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Muscle derived cell mediated ex vivo gene transfer to the lower urinary tract: comparison of viral vectors

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Abstract Gene therapy is a novel form of molecular medicine that may have a major impact on the future of human health care. We explored the efficacy of skeletal muscle derived cells (MDC) transduced with four viruses for ex vivo gene transfer into the lower urinary tract. Primary MDC were isolated from normal neonatal rats and transduced with: (1) adenovirus, (2) herpes simplex virus type-1 (HSV-1), (3) retrovirus or (4) adeno-associated virus (AAV), all of which express the β-galactosidase reporter gene. Adult Sprague Dawley rats ($n=4$ each group-time) were used. The MDC were injected into the right and left lateral bladder walls. The number of injected MDC ranged from 1 to 1.5×10^6. The tissues were harvested after 1, 4, 7, and 15 days, sectioned and assayed for β-galactosidase expression. In the bladder wall, we noted cells expressing β-galactosidase for each viral group. Adenoviral and HSV-1 transduced cells showed strong expression at 1 and 4 days post-injection, but the expression decreased gradually and was not detectable at 15 days post-injection. Retroviral transduced cells were detected at each time point with a strong expression persisting for 15 days but decreasing gradually over time. Although expression of the AAV transduced cells was initially weak, the later time points exhibited a much stronger expression, especially at day 7 post-injection. This expression persisted for at least 15 days post-injection. In conclusion, successful MDC mediated ex vivo gene transfer into the lower urinary tract was achieved with all four viral vectors. Our results suggest that the ex vivo approach may lead to an efficient and persistent viral gene delivery to the lower urinary tract while minimizing exposure of the host to virus.

Keywords Myoblast · Bladder · Gene therapy · Tissue engineering · Stem cell · Muscle · Virus

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Introduction

Gene therapy is a novel form of molecular medicine that may have a major impact on the future of human health care. At its inception, gene therapy focused on the treatment of inherited genetic diseases such as Duchenne muscular dystrophy, cystic fibrosis, and hemophilia [12, 26]. Progress in the field introduced gene therapy for acquired diseases such as cancer, diabetes, and AIDS [16, 22]. Recently, a landmark human clinical trial was initiated for the treatment of a nonfatal musculoskeletal disease, rheumatoid arthritis [8]. In this trial, gene therapy was utilized as a novel approach for drug delivery. The use of gene therapy in this nontraditional manner has prompted consideration toward lower urinary tract applications for such conditions as stress urinary incontinence and impaired detrusor contractility [6, 32]. Advances in cellular and molecular biology have identified a number of proteins, such as insulin like growth factor (IGF-1) and nerve growth factor (NGF) that may potentially improve detrusor contractility [3, 10].

Gene-transfer strategies, including systemic and local delivery, may be used for gene transfer to genitourinary tissues. Systemic delivery consists of injecting the vector into the bloodstream and distributing it to all organs of the body. This technique is preferable when the target tissue cannot be reached directly. Major limitations include the low specificity of gene expression and the large vector concentrations required for therapeutic effects.

Two basic strategies, direct and ex vivo, have been investigated for local gene delivery to tissues. Direct viral gene transfer has been hindered by limitations such as viral cytotoxicity and immune rejection problems.

New mutant vectors have been developed that greatly reduce the toxicity of the viral vectors which in turn have reduced immune rejection problems, but the inability of viral vectors to sustain their expression remains an important issue. This study investigates the use of the ex vivo gene transfer approach in the urinary tract to overcome the limitations of systemic and direct injection of viral vectors.

MDC can be transduced by different viral vectors and then used as a gene delivery vehicle to transfer therapeutic proteins into muscle [13, 15, 27]. MDC are uniquely different from other cells in our body in that mononucleated myoblasts can naturally differentiate to form multinucleated muscle fibers capable of muscle contraction. When myoblasts fuse to form myotubes, these cells become postmitotic with maturation. The postmitotic myotubes can express proteins in a stable fashion for a prolonged period, which may be advantageous for gene therapy [7]. Moreover, a muscle biopsy is a relatively easy and minimally invasive office procedure. From this small muscle biopsy, MDC can be easily isolated and cultivated to produce millions of cells in a few days [13]. Furthermore, the ex vivo approach is safer than direct injection because cells are isolated from the subject and genetically manipulated in a controlled environment.

