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Lentiviral vectors for delivery of genes into neonatal and adult ventricular cardiac myocytes *in vitro* and *in vivo*

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Abstract Vectors based on lentiviruses such as human immunodeficiency virus (HIV) type-1 have many advantages for gene therapy, including the ability to infect non-dividing cells, long-term transgene expression and the absence of induction of an inflammatory/immune response. This study was initiated to determine whether lentiviruses would efficiently transfer genes to both neonatal and adult cardiac cells in culture and, by direct injection, to the heart *in vivo*. A three-plasmid expression system, including a packaging defective helper construct, a plasmid coding for a heterologous (VSV-G) envelope protein and a vector construct harboring reporter genes – *E-GFP* (enhanced green fluorescent protein) and *puro* (puromycin-resistance protein) was used to generate pseudotyped HIV-1 particles by transient transfection of human embryonic kidney 293T cells. We demonstrated efficient gene transfer into neonatal and adult cardiac myocytes *in vitro* and identified conditions in which virtually 100 % of cultured neonatal and 70 % of adult cardiac myocytes express the reporter gene. Transduction of adult cardiac myocytes with high titre lentiviral vectors did not affect the cell number, morphology or viability compared to untransduced cells. We delivered HIV-1-based vectors to the intact heart by direct injection. Hearts transduced with pseudotyped HIV-1 vectors showed levels of transgene expression comparable to that achieved by adenovirus vectors. This study demonstrates for the first time that lentivirus-based vectors can successfully transduce adult cardiomyocytes both *in vitro* and *in vivo*, and opens up the prospect of lentivirus-based vectors becoming an important gene delivery system in the cardiovascular field.

Key words HIV-1 – lentiviral vector – cardiac myocytes – gene transfer – heart

Introduction

Gene delivery to cardiac myocytes has the potential both to therapeutically correct genetic defects and, investigationally, to study cardiac muscle physiology. It has proven difficult to deliver genes to cardiac myocytes as they are terminally differentiated, they do not divide, and, *in vitro*, have a relatively short life span (19). Gene delivery to cardiac myocytes is further hampered by toxicity associated with delivery vectors; adenoviruses, for example, may disrupt the physiology of the cell (54).

Vectors based on lentiviruses have been in use for a number of years and have been shown to be able to transduce cells which are not dividing (35, 42), both *in vitro* and *in vivo*. This is due to the native capability of lentiviruses to infect terminally differentiated cells of the monocyte/macrophage series (7, 27, 59). Transduction involves integration of genetic material into the target cell and this allows for long-term stable gene expression from what has now become a new cellular genetic element (41). Newer pseudotyped lentiviral vectors offer a major advantage over other high efficiency systems in that they appear to cause little or no disruption of the target cell (26). This is concordant with the fact that retroviruses themselves rely on perturbing cell function as little as possible for their own survival (11). Lentiviral vectors also have a relatively large capacity for gene transfer, potentially up to 8 kilobases of sequence.

We and others demonstrated HIV-based vector gene delivery in the early 1990s following our identification of the HIV-1 encapsidation signal (15, 25, 46–48). Since that time, heterologous envelopes have been used to widen the tropism of the vector (8) and, in doing so, have led to the generation of a more stable particle capable of being frozen and thawed and with a degree of independence of lentiviral accessory gene products for infection (1, 16). We have been using three- and four-plasmid lentiviral vector systems to deliver genes to a number of cells. We describe the ability of lentivirus-based systems to deliver and stably express genes in both neonatal and adult cardiac myocytes without any perturbation of cell viability or evidence of cytotoxicity. For the first time, we have demonstrated that a lentiviral vector system is able to transduce the heart *in vivo*. These results suggest that lentiviruses could offer a promising new approach for treatment of cardiovascular diseases and for cardiovascular studies.

Materials and methods

Plasmid constructs

The vector HVP is based on the HXBc2 isolate of HIV-1 and contains a *Bal* 1-*Eco*R1 deletion (2621–5743) removing the reverse transcriptase and integrase domains of *pol* as previously described (48). It contains a 580 bp *Bgl* II deletion in the *env* gene and a promoterless puromycin resistance gene is inserted in a position analogous to that of the viral *nef* gene. HVPGFP was generated by digestion of HVP with *Apa* I (position 2010) and *Sal* 1 (5786). The E-GFP gene (a kind gift of Dr. Brian Salmons) was removed from pCMVGFP using *Apa*1 (position 945) and *Xho*1 (position 1716). The purified E-GFP containing fragment was then ligated into the purified HVP vector.

The HIV-1 *gag pol* expressing plasmid LΔP1GPH (kindly donated by Dr. Jane Allen) is based on the HXBc2 isolate (48). The 5' LTR drives expression of the *gag* and *pol* genes and the 3' LTR has been replaced by the human cytomegalovirus termination signal. It has a 19bp deletion in the packaging signal. The hygromycin resistance gene has been inserted at the 3' end of the *env* gene and a *Bgl* II fragment (7041 – 7621) within *env* has been removed.

The VSV-G plasmid was kindly provided by T Friedmann (8). This contains a 1.6 kb fragment encoding VSV-G driven by a CMV promoter.

