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ADENO-ASSOCIATED VIRAL VECTOR TRANSDUCTION OF HUMAN MESENCHYMAL STEM CELLS

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Abstract

Mesenchymal stem cells (MSCs) have received considerable attention in the emerging field of regenerative medicine. One aspect of MSC research focuses on genetically modifying the cells with the aim of enhancing their regenerative potential. Adeno-associated virus (AAV) holds promise as a vector for human gene therapy, primarily due to its lack of pathogenicity and low risk of insertional mutagenesis. However, the existing data pertaining to AAV transduction of MSCs is limited.

The objective of this work was to examine the efficiency and kinetics of *in vitro* transduction using AAV serotype 2 in human MSCs and to assess whether AAV transduction affects MSC multipotentiality. The results indicated that human MSCs could indeed be transiently transduced *in vitro* by the AAV2 vector with efficiencies of up to 65%. The percentage of GFP-positive cells peaked at 4 days post-transduction and declined rapidly towards 0% after day 8. The level of transgene expression in the GFP-positive population increased 4-fold over a 10,000 fold viral dose increase. This dose-response contrasted with the 200-fold increase observed in similarly transduced 293-cells, indicating a relatively restricted transgene expression in MSCs following AAV mediated gene delivery. Importantly, transduced MSCs retained multipotential activity comparable to untransduced controls.

Key Words: Mesenchymal stem cells, adeno-associated virus, transgene expression, multipotential activity.

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Introduction

Adult mesenchymal stem cells (MSCs) have received considerable attention in the emerging field of regenerative medicine (Barry and Murphy, 2004). MSCs can differentiate into a variety of mesenchymal lineages, including chondrocytes, osteocytes, adipocytes, tenocytes and myocytes (Wakitani *et al*., 1995; Awad *et al*., 1999; Pittenger *et al*., 1999). This multipotentiality combined with their ease of isolation and high capacity for *in vitro* expansion has attracted considerable interest in their use in tissue engineering applications.

A remarkable feature of MSCs is their apparent ability to migrate towards and engraft at sites of injury *in vivo*. Following engraftment, they elicit a regenerative effect on the injured tissue. This activity underpins the strong interest in the therapeutic use of MSCs in diseases such as myocardial infarction (Oulic *et al*., 2001; Woller *et al*., 2004), neuronal injury (Wang *et al*., 2002) and osteoarthritis (Murphy *et al*., 2003; Wakitani *et al*., 2003).

However, there are several questions concerning the use of MSCs that need to be addressed. Firstly, both *in vitro* and *in vivo* studies show that differentiated MSCs can transdifferentiate or dedifferentiate (Muraglia *et al*., 2003; De Bari *et al*., 2004) and a method of securing their phenotypic stability following differentiation will be useful. Secondly, although intravenously injected MSCs engraft in damaged tissues, a majority lodge in the vasculature of the lung and other highly vascularized organs (Gao *et al*., 2001). A method of achieving tissue-specific homing and engraftment of MSCs would be a major step towards a potent and cost-efficient therapy.

We and others have looked at gene therapy to overcome the above mentioned limitations. In a tissue engineering context MSCs can be genetically modified to express transcription factors or growth factors that induce differentiation along a desired lineage (Carlberg *et al*., 2001; Gels *et al*., 2001; Katayama *et al*., 2004). Tissue-specific homing and engraftment of MSCs could be achieved by the introduction of genes for tissue-specific cell surface adhesion molecules. Furthermore, as MSCs engraft in damaged tissues, genetically engineered MSCs could be employed as a means of delivering therapeutically active proteins to these tissues (Stagg *et al*., 2004; Reiser *et al*., 2005; Kim *et al*., 2006; Goncalvos *et al*., 2006).

Most efforts have relied on adeno- and lentiviral vectors for delivering genes to MSCs. Effective as these vectors may be, concerns regarding their immunogenicity and, in the case of lentivirus, the risk of insertional mutagenesis, have led to the pursuit of safer alternatives.

