

RESEARCH ARTICLE

Seminiferous tubule cannulation (STC): a new, sensitive technique for detecting gene transfer in developing sperm

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As gene therapy vectors, strategies, and disease targets continue to expand and diversify, the likelihood that developing germ cells will be exposed to gene transfer vectors increases. Insertion of exogenous genetic material into the germ line might have devastating effects on normal development which could be heritable. Accordingly, it is important that vectors be tested for their potential to insert genes into developing gametes. Such tests are most difficult in males, where differentiating sperm are sequestered behind the blood–testis barrier. In this communication we report the development of a new technique, which we call seminiferous tubule cannulation (STC). We demonstrate that STC allows

delivery of high quantities of gene therapy vector directly to spermatogenic cells without significantly disturbing the cytoarchitecture of the seminiferous tubule. To demonstrate the effectiveness of this technique, three promoters driving lacZ gene expression in adenovirus vectors were tested for their ability to transduce cells within the seminiferous tubule. Results indicate that the cytomegalovirus promoter, but not the Rous sarcoma virus or elongation factor 1 α promoters, is active within the seminiferous tubule. Further development of this technique promises to lead to a standardized test for male germ cell transduction by gene therapy vectors. Gene Therapy (2003) 10, 43–50. doi:10.1038/sj.gt.3301850

Keywords: germ cell transduction; seminiferous tubule; adenovirus

Introduction

As with any other therapy, gene therapy can have adverse effects. However, unlike other treatments, a potential risk of gene therapy is genetic modification of the germ line by addition of new genetic material. While some conventional remedies can alter the germ line through mutagenesis, gene therapy has the potential to add new coding functions to the genome. Expression of new genetic material in the embryo, fetus, or adult could have serious harmful effects which might be heritable. For this reason it is important that experimental systems be developed which sensitively test for the potential of gene therapy vectors to access the germ line.

A variety of tests have thus far been developed to address this problem. Most of these assays have been indirect, relying upon tests of progeny after systemic administration of vectors,^{1–4} tests for reporter gene expression or genetic material in gonads at various times after vector administration,^{2,4,5–9} or statistical analyses which extrapolate probabilities of germ line integration based upon negative DNA assays of gonadal tissues.¹⁰ Unfortunately, none of these testing paradigms addresses the question of whether direct exposure of gametogenic cells, gametes, or early embryos will lead to gene transfer. Such direct exposure is likely to occur as gene therapy vectors, routes of administration, and

therapeutic strategies diversify and become promulgated. Assessment of germ line integration is more problematic for spermatogenic cells than for developing eggs. Development of oocytes within preovulatory follicles mainly involves growth; however, spermatogenesis is characterized by a complex differentiative process whereby the nuclear material condenses, chromosomal proteins are replaced, and a flagellum forms. A cell at any of the many stages of spermatogenesis might have unique susceptibility to insertion of gene therapy vectors. Further complicating the problem is that spermatogenesis takes place behind the blood:testis barrier, which prevents large molecules from accessing developing sperm from the bloodstream.¹¹

Some laboratories, including our own,^{12,13} have performed direct testicular injection of adenovirus vectors and examined seminiferous tubules for expression of the reporter gene *lacZ*, which was encoded in the vectors. Expression within tubules is seen in some¹³ but not all¹² experiments, and these tests are crude for several reasons: even the smallest injection needles are far larger than the lumen of the mouse seminiferous tubule. Thus, it is necessary to rupture the wall of the tubule with the sharp edge of the needle in order to access the lumen, and the lumen can be accessed only at sites immediately adjacent to the rupture point. Because spermatogenesis proceeds in waves longitudinally along the seminiferous tubule, such cross-sectional approaches are unlikely to expose cells at all stages of spermatogenesis to the vector. Moreover, these procedures are traumatic, and can disrupt the normal relationship between gametogenic cells and Sertoli cells of the tubule. Experimental models

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Received 26 August 2001; accepted 15 June 2002

wherein vectors are inserted into the male sex ducts as models for retrograde transport of vectors to the testis in the course of gene therapy for prostate or bladder disease have been utilized.^{4,7,8} However, these methods require successful cannulation of ductular tubules, with retrograde flow of substantial material back to the testis. As such they are technically challenging and do not guarantee that high quantities of vector will reach the lumen of the seminiferous tubule.