Adenovirus type 5 mutants were used in this study. EGFP fragments of the E. coli nls Lac 2 gene were cloned between the enhancer/promoter of the cytomegalovirus immediate-early genes and the Simian virus 40 polyadenylation signal of the pACCMV pLpA shuttle vector (33). Replication-deficient adenovirus was generated through homologous recombination of two plasmids (pJM17, a bacterial plasmid that contains the full-length adenoviral genome, and the shuttle vector) after cotransfection into E1 transformed human embryonic kidney 293 cells to produce E1-deleted adenovirus. Viral stocks were generated by infecting confluent 293 cells, harvesting the cells, and concentrating the cells through CsC1 ultracentrifugation. Viral stocks were then desalted through a Sepharose CL4B (Sigma Chemical Co) column into a Tris-buffered solution, plaque-titered, aliquoted, and stored at –70 °C with 10% glycerol until use.

Vector production and transduction

Pseudotyped vectors were generated by transfection of plasmid DNA into 293T cells using a modified calcium phosphate method (9). Transfections were done in 100-mm dishes using optimized ratios of constructs: 7 μg vector construct, 8 μg of the packaging construct and 4 μg of the VSV-G *env* plasmid DNA per 10 ml of Dulbecco's modified Eagles medium (DMEM) including 10% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin. The medium was changed 12 – 14 h later. Sixty-two hours after the start of transfection, the medium was removed and filtered through a 0.45 μm Millex-HA filter (Millipore). Vectors were concentrated by ultracentrifugation for 90 min at 25,000 rpm (4 °C) in a Beckman centrifuge. Virion stocks were resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and stored at –80 °C.

Virus vector titers were determined by limiting dilution. SV2 target cells were split into six-well plates, which were plated to give approximately 50% confluence at the time of transduction. Transductions were performed with serial dilutions of vector stock in a total of 0.5 ml medium. After 6 h at 37 °C, 1.5 ml of medium was added and the plates were incubated at 37 °C for 3 days. The medium was

then aspirated and 2 ml of medium supplemented with puromycin (1.5 μg/ml) was placed into each well. The medium was changed every 3 to 4 days, and the colonies were counted on day 14 after staining with crystal violet (0.2% in 20% ethanol). In some cases FACS analysis of GFP transduced cells was used to estimate titer.

Isolation and culture of neonatal and adult myocytes

Neonatal rat ventricular cardiac myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats as described previously (64). Briefly, cells from neonatal rat ventricles were dispersed in a series of incubations at 37 °C in N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffered salt solution containing 0.6 mg/ml pancreatin and 0.5 mg/ml collagenase. The dispersed cells were preplated for at least 30 minutes to minimize fibroblast contamination and the unattached cells replated on six-well gelatin-coated plates at a density of $2–5 \times 10^5$ cells/well. The cardiac myocytes were cultured at 37 °C, in room air with 5% CO_2 in 4:1, DMEM:medium 199 (M199), supplemented with 10% horse serum (HS), 5% FCS and 100 U/ml penicillin/streptomycin for the first 24 hours. Thereafter cells were maintained in an identical medium with a reduced serum concentration of 1% FCS. Under these conditions, in excess of 90% of cells beat spontaneously for the duration of the experiment. Experiments were performed after 2 to 3 days in culture.

Adult ventricular cardiac myocytes were isolated from the ventricle of rabbit hearts. Male New Zealand White rabbits were killed by intravenous injection of pentobarbitone sodium (200 mg/kg). Hearts were rapidly excised and Langendorff perfused with a calcium-free medium for 5 min, followed by perfusion with a collagenase/protease digestion medium for 8 – 10 min as previously described (60). Isolated myocytes were washed 3 times with DMEM supplement with 100U/ml penicillin/streptomycin, and then submitted to primary culture by a modified method of Singh Kent et al. (55). Under these conditions, 60 – 70% of cells displayed rod-shaped morphology and sarcomeric cross-striations. The cells were plated on six-well plates at a density of $2–5 \times 10^4$ cells/well. The adult cardiac myocytes were cultured at 37 °C, in room air with 5% CO_2 in M199, supplemented with 0.2% BSA, 0.1 mM L-ascorbic acid, 5 mM creatine, 5 mM taurine, 2 mM DL-carnitine and 100 U/ml penicillin/streptomycin. Experiments were commenced 2 h after plating.

In vivo studies

Inbred male Lewis rats (250 – 300 g) were used as donors and recipients. Heterotopic cardiac transplants into the abdomen were performed as described previously (43). Rats were cared for according to the standard guidelines using a protocol approved by the University of Cambridge Central Biomedical Services.

Direct injection of HIV-1 pseudotyped vector or adenoviral vector was performed immediately after reperfusion of cardiac transplants and restoration of the heartbeat. 200 μl viral vectors were injected between the two muscle layers of the apex of the beating heart, at 3 – 4 points using a 29-gauge needle.

Histochemical staining of GFP

Recipients were killed 7 days after transplantation and the hearts were removed and frozen in liquid nitrogen. 5 μm thick cryostat sections of the hearts were fixed with acetone. Non-specific binding was blocked with fatty acid free-BSA in triethanolamine buffered saline solution. The sections were then incubated overnight with 1:50 (polyclonal) rabbit anti-rat GFP antibody (AbCam, UK) in a humidified chamber at 4 °C. 1:200 donkey anti-rabbit antibody (AbCam, UK) was used to detect bound primary antibody.

Histochemical staining of β-galactosidase

Recipients were killed 7 days after transplantation and the hearts were removed and frozen in liquid nitrogen. 10 μm thick cryostat sections were cut along the axis of the hearts. The sections were then fixed with 0.25% glutaraldehyde in PBS. β-galactosidase activity was detected using 5-bromo-4-chloro-3-indolyl-β-d-galactosidase (X-Gal, Sigma). All the sections were counterstained with hematoxylin and eosin.