Among these, adeno-associated virus (AAV), a small parvovirus, holds several advantages as a vector for human gene therapy, the most obvious being its lack of pathogenicity. For a recent review of past and ongoing clinical trials with AAV, see Carter (2005).

The few published studies that utilize AAV transduction of MSCs agree on the feasibility of this (Ho *et al*., 2004; Kumar *et al*., 2004; Kumar *et al*., 2005; Bosch *et al*., 2006; McMahon *et al*., 2006). However, these studies leave several basic questions regarding transduction efficiency and kinetics unanswered. In the present study, flow cytometry and fluorescence microscopy were used to examine the *in vitro* transduction efficiency of AAV serotype 2 in human MSCs. Furthermore, we evaluated whether transduction with AAV altered the differentiation potential of the cells.

Materials and Methods

Isolation of human MSCs

MSCs were isolated by direct technique as described previously (Murphy *et al*., 2006). In brief, heparinized bone marrow aspirates (10 mL) were taken from the iliac crest of healthy donors. Marrow was obtained after informed consent and all procedures were approved by the Clinical Research Ethical Committee at University College Hospital, Galway. Marrow was diluted 10-fold in phosphate-buffered saline (PBS). The cell fraction was separated by centrifugation and seeded on T-175 flasks in DMEM-LG containing 1 g/L glucose with 10% FBS (Gibco, Invitrogen, Dun Laoghaire, Ireland)) of a batch previously determined to support MSC growth at ~300,000 cells/cm^2 for culture at 37°C with 95% humidity and 5% CO_2 in the same medium. After 5 days red blood cells were washed off with PBS and fresh medium was added. Colonies of adherent cells formed within 9 days. The colonies were trypsinized from the flasks when they covered 60% to 90% of the plate and were cryopreserved for later use. Upon thawing, MSCs were expanded through several passages prior to viral transduction. All MSCs used in this study were from passage 5. Flow cytometry (FACSCalibur, Beckton Dickinson (BD), Oxford, UK) demonstrated that the cells were homogenously CD105+, CD34-, CD45-, CD14-, which is a typical MSC surface antigen profile (Tuli *et al*., 2003) (data not presented).

Preparation of rAAV-eGFP vector

The enhanced green fluorescent protein (eGFP) gene under transcriptional control of a TRUFR promoter was cloned into the rAAV vector backbone. The rAAV–eGFP virus was prepared using the adenovirus-free system by the Gene Core Facility, University of North Carolina at Chapel Hill, NC, USA. The concentration of infectious rAAV–eGFP particles was approximately $4.5x10^{10}$ per mL determined by titration on human embryonic kidney cells.

Transduction of hMSCs or 293-cells

MSCs were plated in 24-wells at 10,000 cells/well and 500 µl MSC medium was added per well. Cells were allowed to attach for 24h before virus was added directly to the medium at 1, 10, 100, 1,000 and 10,000 multiplicities of infection (MOI). Media were changed after 24h and every 48h for the remaining culture period. The procedure for transducing 293-cells (human embryonic kidney cells) was identical, except that 20,000 cells were seeded per well.

Harvest of MSCs and preparation for flow cytometry

Cultures were trypsinized and collected in separate Eppendorf tubes. Cells were stained with 7-AAD (Guava Technologies, Hayward, CA, USA; cell viability kit) after which they were left in the dark for 20 minutes at 4°C to allow for staining of dead cells. The cells were resuspended in BD Cellwash (BD Biosciences) and analysed for eGFP expression and 7-AAD staining on a GUAVA Easycyte R, (Guava Technologies). 5,000 cells were counted per acquisition. The percentage of viable cells expressing eGFP and the mean fluorescence intensity (MFI) in this population was assayed.

