad5-*Prm1-nlacZ*) (J–L) testes. (M–O) Cells were mixed with Sertoli-cell-like 15P-1 cells after Ad5-CMV-GFP exposure and before seeding. Large Sertoli cell (A–C), intermediate-sized Leydig cell (D–F), Leydig or myoid cell (G–I), GFP-positive Leydig cell (J, K, arrow), and X-gal positive transgenic spermatids (arrowheads), GFP-positive 15P-1 cells (M–O, arrowheads), and an unidentified, GFP-positive cell (arrow) are shown.

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mination and DIC optics. X-gal staining was performed with 1 mg/ml X-gal in staining solution as described below.

Ventricular injection. We conducted two blinded, randomized controlled studies. In one, two groups of 16 two-month-old FVB/N males (Taconic Labs, Germantown, NY) were analyzed for successful gene transfer to the heart and biodistribution to the testes. To half the animals in each group we administered 1.85×10^8 Ad5-*FGF4* viral particles per gram (vp/g) body weight. To the remaining animals we injected 100 μl of saline. Virus was delivered into the left ventricular cavity of the heart. Briefly, mice were sedated with ketamine and xylazine by intraperitoneal injection. The left side was shaved and the mouse was placed with the left side up on a heated body pad with supplemental O_2 supplied with a face mask. A small incision was made over the left thorax exposing the underlying muscle and intercostal space overlying the left ventricle of the heart. A 28-g needle attached to a 1 ml syringe was used to deliver virus or saline (100 μl in each case) into the left ventricle of the heart. Delivery into the left ventricular cavity was initiated only after arterial blood flash into the syringe was observed before injection of the virus and confirmed by oxygen saturation (I-stat blood-gas analyzer; $PO_2 \geq 100$ mmHg) of withdrawn blood after viral injection. Subsequently, the skin was closed using tissue adhesive. Animals were sacrificed with ketamine/xylazine at day 4 (group 1) or day 14 (group 2). Testes and heart were collected and frozen for PCR analysis of Ad5-*FGF4* as described below.

In the second study, we analyzed three groups of 30 two-month-old FVB/N males for germline infection. For Ad5-*Prm1-nlacZ* we administered 1.85 3 10^7 (low) or 1.85×10^8 (high) vp/g body weight into the left ventricular cavity of 10 males in each group. As a negative control, we administered Ad5-*FGF4* in the high dose of 1.85×10^8 vp/g body weight to the remaining 10 males in each group. Mice were sacrificed by CO_2 asphyxiation at 16, 27, and 50 days post-injection. The presence of β-galactosidase positive seminiferous tubules and epididymal sperm was determined as described below.

Mice studied for germline infection were housed individually before and after administration of the virus under specific pathogen-free conditions at the University of Washington. All animals were handled using an approved Institutional Animal Care and Use Committee (IACUC) protocol and euthanized according to American Veterinary Medical Association (AVMA) guidelines. Mice used for biodistribution studies were housed at Collateral Therapeutics Inc. under an IACUC-approved protocol.

Quantitative PCR analysis of virus dissemination. Quantitative PCR analysis was performed by Althea Technologies, Inc., San Diego, CA. A TaqMan (Perkin Elmer Inc., Gaithersburg, MD) based assay was used to detect a 71-nt Ad5-*FGF4* sequence with a lower limit of detection of 10 copies of Ad5-*FGF4* per μg genomic heart and testis DNA. Reactions were performed using an ABI PRISM 7700 sequence detector: 5′ primer, 5′-AGCAGCAAGGGCAAGCTCTA-3′; 3′ primer, 5′-GGAAGGAGAATCTC-CTTGAACGT-3′; and fluorogenic probe, 5′-6FAM-TCGCCCTTCTTCACC-GATGAGTGC-TAMRA-3′. The 5′ primer bridges an exon junction. Reactions were performed in a volume of 100 μl. Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 62°C for 1 min.

X-gal staining of seminiferous tubules, epididymal sperm, and testes cryosections. To increase accessibility of seminiferous tubules for the X-gal substrate, tubules were first incubated with 50 μg/ml collagenase IV (Sigma) in Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F12 medium (GibcoBRL) for 25 min at 37°C. Tubules were subsequently washed with PBS, pH 7.4, containing 2 mM $MgCl_2$ (PBS/$MgCl_2$) and fixed in ice-cold 2% paraformaldehyde/0.8% glutaraldehyde in PBS/$MgCl_2$ for 5 min at room temperature. Tubules were again washed with PBS/$MgCl_2$ and stained with 1 mg/ml X-gal in staining solution (PBS pH7.4 containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 100 mM Hepes, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6\,3H_20$) for approximately 12 h at 37°C. Tubules were subsequently washed with PBS and stored in storage solution (PBS, 2 mM $MgCl_2$, 20 mg/ml BSA, and 0.02% sodium azide) at 4°C until visual analysis.

Spermatozoa were isolated from caudal epididymi as follows. After making several small incisions into both caudal epididymi, sperm were allowed to swim out of the tissue in sperm motility medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30 mM HEPES, pH 7.4, 10 mM sodium lactate, 1 mM sodium pyruvate, 20 mg/ml BSA, and 25 mM $NaHCO_3$, pH 7.4, for several hours at 37°C. After the solution had obtained a milky appearance, the remaining epididymi were removed from the suspension and the sperm were centrifuged for 5 min at 3000 rpm. Subsequently, almost all of the supernatant was removed and the spermatozoa were directly stained with 1 mg/ml X-gal in staining solution for approximately 12 h at 37°C. Sperm samples were subsequently transferred into storage solution and kept at 4°C until further analysis.

Cryosections were stained with X-gal as follows. Testes were prefixed with 2% paraformaldehyde/0.8% glutaraldehyde in PBS/$MgCl_2$ for 5 min at room temperature followed by washing in PBS/$MgCl_2$. Testes were subsequently embedded in OCT freezing medium and frozen in isopentane precooled with liquid nitrogen. Individual seminiferous tubule fragments were frozen in a similar way. Cryosections (10 μm) were stained with 1 mg/ml X-gal for 1 to several hours at 37°C. After repeated washing with PBS, sections were briefly stained with DAPI, rinsed in PBS, and mounted in Vecta Shield antifade solution.

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