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In vivo gene transfer to kidney by lentiviral vector

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Background. The growing understanding of the molecular basis of renal diseases makes the development of gene therapy for kidney disorders a potential treatment alternative. Work aimed at determining the feasibility and the efficiency of gene transfer to the kidney using different viral and nonviral transduction systems is a necessary component to understanding the full potential. Lentiviral vectors have been shown to transduce stably different tissues and cell types that are refractory to other gene transfer approaches. To date, the potential of lentiviral vectors to transfer genes in kidney has not been investigated. The scope of this work was to analyze the efficiencies of in vivo transduction of kidney by a lentiviral vector.

Methods. A pseudotyped lentiviral vector carrying the gene for the enhanced green fluorescent protein (EGFP) was delivered into one kidney of experimental mice by retrograde infusion through the ureter. The presence of the virus and the expression of the reporter protein were monitored over time.

Results. Both viral DNA and EGFP expression were measurable in the kidney infused with the lentiviral vector but not in the contralateral kidney. Protein expression was detected by immunostaining, as EGFP fluorescence was masked by the high background fluorescence of the kidney. Expression of EGFP persisted for the entire two-month duration of the experiments.

Conclusions. Lentiviral vectors can effectively deliver exogenous genes to the kidney in vivo, resulting in persistent expression of the introduced gene.

Gene transfer into cells in solid organs is a fundamental goal and presents a major challenge for gene therapy. The identification of genes involved in renal diseases has elicited great interest in gene therapy as an attractive potential treatment for many genetic and metabolic disorders that affect the kidney [1]. Unfortunately, the anatomical complexity of the kidney and the heterogeneity of the cell types that contribute to the specialized function of the nephron make in vivo transduction of kidney a challenging task.

To date, in vivo gene transduction of kidney cells has been addressed using different gene transfer systems based on naked DNA, cationic lipid complexed-DNA, and viral vectors [2]. Adenoviral vectors [3–5], as well as synthetic DNA transduction methods [6–9], result in transient expression of the transgene. Murine retroviruses transduce tubular epithelial cells only following a regenerative response induced by nephrotoxic injury [10]. The dependence of murine or avian retroviruses on cell proliferation for stable integration may limit their application in renal gene therapy because most of the cells in the kidney are postmitotic. Recombinant adeno-associated virus (AAV) vectors result in stable expression of transgene following renal parenchymal injection [11]. Nevertheless, some limitations are inherent to recombinant AAV vectors such as the loss of site-specific integration characteristic of the wild-type AAV and the relatively small cloning capacity. Therefore, the further analysis of gene transfer methods and vector systems is essential for the improvement of renal gene therapy.

Gene delivery systems based on lentiviral vectors have been shown to transduce a wide variety of organs and tissues, including the brain [12–14], liver [15], hematopoietic cells [16], and myocytes [17] either in vitro or in vivo. However, the transduction of kidney using lentiviral vectors has not yet been examined. Lentiviral vectors may offer unique advantages as compared with other delivery systems in targeting kidney cells. They provide persistent transduction through stable integration into the host genome of proliferating as well as nondividing and terminally differentiated cells (for example, adult kidney cells). These are essential characteristics, as the correction of the diseased phenotype in many cases will require continuous expression of the transgene. Moreover, the recent developments in vector design [18, 19] and the establishment of stable packaging cell lines [20] have significantly increased the safety and the ease of production of lentiviral vectors, respectively.

The aim of our studies was to determine whether lentiviral vectors could transduce renal cells in vivo. We explored the lentivirus-mediated delivery of a reporter gene, enhanced green fluorescent protein (EGFP), in kidney in vivo following intraureteral injection. We show that both transgene DNA and transgene expression are detectable in the lentiviral-transduced kidney for the entire

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duration of the experiments. These results prove that lentiviral vector can effectively deliver exogenous genes in renal cells in vivo, resulting in persistent gene expression, and that they can be a valuable tool for the gene therapy of kidney diseases.