In an effort to develop direct tests for gene insertion into gametes, our laboratory has exposed sperm directly to adenovirus vector, performed *in vitro* fertilization, and examined early embryos for reporter gene expression.¹² We have also exposed oocytes to vectors and examined subsequent embryos and fetuses for gene expression or integration.¹⁴ These experiments are a step forward because the embryos studied after *in vitro* fertilization are known to be derived from gametes that were directly exposed to the vector. However, because they involve only mature sperm these tests are also imperfect: they do not allow exposure of spermatogenic cells at various stages of meiosis and spermiogenesis to vectors, as these differentiation events occur within the seminiferous tubule.

In this communication we describe a new technique we have developed, which we call seminiferous tubule cannulation (STC). This procedure allows perfusion of substantial lengths of the seminiferous tubule with highly concentrated gene therapy vector preparations without disturbing the structural integrity of the tubule. These attributes indicate promise for development of this methodology into a definitive test for insertion of gene therapy vectors into spermatogenic cells. We describe this technique and use it to test three different promoters for their ability to drive reporter gene expression in the seminiferous tubule. We also discuss improvements in the STC method which must be made in order to

establish it as a standardized test for male germ cell transduction.

Results

Experience with the STC procedure

Twelve STC procedures were performed with the cytomegalovirus (CMV) vector, 13 with the Rous sarcoma virus (RSV) vector, and 14 with the EF-1 vector. The following observations were made from cannulations of seminiferous tubules with microneedles and instillation of concentrated suspensions of adenovirus gene therapy vectors carrying the reporter gene *lacZ*. These observations were facilitated by addition of a small amount of trypan blue to the vector solution (see Methods):

(1) Even the smallest conventional injection needles are far too large to access the lumen of the seminiferous tubule. Figure 1 shows seminiferous tubules exposed through an incision in the testis capsule. Included in this photograph is a 30g injection needle. These needles have been used in previous attempts to expose spermatogenic cells to gene therapy vectors,¹² and they are the smallest commercially available needles. As can be readily appreciated from this photograph, a 30g needle is far too large to access the lumen of the seminiferous tubule, and can only expose spermatogenic cells to gene therapy vectors by cutting through the tubule wall.

(2) STC allows perfusion of substantial lengths of the seminiferous tubule with gene therapy vector preparations.

Figure 1 shows a testis subjected to a single STC injection. In order to better demonstrate the effectiveness of this procedure, blue ink was instilled into the tubules for improved visualization. As demonstrated here, several loops of the seminiferous tubule have been

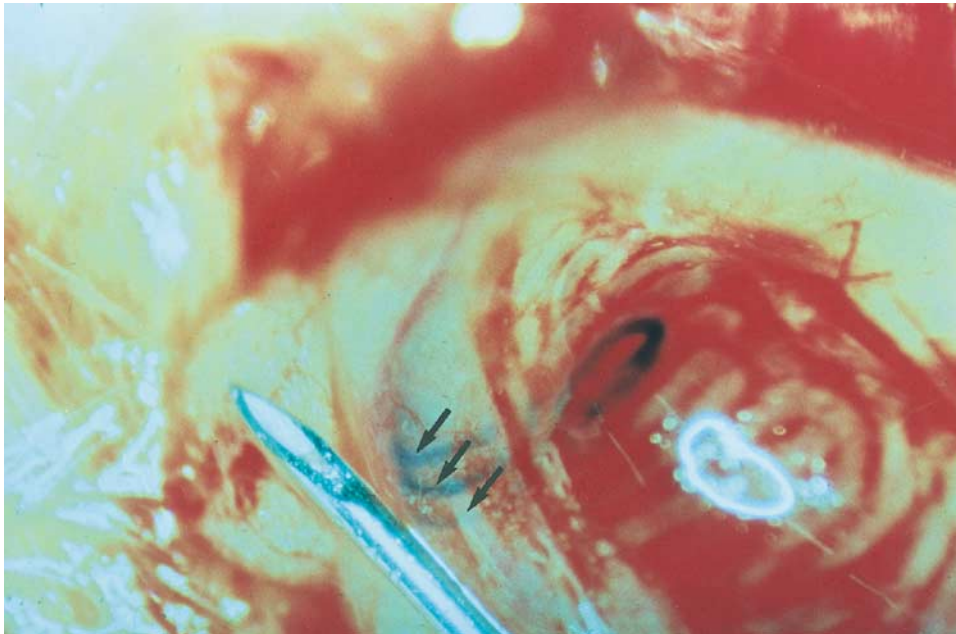


Figure 1 View through a dissecting microscope of seminiferous tubules exposed through an incision in the testis capsule and subjected to STC. The tubules were perfused with a single injection of blue ink (arrows) to indicate that length of tubule that can be perfused in one injection. Note that several loops of tubule are perfused. Also included in this figure is a 30g injection needle. This needle is far too large to access the lumen of the seminiferous tubule.

perfused (arrows). This length of tubule is equivalent to several thousand cell diameters, and thus STC can expose germ cells at all stages of spermatogenesis to high amounts of gene therapy vector. The effectiveness of STC can be further enhanced by performing more than one injection of the exposed tubules. Multiple injections facilitate location of the injected tubules when histological evaluation is performed while at the same time assuring that spermatogenic cells at all stages of differentiation are exposed to vector.