The transplantation of donor muscle cells into host muscle is still hindered by immune rejection, which consequently leads to poor spreading and survival of injected cells [4, 13, 17, 21, 23, 29]. The success of cell transplantation may be dramatically improved by using a specific population of cells that has the capacity to overcome these hurdles. Indeed, we have recently isolated highly purified MDC that have been shown to dramatically improve cell survival post-transplantation when injected intramuscularly [23].

Through the preplate technique, we were capable of obtaining two distinct populations of cells that are characterized by their morphology, adherence properties to collagen-coated flasks, and immune tolerance [23, 24]. One population adhered to the flasks (preplates 1–5) within the first 4 days of isolation. These cells presented characteristics of typical satellite cells [2, 5, 28]. Previous studies in our lab, as well as others, have demonstrated that the majority of these cells do not survive post-injection [23]. The other population of cells that have been identified by the preplate technique took approximately 5–6 days to adhere to the collagen-coated flask (preplate 6). They were round morphologically and desmin positive. More importantly, preplate 6 cells (pp6) have previously demonstrated a much greater ability to survive post-transplantation when compared to satellite cells [23]. Although the mechanisms are unclear, one explanation for the improved survival rate post-transplantation is that these cells do not trigger the same immune response that normal satellite cells do upon injection. This is witnessed by a higher number of CD4 and CD8 lymphocytes in the area injected with satellite cells than in the area injected with the MDC [23].

In this study, we explored the characteristics and efficacy of MDC transduced with four viral vectors (adenovirus, herpes simplex virus, retrovirus, and adeno-associated virus) encoding LacZ reporter gene for ex vivo gene transfer into the lower urinary tract.

Materials and methods

Purification of MDC

A muscle biopsy was obtained from the whole hind limb muscle of a neonatal Sprague Dawley (S.D.) rat under sterile conditions. The muscle was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by the addition of collagenase-type XI 0.2% for 1 h at 37°C, dispase (grade II 240 unit) for 45 min, and trypsin 0.1% for 30 min. The dissociated cells were preplated on collagen-coated flasks, and the cell population in each flask was evaluated by desmin staining. Briefly, the cell suspension was placed on a collagen-coated flask for 1 h.

The first preplate flask (pp1), which represented a population of MDC that adhered in the first hour after isolation, contained a majority of fibroblasts. The non-adhering cells were then transferred to another collagen-coated flask for 1 h to obtain pp2. This procedure was then repeated in 24-h intervals until pp6. The pp6 population that was used in this study was highly enriched for desmin, a marker of myogenic cells. The proliferation medium was DMEM with 10% FBS, 10% HS, 0.5% chick embryo extract, and 1% penicillin/streptomycin. All culture media and other supplies were purchased from Gibco Laboratories (Grand Island, N.Y.).

MDC preparation for viral transfection

MDC were divided into flasks containing $1\sim1.5\times10^6$ cells. Each set of cells was infected with either adenoviral-LacZ vector (3×10^{10} pfu/ml; 25 M.O.I.), retroviral MFG-NB vector (1×10^7 pfu/ml), herpes simplex type-1 vector (1×10^9 pfu/ml; 5 M.O.I.), or adeno-associated viral vector (7×10^9 pfu/ml; 50 M.O.I.). For viral transduction, MDC were rinsed in HBSS and incubated with either the adenovirus, herpes simplex virus, retrovirus, or adeno-associated virus for 2 h at 37°C (the retrovirus infection was performed in the presence of 8 μg/ml polybrene). Following the initial incubation period, 2 ml of proliferating medium was added to the cells for an additional 12 h of incubation. After viral infection, the cells were incubated with fluorescent latex microspheres (FLMs), in order to serve as another marker by which we could follow the fate of the injected cells. At 24 h post-infection, the viral suspensions were removed and the cells were rinsed with HBSS. MDC were detached using trypsin (0.25%) for 1 min, centrifuged for 5 min at 3,500 RPM and the MDC pellet was reconstituted in 20 μl of HBSS.

Adenoviral vectors

The adenovirus, an E1-E3 deleted recombinant adenovirus kindly obtained through I. Kovesdi (Gene Vec, Rockville, Md.), had the LacZ gene under the control of the human cytomegalovirus (HCMV) promoter and followed by the SV40 t-intron and polyadenylation signal (viral titer 3×10^{10} pfu/ml).