Osteogenesis

20,000 AAV-eGFP (MOI 100) transduced or untransduced MSCs/well were seeded in a 6-well plate, N=3. 2 mL MSC medium was added per well. After 24h, medium was removed and 2 mL osteoinductive medium (MSC medium, 100 nM Dexamethasone, 10 mM Beta-Glycerophosphate, 50 µM Ascorbic acid 2-phosphate added. Unless otherwise noted, all reagents were acquired from Sigma Aldrich (Dublin, Ireland). Medium was changed every 3-4 days. Cultures were harvested at day 18. Calcium deposition was evaluated quantitatively with a StanBio Calcium Liquicolour Kit (Stanbio, Boerne, TX, USA) and qualitatively by Von-Kossa staining of formalin fixed cultures. Cultures grown in MSC medium for the entire period served as negative controls, N=3.

Adipogenesis

200,000 AAV-eGFP (MOI 100) transduced or untransduced MSCs/well were seeded in a 6-well plate, N=3. Cells were cultured to confluence in MSC medium (4 days). The cultures were subjected to 3 cycles of 72h in adipogenic induction medium (DMEM-HG (4, 5 gm glucose/L), 10% FBS, 1 µM dexamethasone, 500 µM methyl-isobutylxanthine, and 10 µg/ml insulin), followed by 24 hours in maintenance medium (DMEM-HG, 10% FBS, and 10 µg/ml insulin). After cycle 3, cells were grown an additional week in maintenance medium, after which they were fixed with 10% formalin and stained with Oil Red O for detection of lipid vacuoles. MSCs grown in MSC medium served as negative controls, N=3.

Chondrogenesis

MSCs in monolayer were transduced with AAV2-eGFP at MOI 100, N=5. Untransduced cells served as controls, N=5. 24h post transduction, the cells were trypsinized, spun at 100 x g for 5 min and resuspended in complete chondrogenic medium (CCM) (Dulbecco's Modified Eagle's Medium (DMEM) high glucose, 10 ng/mL TGF-β3, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/

mL selenous acid, 5.33 µg/mL linoleic acid, 1.25 µg/mL bovine serum albumin (BSA), 100 nM dexamethasone, 50 µg/mL Ascorbic acid-2-phosphate, 40 µg/mL proline, 1 mM sodium pyruvate, antibiotic/antimycotic). 2x10e5 cells/tube were transferred to 15 mL polypropylene tubes. Tubes were spun at 100 x g for 5 min. Medium was aspirated and the pellets were subsequently cultured in CCM which was changed every 2-3 days. Cells resuspended and cultured in incomplete chondrogenic medium (CCM minus TGF-β3) served as negative controls. Glycosaminoglycan (GAG) content was quantified after 21 days of culture using the DMMB assay as previously described (Murphy *et al*., 2002). DNA content was measured by the DNA Quant-It Kit (Molecular Probes, Eugene, OR, USA).

Microscopy

Microscopic images of eGFP-positive cells were acquired using an Olympus (Hamburg, Germany) IX71 inverted fluorescent microscope.

Statistics

Transduction efficiencies and MFIs were directly acquired from the GUAVA Easycyte software after gating for dead cells and autofluorescence. 5 samples were acquired for each time point and MOI. Means and standard error of the mean (SEM) are depicted in bar graphs. Statistical analysis was carried out using Student's *t*-test as data could be approximated to normal distribution.

Results

Transduction efficiencies ranged from 1% for MOI 1 to 65% for MOI 10,000 (Fig. 1). A peak in percentage of GFP-positive cells was observed at 4 days post transduction for MOI≥100. Long term expression of MOI 1000 transduced cultures was assayed at day 8 to 32 post transduction. A dramatic time related reduction in percentage of GFP-positive cells was observed with ~2% green cells between day 16 and 24, and a further decrease to ~0.5% at day 32 (data not shown). The decline of GFP-positive MSCs over time can be explained by the replication deficiency of the AAV vector and by the fact that AAV does not integrate in the host cellular chromosome which prohibits vertical vector transmission in dividing cell cultures. Viability assayed by trypan blue exclusion or 7-AAD staining was high (>95%) in all samples studied (data not shown).