(3) Some regions of the testis were more amenable to the STC procedure than others. When the tubules were visualized through the testis capsule, there were areas where several segments of the tubule lay in parallel. These regions were far more readily cannulated than other areas where the tubules exhibited a tortuous course. In these latter regions, resistance to flow around sharp 'hairpins' in the tubules was substantial, and reflux of solution through the cannulation site was significant. When the tubule lay in straight parallel segments, several loops could be instilled with vector before resistance to flow led to significant reflux of material (Figure 1).

(4) Reflux of vector solution occurred in all STC attempts. Because of the high resistance to flow through this long narrow conduit, reflux of solution from the seminiferous tubule occurred with every attempt to perform STC. Reflux into the interstitial tissue did not complicate evaluation of tubular expression of gene therapy vectors, but it did lead to expression of the reporter gene in interstitial regions.

(5) Flow of solution through the tubule was bidirectional. After cannulation of the seminiferous tubule and expulsion of vector solution in the lumen, flow of the solution in both directions from the insertion site of the microneedle was readily appreciated. When the needle was advanced a substantial distance into the lumen prior to release of solution, retrograde flow was less pronounced. Resistance to flow was always greater in one direction within the tubule. To assure adequate perfusion in the direction of insertion of the microneedle, several punctures were made with each STC procedure.

STC does not disturb the normal process of spermatogenesis

Opening of the testis capsule causes extrusion of seminiferous tubules through the opening, and insertion of microneedles during STC creates a small hole in the tubule wall and temporarily increases fluid pressure within the tubule. These effects could impair the process of spermatogenesis and/or damage cells within the tubule. To assess these possibilities, testes subjected to STC were evaluated histologically for abnormalities at 1, 2, 3, and 4 weeks after STC, with a final evaluation at 14 weeks. Regions of the testis that were manipulated by STC were readily identified because they protruded through the testis capsule. These regions were studied carefully for abnormalities. No abnormalities were seen in the sectioned material as compared to testes that were not subjected to STC. To confirm that the very same tubules that received STC were those that were evaluated for abnormalities, tubules were stained immunohistochemically after instillation of the CMV vector. This procedure allows for microscopic analysis at a higher

resolution. Figure 2 shows the results of this experiment. The distribution of expression was similar to that seen for this vector when sections were analyzed for LacZ enzymatic activity: expression in interstitial cells was seen (Figure 2a) with no such expression seen in the control sample (Figure 2b). Very high quantities of LacZ protein were seen within some tubules, with no significant abnormalities of spermatogenesis seen (Figure 2c and d). In tubules with the very highest expression, there was a relative paucity of mature sperm. We believe this result is due to flushing of these free-floating mature cells through the lumen of the tubule in regions where STC produced the highest flow of vector solution. However, a toxic effect which destroys late spermatogenic cells cannot be ruled out with our data.

Vector gene expression within the seminiferous tubule was high with the CMV promoter but absent with the RSV or EF-1 promoters

In order to minimize the chance of failing to identify seminiferous tubules that received virus, frozen sections were performed at 14 μm and stained for LacZ enzymatic activity. Although this procedure has a lower resolution than immunohistochemistry, it allows for more cells to be studied per experiment. Figure 3a shows LacZ staining of testis sections 1 week after performing STC with an adenovirus vector carrying the *lacZ* gene driven by the human CMV promoter. As this figure demonstrates, extremely high levels of expression are apparent. Although procedures with higher resolution than the frozen section analyses performed here are clearly required to definitively determine if germ cells are infected with the virus, the results clearly demonstrate that STC can deliver extraordinarily high quantities of vector directly to spermatogenic cells, and thus that it can provide a sensitive test of the ability of these cells to be infected by any of the available gene therapy vectors. Reflux of vector material into the interstitial regions is again manifest as LacZ staining within these regions (Figure 3b). Expression of the same *lacZ* gene from the RSV and EF-1 promoters was generally lower than with the CMV vector, and expression was seen only within the interstitial regions (Figure 3c and d). Several lines of evidence indicate that failure to observe intratubular expression with the RSV and EF-1 promoters was not due to failure to successfully identify the injected tubules: in all of 11 experiments with CMV, expression within tubules was seen, whereas expression was not seen in 13 experiments with RSV and 14 experiments with EF-1. These results indicate that the tubules subjected to STC were not overlooked in the analysis. Moreover, sections through the adjacent caput epididymis in one experiment with RSV revealed enormous expression within cells lining the lumen of the epididymis (Figure 3e). This finding demonstrates that the tubule lumen was successfully perfused in the experiment, but that intraluminal expression of the RSV promoter takes place only in the ducts. We conclude from these studies that the RSV and EF-1 promoters are not as useful as the CMV promoter for reporter gene studies of gene transfer within the seminiferous tubule.