METHODS

Viral vector preparation

To construct the pHR′PGK, KS-PGK, a bluescript KS plasmid (Stratagene, La Jolla, CA, USA) containing the murine phosphoglycerate kinase (PGK) promoter in the EcoRI site was digested with BamHI, filled in with Klenow polymerase, and self-ligated to generate a ClaI site. The ClaI-HindIII fragment containing the PGK promoter was inserted into the corresponding sites of pcD31/C2 derived from the insertion of a ClaI linker into the NruI site of the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). The PGK promoter with the adjacent 3′ polylinker site was excised with ClaI and XhoI and inserted into the corresponding sites of pHR′ [21] (kindly provided by Dr. D. Trono) to generate the pHR′PGK vector. The EGFP gene from the pEGFP1 (Clontech, Palo Alto, CA, USA) digested with BamHI and NotI was ligated into the corresponding sites of pHR′PGK to generate pHR′PGK/EGFP. Supernatants containing the infectious lentiviral vectors pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) were produced by transient transfection of 293T cells using pHR′PGK/EGFP, pCMVΔR8.2 containing all of the transcomplementing viral proteins except *env*, and pMD.G plasmids, which codifies for the VSV-G protein [21] in a 4:3:1 ratio and Lipofectamine 2000 lipid transfection system (Life Technologies, Gaithersburg, MD, USA) according to manufacturer's instruction. Cells were seeded at 8×10^6 in T75 flasks in complete Dulbecco's modified Eagle's medium (DMEM supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin) 24 hours before the transfection. A total of 20 μg of plasmid DNA was used per flask. Medium was replaced after 16 hours following transfection with complete medium supplemented with 4 mmol/L sodium butyrate (Sigma, St. Louis, MO, USA). Supernatants were harvested 24 and 48 hours later and filtered though a 0.45 μm pore-size filter, and viral particles were concentrated by centrifugation for five hours at 15,000 rpm. Viral pellets were resuspended in 1% of the starting volume of medium in sterile phosphate-buffered saline (PBS) and titer determined as transduction units (TUs). Briefly, serial dilutions of each supernatant were used to infect 10^4 HeLa-Tat cells per well in 24-well plates in the presence of complete medium supplemented with 10 μg/mL Polybrene (Sigma). The TU was determined 48 hours following infection by scoring the number of positive cells expressing the EGFP transgene detected by fluorescence microscopy.

Retrograde ureteral infusion

All animal experiments were conducted according to the Institutional Animal Care and User Committee of Mount Sinai School of Medicine. One-month-old FVB/N mice were anesthetized with Avertin (2,2,2-tribromoethanol in 2 mL 2-methyl-2-butanol, 375 μg/kg intraperitoneally). The left kidney was exposed via a flank incision and gently separated from the surrounding fat. The ureter was clamped distal to the infusion site, and the left uretero-pelvic junction was cannulated with a 30-gauge needle under stereomicroscopic guidance. Approximately one 5×10^8 TUs of lentiviral vector in 80 μL of PBS were infused under gentle pressure to avoid renal pelvic distention and hematuria or leaking of the injected solution from the injection site. After five minutes, the catheter was removed, and urine flow was restored. The abdominal muscles were then sutured, using absorbable suture material, and the skin was closed with surgical staples. The animals were placed in individual cages, and their recovery was monitored.

Tissue processing

After one or two months, the animals were anesthetized with Avertin and killed by decapitation. Kidney tissue obtained from experimental animals was bisected and fixed in 4% paraformaldehyde in PBS for four hours at 4°C. One half of each fixed kidney was embedded in paraffin and 5 μm-thick sections were cut. Sections were then dewaxed and taken through a series of graded alcohol rehydration steps. The other halves of fixed kidney tissue were washed in PBS, transferred into 15% sucrose for 12 hours, and snap frozen using Gentle Jane Snap-Freezing system (Instrumedics Inc., Hackensack, NJ, USA), and 5 μm-thick sections were cut using sliding microtome cryostat. The sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and used for fluorescence microscopy. To reduce the background fluorescence, some of the frozen sections were immersed for 45 minutes in a solution of 0.1% $NaBH_4$ in PBS in low light [22]. The sections were then rinsed three times in PBS and mounted in Vectashield mounting medium (Vector Laboratories) before fluorescence microscopy analysis.

