in vivo *23*: 885-894 (2009)

Efficient Liver-directed Gene Transfer by *In Situ* Generation of Retroviral Vector from Adenoviral Templates

JULIAN D. HARRIS[1], MICHAEL L. ROBERTS[3], DHAYANEETHIE PERUMAL[2], IAN R. GRAHAM[1], STEVE PATTERSON[2] and GEORGE DICKSON[1]

[1]*SWAN Institute of Biomedical and Life Sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX;*
[2]*Department of Immunology, Division of Investigative Science, Faculty of Medicine, Imperial College of Science, Technology and Medicine, The Chelsea and Westminster Hospital, London, U.K.;*
[3]*Regulon A.E., Athens 17455, Greece*

Abstract. *To improve liver-directed retroviral-mediated gene transfer, we injected C57/BL10 mice intravenously with three adenoviral vectors encoding retroviral vector genome and structural components: AdGagPol expressing the respective structural genes of Moloney murine leukaemia virus, Ad10A1Env expressing the 10A1 envelope protein of 10A1-MuLV, and AdLEIN, encoding the LEIN retrovirus genome, expressing green fluorescence protein (eGFP) and the neomycin resistance gene. Materials and Methods: The extent of eGFP expression was determined after 1 and 15 weeks by fluorescence microscopy and FACS analysis. Proviral integration was determined by a novel PCR-based technique. Results: Hepatocytes infected with all three Ad vectors generated LEIN retrovirus after one week and in situ transduction of neighbouring cells resulted in stable proviral integration associated with eGFP expression ranging from 4.3% to 20.5% in different liver cell populations 15 weeks post-infection. Conclusion: Hybrid adeno-retroviral vectors can be efficiently used to improve the efficiency of retroviral-mediated gene transfer to the liver.*

Despite advances in non-viral and viral vector technology over the past decade, efficient and stable gene transfer of therapeutic transgenes into diseased tissues is still a confounding issue in many gene therapy protocols. The liver is an ideal target for somatic gene therapy of many disorders, as its highly vascularised structure could potentially be harnessed to deliver secreted proteins into the circulatory system. Retroviruses provide an excellent vehicle to express therapeutic transgenes stably in the liver, given the well-documented regenerative capacity of this organ. The retrovirus was first described as an efficient gene transfer vector in hepatocytes over two decades ago (1-3). However, when liver cells are transduced with retrovirus *in vivo*, long-term gene expression has been difficult to sustain due to the need to administer high-titre vector necessary for efficient gene transfer (4, 5), induction of a large primary immune response to the vector and transgene (5) and complement-mediated particle lysis (6). To overcome these problems, most retroviral vector gene therapy protocols adopt an *ex vivo* approach, where cells are genetically modified by retroviral vector *in vitro*, selected and expanded before being implanted back into the patient (7-9), or where retroviral producer cells are directly transplanted in the target tissue (10, 11). These *ex vivo* procedures are unwieldy and costly, resulting in the transduction of only a small population of target cells, the induction of a severe immune response and the formation of palpable tumors (11). One way to increase the efficiency of retroviral vector transduction *in vivo* is to produce retroviral vector *in situ*, so that lower titres of vector are concentrated in the target tissue. This approach may conceivably reduce the host immune response to retrovirally-transduced cells, as retroviral production originates from host tissue. We and others have developed hybrid adeno-retroviral vectors in order to achieve *in situ* retroviral vector production, and have found that adenoviral vectors are capable of mediating efficient retroviral production both *in vitro* (12-17) and *in vivo* (12, 14, 18). Production of functional retroviral vector using this hybrid system is a two-step process: target cells are infected with adenoviruses expressing retrovirus structural genes and provirus sequences; infected cells release functional retroviral vector, which then transduces neighbouring cells, resulting in the stable integration of the therapeutic gene.

Correspondence to: George Dickson, SWAN Institute of Biomedical and Life Sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, U.K. e-mail: g.dickson@rhul.ac.uk

Key Words: Hybrid vector, adenovirus, retrovirus, liver, gene therapy.

To assess the efficiency of *in situ* retroviral vector production from the liver, we employed a hybrid adeno-retroviral vector system based on three adenoviral vectors: AdGagPol expresses the respective structural genes of Moloney murine leukaemia virus (Mo-MuLV); Ad10A1Env expresses the 10A1 envelope protein of 10A1-MuLV that can mediate infection *via* the gibbon ape leukaemia virus (GaLV) as well as the amphotropic-MuLV (A-MuLV) receptors (19); AdLEIN, containing the recombinant retrovirus genome LEIN, expressing enhanced green fluorescence protein (eGFP) linked to a neomycin resistance gene (17). We demonstrate that hepatocytes are capable of generating retroviral vector subsequent to infection with hybrid adeno-retroviral vectors. *In vitro*, we generated retroviral titres of 5.3×10^6 cfu/ml in HepG2 cells, and *in vivo* we detected expression of the reporter eGFP cDNA in some 4.3 to 20.5% of hepatocytes 15 weeks after infection, a proportion of which contained integrated retroviral provirus. In conclusion, we demonstrate that the liver can be considered a suitable organ from which to produce retroviral vector *in situ* by using this system.

Materials and Methods

The adeno-retrovirus chimeric vector system. AdLEIN was constructed by a homologous recombination procedure as described previously (17). Construction of the AdGagPol and Ad10A1Env vectors has also been previously described (16).

Optimization of retroviral vector production in HepG2 cells. The human hepatoblastoma cell line HepG2 was used to assess the ability of hepatocytes to produce retroviral vector *in vitro* from adenoviral templates by using a procedure described elsewhere (17). To assess the effect of methylation silencing on retroviral transgene expression, NIH3T3 G418-resistant clones transduced with retroviral vector produced in HepG2 cells were treated with growth medium supplemented with 5 μM 5-azacytidine (AzaC) (Merck). After three days treated cells seeded into growth medium containing 1 mg/ml G418 and resistant colonies were then giemsa-stained and counted after a further eight days.