Retroviral vectors

The retroviral vector that we used for this study was the MFG-NB [9]. The vector contains a modified LacZ gene (nls-LacZ) that includes a nuclear-localization sequence cloned from the simian virus (SV40) large tumor antigen and is transcribed from the long terminal repeat (LTR). The titer of the viral stock was 1×10^7 pfu/ml. The viral stock was grown in the laboratory of J. Huard.

Herpes simplex type 1 vectors

The herpes simplex virus vector used for this experiment was a new mutant HSV-1 vector deleted for multiple immediate early genes (ICP4, ICP22 and ICP 27) that carried the LacZ gene under the control of the HCMV promoter (viral titer 1×10^9 pfu/ml) [14].

Adeno-associated viral vectors

The adenovirus-free AAV-LacZ viral stocks (pXX2-LacZ) were made by two-plasmid cotransfection and double CsCl purification according to the published method [31]. The viral doses used for these experiments ranged from 7×10^9 to 7×10^{10} pfu/ml. pXX2-LacZ is an AAV-LacZ vector containing the cassette with the human cytomegalovirus promoter driving the LacZ reporter gene followed by the simian virus 40 (SV 40) polyadenylation signal, which is flanked by the AAV inverted terminal repeats (ITRs). In addition, this plasmid also contains the AAV rep and cap genes. After coinfection of 293 cells with various dilutions of the AAV stocks and adenovirus 5 (Ad5 dl309) at a M.O.I of 1 for 24 h, the titers of the AAV-LacZ viruses were determined by counting the blue cells after 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) staining [31].

Virally transduced MDC injection in animals

All experiments were performed on adult female S.D. rats in accordance with the requirements and recommendations in the Guide for the Care and the Use of Laboratory Animals (US Public Health Service). This project was approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh and the University of Pittsburgh. Only certified viral free animals were used. The animals were housed in an approved viral gene therapy facility at the Children's Hospital of Pittsburgh. After anesthesia and surgical preparation, a low midline incision was made to expose the bladder wall. A total of 20 µl of a virally transduced MDC suspension in HBSS solution ($1\sim1.5\times10^6$ cells per 20 µl) was injected into the bladder walls of S.D. rats.

Tissue harvest and histology

The animals were sacrificed at 1, 4, 7 and 15 days after MDC injection, and the bladder was removed. The tissues were then snap frozen using 2-methylbutane, pre-cooled in liquid nitrogen. Analysis of the sections included hematoxylin/eosin staining and X-gal staining for the β-galactosidase reporter gene to determine the location of the gene transfer area and the viability of the injected MDC. The area around each injection site was stained, examined microscopically, and photographed.

LacZ staining by histochemical technique

The cryostat sections of the injected tissue were stained for LacZ expression as follows: they were first fixed with 1% gluteraldehyde (Sigma) for 1 min and rinsed twice in phosphate-buffered saline (PBS). They were finally incubated in X-gal substrate [0.4 mg/ml 5-bromo-chloro-3-indolyl-β-D-galactoside (Boehringer-Mannheim, Indianapolis Ind.), 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$] in PBS 3 h-overnight (37°C).

Immunohistochemistry for desmin on cultured cells

A monoclonal antibody specific for desmin was used. The cultured cells were fixed with cold methanol (–20°C) for 1 min and blocked with 5% horse serum for 1 h. The cells were incubated overnight at room temperature in a humid chamber with primary antibodies (1/200 monoclonal mouse anti-desmin; Sigma, St. Louis, Mo.) in PBS, pH 7.4. After three rinses in PBS, the cells were incubated with a second antibody, anti-mouse conjugated to Cy3 immunofluorescence (1/200; Sigma) for 1 h. Following three rinses in PBS, the cells were visualized under fluorescent microscopy.

Data processing

The photographs of LacZ staining were taken with a Nikon E 800 microscope. The image was transferred via Photoshop software (Adobe System) and saved on a TIFF format. The intensity of X-gal staining was evaluated by measuring the square pixels with NIH-Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/), where black and white binary images were obtained for quantification.