Immunohistochemistry

Immunostaining was performed on paraffin sections to detect EGFP. For each specimen, multiple sections were first incubated in a blocking solution consisting of PBS containing 10% normal goat serum for 40 minutes at room temperature and then with anti-EGFP polyclonal rabbit antibodies (Clontech) at 1:50 dilution. Goat anti-rabbit biotinylated secondary antibodies diluted 1:200 in

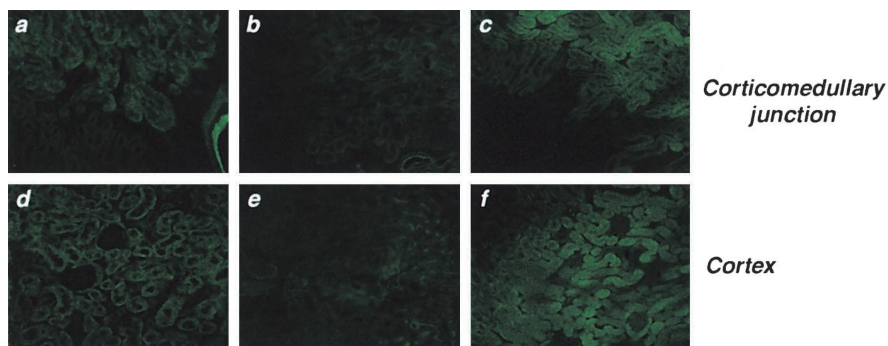

Fig. 1. Fluorescence microscopy of untreated (*c* and *f*) and HR′PGK/EGFP-infused kidney (*a*, *b*, *d*, and *e*) at one month. The corticomedullary junction (a–c) and the cortex areas (d–f) are shown. Sections in b and e, from virus-treated kidney, were treated with sodium borohydride as described in the **Methods** section. The treatment resulted in a general decrease of the autofluorescence without, however, improving the specific signal.

PBS and avidin-biotin-horseradish-peroxidase were used for detection using the ABS kit (Vector Laboratories) according to the manufacturer's instruction. Sections were developed in 3-amino-9-ethylcarbazole (AEC) solution (Vector Laboratories). For a negative control, immune serum was replaced by PBS. The tissues were counterstained with hematoxylin (Vector Laboratories) for easier visualization and mounted using Aqua Poly/Mount medium (Polysciences, Inc., Warrington, PA, USA) for microscopic analysis.

DNA extraction and polymerase chain reaction

Total DNA from fixed frozen kidney was extracted using the QIAamp tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Equal amounts of DNA from each sample were used in the polymerase chain reaction (PCR) amplification. For the amplification of EGFP, the PCR reactions were performed in the following PCR mixture: PCR buffer 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 5 U Taq, 100 ng/μL of the following primers: primers 5E, 5′-TGACCCTGAAGTTCATCTG CACCACCGGCA-3′, and 3E, 5′-CGAGCTGCACGC TGCCGTCCTCGATGTTGT-3′. PCR was performed for 45 cycles at 94°C for 30 seconds and at 72°C for 1 minute. A final elongation step at 72°C for two minutes was added. The amplification of the cellular gene G3PDH was carried out in the same PCR reaction mixture for 20 cycles at 94°C for 1 minute, 58°C for 30 seconds, and 72°C for 45 seconds using the primers: G3PDH/forward, 5′-ACCACAGTCCATGCCATCAC-3′, and G3PDH/reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

To determine whether lentiviral vectors could transduce renal cells in vivo, retrograde ureter injection was performed on one-month-old FVB/N mice to infuse either control PBS or five 10×10^7 TUs of the lentiviral vector HR′PGK/EGFP. In this vector, the expression of the EGFP gene is driven by the constitutive murine PGK promoter.