In vivo administration of the hybrid adeno-retroviral vector system. In order to assess the efficiency of LEIN-mediated eGFP expression in the liver by the adenovirus vector AdLEIN alone, eight-week-old C57BL/10 mice were tail vein-injected with AdLEIN. Virus stock was diluted in 200 μl of 10 mM Tris HCl pH 8.0, 2 mM $MgCl_2$ and 0.9% (w/v) NaCl. For the co-administration of the adeno-retroviral chimeric vectors AdLEIN, AdGagPol and Ad10A1, the optimal ratios for the generation of the LEIN retrovirus *in vitro* were applied to the *in vivo* studies in which 16-week-old C57BL/6 mice were tail vein-injected with a 200 μl adenovirus vector dose. A first group of animals (n=8) were treated with AdLEIN, with a vector dose of 3×10^{10} virus particles. A second group of animals (n=8) were treated with AdLEIN and AdGagPol in the same inoculum, with vector doses of 3×10^{10} and 1.5×10^{10} virus particles, respectively A third group (n=16) were given AdLEIN, AdGagPol and Ad10A1 with vector doses of 3×10^{10}, 1.5×10^{10} and 1×10^{10} virus particles, respectively. A group of untreated animals (n=8) were used as negative controls. After one week, four animals from groups one and two, and eight animals from group three were sacrificed, and after 15 weeks, the remaining animals were sacrificed.

Processing of tissues for microscopy, FACS and PCR. A portion of liver from each animal was soaked in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.6) for 6 hours at 4˚C, then 30% sucrose overnight and the next day embedded in OCT compound (Tissue-TEK), snap-frozen in liquid nitrogen-cooled isopentane and stored at –80˚C. Tissue sections (10 μm thickness) were observed directly under fluorescence microscopy. To examine eGFP expression by FACS analysis, the majority of the livers were disaggregated in 0.1% Collagenase Type II, followed by clarification of the cell suspension through a 100 μM cell strainer (Becton Dickinson Labware), then fixed in 4% paraformaldehyde at a density of 5×10^5 cells/300 μl prior to analysis. Liver genomic DNA was extracted from 50-100 mg liver tissue using DNAzol reagent (Invitrogen) for the detection of proviral integration by PCR.

Detection of proviral sequences in LEIN-infected cells. Detection of provirus-specific sequence in the LEIN-infected NIH3T3 and HepG2 cells, and in the livers of animals treated with the adeno-retrovirus chimeric system, was performed by PCR at 45 and 70 cycles utilising integrated provirus-specific primers as described elsewhere (18). For the detection of adenovirus sequences only, amplification of a 1042 bp product derived from the adenovirus fibre gene was performed as previously described (20). The PCR reactions were subjected to 1.5% agarose gel electrophoresis and products visualised by ethidium bromide staining.

Detection of eGFP cDNA and Ad Fiber gene copy numbers in livers. To determine the number of eGFP cDNA copies in the livers of treated animals normalised against the number of Ad fibre gene copies, real-time PCR was performed on isolated liver genomic DNA using Sybr Green I in conjunction with the melting temperatures of the amplified products to demonstrate the specificity of the analysis. Real-time PCR was performed using the Sybr Green I 2 x Reaction System (Eurogentec, Seraing, Belgium). Reactions were performed with either the adenovirus fibre gene primer set (Ad fibre primer 1: 5'-CCGCACCCACTATCTTCATG-3'; Ad fibre primer 2: 5'-AACTAGAGGTTCG GATAGGC-3') yielding an amplified product of 196 bp, or the eGFP cDNA primer set (eGFP primer 1: 5'-GCGACGTAAACGGCCACAAG-3'; eGFP primer 2: 5'-CGACGTAAACGGCCACAAG-3') yielding an amplified product of 177 bp. The standards employed were pEGFP-N3 (Clontech TakaraBio, California, USA) to determine eGFP copy number and pAd-CMV/apoE (20) for Ad fibre gene copy number. Target sequences were amplified using a Cepheid Smart Cycler (Oswel, Southampton, UK) and the reaction conditions were 50˚C for 2 min to allow UNGase digestion of any carryover amplified product; 95˚C for 10 min to activate the Hot Goldstar DNA polymerase and denature the DNA. For Ad fibre gene amplification, 40 cycles of 95˚C for 15 s, 60˚C for 30 s and 79˚C for 15 s, with optical fluorescence monitoring switched on for the latter extension temperature was performed. In the case of eGFP cDNA amplification, 45 cycles of 95˚C for 15 s, 64˚C for 45 s, with optical fluorescence monitoring switched on for the latter extension temperature. The amount of eGFP cDNA copies was normalised against the amount of Ad fibre gene copies present in the livers of treated animals.

Results

Titration of the retrovirus LEIN on HepG2 indicator cells. In order to determine the optimal ratio of AdLEIN:AdGagPol: Ad10A1 required to generate recombinant retroviral vector LEIN in the liver, HepG2 liver cells were infected *in vitro* with varying amounts of each adenoviral vector. HepG2 cells were infected efficiently with adenoviral vectors and supernatant from these cells harvested 48 h post-infection contained sufficient quantities of the LEIN retrovirus to efficiently transduce NIH3T3 and parental HepG2 indicator cells (Figure 1). We found that a ratio of 3:1.5:1 (AdLEIN:AdGagPol:Ad10A1) generated the highest quantity of LEIN retrovirus when titred on parental HepG2 cells at 5.3×10^6 cfu/ml and that all G418-resistant HepG2 colonies expressed eGFP (Figure 2A). LEIN titration on NIH3T3 cells demonstrated optimal retrovirus generation at a ratio of 3:1.5:3 (AdLEIN:AdGagPol: Ad10A1) with a titre of 2.6×10^6 cfu/ml (Figure 2A). In order to assess if the efficiency of retrovirus-mediated gene transfer is influenced by retroviral LTR promoter shutdown in response to methylation, we prevented promoter methylation with the addition of 5 μM AzaC. We found that the addition of AzaC had no effect on the formation of G418-resistant colonies subsequent to retroviral vector transduction *in vitro*, thus indicating that methylation of the retroviral LTR was not hindering expression of GFP (Figure 2B).

In vitro detection of proviral sequences by a PCR-based assay. To confirm that retrovirus-mediated gene transfer resulted in stable integration of provirus into the indicator cells, we employed a PCR-based approach that specifically amplifies integrated proviral sequences (Figure 3A) (18). Using the P1/P2 primer set his technique was sensitive enough to detect plasmid pLEIN spiked in genomic DNA to a copy number of 0.1 (Figure 3B). As expected, when the integration-specific P3/P2 primer set for provirus detection (forward primer P3 and reverse primer P2) was used with pLEIN at the same copy numbers, no product was generated (Figure 3B). To demonstrate the ability of the P3/P2 primer set to detect provirus, genomic DNA isolated from a G418-resistant/eGFP-expressing NIH3T3 clone (NIH3T3-LEIN), derived from LEIN retrovirus transduction, was used as a positive control. Moreover, a number of NIH3T3-LEIN and HepG2-LEIN clones stably transduced with LEIN retroviral vector were also tested for integrated proviral sequences, and were found to amplify the 500 bp product indicative of retroviral integration (Figure 3C).