Higher resolution images obtained from immunohistochemical staining (Figure 2) allowed us to make some assessment of the cells within the tubule which were

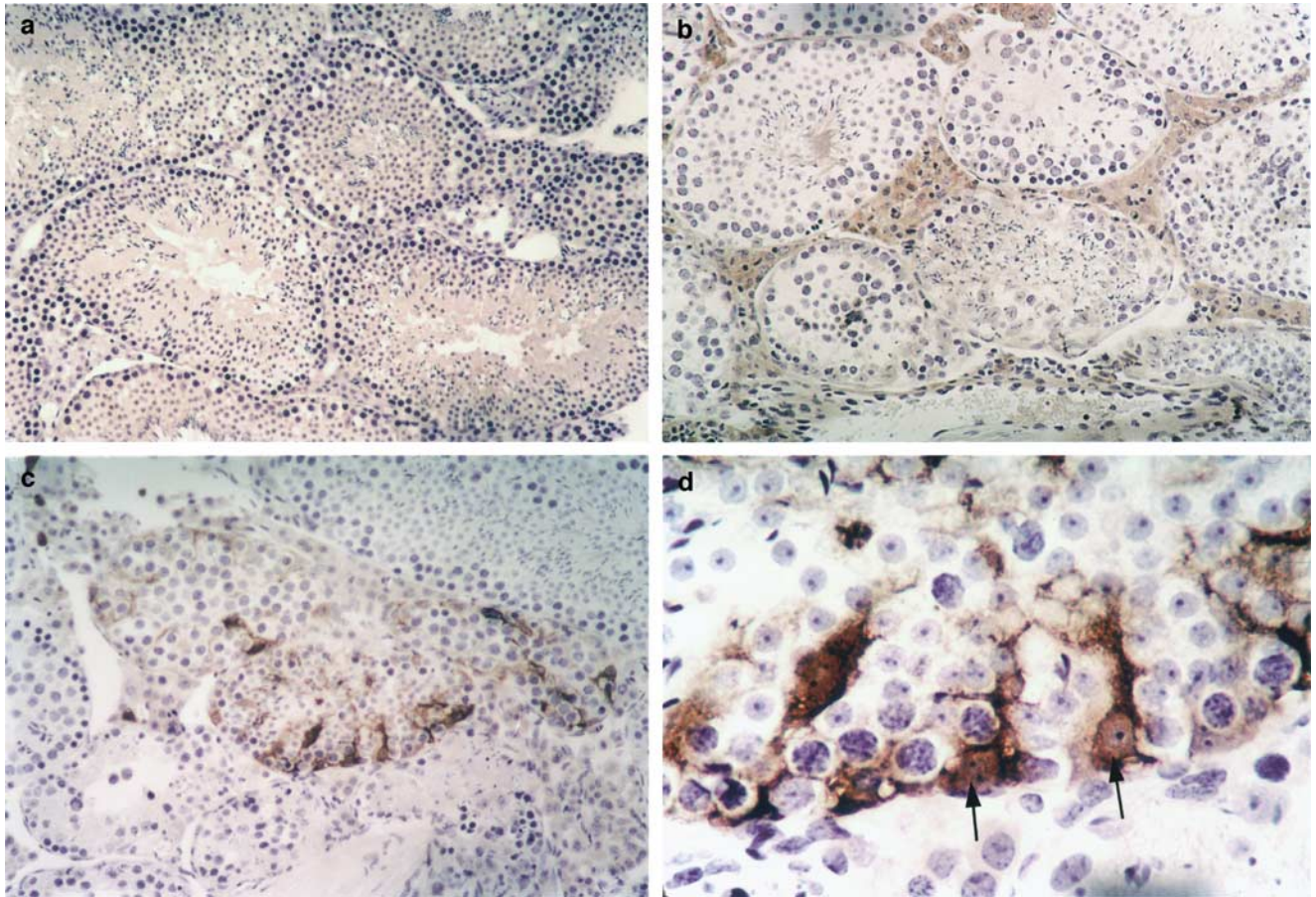


Figure 2 Staining for LacZ protein by immunocytochemistry 1 week after STC using the CMV vector. (a) Sham-operated control stained for LacZ. No staining is observed ($\times 10$ objective). (b) Staining as in (a) in the testis subjected to STC. Marked interstitial staining is seen ($\times 20$ objective). (c) Staining of seminiferous tubules for LacZ after STC. Tubule architecture appears normal ($\times 20$ objective). (d) Closeup view of (c), showing heavy staining within the tubule. Two Sertoli cell nuclei are noted with arrows. This pattern of staining is consistent with expression in Sertoli cells but does not preclude expression in germ cells ($\times 40$ objective).

transduced by the vector. Sertoli cells were clearly transduced, as determined by the pattern of staining. Sertoli cells are anchored to the basement membrane of the tubule and their cytoplasm surrounds sperm as they progress toward the tubule lumen. Thus, the Sertoli cell has an elongated cytoplasm that extends toward the tubule lumen in a pattern consistent with it being invested within the clusters of developing germ cells. A pattern of staining typical of the position and morphology of the Sertoli cell cytoplasm was clearly seen in some sections, and in these cells a very prominent nucleolus of the Sertoli cell was also identified (Figure 2d, arrow). These studies thus established expression within the Sertoli cell