Analysis of transduced cells

Cells were detached from the plate by using PBS containing trypsin/EDTA. The cells were washed twice and resuspended in PBS, and then subjected to fluorescence-actived cell sorting (FACS) analysis. Alternatively cells were analysed by fluorescence microscopy (Olympus 1x70) with GFP exciter 435– 495 nm; barrier filter 510 – 550 nm (Chroma).

Statistical analysis

All values are expressed as mean ± SE. The "n" numbers in the result section relate to the number of animals used. Within each independent experiment, at least duplicate measurements were performed. The values from each experiment were pooled to allow statistical comparisons.

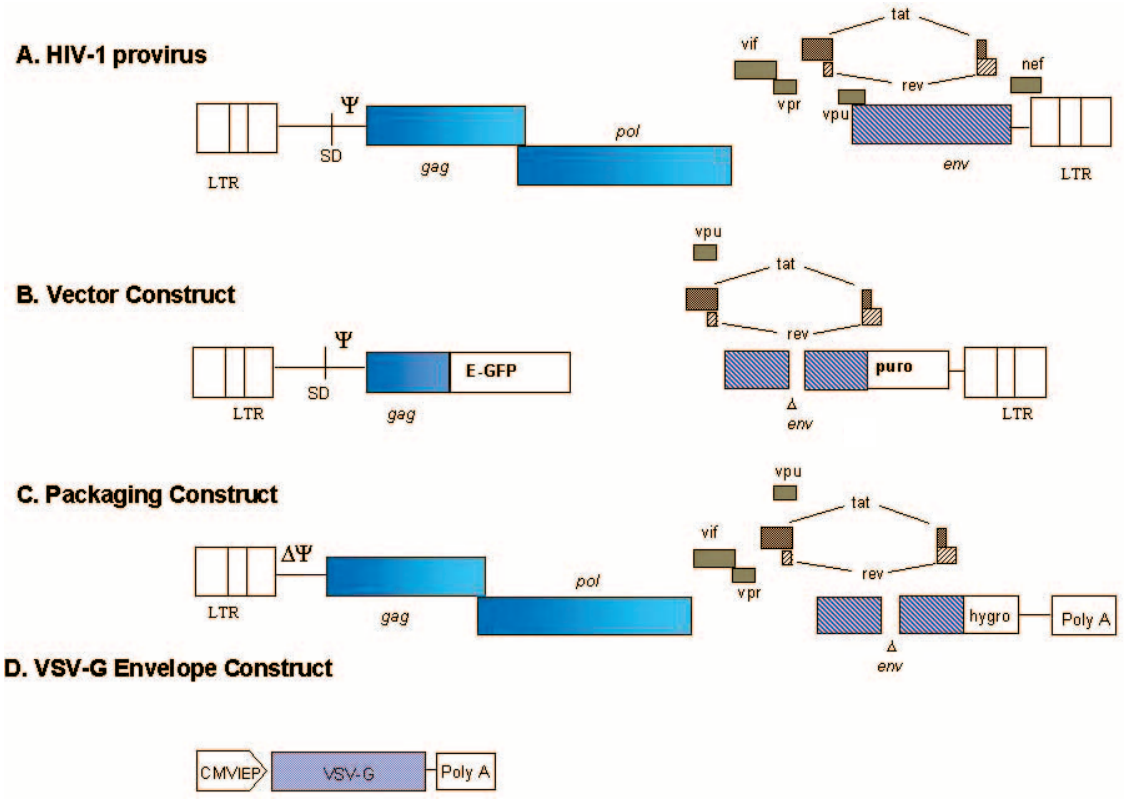


Fig. 1 Schematic representation of the HIV provirus (**A**) and components of the HIV-1 three plasmids expression system. **B** Transducing vector construct. **C** Packaging construct. The large open triangle symbolizes a 19-bp deletion in packaging signal between 5′ splice donor site and the beginning of the gag sequence. **D** VSV-G Env expressing construct.

Statistical comparisons were performed using a single factor analysis of variance (ANOVA) with post-hoc comparisons made using the Fischer Protected Least Significant Difference method. All analyses were performed using the Statview v4.0 statistical package (Abacus Concepts Inc, Berkley, CA). A probability value $P < 0.05$ was considered significant.

Results

Construction of HIV-1 based vector

To generate safe and replication-defective virus, the HIV-1 genome (Fig. 1A) was segregated into three components: a packaging construct, a vector construct, and an *env*-encoding plasmid. Our packaging construct (LΔP1GPH) (Fig. 1C) expresses the Gag, Pol, Tat, and Rev proteins, with a large deletion within the *env*-coding region. The 3' LTR was replaced by a heterologous polyadenylation signal. The packaging construct lacks *cis*-acting sequences that have been implicated as important for efficient HIV-1 RNA packaging (21), precluding RNA transfer to target cells. The vector construct (HVP-GFP) (Fig. 1B) carries a 600 bp deletion within the *env*-coding region. It contains the HIV-1 packaging signal and sequences necessary for reverse transcription and vector integration. The Rev response element (RRE) was retained in *env*. The truncated Gag gene and the EGFP coding region were expressed from unspliced transcripts; the *tat* and *rev* genes and bacterial *puro* gene as a reporter gene were expressed from spliced transcripts. The viral accessory proteins Vif, Vpr and Nef were absent. The formation of replication-competent HIV-1 was precluded because a large region of the *env*-coding region was missing in these vectors and only the vector has a 3' LTR. The envelope construct (Fig. 1D) contains the vesicular stomatitis virus G glycoprotein (VSV-G), a heterologous *env* protein that leads to formation of HIV-1 pseudotypes. Gene transfer without generation of replication competent virus has been validated extensively in our laboratory using this system (48).