Detection of eGFP expression in mouse livers following administration of AdLEIN. In order to assess the efficiency of LEIN-mediated eGFP expression in the liver, C57BL/10 mice (n=2) were tail vein-injected with 3×10^{10} viral particles of AdLEIN. After 5 days, animals were sacrificed and the livers analysed for eGFP expression. The phase-contrast images of liver sections (Figure 4), facilitate the identification of liver structures, where (a) represents a portal vein, (b) is a hepatic artery and (c) indicates a bile duct. We noted that GFP expression in AdLEIN-treated animals was detectable throughout the liver, with the most intense staining evident around the portal vein and bile duct (Figure 4). FACS analysis was used to quantify the extent of eGFP expression in the livers of C57BL/10 mice five days post-injection. Figure 5A is representative of a forward scatter-side scatter plot and identifies the different cell types in the disaggregated liver cell populations. Examples of histograms for the R1, R2 and R3 gated cell populations in Figure 5B show a proportion of cells expressing eGFP in the AdLEIN-treated mice. The proportion of cells expressing eGFP and the mean fluorescence intensity (MFI) of the gated cell populations is shown in Figure 5C. In all three cellular populations in the mouse liver, 14-21% of cells expressed eGFP (Figure 5C). This corresponded to a significant increase in the MFI of 67.6%±0.1% (±SEM, p=0.002, Mann-Whitney rank sum test). From these results we concluded that eGFP was efficiently expressed from the LEIN retroviral expression cassette in livers of C57BL/10 mice.

Hybrid adeno-retroviral vectors mediate long-term eGFP expression in the liver. To assess the efficiency of hybrid vector-mediated retroviral production *in vivo*, C57BL/6 mice were tail vein-injected with either AdLEIN, AdLEIN: AdGagPol, or AdLEIN:AdGagPol:Ad10A1 at virus particle ratios determined *in vitro* (Figure 2A). After 15 weeks, eGFP expression was evident in single cells distributed throughout the livers from animals treated with AdLEIN or AdLEIN:AdGagPol (Figure 6A). In livers from animals treated with AdLEIN:AdGagPol:Ad10A1, the majority of eGFP expression was detectable as colonies of fluorescent hepatocytes, which were present in close proximity to the blood vessels of the liver, identifiable by the presence of red corpuscles (see arrows in Figure 6B).

We proceeded to quantify the efficiency of transduction in the three populations of gated hepatocytes using FACS (R1, R2 and R3, as described in Figure 5A) after 15 weeks (Figure 6C). In livers from mice treated with AdLEIN: AdGagPol:Ad10A1, the percentage of eGFP-expressing hepatocytes in gate R2 was higher compared to AdLEIN- and AdLEIN:AdGagPol-treated mice, whereas no difference was observed in populations R1 and R3 (Figure 6C, Table I). Mouse H demonstrated the highest expression of GFP in all gated populations, with 57.5% of hepatocytes in gate R3 and 48.1% of hepatocytes in gate R2 expressing the transgene. Overall, these results demonstrate that the hybrid adeno-retroviral vector system can efficiently express eGFP after 15 weeks in the livers of immuno-competent C57BL/6 mice.

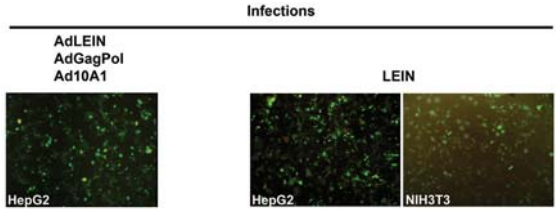


Figure 1. *Fluorescence microscopy demonstrating production of the LEIN retrovirus from HepG2 cells infected with AdLEIN:AdGagPol: Ad10A1Env. HepG2 cells were infected with AdLEIN:AdGagPol: Ad10A1Env (3:1.5:1), and two days later, culture supernatant was harvested from the infected HepG2 cells (left panel) and used for retroviral transduction of NIH3T3 and parental HepG2 indicator cells (right panels, two days post infection).*

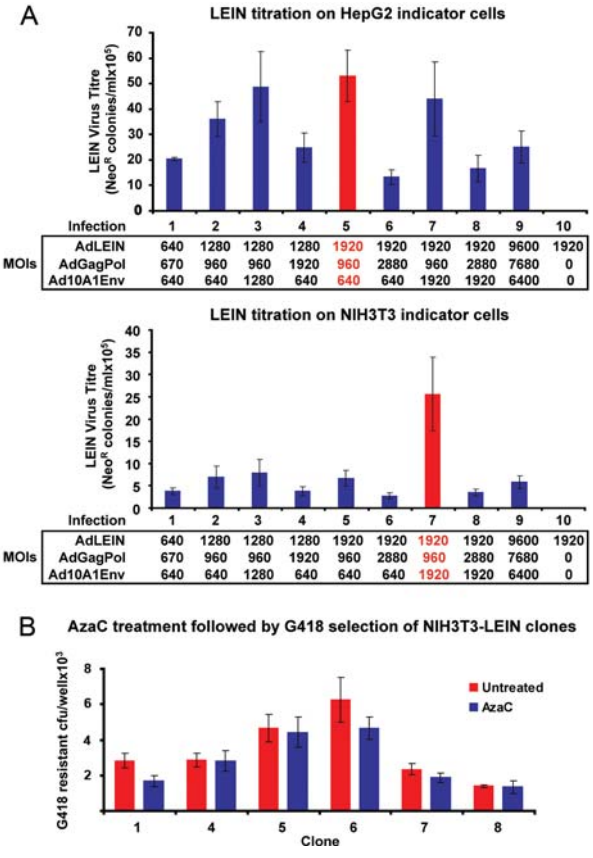


Figure 2. *Optimization of LEIN retrovirus production from HepG2 cells infected with the adeno-retrovirus chimeric vector system. A) In order to determine the optimal ratio of adenovirus vectors required for LEIN retrovirus generation, HepG2 cells were infected with varying proportions of AdLEIN, AdGagPol and Ad10A1Env, and after 2 days, culture supernatants containing the LEIN retrovirus was titred on HepG2 (upper graph) and parental NIH3T3 (lower graph) cells. The panels below the x-axes indicate the virus particle multiplicity of infection for each adenoviral vector used for transduction of HepG2 cells. B) NIH3T3 cells transduced with LEIN retroviral vector derived from the AdLEIN:AdGagPol:Ad10A1Env-infected HepG2 cells were treated with 5 μM AzaC for 72 hours prior to selection in 1 mg/ml G418 for 8 days. The number of G418-resistant/eGFP expressing colonies per well is shown graphically for each clone.*