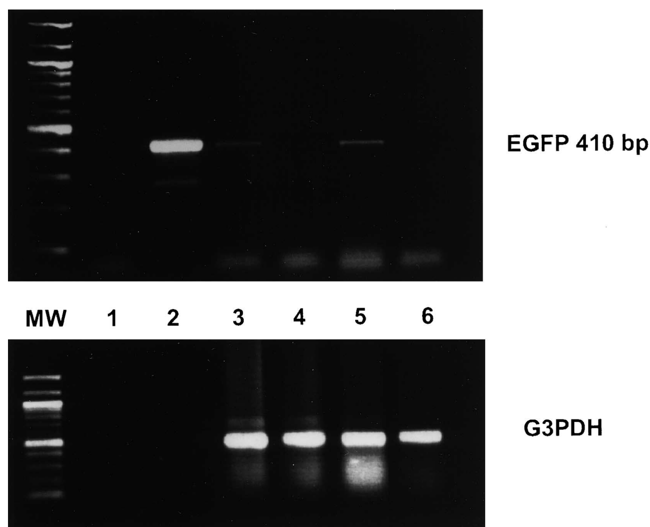

Fig. 2. Detection of EGFP transgene DNA in lentiviral transduced kidneys. Kidneys were collected one month (lanes 3 and 4) or two months (lanes 5 and 6) after viral infusion. Total DNA was extracted from kidneys infused with HR′PGK/EGFP (lanes 3 and 5) and contralateral kidneys (lanes 4 and 6) and subjected to PCR amplification for EGFP or G3PDH. Lanes 1 and 2 show the reaction products of the negative control, in which the template DNA was omitted, and the positive control, in which a plasmid containing the EGFP gene was used as template, respectively. MW, DNA molecular weight markers.

Animals were sacrificed one or two months following viral injection, and both injected and contralateral control kidneys were harvested and snap frozen to analyze EGFP expression. Each kidney was cut transversally in two halves that were used either for microtome dissection or for PCR analysis. The detection of EGFP by fluorescence microscopy proved particularly difficult in the kidney because of the very high background fluorescence of kidney sections, particularly in the cortex or possibly because of the relatively weak intensity of the expressed EGFP (Fig. 1). Alternative methods to reduce

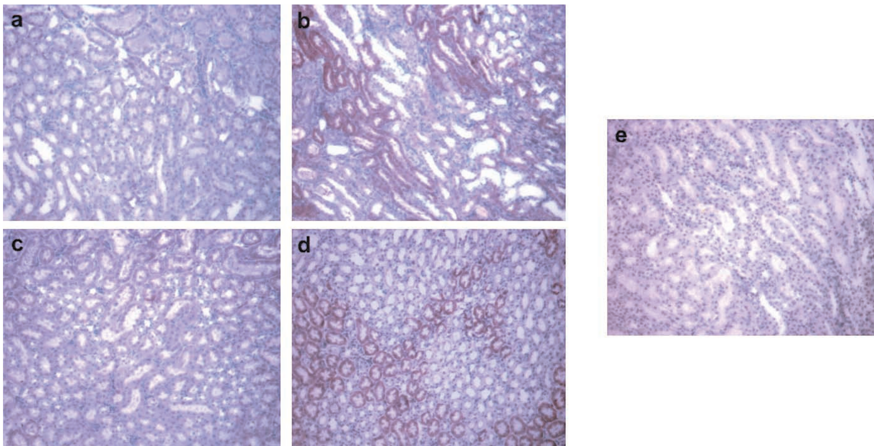

Fig. 3. Expression of EGFP in lentiviral transduced kidneys. Sections were prepared from kidneys collected one month (*a* and *b*) or two months (*c*–*e*) following retrograde lentiviral infusion or PBS infusion. The immunodetection for EGFP showed clear positive staining in the corticomedullary junction of the HR′PGK/EGFP-infused kidneys (b and d). Control contralateral kidneys (a and c) and a PBS-injected kidney (e) were used as control and showed no specific staining.

spontaneous fluorescence, such as sodium borohydride treatment [22], were not effective.

In order to assess whether viral infection had occurred, the presence of EGFP gene was analyzed by PCR amplification on total DNA extracted from the frozen half of each kidney. Equal amounts of total DNA were used in the amplification reaction. A 410 bp band corresponding to the amplified EGFP DNA was detectable in the kidneys both after one month and two months following the viral infusion (Fig. 2). No amplification product was detected in the kidneys of animals injected with PBS alone or in the contralateral kidneys of virus-infused animals. The G3PDH gene was readily amplifiable in all of the samples confirming the integrity of all the DNA specimens.