Transduction of neonatal myocytes

Primary neonatal rat ventricular myocytes were transduced with EGFP-encoding HIV-1 pseudotyped vectors

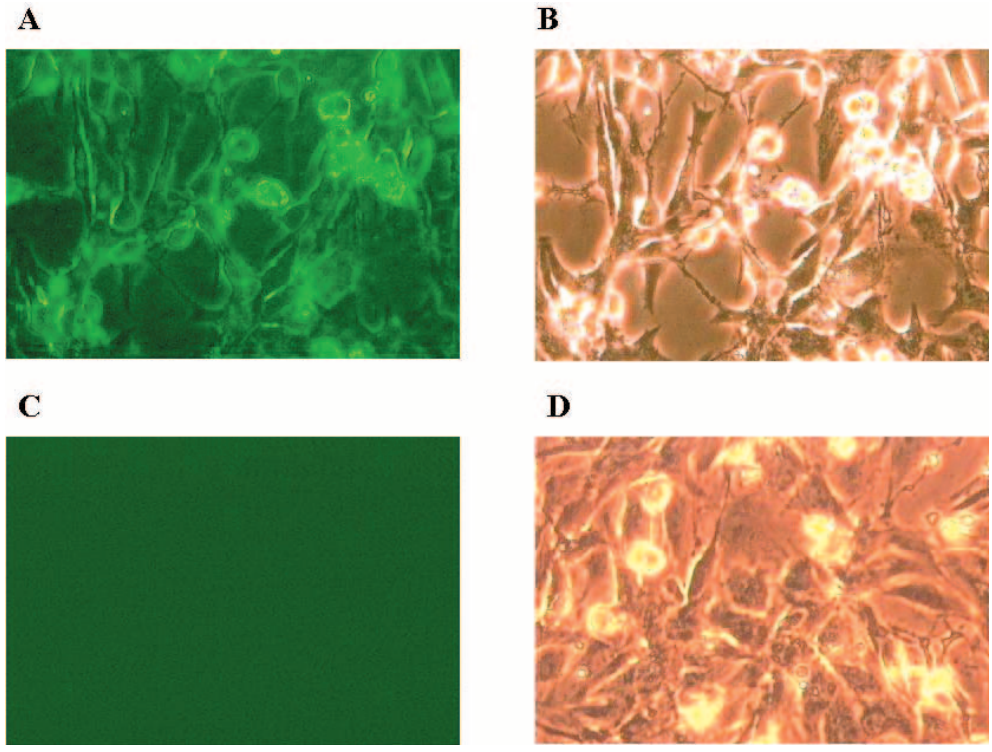


Fig. 2 Transduction of rat neonatal myocytes. Cardiac myocytes transduced with HVP-GFP pseudotyped vector (**A, B**). Cardiac myocytes transduced with HVP-GFP vector without VSV-G env plasmid (**C, D**). Approximately 2–5 × 10^5 myocytes in six-well plates were transduced with 10^6 cfu vectors. The cells were viewed 48 h after transduction under the fluorescence microscope (**A, C**) and light microscope (**B, D**).

(HVP-GFP) two days after isolation (in 1% FCS) to ensure the neonatal cells were permanently withdrawn from the cell cycle. Gene transfer was assessed by GFP reporter expression 48 h after transduction (see Fig. 2). As seen in Fig. 2A, neonatal myocytes expressed GFP after transduction of HVP-GFP. In contrast, HVP-GFP in the absence of cotransfecting *env* plasmid failed to infect the cells (Fig. 2C), suggesting specific transduction by pseudotyped vectors. GFP expression was maximal by 48 hours and maintained at stable levels through the maximal culture period (data not shown). To determine the percentage of transduced cells, cells were collected 48 h after transduction and processed for quantitative FACS analysis (Fig. 3). In high multiplicity transduction (10^6 cfu measured on the target cells), virtually 100% of myocytes expressed GFP. The proportion of transduced cells varied with vector multiplicity (Fig. 3). This suggests that GFP expression in the transduced cells is due to the vector transduction, rather than GFP-positive vector labelling the cell surface, since in the latter case only a single population would be expected in FACS analysis. The vector transduction included both myocytes and a small proportion of non-myocyte fibroblasts (< 10 %) which remained in the culture following initial myocyte purification (data not shown).

GFP expression was quantified by measuring the GFP median fluorescence intensity in FACS (Fig. 4). GFP expression of HVP-GFP pseudotyped vectors correlated with vector titres. In contrast, in myocytes transduced

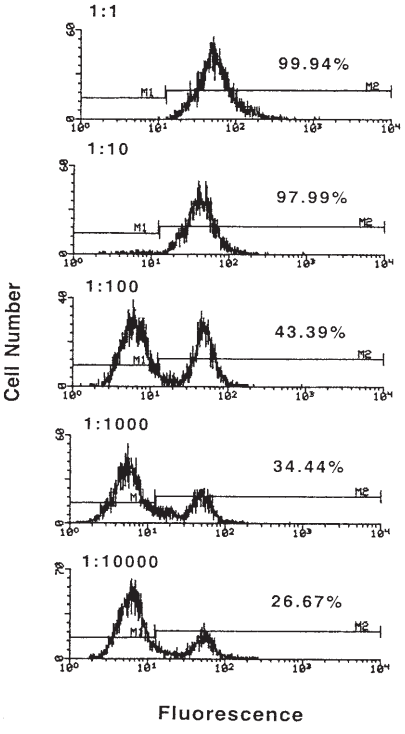


Fig. 3 Efficiency of EGFP reporter gene expression in neonatal cells. Cells were transduced with HVP-GFP pseudotyped vector and tested for the reporter gene expression by FACS analysis. Approximately 2 × 10^5 myocytes were transduced with 10^6 cfu vectors. For low- and high-multiplicity transductions, 1:1, 1:10, 1:100, 1:1000 and 1:10000 respectively of HVP-GFP pseudotyped vector stock were used. Transduced cells were processed for FACS analysis 48 h later.