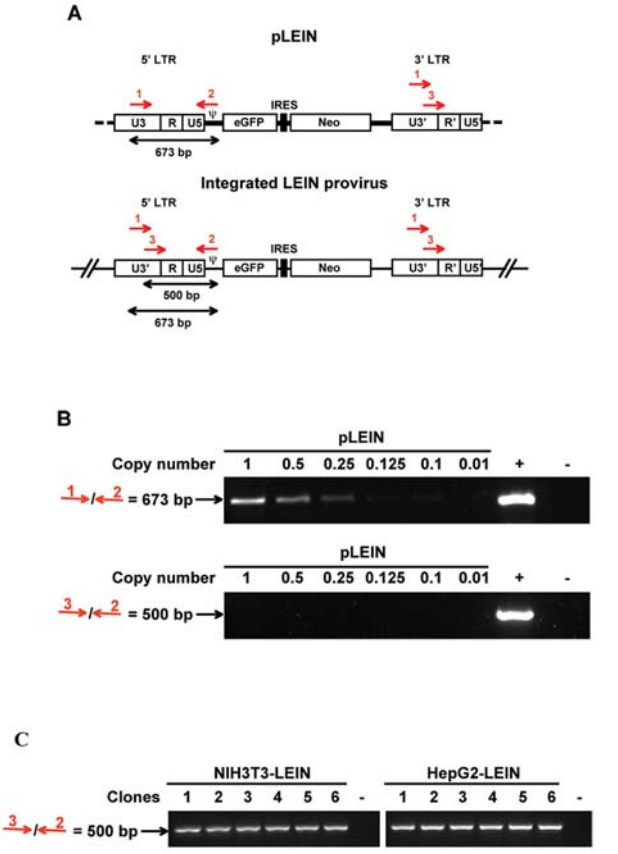


Figure 3. *The principle and sensitivity of the PCR-based assay for the detection of integrated retroviral sequences in genomic DNA. A) Schematic diagram showing the location of forward primer P1, which hybridises to the U3 regions within the 5'LTR and 3'LTR of the retroviral genome; reverse primer P2, which hybridises to the retroviral packaging sequence (ψ); and forward primer P3, which hybridises to U3' of the 3'LTR. Primers P1 and P2 generate a 673 bp product from both integrated and unintegrated retroviral sequences. Subsequent to retroviral integration, the U3' region of the 3'LTR is duplicated to the 5'LTR allowing primer P3 to generate a 500 bp product with reverse primer P2. B) Upper panel. The plasmid pLEIN was used to spike NIH3T3 genomic DNA at 1.0 down to 0.01 copies per cell, and 40 cycles of PCR were performed using forward primers P1 and P2 that recognises both unintegrated and integrated sequences (673 bp). Lower Panel. A second PCR was performed using primers P3 and P2 that generates a product derived specifically from integrated sequences (500 bp). Genomic DNA isolated from a LEIN infected G418-resistant/eGFP-expressing 3T3 clone (NIH3T3-LEIN), derived from a LEIN retrovirus infection was used as a positive control (+). Negative control (–), NIH3T3 parental genomic DNA. C) To demonstrate the ability of the retroviral integration-specific PCR primers P3 and P2 to detect provirus, genomic DNA isolated from G418-resistant/eGFP-expressing NIH3T3 (NIH3T3-LEIN) and HepG2 (HepG2-LEIN) clones infected with LEIN generated from AdLEIN:AdGagPol:Ad10A1Env infection of HepG2 cells. The negative controls (–) are the respective NIH3T3 and HepG2 parental genomic DNAs.*

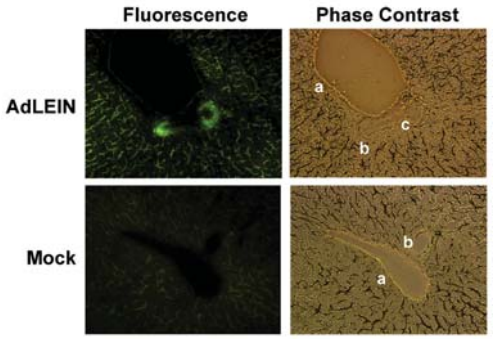
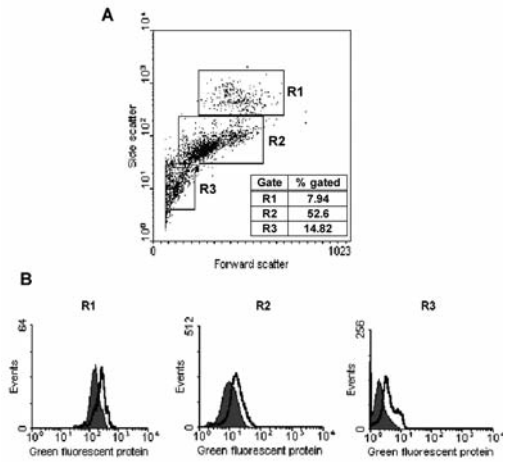


Figure 4. *Detection of AdLEIN-mediated eGFP expression in liver sections. Upper panel. The adeno-retrovirus chimeric vector AdLEIN expressing eGFP was administered to two C57BL/10 mice by tail vein injection at a dose of 3×10^{10} vp in 200 µl. Lower panel. A single animal was given a mock injection of 200 µl virus stock dilution buffer. Animals were sacrificed 5 days later, and livers removed. A portion of liver from each animal was fixed and embedded in OCT compound, snap frozen in liquid nitrogen, and sections of 10 µm thickness were observed directly under fluorescence (left panel) and phase contrast microscopy (right panel). The portal vein (a), hepatic artery (b) and bile duct (c) are identified in the phase contrast images of the liver sections.*



C

Treated Animals	Gate	Cells expressing eGFP (%)	Mean fluorescence intensity Mock	Treated
1 (n =4)	R1	15.68 ± 1.37	146.98	206.94 ± 4.87
	R2	13.48 ± 0.65	9.09	15.16 ± 0.21
	R3	19.36 ± 0.98	2.08	3.95 ± 0.04
2 (n = 3)	R1	21.14 ± 1.97	146.98	232.22 ± 3.92
	R2	14.37 ± 0.80	9.09	15.45 ± 0.15
	R3	15.71 ± 1.25	2.08	3.75 ± 0.07

Figure 5. *Detection of AdLEIN-mediated eGFP expression by FACS analysis. The adeno-retrovirus chimeric vector AdLEIN expressing eGFP was administered to two C57BL/10 mice by tail vein injection at a dose of 3×10^{10} vp in 200 µl. A single animal was given a mock injection of 200 µl virus stock dilution buffer. A) Forward scatter-side scatter plot showing three distinct populations of disaggregated liver cells: R1, R2 and R3. B) Histograms showing mean intensity of fluorescence of disaggregated liver cells isolated from animals untreated (blue plot) or injected with AdLEIN (white plot) for each population of cells. C) Table showing percentage of cells expressing eGFP from the three populations and the mean fluorescence intensity of (MFI) given for two treated animals.*