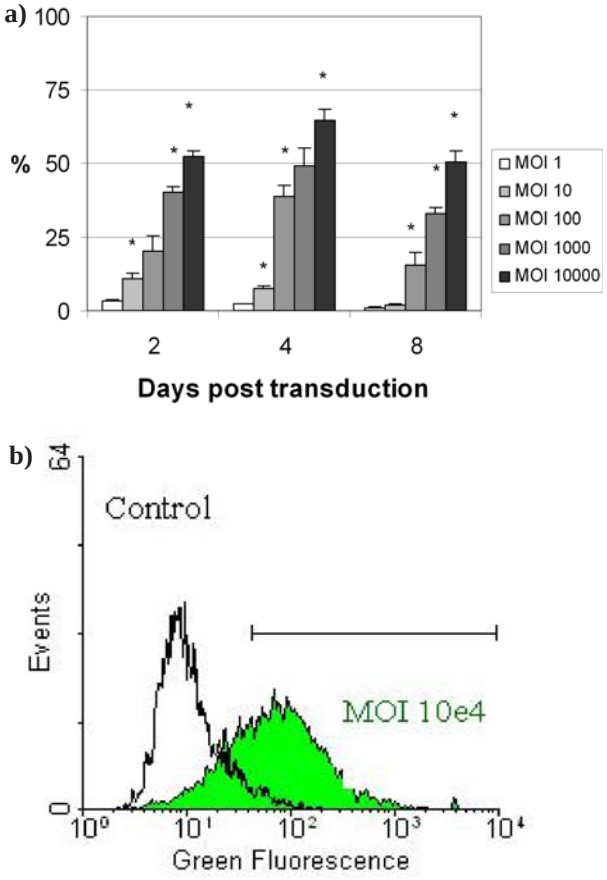

Figure 1. a) Mean + sem transduction efficiency of AAV2 in hMSCs. Each bar is based on flow cytometry of 5 samples. * represent $p<0.05$ compared to 10-fold lower MOI dose at the same time point. b) Representative histogram of MOI 10,000 transduced MSCs *vs* control, 4 days post transduction. Bar indicates 65% of transduced cells, 5% of untransduced cells (autofluorescence).

Microscopy

Intensity of fluorescence in transduced MSCs was low with MOI 1-100 transduced cultures virtually indistinguishable from controls. In contrast, numerous bright fluorescent cells were visible in MOI 1,000 and 10,000 transduced cultures (Fig. 2). This fluorescence was completely absent in uninfected controls.

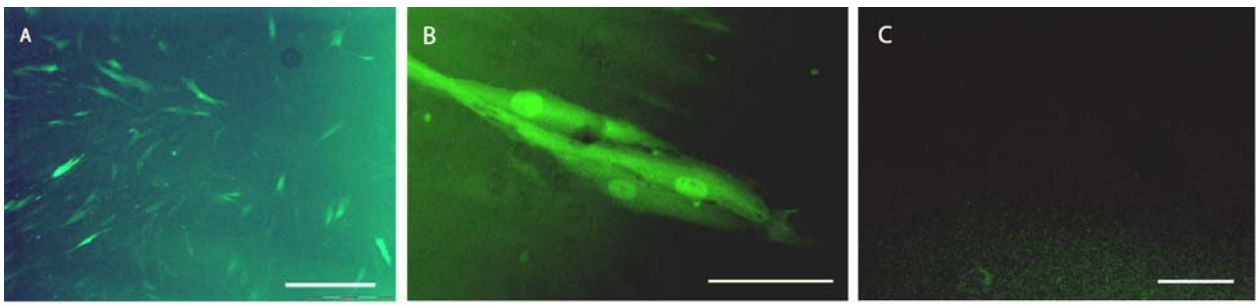

Figure 2. GFP expression at day 4 post transduction in MOI 10,000 transduced MSCs (A + B) and untransduced controls (C).