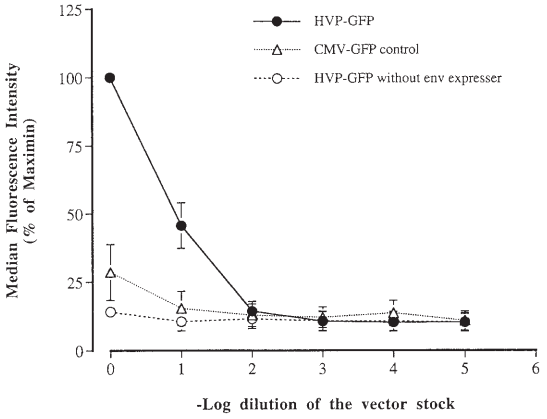


Fig. 4 Dosage-dependent expression of HVP-GFP pseudotyped vector transduced neonatal cardiac myocytes. Approximately 2×10^5 myocytes were transduced. For low- and high-multiplicity transductions, 1:1, 1:10, 1:100, 1:1000 and 1:10000 respectively of HVP-GFP pseudotyped vector stock were used. Transduced cells were processed for FACS analysis 48 hr late GFP median fluorescence intensity was normalized by cells transduced with high-multiplicity of HVP-GFP pseudotyped vector particles. Cells transduced with HVP-GFP pseudotyped viral particles (closed circle), cells transduced with plasmid pCMV-GFP and empty pseudotyped viral particles (open triangle) and cells transduced with HVP-GFP viral particles without VSV-G envelope (open circle).

with HVP-GFP vector without the VSV-G expressor, GFP expression was negative at all vector titres tested. In order to eliminate passive protein transfer as being responsible for fluorescence in the target cells, a CMV-driven GFP control plasmid was used. CMV-GFP together with the packaging construct and the VSV-G *env* plasmid, were transfected into 293T cells, giving rise to a 9-fold (n = 2) higher level of GFP expression than the HVP-GFP vector. However, cells transduced with the supernatants from this transfection showed a 4-fold lower level of GFP expression compared to those transduced with a pseudotyped HVP-GFP vector (n = 3) (Fig. 4) indicating retroviral transduction was responsible for virtually all the gene expression in the target cells. Taken together, the correlation between GFP expression and vector multiplicity indicated that GFP expression was strictly a result of the GFP vector sequence being transduced and reverse transcribed by HIV-1 pseudotyped particles (Fig. 4). This was also confirmed by the observation of maximum fluorescence occurring 48 h post-transduction, consistent with a viral RT and integrase-mediated event rather than passive protein transfer.

The apparent vector titre estimated from FACS analysis shown in Fig. 4 is less than that obtained with puromycin selection. The FACS analysis is a gated measurement assessing the total GFP expression of the cell population. Thus, this may not be detectable when GFP expression is below a certain threshold. Single or low copy number integration might possibly give expression below the sensitivity threshold of FACS analysis. This is currently under study.

Necrotic damage to the cultured neonatal cardiac myocytes caused by the transduction procedure was examined by measuring creatine kinase (CK) release into the culture medium. There was no difference between

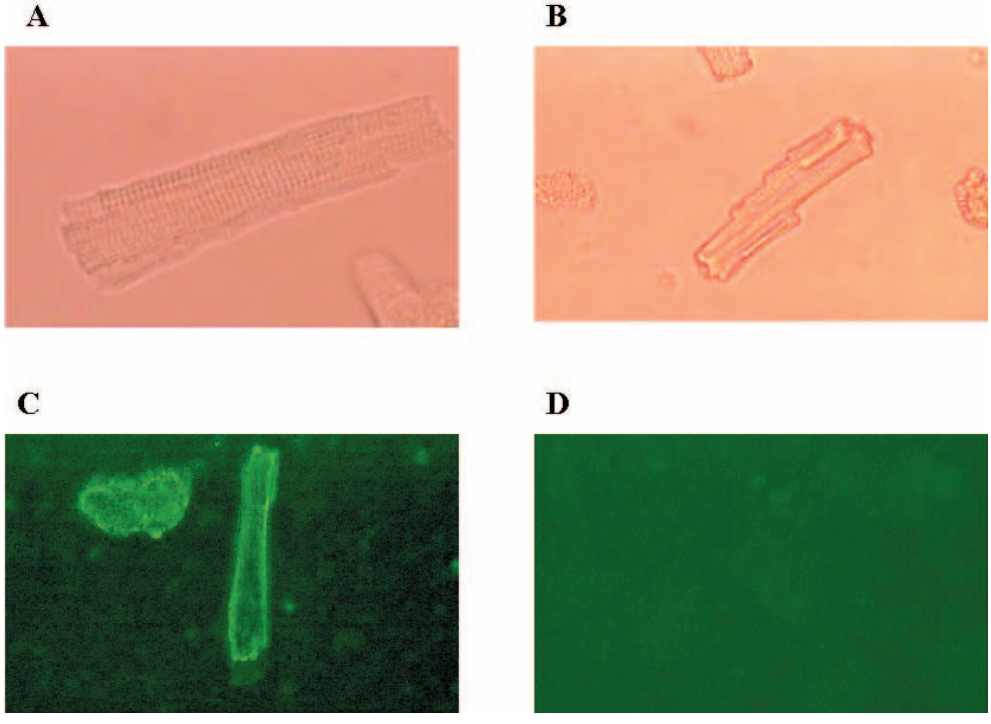


Fig. 5 HIV-GFP pseudotyped vector mediates efficient transfer to adult rabbit ventricle myocytes. Representative fields are shown for (**A**) immediate after isolation and (**B**) 48 h after isolation and GFP fluorescence 48 h after transduction with (**C**) 10^6 cfu virus or with (**D**) control vector (HVP-GFP without cotransfection with VSV-G *env* plasmid).