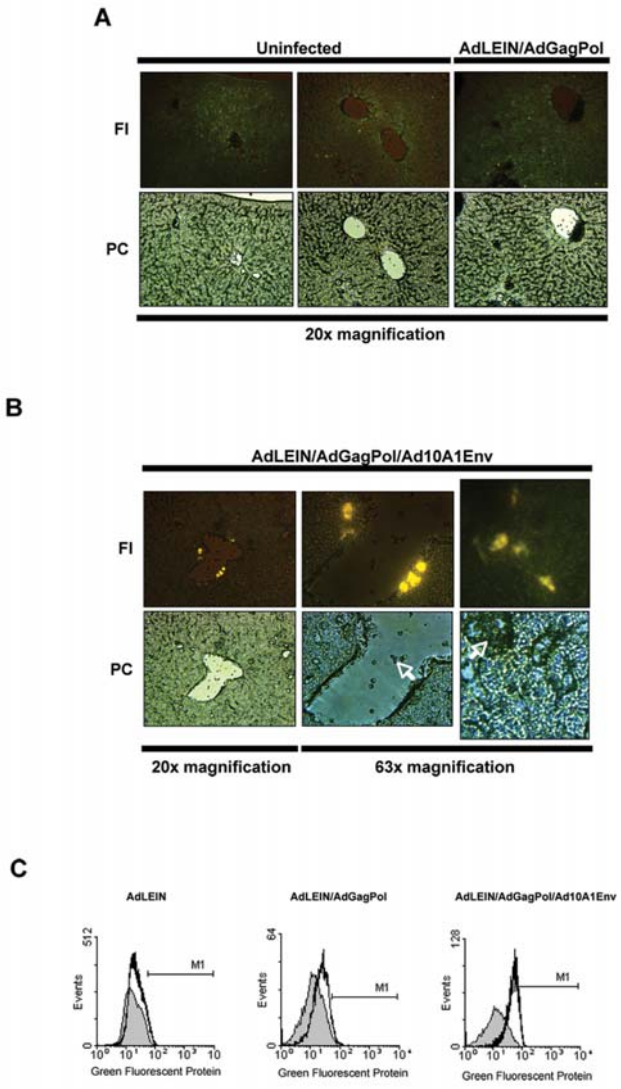


Figure 6. *Liver in situ generation of the LEIN retrovirus following administration of the hybrid adeno-retrovirus vector system. A) Animals were either untreated (left two panels) or injected with AdLEIN and AdGagPol only (right-most panel), then sacrificed 15 weeks later. B) To examine the ability of livers to produce retroviral vector, animals were also injected with AdLEIN, AdGagPol and Ad10A1, and sacrificed after 15 weeks. Portions of fixed liver tissue from the C57BL/6 mice were embedded in OCT compound, sliced at 10 µm thickness and observed directly under fluorescence (Fl) and phase contrast (PC) microscopy. The majority of eGFP expression in the AdLEIN:AdGagPol:Ad10A1Env-treated animals was present in tissue in close proximity to blood vessels of the liver that could be identified by the presence of red corpuscles (white arrows). Sections were photographed at ×20 and/or ×63 magnification. In the case of the ×20 magnification of a AdLEIN:AdGagPol: Ad10A1Env-treated animal, a ×63 magnification (middle panel) is included. C) Representative histogram plots of transduced liver cells showing animals injected with either AdLEIN, AdLEIN:AdGagPol or AdLEIN:AdGagPol:Ad10A1. FACS analysis of the treated animals (from gated population R2) is shown by the black histogram plot and overlaid on a representative plot from a mock-injected animal (shaded in grey).*