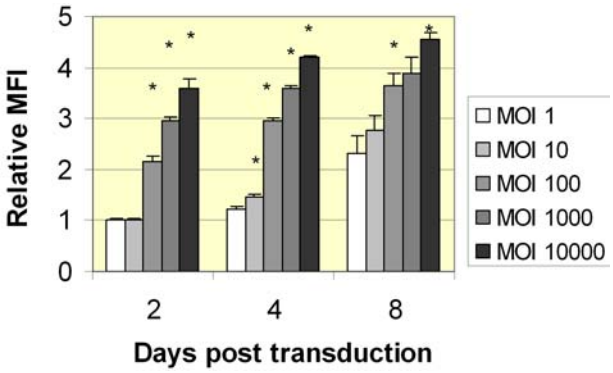

Figure 3. Relative MFIs + sem of GFP-positive AAV-GFP transduced MSCs. * indicate $p<0.05$ compared to a 10-fold lower MOI dose at the same time point.

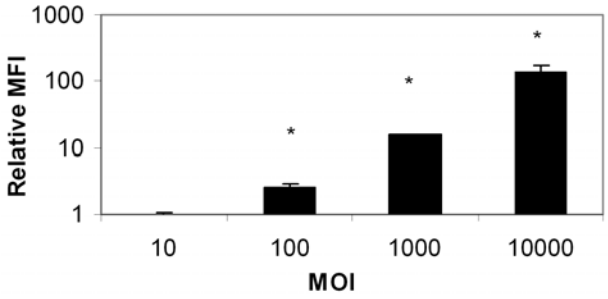

Figure 4. Relative MFIs + sem of AAV-GFP transduced 293-cells, day 4 post transduction. * indicate $p<0.05$ compared to a 10-fold lower MOI dose.