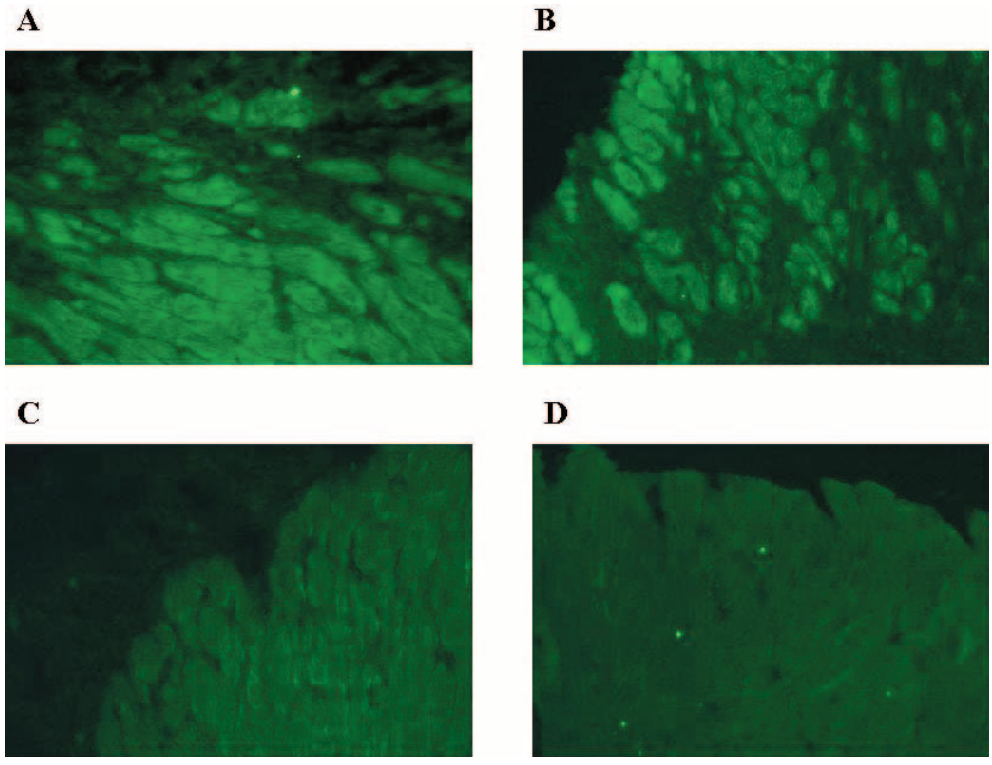


Fig. 6 Immunohistochemical staining for GFP in transduced heart. Representative fields are shown for (**A**) heart transduced with adenoviral vector, (**B**) heart transduced with HIV-1 pseudotyped vector, (**C**) heart transduced with HIV-1 pseudotyped vector without VSV-G envelope and (**D**) heart transduced with empty pseudotyped viral particles.

transduced and untransduced cells (CK release in transduced cells was 91.5 ± 7.2% of that seen in untransduced cells). Almost all cells beat spontaneously throughout the culture, even during the transduction period. Two days after transduction, these cells were confluent and beating synchronously, suggesting the HIV-1 vector had not disturbed the electrical coupling that is essential for synchronous beating. The results suggest that HIV vector transduction was not cytotoxic.

Transduction of adult myocytes

After isolation from adult ventricular myocardium, 60 – 70% of the cardiac cells displayed the rod-shaped morphology and sarcomeric cross-striations characteristic of myocardium in situ. The morphologic phenotype of the adult cardiac myocytes was stably maintained in culture (Fig. 5A, B). Ventricular myocytes transduced after 2 h in culture were analysed 24 – 48 h later. Ventricular myocytes that were transduced with EGFP-encoding HIV-1 vector efficiently expressed the exogenous GFP (see Fig 5C). Myocytes which were rounded up also stained positive for GFP. To eliminate uncertainties over whether viral particles caused cell damage and therefore increased autofluorescence, EGFP-free viral vector was used as a control and fluorescence was not observed (data not shown). Fluorescence was not due to passive DNA transfer since HVP-GFP vector particles without *env* could not successfully transduce the myocytes (Fig. 5D). HVP-GFP vector transduction provides an efficient means of gene transfer into adult cardiomyocytes *in vitro* which has not been possible with other transfection strategies except adenovirus. However, due to the small cell number, it was not possible to quantitate the GFP expression in rod-shaped ventricular myocytes.