Possible explanations for the lack of easily detectable EGFP fluorescence included promoter inactivation or the masking of EGFP by the high background fluorescence. To address these hypotheses, we analyzed EGFP expression by immunohistochemistry using a specific anti-EGFP polyclonal rabbit antibody. The immunodetection showed EGFP-specific staining in virus-infused kidneys but not in control contralateral kidneys or in the kidney of animals infused with PBS (Fig. 3). In the kidney infused with HR′PGK/EGFP, the signal was distributed along the corticomedullary junction with prominent localization in the proximal straight tubules. EGFP expression was still readily detectable up to the termination of the experiments, two months after lentivirus infusion. These data also indicated that the PGK promoter was active during the duration of the experiments and suggested that EGFP fluorescence may not be a reliable readout for the determination of EGFP expression in kidney.

DISCUSSION

The development of an efficient gene transfer approach to address many inherited renal disorders such as Alport's syndrome, renal tubular acidosis, and polycystic kidney disease or to prevent localized renal inflammatory processes will depend on effectively delivering the therapeutic gene to the kidney. Here we have reported that lentiviral vectors successfully transduced kidney cells in vivo following retrograde infusion through the ureter and resulted in the persistent expression of the reporter transgene, EGFP, up to the two-month duration of the experiments.

Despite the initial difficulties we encountered in directly analyzing EGFP expression through fluorescence microscopy, the detection of retrotranscribed viral DNA, measured by PCR amplification of the transgene, indicated that the lentiviral vector entered the renal cells. Transduction was confirmed by the immunohistological detection of the expressed EGFP. The expression of the transgene delivered by the lentivirus was predominantly detected in the corticomedullary junction and appeared to involve mostly proximal straight tubules. The localization of EGFP expression within the bright autofluorescent cortical boundary may in part explain the difficulty in detecting the reporter by fluorescence. This distribution differs from that previously observed in rats with adenoviral vectors administered through the same route [3]. In fact, adenovirus-delivered transgenes were expressed predominantly in the papilla and medulla following retrograde infusion, whereas expression in proximal tubules in the cortical area resulted from perfusion via the renal artery [23]. However, similarly to lentivirus, liposome complexed-DNA infused retrograde via the ureter targeted the transgene primarily to the outer medulla and cortex [24]. The reasons for these differences remain to be elucidated, and more comparative experiments will be needed to determine whether the differential distribution depends on the delivery system, the promoter used, the physical parameters of delivery, technical procedures, or animal models. The possibility of selectively accessing different segments of the nephron,

using alternative gene transfer systems, is particularly appealing, as it would allow the delivery of the therapeutic genes specifically to the affected sites. In this regard, the nature of the targeted cells within the positive tubules is an important question, and work is in progress to address this issue.

Importantly, during these studies, no apparent toxicity was observed in mice treated with the lentiviral vector, and survival was the same in lentivirus-injected as compared with PBS-injected animals. Furthermore, the persistence of the transgene expression exceeded those reported for adenoviral or lipid gene transfers and were comparable with what was previously shown for AAV vectors delivered through parenchymal injection [11].

Similar to recombinant AAV vectors, the random integration of lentiviruses into the host genome raises some concern for insertional mutagenesis. On the other hand, lentiviral vectors can accommodate transgenes of 8 kb or larger. The ability to deliver large DNA fragments is highly desirable, as some therapeutic genes may exceed the limit allowed by AAV. The larger cloning capacity of lentiviruses may also become necessary for the inclusion of regulatory elements that confer tissue-specific expression of the transgene. This characteristic may become essential for renal gene therapy because of the highly specialized nature of the cell types resulting in the normal function of the nephron. Specificity could be achieved by placing the transgene in the lentivirus under the control of a cell-specific promoters. To take full advantage of this possibility, it will be important to compare different administration routes and further characterize the renal cells targeted by the lentiviral vectors engineered with specific promoters.

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