but did not definitively rule out expression in germ cells. The LacZ gene in this CMV vector did not have a nuclear localization signal, and the extensive staining within the cytoplasm of the large Sertoli cells which surround spermatogenic cells made it impossible to rule out expression within germ cells.

The timing of testicular expression varies among the promoters tested

In addition to comparing sites of activity of the CMV, RSV, and EF-1 promoters, we used the STC technique to

assess the timing of activation and strength of function of the three promoters. Testes were harvested at 1,2,3, and 4 weeks after STC and stained for LacZ (Table 1). The promoter with activity detected earliest was EF-1, with clear LacZ staining 3 days after STC (Figure 3d). CMV expression was highest at 1 week of vector insertion, while RSV activity peaked at 3 weeks. These results, summarized in Table 1, indicate that tests for vector expression within the seminiferous tubule using the CMV promoter tubule should be performed about 1 week after vector administration. Somewhat surprisingly, the EF-1 promoter, though active soonest after vector administration, was the weakest promoter tested (Figure 3d, Table 1).

Discussion

In this communication we describe a new technique, which we call STC, for exposing spermatogenic cells to high concentrations of gene therapy vectors, with the ultimate purpose of determining the risk that exposure of such cells to these vectors will lead to germ line gene insertion. This technique assures that high amounts of vector will reach cells that are normally sequestered