To address the potential impact of high titre transduction itself on adult ventricular myocytes, cell number, morphology and viability were compared in cultures subjected to 10^6 cfu of HIV-GFP vector versus control cells. After 48 h, the total cell number was 54.0 ± 4.4% of original number in untransduced cells and 58.7 ± 7.8% in transduced cells ($n = 3$, $p > 0.05$, no significant difference). The proportion of myocytes retaining the differentiated, rod-shaped morphology was 43.4 ± 3.3% in untransduced cells and 36.2 ± 1.9% in transduced cells (no significant difference $p > 0.05$). Of the cells which had rod-shaped morphology, 75.3 ± 10.6% were viable in untransduced cells and 67.0 ± 6.2% were viable in transduced cells, as determined by trypan blue exclusion ($n = 3$, $p > 0.05$, no significant difference). Necrotic damage to the cultured adult cardiac myocytes by the transduction procedure was also examined by measuring CK release into the culture medium. CK release in transduced cells was 88.9 ± 9.3% of the value in untransduced ($n = 3$, $p > 2.05$). Thus, under the conditions tested, HIV vector transduction by itself had no deleterious effects on the survival or morphology of adult ventricular muscle

cells. After 48 hours in culture, it is difficult to perform electrophysiological responses using standard whole-cell patch clamping recording methods (45), even in non-transduced cells. Thus, attempts to study the contractile function of transduced cardiomyocytes at a single cell level failed.

In vivo transduction of transplanted heart

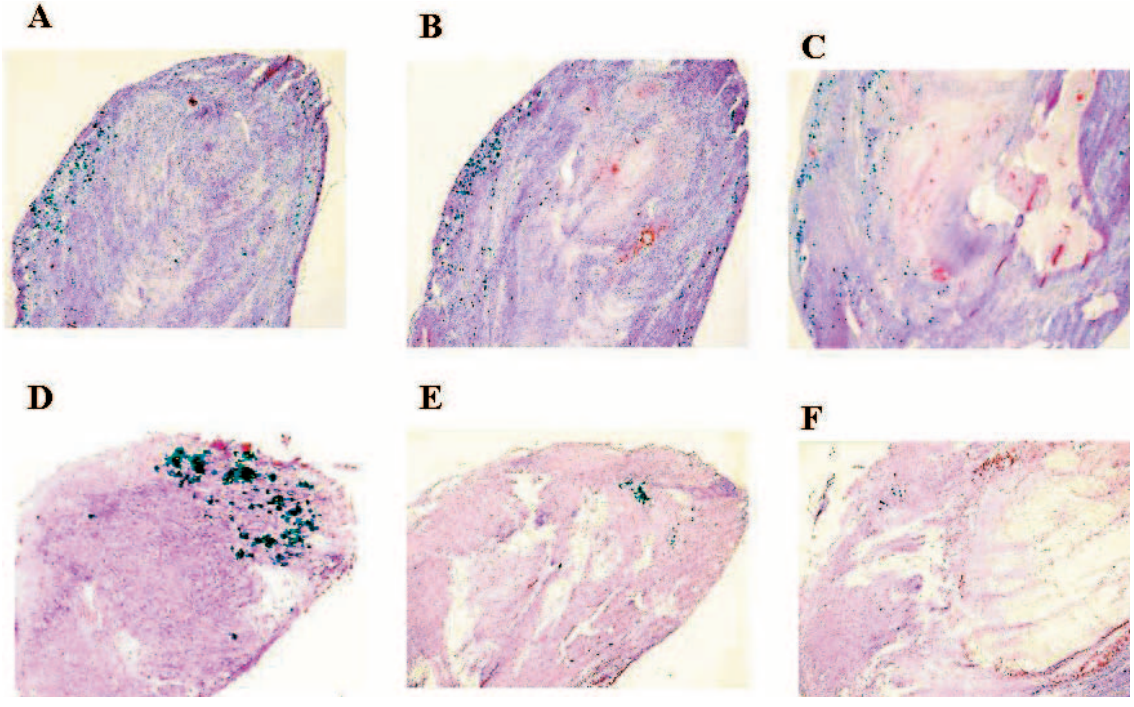
In this study, a transplantation model was used since our ultimate goal is to modulate the host immune response in organ transplantation. Moreover, it is the only model that allows us to handle the heart *ex vivo*. It offers the unique opportunity of allowing us to precisely study different strategies of gene transfer in the future. Due to the higher background autofluorescence of the intact adult heart, an alternative HIV-1 vector with a stronger promoter for GFP, pHCMV-GFP, was used (a kind gift of Dr. Didier Trono, Department of Genetics and Microbiology, CMU, Geneve, Switzerland). In this HIV-1 vector system, the strong CMV promoter drives EGFP. Seven days after the transplantation, the level of gene transfer and expression achieved with HIV-1 vector was comparable to that obtained using an adenoviral vector (n = 3) (Fig. 6). No positive GFP stain was seen in the hearts transduced with HIV-1 vector without *env* or with empty vector.

There was no histological evidence in the transplanted hearts of any direct injury or inflammatory infiltrate in the heart caused by both viral vectors (data not shown).

To confirm the results regarding gene expression with HIV-1 vector and to localise gene expression *in vivo*, another HIV-1 vector encoding β-galactosidase (a kind gift of Dr. N Deglon, Division of Surgical Research and Gene Therapy Center, Lausanne Medical School, Switzerland) was delivered into the heart by direct injection. Transplanted hearts were removed for histological examination after 7 days. Although the injection points were only at the apex, the β-galactosidase activity was detected throughout the myocardium in all of the transplanted hearts with direct injection of HIV-1 vectors (n = 3) (Fig. 7).

The efficiency of β-galactosidase expression was less than that of GFP for both vectors. This could be that the staining of nuclear-localised β-galactosidase is relatively insensitive and is detectable only in highly expressing cells. The staining, therefore, may underestimate the extent of transgene expression. Lower levels of expression may not be detected by this method but may be sufficient to induce physiological changes, depending on the transgene expressed.