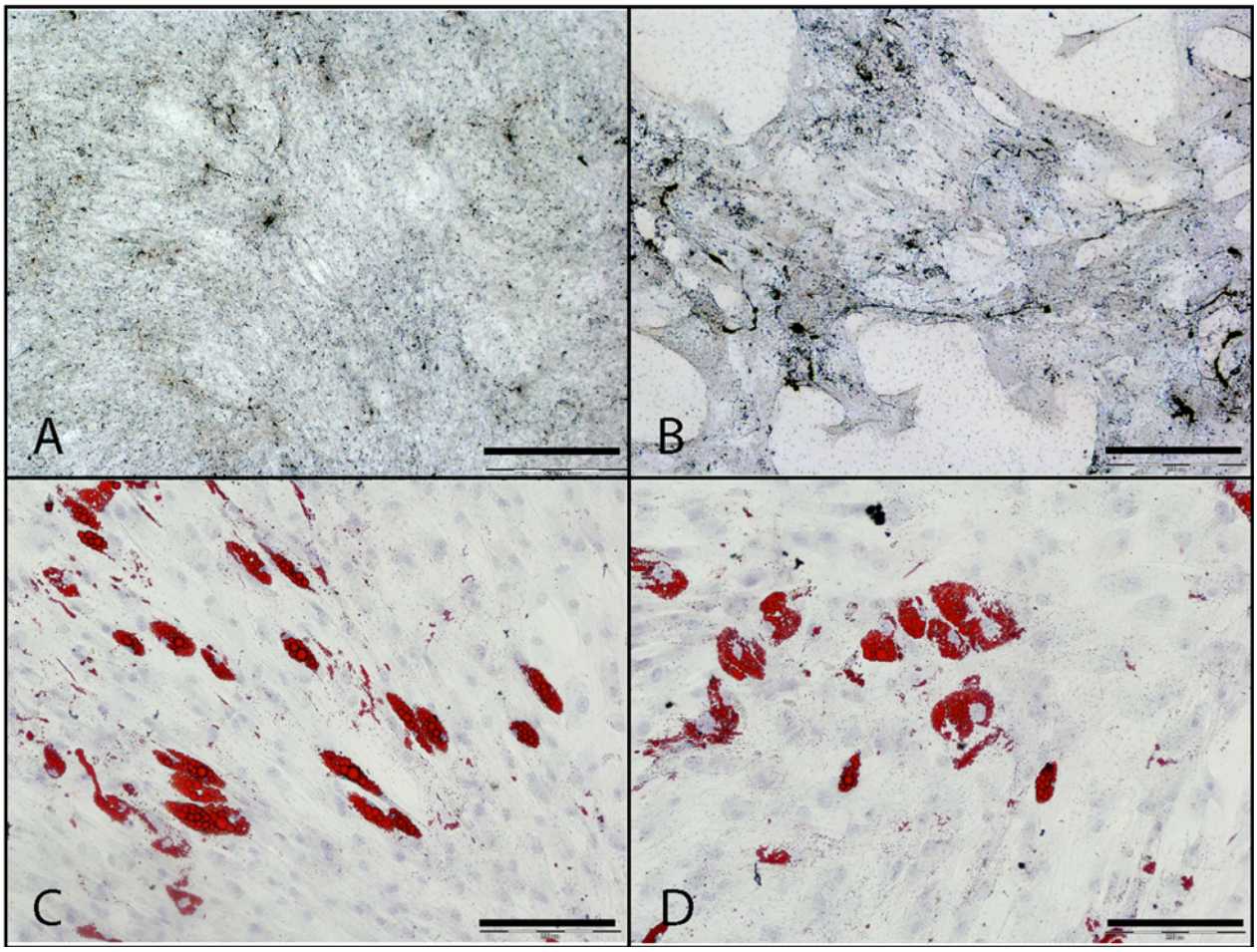

Figure 5. Von Kossa staining of untransduced (A) and AAV-GFP transduced (MOI 100) (B) osteogenic differentiated MSCs, bar = 500 µm. Adipogenesis of untransduced (C) and AAV-GFP transduced (MOI 100) MSCs (D), bar = 200 µm.

Mean fluorescence intensity (MFI)

Mean fluorescence intensity (MFI) is a parameter for the cellular synthesis of transgene GFP in the AAV-GFP transduced cell population. In order to evaluate the level of transgene expression, cells positive for GFP were analyzed for MFI (Fig. 3).

A 10,000 fold increase in viral dose gave a ~4 fold increase in MFI of GFP-positive MSCs at day 4 post transduction. We compared this result to the results from a similar transduction experiment conducted on 293-cells, a cell-line known to be permissive for AAV transduction. MFIs of GFP-positive 293-cells increased ~200 fold over the same range of viral doses that only produced a ~4-fold MFI increase in MSCs (Fig. 4). Thus, although it is possible to achieve high transduction efficiencies with AAV in hMSCs, the level of transgene expression in the transduced population appears to be severely restricted, at least compared to 293-cells.

Differentiation assays

Importantly, AAV-GFP transduced hMSCs retained multipotential activity comparable to untransduced cells.

Osteogenesis
AAV-GFP transduced (MOI 100) MSCs retained the ability to differentiate along the osteogenic lineage as determined by Van Kossa staining of deposited calcium (Fig. 5A+B). There was no difference in the amount of deposited calcium between transduced and untransduced cultures, N=2 (Fig. 6).

Adipogenesis
AAV-GFP transduced (MOI 100) MSCs underwent adipogenesis with the same efficiency as untransduced cells, indicated by the production of lipid vacuoles after 21 days of culture in adipogenic medium (Fig. 5 C+D).

Chondrogenesis
AAV-GFP transduced (MOI 100) MSCs produced glycosaminoglycans (GAG) at comparable levels to untransduced cells, N=5 (Fig. 7).