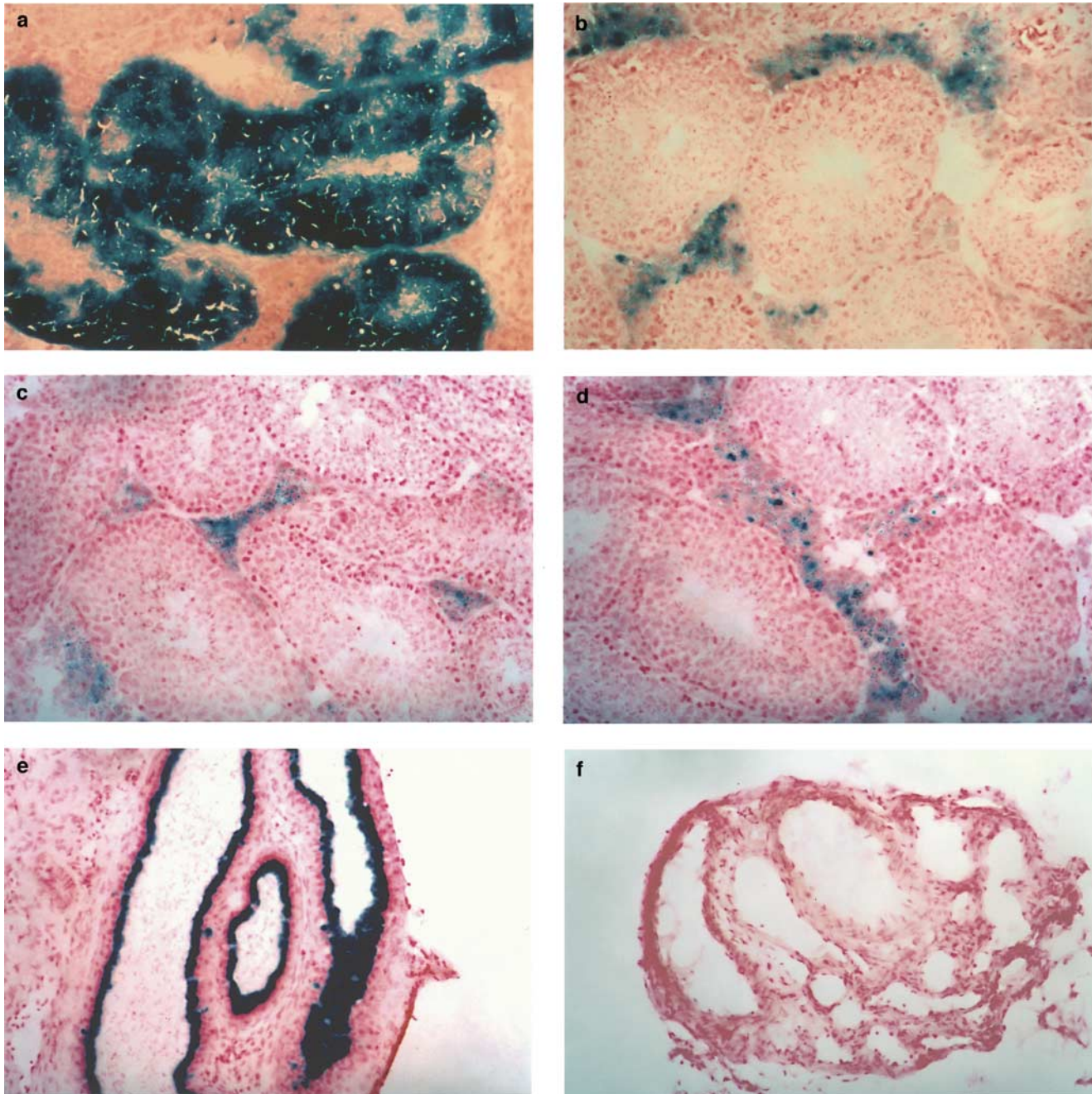


Figure 3 LacZ staining of testicular tissues after STC. (a) Testicular tubules stained 1 week after STC using the adenovirus vector with the CMV promoter. Note intense staining within seminiferous tubules ($\times 40$ objective). (b) Interstitial staining 1 week after STC with the adenovirus vector carrying the CMV promoter. Some regions are intensely stained, indicating reflux of vector into the interstitial space ($\times 10$ objective). (c) Tubules stained for LacZ 3 weeks after STC using the vector with the RSV promoter. Interstitial staining, but no tubular staining is apparent ($\times 10$ objective). (d) Interstitial LacZ staining 3 days after STC with the vector carrying the EF-1 promoter. Staining is less strong than with the DMV or RSV promoters but is clearly apparent in interstitial regions. (e) LacZ staining of the lining of epididymal tubules 3 weeks after STC using the RSV vector. Intense staining of cells lining the tubule lumen demonstrates that STC effectively accessed the lumen of the tubule ($\times 10$ objective). (f) A region of epididymis from a mouse subjected to STC with saline. No staining of luminal cells is observed ($\times 10$ objective).

behind the blood–testis barrier, without damaging those cells or the architecture of the seminiferous tubule. Further development of this methodology should lead to a standardized testing procedure for male germ cell transduction. Such a standardized test is very much

needed to address an important potential risk of gene therapy that has heretofore not been studied systematically.

As demonstrated in Figures 2 and 3, cells within the seminiferous tubule can be exposed to extremely high

Table 1 Timing and location of LacZ staining with vectors carrying the CMV, RSV, and EF-1 promoters after STC

Promoter	3 days–1 week	1–2 weeks	2–3 weeks	3–4 weeks	4 weeks
CMV					
Tubules	+	+++	+	—	—
Interstitial	+	+++	+	—	—
RSV					
Tubules	—	—	—	—	—
Interstitial	—	+	++	+	—
EF-1					
Tubules	—	—	—	—	—
Interstitial	+	—	—	—	—

amounts of gene therapy vector, in this case exemplified by an adenovirus vector. Because a very fine microneedle is inserted through the wall of the tubule, and because solution is instilled with minimal pressure over the resistance to flow that exists within the tubule lumen, STC is likely to be far less disruptive to spermatogenesis than any technique used previously.