Fig. 7 Histochemical staining for β-galactosidase in transduced heart. Representative fields are shown for (**A**) heart transduced with HIV-1 pseudotyped vector in the apex of the heart, (**B**) heart transduced with HIV-1 pseudotyped vector in the middle section of the heart, (**C**) heart transduced with HIV-1 pseudotyped vector in the base section of the heart, (**D**) heart transduced with adenoviral vector in the apex of the heart, (**E**) heart transduced with adenoviral vector in the middle section of the heart and (**F**) heart transduced with adenoviral vector in the base section of the heart.



Discussion

Cardiac myocytes are terminally differentiated cells, which withdraw from the cell cycle soon after birth. Subsequent myocardial growth occurs through enlargement of individual cardiac myocytes, and cell loss following injury is not replaced by cell proliferation. This makes them particularly problematic in terms of gene delivery. Gene therapy offers a potential valuable therapeutic avenue to treat primary cardiac diseases, to correct defective cells and possibly to differentiate fibroblasts into myocytes (36, 52).

The delivery of genetic material into intact adult myocardium is currently achieved by one of two methods, either virally mediated or non-viral (physical) delivery. The latter has the advantage of involving no other components of infectious organisms and being simple and relatively cheap (38, 39) but the efficiency of delivery is much less than that associated with viral vectors (13, 37). Until recently, adenoviruses were the most favored method of gene delivery to cardiac myocytes as they are highly infectious for non-replicating mammalian cells (5, 23, 27). They can be generated as vectors to very high titre (3, 5) and are capable of delivering genes to cardiac myocytes by direct injection (14, 28), by perfusion into coronary arteries (2, 6) and into the ventricular cavity (15). Their use has, however, been limited by the transient nature of their expression (2, 17, 20) and by the inflammatory response that they engender under some conditions (57, 62, 63). Although the newer "gutless" adenovirus vectors which express no adenovirus gene products (51) may be less prone to trigger inflammatory responses, the adenovirus particle is nevertheless disruptive of cell membranes and capable of provoking inflammation (16, 18, 54). In addition, because of the ubiquitous nature of adenoviral infections, virtually everybody has significant levels of pre-formed antibodies against adenovirus from prior infections (10, 58) which may result in unpredictable variable transfection levels in clinical practice. The recent problems with adenoviral-mediated gene delivery to the liver have emphasized that the efficiency of this system may only be achieved at a price (32). Adeno-associated viruses (AAV) have also been demonstrated as useful for gene delivery to cardiac muscle (30, 49). While they are non-pathogenic and do not trigger inflammation, they are relatively difficult to manipulate (56). Part of the problem is the incomplete understanding that we have of AAV replication. Lentiviruses have the unique ability to introduce and integrate their DNA genome into the chromosomes of cells which are not dividing. The major concern associated with lentiviruses is bio-safety; chiefly, as with other retroviruses, the risk of insertational mutagenesis. However, it has been nearly 10 years since the first retrovirus gene therapy clinical trial (44) and there are no reported cases of insertational mutation. Moreover this phenomenon has not been identified in HIV positive patients. Another concern is formation of replication-competent virus generated during the vector preparation. To minimize the recombination, three-plasmid and four-plasmid systems have been used to generate HIV-1 vector particles. The design of these systems is based on the concept of segregated packaging systems that have been available for retroviruses for over a decade. Naldini et al. (42) were the first to describe an HIV-1-based three-component system that involves heterologous Env proteins. Analogous to those used in other laboratories (22, 66), we have developed an efficient three-component packaging system to produce HIV-1 pseudotyped vectors. The titers obtained were 1×10^6 cfu/ml with a single ultracentrifugation. The use of heterologous Env proteins such as VSV-G is assumed to preclude the formation of replication-competent HIV-1, further adding to the safety of the system.

It has been shown that lentivirus-based vectors can deliver genes to mitotically inactive neurons (4, 12, 24), and neuroglia (34, 41). There have been few reports regarding application of lentivirus-based vectors to cardiac myocytes and all of those studies to date have used neonatal cardiac myocytes (35, 50). There are qualitative and quantitative differences between neonatal and adult cardiac myocytes, including the expression of contractile protein isoforms, G-proteins and ATPase (40), growth factor and their receptors (31, 53), and transcription factors (29, 65). This is the first study demonstrating that an HIV-1-based pseudotyped vector is able to infect adult cardiac myocytes efficiently and that efficient gene transfer to adult ventricular myocytes in culture can occur without adverse effects to the cells. For adult cardiac myocytes, transduction studies were limited to 48 hours due to the difficulty in maintaining healthy differentiated adult cardiac myocytes in culture, independent of vector transduction. In neonatal cardiac myocytes, GFP expression was maximal at 48 hours after transduction and maintained through the maximal culture period. We have demonstrated, for the first time, that HIV-1 vector mediated gene transfer using direct injection of heart transplants results in significant gene expression. Adenoviral vector mediated gene transfer results in more β-galactosidase positive cells than HIV-1 vector in the apex of the heart. The adenoviral vector titer used in this study was 1×10^{10} cfu/ml, whereas that of the HIV-1 vector was 10^6 cfu/ml. The effect of viral titer on adenovirus-mediated gene transfer has been shown previously (61). Adenoviral-mediated transgene expression was markedly diminished by lower titer and the timing of peak gene expression was also shifted with virus titer. With improvement in HIV-1 vector purification there are likely to be further increases in the attainable concentrations and therefore increase its efficiency. Further experiments are currently being conducted to compare long-term stable expression between these two viral vectors

and to quantitate more accurately their relative efficiency in accessing cardiac myocytes under the relevant conditions.

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