Results

In these experiments, we investigated the feasibility of establishing an ex vivo gene delivery approach using virally transduced MDC injected into the bladder wall of adult S.D. rats. All animals survived without any complications. We were able to isolate MDC via the preplate technique. Immunohistochemistry for desmin was used to characterize the MDC in culture (Fig. 1). We have observed that different populations of primary MDC at different preplates contain different percentages of desmin positive cells [23]. A phase contrast and desmin stain of pp6 MDC showed that more than 90% were desmin positive cells (Fig. 1A,B).

For the animal experiments, we were able to identify cells expressing β-galactosidase for each viral group in

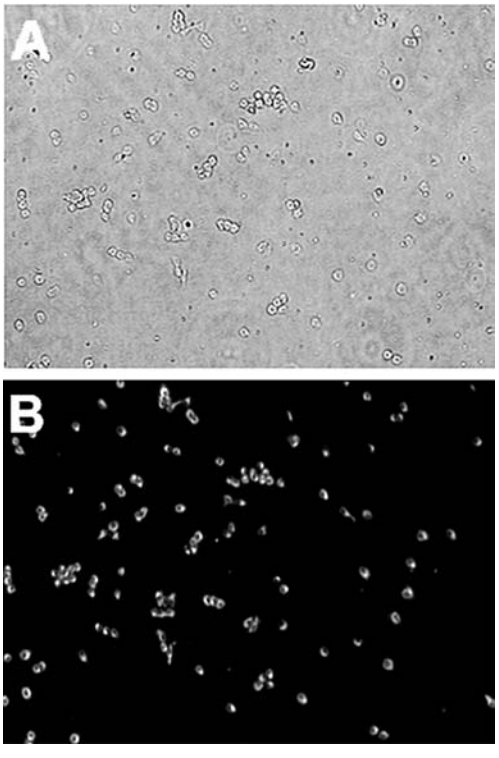

Fig. 1. Phase contrasts of cultured MDC **A** were over 90% positive for desmin **B**. Magnification ×200

the injected bladder. Fluorescent microscopy for FLM's gave us a quick assessment of the viability of the injected MDC and also the migration and distribution of injected MDC (Fig. 2).

Although we could observe many FLMs in the injected site, LacZ positive cells were not always detected. This means that the injection was successful, but β-galactosidase expression was not demonstrated. Positive blue staining for LacZ demonstrated that injected MDC survived and were capable of protein synthesis due to the expression of the transduced LacZ reporter gene.

In the study comparing the four viral vectors, adenoviral transduced cells showed a strong expression at 1 and 4 days post-injection (Figs. 3A, B). However, a gradual decrease in expression led to a weakened signal at 7 days (Fig. 3C) that was undetectable at 15 days post-injection (Fig. 3D). HSV-1 transduced cells demonstrated strong expression at 1, 4, and 7 days post-injection (Figs. 4A–C), but the expression decreased gradually and was almost non-detectable at 15 days post-injection (Fig. 4D). Retroviral transduced cells were detected at each time point with a strong expression persisting for 15 days that decreased gradually over time. The expression of retroviral transduction at 7 days was the strongest among the four viral vectors (Fig. 5).

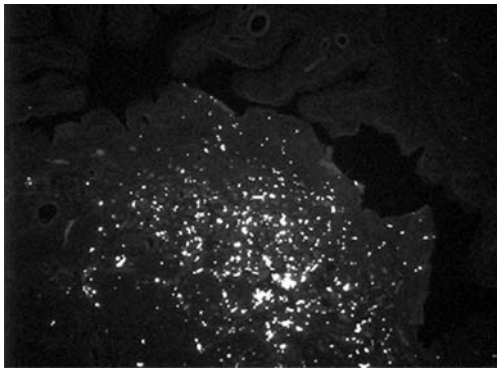

Fig. 2. Photomicrographs of FLM incubated MDC injected into bladder sections. *Green* shows FLM-positive MDC in the injected bladders. Magnification ×100