Rupture of the tubule, as almost certainly occurs when injection needles are thrust through the capsule of the testis (see Figure 1), is likely to disturb the normal relationship of cells within the injected region. Since close interaction between spermatogenic cells and Sertoli cells occurs for several weeks and is critical for spermatogenesis, perturbations of the tubule architecture could damage spermatogenic cells and lead to false positive or negative tests for gene insertion. For these reasons, STC has the potential to provide the most reliable test yet developed for gene transfer into developing sperm. Moreover, because this procedure can be readily used in the mouse, the STC test can be inexpensive.

In order to demonstrate some of the applications of STC to the problem of male germ cell genetic transduction, we have compared three promoters in LacZ-expressing adenovirus vectors. We find that the CMV promoter, but not the RSV or EF-1 promoters, is expressed within the tubule lumen. It is unlikely, though not impossible, that these findings are due to failure to identify for examination those tubules that were successfully instilled with vector. Although these experiments were performed for the purpose of illustrating the usefulness of STC, the findings clarify a discrepancy in the published literature in this field. Direct injection of rat testes with a vector carrying the CMV promoter¹³ showed expression within seminiferous tubules, while previous work from our laboratories with the mouse showed failure of expression within tubules after epididymal instillation of a vector carrying the RSV promoter.¹² We interpreted our results as indicating that seminiferous tubules of the mouse cannot be infected by adenovirus, but the findings with STC clearly demonstrate infection within the mouse seminiferous tubule and indicate that infection was not detected previously because the RSV promoter used is not functional in cells within the tubule. The published differences between rat and mouse in the pattern of adenovirus infection within the testis was therefore reconciled by the results obtained here. It is important that such discrepancies be resolved

in order that a better appreciation of interspecies differences in susceptibility to vector entry be obtained. Dramatic interspecies differences in the ability to insert vectors into spermatogenic cells could undermine the validity of any animal test for germ cell transduction. While this concern is somewhat alleviated by our present reconciliation of the discrepancies between mice¹² and rats¹³ injected with adenovirus, more extensive investigation of this issue is warranted. We believe STC can be used to address this important issue (see below).

We have also determined that the timing of activation of the CMV, RSV, and EF-1 promoters within male reproductive tissues differs. Although these studies were again performed simply to illustrate the utility of STC, they provide important information on the behavior of these promoters, and identify an optimal time interval after vector delivery in which to evaluate testicular tissue for reporter gene expression. They also demonstrate marked differences in promoter strength, with CMV being the most active and EF-1 the least.

Although this STC procedure has potential for providing a definitive, standardized test for genetic transduction of spermatogenic cells by any method of gene transfer, some issues must be addressed before it can fulfill this potential. We must attain further assurance that the injected loops of tubules, which are a small minority of those present in each testis, do not escape analysis when preparations are sectioned and examined microscopically. Our data indicate that the injected tubules can readily be found, as we detected tubular expression in 12/12 experiments with the CMV promoter, and we were able to locate the perfused tubules in 4 μ m paraffin sections (Figure 2). However, instillation of vector can perfuse loops of tubule that remain under the testis capsule (Figure 1). While location of the injected tubules is not challenging when frozen sections are performed, identification of appropriate regions for analysis may be more difficult when higher resolution studies, which require thinner sections and high magnification views, are performed. For unequivocal identification of gametogenic cells that might take up and express gene therapy vectors, paraffin sections using vectors with a nuclear localization signal on the reporter gene, or even electron microscopic analysis might be required. Failure to analyze the appropriate regions of the testis could yield false negative results.

Another important issue involves the choice of promoters when reporter gene studies are done. Our

present findings indicate that the EF-1 and RSV promoters will not drive reporter gene expression within the seminiferous tubule and thus that use of these promoters for reporter gene studies of male germ cell transduction is likely to give a false negative result. The CMV promoter is clearly active within the seminiferous tubule (Figure 3), but we have not confirmed that this promoter is active in gametogenic cells. Previous studies with the rat have found LacZ gene expression only in Sertoli cells when this promoter is used.¹³ This result may be due to the absence of vector insertion in germ cells, but could also result from inactivity of the CMV promoter in germ cells. Our laboratory is currently preparing vectors that express reporter genes from promoters known to be active in the gametogenic cells themselves.^{15,16} In our view, an ideal test for the presence of genetic material inserted into gametes would entail direct nucleic acid hybridization, as such tests would circumvent problems of sensitivity when promoter function is low. Nucleic acid hybridization will again require efficient identification of the regions of the tubule which receive genetic material.