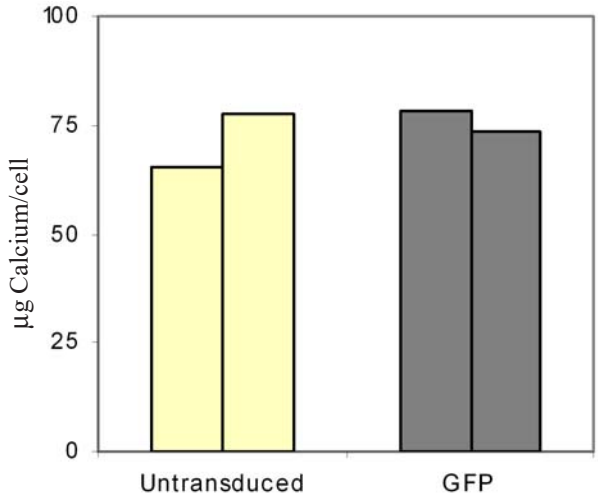

Figure 6. Calcium deposition in untransduced versus AAV-GFP transduced osteogenic differentiated MSCs, N=2.

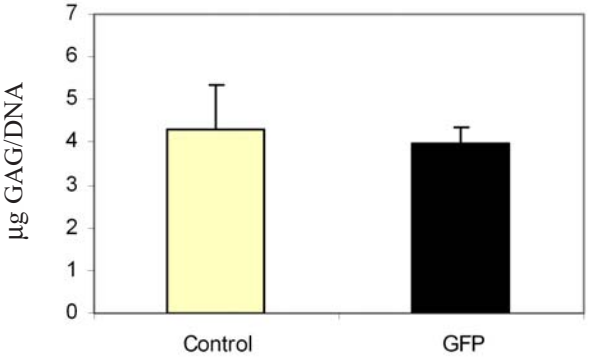

Figure 7. Production of GAG + sem in control and MOI 100 transduced MSCs, N=5. There was no statistical difference in levels of GAG between the two groups (p = 0.893).

Discussion

We found that high doses of AAV2 could transduce MSCs *in vitro* with efficiencies of up to 65%. In order to assess the level of transgene expression in the transduced population, we compared MFIs of transduced hMSCs to similarly transduced 293-cells. Whereas MFIs of GFP-positive 293-cells increased ~200 fold upon increasing the viral dose 10,000 fold, MFIs in similarly transduced MSCs only increased ~4 fold. This indicates that although AAV2 can transduce MSCs, levels of transgene expression remain severely restricted. Consistent to our results, Ito *et al.* reported a tiny increase (approximately +5% compared to untransduced cells) in extracellular TGFβ1 concentrations following AAV mediated delivery of this gene to MSCs (Ho *et al*., 2004). These results and our own failure to induce chondrogenic differentiation in MSCs by transduction with the same AAV-TGFβ1 vector (data not presented here) lead us to suggest that in MSCs, AAV is better suited to introduce genes for proteins that exert their function at lower concentrations. Transcription factors or cellular adhesion molecules are obvious candidates.

There may be several reasons for the low transgene expression following AAV mediated gene delivery to MSCs. A scarcity of the primary cell surface receptor for AAV, heparin sulfate proteoglycan (Summerford and Samulski, 1998), on the cellular surface of MSCs might limit the number of endocytosed virions. Uncoating of virions following cellular uptake was recently described as an important barrier to efficient AAV transduction (Hauck *et al*., 2004). Second strand synthesis following virion uncoating and nuclear delivery of the single stranded viral genome is another rate limiting step for AAV transduction (Ferrari *et al*., 1996; Fischer *et al*., 1996). Presumably, a yet-to-be-identified cellular DNA repair mechanism is responsible for this second strand synthesis. It is interesting to note that the DNA repair in undifferentiated stem cells is less error-prone than in more differentiated cells (Bill *et al*., 1991; Saretzki *et al*., 2004). Hypothetically, cells that exert a more stringent control over their DNA-repair enzymes will be less likely to mistake single stranded viral DNA for damaged genomic DNA in need of repair. Thus, a highly stringent DNA-repair mechanism in MSCs could play a role in the low transgene expression observed in this study.