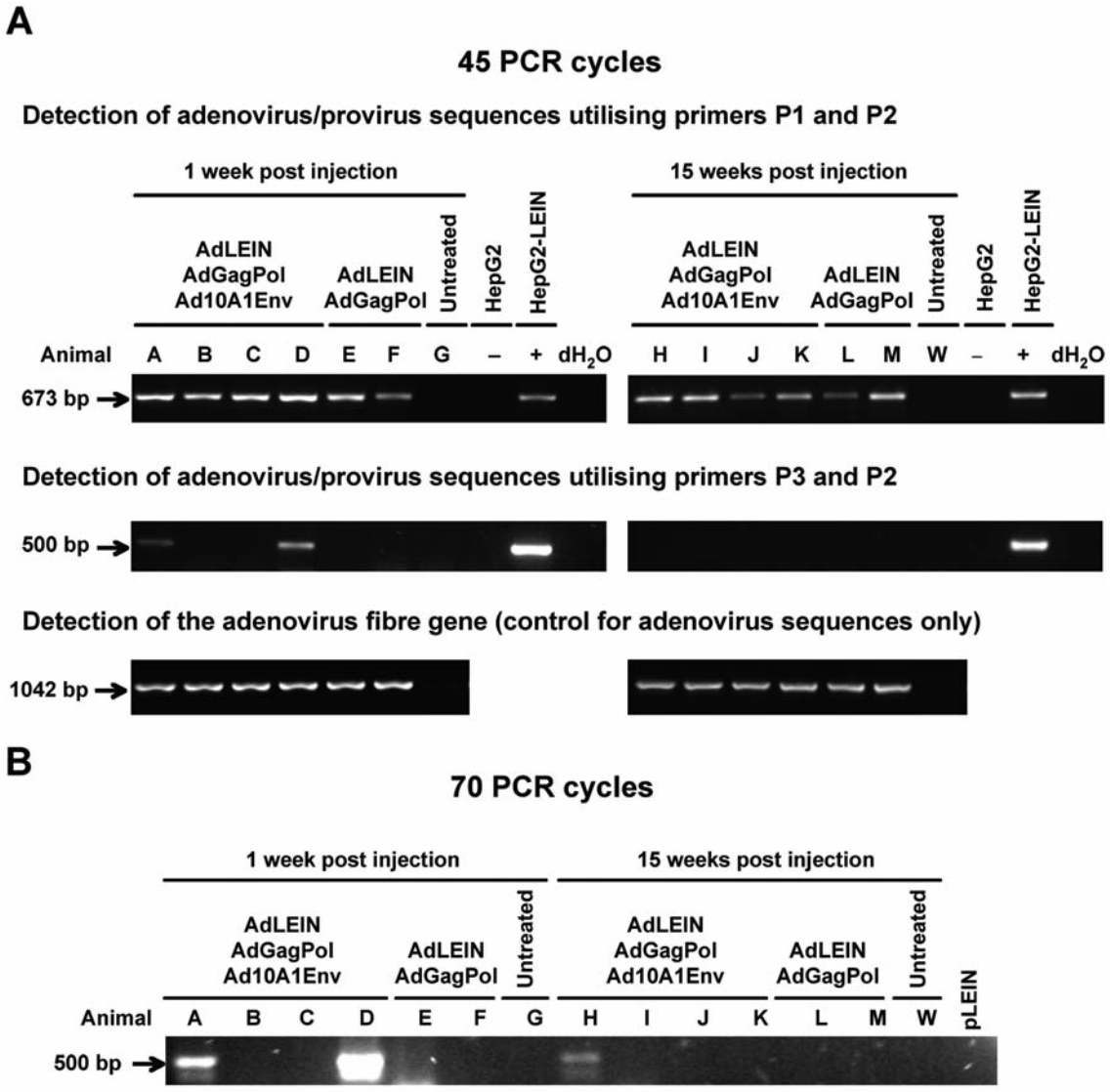


Figure 7. *Detection of in situ retroviral integration in the livers of C57BL/6 mice by PCR, following liver-directed administration of the hybrid adeno-retrovirus vector system. A) Detection of a provirus-specific sequence in liver genomic DNA from animals sacrificed one week (left panel) and animals sacrificed 15 weeks (right panel) post-injection. Upper gel. Genomic DNA from animals were subjected to 45 cycles of PCR using non-specific forward primer P1 and reverse primer P2 to detect integrated and non-integrated templates at 673 bp. Middle gel. Genomic DNA from animals were subjected to 45 cycles of PCR using integrated provirus-specific forward primer P3 and reverse primer P2 to detect integrated retroviral templates at 500 bp. Non-transduced HepG2 cells were used as a negative control (lane 8), and as a positive control genomic DNA from HepG2-LEIN cells was used (lane 9). Lower gel. To detect adenoviral vector sequences, two primers were used to amplify a 1042 bp segment of the adenovirus fibre gene (20). B) Detection of a provirus-specific sequence in liver genomic DNA from animals sacrificed one week and 15 weeks post-injection. PCR was performed at 70 cycles utilising provirus-specific forward primer P3 and reverse primer P2 that generates a 500 bp PCR product. pLEIN was used to demonstrate the lack of a 500 bp signal with unintegrated retroviral sequence (lane 15).*

Moreover, the presence of colonies of eGFP-expressing cells that we detected near blood vessels in these livers suggests that sufficient amounts of retroviral vector may be produced to stably transduce dividing hepatocytes (Figure 6B).

We employed the PCR-based approach to confirm that retroviral-mediated gene transfer resulted in stable integration of provirus into the liver cells *in vivo*. We were able to detect LEIN sequences in genomic DNA preparations by using forward primer P1 (Figure 3A) after one and 15 weeks post-injection (Figure 7A). By using 45 cycles of PCR we were able to detect the presence of integrated proviral sequences after one week in two of the four animals analysed (animals A and D; Figure 7A). Using these conditions we were unable to detect integrated sequences in the genomic

DNA of animals sacrificed after 15 weeks. However, we were able to detect the presence of proviral sequences in one animal sacrificed after 15 weeks by amplifying the template using 70 cycles of PCR (mouse H; Figure 7B). Interestingly, this animal also displayed the highest level of eGFP expression after 15 weeks compared to the other three in all gated populations as determined by FACS analysis (Table I). From these results we were able to show that a proportion of retroviral vector generated in the liver from the hybrid vector system, is able to integrate into the host cell genome as early as one week persisting to at least 15 weeks post-injection.

eGFP transgene amplification in the liver following administration of the Ad/retrovirus chimeric vector system. To further examine whether the reporter gene was incorporated into the genome of hepatocytes, we quantified the number of copies of GFP cDNA in the livers of mice treated with the hybrid virus system. The values obtained for the eGFP cDNA were normalised against the Ad fibre gene copy numbers and we postulated that a significant increase in the eGFP:Ad fibre gene ratio may reflect an increase in the proportion of eGFP expression from integrated LEIN provirus (Figure 8). Comparison of animal groups receiving AdLEIN, AdGagPol and Ad10A1 demonstrated a statistically significant 2.7-fold increase in the eGFP:Ad fibre gene ratio between 1 and 15 weeks after the injections ($p<0.00004$, students *t*-test, n=4). There was no significant increase in this ratio in livers from mice treated with AdLEIN only ($p=0.47$, student's *t*-test, n=4). Interestingly, we observed a 1.8-fold increase in the eGFP:Ad fibre gene ratio in the livers of mice treated with AdLEIN and AdGagPol, which was not statistically significant ($p=0.118$, student's *t*-test, n=4). In addition, a moderate further increase in eGFP cDNA amplification was achieved by the addition of DEAE-dextran (5 mg/ml) to the virus inoculum. The observation that the copy number of eGFP cDNA increases from one to 15 weeks in a statistically significant manner only in animals treated with all three viruses, despite the loss of adenovirus template DNA may indicate that the integrated LEIN provirus is the main source of eGFP expression by 15 weeks.

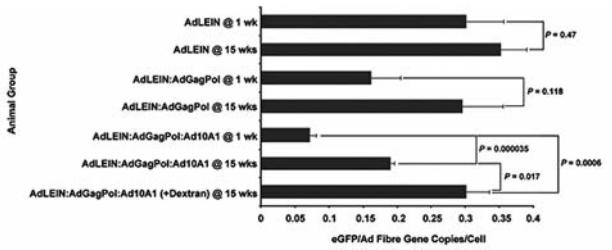


Figure 8. *eGFP transgene amplification in the liver following administration of the Ad/Retrovirus chimeric vector system. The Ad vectors AdLEIN, AdGagPol and Ad10A1 were administered to female C57BL/6 mice by tail-vein injection with and without DEAE-Dextran at a ratio of 3:1.5:1, respectively (n=8). In addition, groups of animals were treated with AdLEIN-only (n=8) and AdLEIN:AdGagPol (n=8). For each group, animals were sacrificed 1 week (n=4) and 15 weeks (n=4) later. Livers were removed and genomic DNA was isolated for real-time PCR to determine the copy number of eGFP cDNA and Ad fibre gene copies per cell. The values obtained for the eGFP cDNA were normalised against the Ad fibre gene copy number per cell and a significant increase in the eGFP:Ad fibre gene ratio may reflect the in situ generation of the LEIN retrovirus.*