Even at its present state of refinement, STC can help address another important issue: that of possible interspecies differences in susceptibility of germ cells to vector insertion and the ability of transduced cells to support function of promoters that drive reporter gene expression. To our knowledge, no rigorous study has yet been conducted in which one gene transfer protocol is performed identically in several different species for a comparative analysis of gene transfer in testicular germ cells. STC requires less technical skill in larger animals than in the mouse, and we believe this technique can be used effectively to make an interspecies comparison of germ cell genetic transduction. Even if differences are found, it will be important to establish an animal test for gene transfer. However, a comparative study of several mammals would provide an important index of reliability of this assay for assessing the risk of human germ line gene insertion.

In summary, we herein report the development of a new, potentially powerful method for testing the potential of any gene therapy vector to insert foreign genes into developing sperm within the testis. This technique, which we call STC, can deliver highly concentrated vector preparations directly to germ cells behind the blood–testis barrier with minimal disturbance to the cells of the testis or to the tubule integrity. The procedure can be performed in the mouse. Further development of this technique could lead to a standardized and rigorous test for the potential of gene therapy vectors to genetically transduce male germ cells.

Methods

Mice: Adult (8–10 weeks) CD-1 male mice were obtained from Taconic Farms, Germantown, NY, USA. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee, Mt Sinai School of Medicine.

Vectors: An adenovirus vector in which the bacterial *lacZ* gene is driven by the human CMV promoter was obtained from the Vector Core of the Institute for Human Gene Therapy of the University of Pennsylvania Health

System, Philadelphia, PA, USA. Adenovirus vectors with *lacZ* driven by the RSV promoter¹² and the protein elongation factor one alpha promoter¹⁷ were obtained from the Vector Core of the Institute for Gene Therapy and Molecular Medicine, Mt Sinai School of Medicine, New York, NY, USA. All adenovirus vectors were obtained from the core facility of the Institute for Gene Therapy and Molecular Medicine, Mt Sinai School of Medicine. Vector concentrations were adjusted by dilution in KSOM¹⁸ mouse embryo culture medium (Specialty Media) to a concentration of 5×10^7 PFU/ml. Prior to STC, 0.4% trypan blue (Sigma No. T-81540) was added as 5% vol/vol of the virus suspension volume.

STC: Under general anesthesia with sodium pentobarbital, the testes were exposed through an abdominal incision. An opening in the testis capsule was made with watchmakers' forceps and extended until several loops of tubule protruded through the capsule (Figure 1). A glass microneedle was produced from capillary tubing (World Precision instruments, No. TW 100F-4) using a vertical pipet puller (David Kopf Instruments, Model 700C). The tip of the capillary pipet was filled first from below, then the barrel was filled from behind with a pasteur pipet that was tapered in a bunsen flame. For STC, the micropipet was fitted to flexible tubing with adaptors for mouth pipetting.

Prior to STC, flow from the micropipet was initiated by brushing the tip against the shaft of the forceps. After free flow of solution was established, the microneedle was inserted into an exposed tubule and advanced into the lumen. Forward pressure was then applied by mouth until solution could be seen perfusing the tubule. Constant pressure was applied until solution was advanced through several loops. This procedure was repeated for adjacent regions that were not immediately contiguous with the initial puncture site, such that many loops of tubule were perfused with each procedure. After STC, the testis was returned through the incision and the animal sutured. This process required 5–10 min for completion.

LacZ staining: Testes were embedded frozen-sectioned at a thickness of 14 μM and stained according to standard procedures and exactly as previously published¹⁴ by the Morphology and Assessment Core of the Institute for Gene Therapy and Molecular Medicine, Mt Sinai School of Medicine, New York, NY, USA. In one experiment designed to obtain a higher resolution view of the distribution of LacZ staining within the tubule, testes subjected to STC with the CMV-*lacZ* vector were harvested 1 week after STC, fixed overnight in 10% buffered formalin, embedded in paraffin, sectioned at a thickness of 4 μM , and stained immunohistochemically exactly as previously described.¹²

Paraffin embedding, sectioning and staining: Tissues were fixed overnight in 10% buffered formalin at 4°C. They were then embedded sectioned and stained with hematoxylin and eosin as published previously¹² according to standard procedures by the Department of Pathology, Mt Sinai School of Medicine, New York, NY, USA.

Acknowledgements

I would like to thank G Pohorenc for invaluable technical assistance. JWG is Mathers Professor.

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