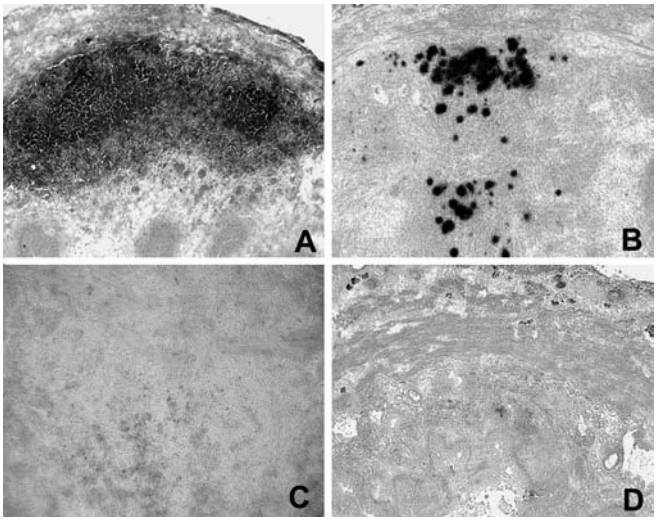

Fig. 3. Photomicrographs of adenoviral transduced MDC injected into bladder sections showing LacZ staining at 1, 4, 7, and 15 days post-injection (**A**, **B**, **C**, **D**, respectively). The *blue* area shows the injected LacZ positive MDC. Adenoviral transduced MDC showed strong expression at 1 and 4 days post-injection (**A**, **B**), but gradually decreased to a weak signal at 7 days (**C**) and was undetectable at 15 days post-injection (**D**). Magnification ×100

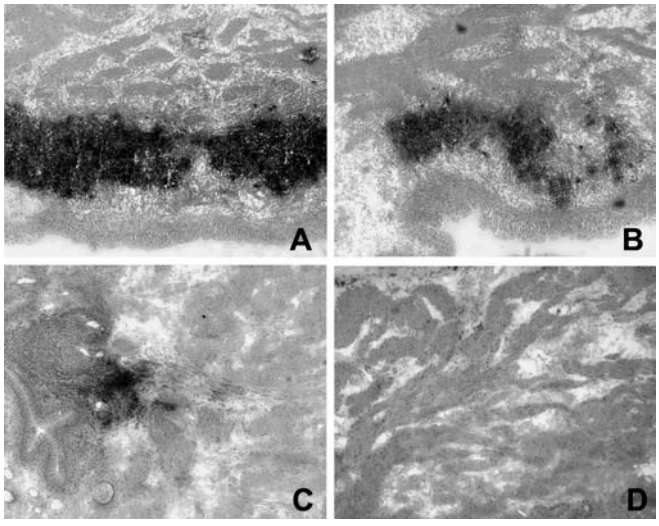

Fig. 4. Photomicrographs of HSV-1 transduced MDC injected into bladder sections showing LacZ staining at 1, 4, 7 and 15 days post-injection (**A**, **B**, **C**, **D**, respectively). The *blue* area shows the injected LacZ positive MDC. HSV-1 transduced MDC demonstrated strong expression at 1, 4 and 7 days post-injection (**A**, **B**, **C**), but the expression decreased gradually and was almost non-detectable at 15 days post-injection (**D**). Magnification ×100

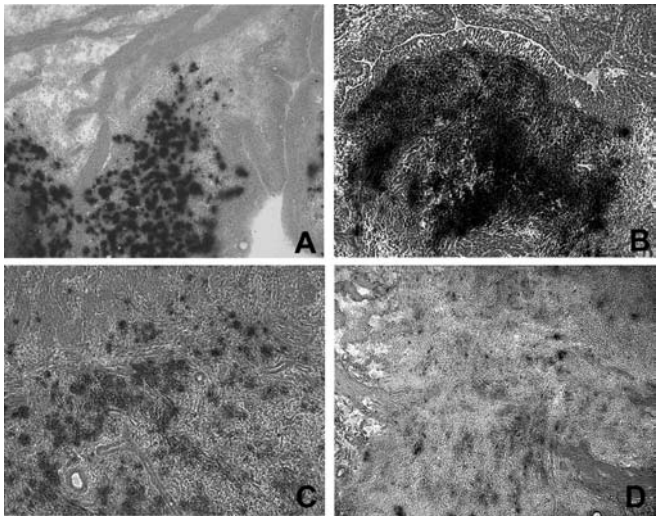

Fig. 5. Photomicrographs of retroviral transduced MDC injected into bladder sections showing LacZ staining at 1, 4, 7 and 15 days post-injection (**A**, **B**, **C**, **D**, respectively). The *blue* area shows the injected LacZ positive MDC. Retroviral transduced MDC were detected at each time point with a strong expression persisting for 15 days but decreasing gradually over time. Magnification ×100