Discussion

In this study, we describe the liver-directed administration of a hybrid adenovirus-retrovirus vector system, resulting in *in situ* hepatic generation of recombinant retrovirus from hepatocytes and subsequent retroviral infection of and stable integration into the genomes of neighbouring cells. Indeed, the level of expression achieved using hybrid adeno-retroviral vectors exceeds that normally obtained using conventional retroviral vector technology, where the degree of retrovirally transduced hepatocytes *in vivo* does not tend to exceed 1% when infusing retroviral vector without stimulating hepatocyte proliferation (21, 22). *In vitro*, the infection of HepG2 cells with the adenovirus-retrovirus chimeric vector system can generate recombinant retroviral vector with comparable virus titres to those obtained from stable retroviral producer cell lines (23, 24). Optimization of retroviral production on HepG2 cells demonstrated an adenoviral vector dose-dependent effect on the generation of retrovirus, *i.e.* higher multiplicities of infection of AdLEIN tended to result in higher retrovirus titres. However, our results more clearly indicated that the gagpol product has a negative effect on the generation of retrovirus (Figure 2A). This is presumably due to high levels of the Gag protein impairing the incorporation of Envelope protein into budding retrovirus particles, as has been discussed elsewhere (16).

To confirm that the retrovirus LEIN, and not adenoviral AdLEIN, was responsible for conferring eGFP expression and neomycin resistance to the HepG2 and NIH3T3 indicator cells, a PCR-based approach was employed (18). The assay can distinguish between the episomal non-replicating adenovirus vector DNA and the integrated retroviral sequences (provirus). We demonstrated that this system was sensitive enough to detect proviral templates at a copy number of 0.1 and could further be used to detect integrated proviral genomes from *in vivo* transduced liver cells. We then proceeded to examine the number of copies of eGFP cDNA by quantitative real time PCR in transduced livers and found that treatment with all three viruses was required in order to observe an increase in copy number over time. Thus, further implying that integrated LEIN provirus is the main source of eGFP template 15 weeks post-injection.

Table I. *Expression of GFP in individual animals by FACS 15 weeks post-treatment. *Integrated pro-viral LEIN was detected in mouse H by using PCR (see Figure 7B).*

AdLEIN				AdLEIN, AdGagPol				AdLEIN, AdGagPol, Ad10A1			
Mouse	GFP Expression (%)			Mouse	GFP Expression (%)			Mouse	GFP Expression (%)		
	R1	R2	R3		R1	R2	R3		R1	R2	R3
P	2.5	6.0	9.0	L	1.5	4.5	18.4	H*	2.5	48.1	57.5
Q	3.4	3.5	6.1	M	2.5	9.6	34.0	I	4.2	4.2	11.0
R	5.1	1.9	4.4	N	5.8	4.1	11.9	J	6.2	11.0	33.3
S	1.6	3.8	7.1	O	5.2	6.4	20.4	K	6.8	3.2	6.1
								T	3.6	1.4	2.6
								U	5.0	10.8	15.8
								V	1.7	6.5	17.5
Average	3.1	3.8	6.6	Average	3.7	6.1	21.2	Average	4.3	12.2	20.5

Tail-vein injection of AdLEIN alone resulted in efficient infection of C57BL/10 mouse livers, with up to 20% of hepatocytes expressing eGFP after five days, as assessed by FACS analysis. We chose to analyse eGFP expression in hepatocytes by FACS, as detection by fluorescence microscopy was difficult to quantify due to the solubilty of the eGFP protein. However, by using fluorescence microscopy we were able to ascertain that the most intense eGFP-expressing hepatocytes were adjacent to blood vessels. This indicated that transduced hepatocytes were in an optimal location to secrete therapeutic transgene into the circulation.

One week after liver-directed administration of AdLEIN:AdGagPol:Ad10A1 to C57BL/6 mice, we found that two of the four animals analysed contained integrated LEIN provirus in the genomic DNA of their hepatocytes. However, it was more difficult to detect proviral templates in animals examined 15 weeks post-injection with 70 cycles of PCR being required to amplify the proviral sequences detected in only one animal. This implies that an immune response is removing a proportion of the retrovirally-transduced hepatocytes, as has been described elsewhere (5). However, eGFP expression was detected in an average of 4.3% to 20.5% of hepatocytes from each liver at this time, and in the animal that contained detectable integrated LEIN template, half of the hepatocytes from gated populations R2 and R3 expressed eGFP. Furthermore, examination of eGFP-expressing hepatocytes by fluorescence microscopy in animals sacrificed after 15 weeks revealed that most of the eGFP expression was concentrated in colonies of hepatocytes adjacent to blood vessels. This is in itself indicative of retroviral vector transduction, as at this stage in animals injected with AdLEIN or AdLEIN:AdGagPol, no eGFP expressing colonies were detected in these livers using fluorescence microscopy. FACS analysis revealed that animals treated with AdLEIN contained much fewer GFP-expressing hepatocytes compared to AdLEIN:AdGagPol- and AdLEIN: AdGagPol:Ad10A1-treated mice.

Surprisingly, injection of AdLEIN:AdGagPol or AdLEIN:AdGagPol:Ad10A1 resulted in a similar proportion of transduced hepatocytes in gated populations R1 and R3, with only the R2 population showing enhanced GFP expression after the addition of all three components of the retroviral vector. Given that we also observed a small increase in the number of eGFP cDNA copies from one to 15 weeks in the livers of mice treated with AdLEIN:AdGagPol, it is possible that endogenous expression of retroviral Pol proteins, including reverse transcriptase and integrase, concomitant with retroviral vector transcription from AdLEIN, might have resulted in some degree of integration in the adenovirus-infected cells, as has been discussed elsewhere (25).

We have shown that hepatocytes are capable of producing recombinant retroviral vector transiently using a hybrid adeno-retroviral vector system, achieving titres normally only possible when using stable retrovirus producing cell lines. We further show that the retroviral vector produced is fully functional and able to integrate its DNA into the genome of host indicator cells. Upon injection into the tail vein of immune-competent mice, sufficient quantities of hybrid adeno-retroviral vector components infect the liver and began to generate recombinant retroviral vector after one week, resulting in the production of retroviral vector that stably transduces hepatocytes in the surrounding milieu. After 15 weeks, colonies of GFP-expressing hepatocytes are visible throughout the liver, which represent an efficiency of transduction in the region of 4-20%, as assessed by FACS analysis. Furthermore, a proportion of these hepatocytes contained integrated LEIN provirus that was detectable by PCR and the copy number of GFP increases over time. In

conclusion, by using the hybrid adeno-retroviral vector system, we have been able to demonstrate a degree of transduction that exceeds that obtained by conventional non-invasive retroviral-based methods.