The duration of transgene expression is a clinically highly relevant parameter. We observed a peak of GFP-positive cells at 4 days post transduction followed by a rapid decline to near control levels after day 16. It is worth noting that many MSC-based therapies currently under investigation aim at healing, which is ideally a transient process. With this in mind, AAV-mediated gene therapy of MSCs could be utilized as a way of temporarily supplying the MSCs with beneficial genes while the healing is ongoing without risking potential adverse effects of long term transgene expression. Finally, it is important to note that the transient expression observed *in vitro* in this study might not correlate to the *in vivo* situation. For instance, the duration of transgene expression for haematopoietic stem cells transduced with AAV was much longer *in vivo* than *in vitro* (Ponnazhagan *et al*., 1997), presumably due to more frequent cellular divisions *in vitro*.

In conclusion, high titers of AAV2-GFP transiently transduce human MSCs *in vitro* with efficiencies of up to 65%. A low level of transgene expression is a drawback

that needs to be taken into consideration for future studies utilizing AAV-mediated gene delivery to MSCs. Importantly, AAV2-GFP transduced MSCs retained multipotential activity comparable to untransduced cells.

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Discussion with Reviewers

B. Johnstone: Since the cells are not selected by means other than plate adherence and passage, it is possible that there is still heterogeneity in the transduced cultures. With evidence of heterogeneity of transduction, could subpopulations of cells be distinguished and differentiation capacity compared?

Authors: Certainly these cultures may be heterogeneous and plate adherence is a crude method of selection. Separation of subpopulations based on transduction efficiency is a useful approach in trying to understand this heterogeneity and could be approached by high speed cell sorting, for instance. It is interesting to contemplate whether there may be a link between transduction capacity and differentiation. The experimental approach is straightforward and it may give rise to useful new information about the microheterogeneity of these cells.

B. Johnstone: Are the authors considering methods to improve transduction and increase the low level of AAV transgene expression in MSCs? For example, other AAV serotypes may provide better transduction levels in MSCs.

Authors: There are other approaches which will lead to a higher efficiency of transduction and these are worth exploring in detail. Recently we published a study (McMahon *et al*., 2006; text reference) using rat MSCs and a range of vectors was tested on the same stem cell preparation. Adenovirus, AAV serotypes 1, 2, 4, 5, and 6, lentivirus, and nonviral vectors were compared. Lentivirus proved to be most effective with transduction efficiencies of up to 95%, concurrent with low levels of cell toxicity. Adenovirus also proved effective, but a significant increase in cell death was seen with increasing viral titer. Interestingly, rat MSCs remained refractory to transduction by all AAV serotypes, in contrast to rabbit MSCs tested at the same time. Lipofection of plasmid DNA gave moderate transfection levels but was also accompanied by cell death. Electroporative gene transfer proved ineffective at the parameters tested and resulted in high cell death. High and moderate levels of cell transduction using lentivirus vectors did not affect the ability of the cells to differentiate down the adipogenic pathway.

M. Stoddart: All the data showing highest efficiency (65%) and highest MFI was carried out using 10,000 MOI. A successful therapy is likely to require high transduction efficiency, high expression efficiency or both. Why did the authors choose a 100 fold lower MOI (100 MOI) for the differentiation experiments?

Authors: This is a valid point and in an ideal context the differentiation potential would be monitored at the MOI that gives the highest efficiency of transduction. The point of the experiment was to determine that the phenotype of the cells was not altered by transduction and this has been demonstrated as reported in the manuscript. If AAV-transduced MSCs were to be used clinically it would be necessary to explore the effect of differentiation in more detail.