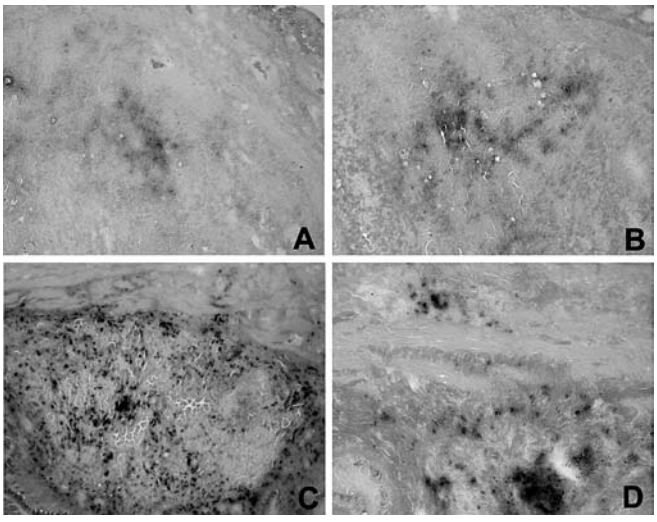

Fig. 6. Photomicrographs of AAV transduced MDC injected into bladder sections showing LacZ staining at 1, 4, 7 and 15 days post-injection (**A**, **B**, **C**, D, respectively). The *blue* area shows the injected LacZ positive MDC. Although AAV transduced cells had an initially weak expression, later time points had a much stronger expression, especially at day 7 post-injection. This expression persisted for at least 15 days post-injection. Magnification ×100

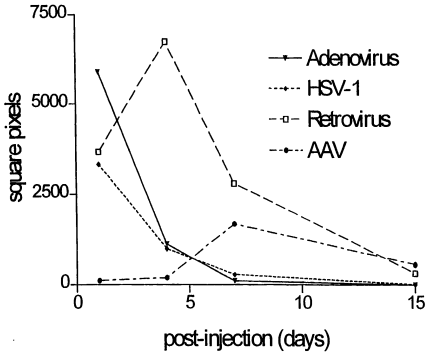

Fig. 7. Line graph demonstrating the intensity of LacZ expression of each viral vector at each time point. Each section was analyzed with a NIH-image program, where black and white binary images were obtained for quantification ($n = 4$ for each group at each time point)

Although AAV transduced cells had an initially weak expression, later time points had a much stronger expression, especially at day 7 post-injection. This expression persisted for at least 15 days post-injection (Fig. 6), but was weaker than other viral vectors. In every viral vector trial, many LacZ positive cells were observed between submucosal and smooth muscle layers. Some of the injected MDC migrated into the smooth muscle layer over the time course (Figs. 4C; 5C, D; 6D).

We compared the intensity of LacZ expressions of each viral vector at each time point by analyzing square pixels with NIH-image program, where black and white binary images were obtained for quantification (Fig. 7). The X-gal expression of adenoviral and HSV-1 vectors was the strongest at 1 day post-injection but decreased over time. Retroviral and AAV vector X-gal expression were both strongest at 4 and 7 days post-injection and persisted for 15 days post-injection.

Discussion

This study demonstrated the feasibility and efficacy of ex vivo gene therapy to the lower urinary tract using skeletal muscle derived cells. Although MDC may not be the intuitive first choice as a gene-delivery vehicle to the bladder wall, it does offer several advantages. A skeletal muscle biopsy to isolate MDC is easier and less invasive than cystoscopic biopsy to isolate detrusor smooth muscle cells. A small quantity of muscle usually contains enough myogenic cells to produce millions of MDC in culture.

The ex vivo MDC mediated gene therapy approach can serve, not only as a vehicle for gene complementation, but also create a reservoir of myogenic cells capable of regenerating myofibers. Once the MDC cells are isolated and grown in culture, it is easy to distinguish a batch of pure muscle cells from those contaminated with other cell types such as fibroblasts and adipocytes using desmin, a myogenic-specific marker, to determine the myogenic index of the cell culture (Fig. 1).