Acknowledgements

Parts of this work were supported by grants from the British Heart Foundation and the National Heart Research Fund. We gratefully thank François-Loïc Cosset and Ghislaine Duisit for providing the AdGagPol and Ad10A1 vectors.

References

1 Ledley FD, Grenett HE, McGinnis-Shelnutt M and WOO SL: Retroviral-mediated gene transfer of human phenylalanine hydroxylase into NIH 3T3 and hepatoma cells. Proc Natl Acad Sci USA *83*: 409-413, 1986.
2 Ledley FD, Darlington GJ, Hahn T and Woo SL: Retroviral gene transfer into primary hepatocytes: implications for genetic therapy of liver-specific functions. Proc Natl Acad Sci USA *84*: 5335-5339, 1987.
3 Wilson JM, Jefferson DM, Chowdhury JR, Novikoff PM, Johnston DE and Mulligan RC: Retrovirus-mediated transduction of adult hepatocytes. Proc Natl Acad Sci USA *85*: 3014-3018, 1988.
4 McCormack JE, Edwards W, Sensintaffer J, Lillegren L, Kozloski M, Brumm D, Karavodin L, Jolly DJ and Greengard J: Factors affecting long-term expression of a secreted transgene product after intravenous administration of a retroviral vector. Mol Ther *3*: 516-525, 2001.
5 Podevin G, Pichard V, Durand S, Aubert D, Heloury Y and Ferry N: *In vivo* retroviral gene transfer to the liver is cancelled by an immune response against the corrected cells. Can it be avoided? Pediatr Surg Int *18*: 595-599, 2002.
6 Welsh RM, Cooper NR, Jenson FC and Oldstone MBA: Human serum lyses RNA tumor viruses. Nature *257*: 612-614, 1975.
7 Aoki M, Morishita R, Higaki J, Moriguchi A, Hayashi S, Matsushita H, Kida I, Tornita N, Sawa Y, Kaneda Y and Ogihara T: Survival of grafts of genetically modified cardiac myocytes transfected with FITC-labeled oligodeoxynucleotides and the beta-galactosidase gene in the non-infarcted area, but not the myocardial infracted area. Gene Ther *4*: 120-127, 1997.
8 Kerr WG: Genetic modification of the hematolymphoid compartment for therapeutic purposes. Hematol Oncol Clin North Am *12*: 503-519, 1998.
9 Overturf K, Al-dhalimy M, Manning K, Ou CN, Finegold M and Grompe M: *Ex vivo* hepatic gene therapy of a mouse model of hereditary tyrosinemia type I. Hum Gene Ther *9*: 295-304, 1998.
10 Culver KW, Ram Z, Walbridge S, Ishii H, Oldfield EH and Blease RM: *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science *256*: 1550-1552, 1992.
11 Fassati A, Wells DJ, Walsh F and Dickson G: Transplantation of retroviral producer cells for *in vivo* gene transfer into mouse skeletal muscle. Hum Gene Ther *7*: 595-602, 1996.
12 Feng M, Jackson WH, Goldman CK, Rancourt C, Wang M, Dusing SK, Siegal G and Curiel DT: Stable *in vivo* gene transduction *via* a novel adenoviral/retroviral chimeric vector. Nature Biotech *15*: 866-870, 1997.
13 Lin X: Construction of new retroviral producer cells from adenoviral and retroviral vectors. Gene Ther *5*: 1251-1258, 1998.
14 Ramsey WJ, Caplen NJ, Li Q, Higginbotham JN, Shah M and Blaese RM: Adenovirus vectors as transcomplementing templates for the production of replication defective retroviral vectors. Biochem Biophys Res Commun *246*: 912-919, 1998.
15 Caplen NJ, Higginbotham JN, Scheel JR, Vahanian N, Yoshida Y, Hamada H, Blaese RM and Ramsey WJ: Adeno-retroviral chimeric viruses as *in vivo* transducing agents. Gene Ther *6*: 454-459, 1999.
16 Duisit G, Salvetti A, Moullier P and Cosset FL: Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines. Hum Gene Ther *10*: 189-200, 1999.
17 Roberts ML, Athanosopoulos T, Pohlschmidt M, Duisit G, Cosset FL and Dickson G: Post-mitotic, differentiated myotubes efficiently produce retroviral vector from hybrid adeno-retrovirus templates. Gene Ther *8*: 1580-1586, 2001.
18 Roberts ML, Wells DJ, Graham IR, Fabb SA, Hill VJ, Duisit G, Yuasa K, Takeda S, Cosset FL and Dickson G: Stable micro-dystrophin gene transfer using an integrating adeno-retroviral hybrid vector ameliorates the dystrophic pathology in mdx mouse muscle. Hum Mol Genet *11*: 1719-1730, 2002.
19 Wilson CA, Farrell KB and Eiden MV: Properties of a unique form of the murine amphotropic leukemia virus receptor expressed on hamster cells. J Virol *68*: 7697-7703, 1994
20 Harris JD, Graham IR, Schepelmann S, Stannard AK, Roberts ML, Hodges BL, Hill VJ, Amalfitano A, Hassall DG, Owen JS and Dickson G: Acute regression of advanced and retardation of early aortic atheroma in immunocompetent apolipoprotein-E (apoE) deficient mice by administration of a second generation [E1−, E3−, polymerase−] adenovirus vector expressing human apoE. Hum Mol Genet *11*: 43-58, 2002.
21 Themis M, Forbes SJ, Chan L, Cooper RG, Etheridge CJ, Miller AD, Hodgson HJF and Coutelle C: Enhanced *in vitro* and *in vivo* gene delivery using cationic agent complexed retrovirus vectors. Gene Ther *5*: 1180-1186, 1998.
22 Forbes SJ, Themis M, Alison MR, Selden C, Coutelle C and Hodgson HJF: Retroviral gene transfer to the liver *in vivo* during tri-iodothryonine-induced hyperplasia. Gene Ther *5*: 552-555, 1998.
23 Cosset FL, Takeuchi Y, Battini JL, Weiss RA and Collins MKL: High-titre packaging cells producing recombinant retroviruses resistant to human serum. J Virol *69*: 7430-7436, 1995.
24 Ory DS, Neugeboren BA and Mulligan RC: A stable human-derived packaging cell line for production of high titre retrovirus/vesicular stomatitis virus G pseudotypes. Proc Nat Acad Sci USA *93*: 11400-11406, 1996.
25 Murphy SJ, Chong H, Bell S, Diaz RM and Vile RG: Novel integrating adenoviral/retroviral hybrid vector for gene therapy. Hum Gene Ther *13*: 745-760, 2002.

Received July 17, 2009
Accepted September 14, 2009