One weakness of this study was the use of allogenic MDC. In theory it would have been better to use autologous MDC. Autologous MDC have been shown to survive over 4 weeks after bladder wall injection and differentiate into myotubes [33]. However, the time requirement for a large number of animals and separate cultures and the potential variation amongst MDC of different animals would be problematic. All the cells that were used came from the same animal and the potential immunogenicity was similar in all four groups.

Adenoviral vectors can infect both mitotic myoblasts and post mitotic immature myofibers, and they can be prepared at high titers (10^9–10^{11} pfu/ml) [1]. However, the stability and long-term expression of transgenes delivered to skeletal muscle using the first generation adenoviral vectors have been hindered, mainly because of immune rejection. The low gene insert capacity (up to 8 kb) of the first generation adenoviral vectors has recently been overcome by the development of new mutant adenoviral vectors that lack all viral genes and have an insert capacity 28 kb [19]. In our experiment, the LacZ expression of the adenoviral vector was strongest at 1 and 4 days post-injection, but the expression was almost gone at 1 week post-injection. This decrease in expression was probably due to immunorejection of adenoviral vector.

Herpes simplex virus (HSV) type-1 based vectors are naturally capable of carrying large DNA fragments (up to 25 kb). However their relatively high cytotoxicity, which hampers long-term transgene expression, has been identified as a major disadvantage of the first-generation HSV vectors. Recently, the deletion of the immediate-early (IE) genes from the HSV vectors was noted to be associated with reduced cyotoxicity [14]. Similar to

adenoviral transduced cells, HSV-1 transduced cells demonstrated a strong expression at 1, 4, and 7 days post-injection. However, the expression decreased gradually and was almost non-detectable at 15 days post-injection.

Retroviruses are eukaryotic RNA viruses. They can infect dividing cells with a high efficiency and are relatively safe [25]. The ability of retroviruses to become integrated into the host cell genome can provide long-term stable expression of the delivered gene. Retroviral vectors have limitations such as low insert capacity (up to 7 kb), relatively low viral titers (10^5–10^6 pfu/ml), and the risk of insertional mutagenesis. In our experiment, retroviral transduced MDC demonstrated β-galactosidase activity strongly at each time point with the peak at day 4. These results suggest that retroviral vectors ex vivo approach should have clinical applications because they have low immunogenicity and thus can achieve long-term persistence.

Adeno-associated viral vectors (AAV) have also been reported as gene delivery vehicles for muscle cells [31]. AAV is a small non-pathogenic DNA virus that can only replicate in cells in the presence of helper viruses such as adenovirus or herpes virus. Although both long-term transgene expression (up to 18 months) and a high efficiency of mature myofiber transduction have been observed in mouse skeletal muscle, the application of AAV vectors for gene therapy might be limited by their restrictive gene insert capacity (less than 5 kb) [30]. In our experiment, positive LacZ staining was observed at each time point with the highest expression at day 7, but the expression was much weaker than other viral vectors.

Although there are few reports of injections of skeletal MDC into the urinary tract [6, 32], there have been several reports of gene therapy and skeletal myoblast therapy for promoting myocardial regeneration [11, 20, 34]. In addition to the ability of MDC to differentiate into myotubes, they may also improve the function of injected organs. We have shown that several viruses can be successfully delivered to the bladder wall using MDC mediated ex vivo gene transfer. MDC are capable of delivering new genes that are engineered to secrete trophic substances. The ability to upregulate trophic factors at the site of MDC injection may improve muscle regeneration leading to improved muscle healing [18]. Injection of MDC and MDC based gene transfer into the lower urinary tract may improve functional properties of the bladder and urethra.

The current approaches to ex vivo gene therapy using MDC are not without limitations. It is clear that immunological problems associated with viral vectors, as well as the bacterial β-galactosidase to follow the fate of the injected cells, still limit this technique. However, with viral vectors continuously being engineered to be less immunogenic, combined with the use of MDC that appear to display an immune privileged behavior, it is quite conceivable that this therapy may be used for urinary clinical applications.

In conclusion, we demonstrated the feasibility of MDC mediated ex vivo gene transfer with four different viral vectors into the lower urinary tract. Our results suggest that the ex vivo approach may someday achieve an efficient viral gene delivery into the lower urinary tract.

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