

RESEARCH ARTICLE

Light-activated gene transduction of recombinant adeno-associated virus in human mesenchymal stem cells

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Deficiencies in skeletal tissue repair and regeneration lead to conditions like osteoarthritis, osteoporosis and degenerative disc disease. While no cure for these conditions is available, the use of human bone marrow derived-mesenchymal stem cells (HuMSCs) has been shown to have potential for cell-based therapy. Furthermore, recombinant adeno-associated viruses (rAAV) could be used together with HuMSCs for in vivo or ex vivo gene therapy. Unfortunately, the poor transduction efficiency of these cells remains a significant obstacle. Here, we describe the properties of ultraviolet (UV) light-activated gene transduction (LAGT) with rAAV in HuMSCs, an advance toward overcoming this limitation. Using direct fluorescent image analysis and real-time quantitative PCR to evaluate enhanced green fluorescent protein (eGFP) gene expression, we found that the optimal

effects of LAGT with limited cytotoxicity occurred at a UV dose of 200 J/m². Furthermore, this UV irradiation had no effect on either the chondrogenic or osteogenic potential of HuMSCs. Significant effects of LAGT in HuMSCs could be detected as early as 12 h after exposure and persisted over 21 days, in a time and energy-dependent manner. This LAGT effect was maintained for more than 8 h after irradiation and required only a 10-min exposure to rAAV after UV irradiation. Finally, we show that the production of secreted TGF β 1 protein from rAAV-TGF β 1-IRES-eGFP infected to HuMSCs is highly inducible by UV irradiation. These results demonstrate that LAGT combined with rAAV is a promising procedure to facilitate gene induction in HuMSCs for human gene therapy.

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Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) show potential for repair and regeneration of a variety of nonhematopoietic tissues including bone, cartilage, fat, marrow stroma and skeletal muscle.^{1–3} Recently, MSCs have been reported to have the potential to differentiate into cardiac muscle,^{4,5} neural tissue,⁶ the hematopoietic lineage, and the epithelium of internal organs, such as liver, lung, gut and vessels.^{7–10} These observations raise the possibility of improved cell-based therapy for both acquired and genetic disorders in a number of tissues. Indeed, clinical trials of MSCs-based therapies have been performed for several diseases including osteogenesis imperfecta,^{11,12} myocardial infarction¹³ and hematopoietic failure after chemotherapy.¹⁴

In contrast to embryonic stem cells (ES cells), MSCs can be selected from autologous bone marrow and either used directly or after genetic manipulation. These cells can be applied in both local and systemic therapies without any serious ethical issues or immunologic consequences. Although MSCs can be obtained from

other types of tissue including muscle and periosteum,^{15–17} MSCs from the bone marrow have several advantages over stem cells from other tissues. These include: (i) their accessibility, (ii) their ease of manipulation and (iii) their potency and reliability for differentiation. These features have prompted researchers to develop clinical methods for the isolation, expansion and characterization of human bone marrow-derived MSCs (HuMSCs).^{3,9,18,19} However, there are still limitations in using these cells. Most notably is the low percentage of HuMSCs that are capable of differentiation into functional tissues and also the low probability of these few cells entering the differentiation pathway.

One of the most promising solutions to improve HuMSCs therapy is the addition of therapeutic gene transduction. Through the combination of gene therapy and stem cell therapy, there is potential for both selecting and also expanding the appropriate cell types for repair and regeneration. Potential target genes include secreted factors, receptors, intracellular signaling proteins and transcription factors that influence either the HuMSCs or host cells. Previously, several researchers have tried to develop MSC-based gene therapy with either adeno-virus^{20–23} or retrovirus.²⁴ However, problems regarding the host immune response and insertional mutagenesis have cast serious doubt on the clinical utility of these vectors. We believe that recombinant adeno-associated

viruses (rAAV) have several empirical advantages for musculoskeletal gene therapy.^{25,26} While extensive research has been done on rAAV gene therapy in hematopoietic and other stem cells,^{27–30} the use of rAAV to transfer genes into HuMSCs has yet to be reported.

Currently, the most frequently cited concerns regarding rAAV-mediated gene therapy are sustained gene expression and transduction efficiency. In the case of a transient process like tissue repair, sustained gene expression is actually undesirable. Thus, if a method to markedly increase rAAV transduction could be developed, it would be a major advance toward clinically useful gene therapies. Based on the knowledge that DNA-damaging agents stimulate transduction,^{31,32} and that rAAV transduction is rate-limited by second-strand synthesis,^{33,34} researchers have been working on ways to capitalize on these principles by inducing the host cell DNA polymerase(s) believed to be critically involved in this second strand synthesis. Koeberl *et al*³⁵ reported that *in vivo* exposure to gamma irradiation prior to administration of rAAV into the tail vein increased the transduction rate in mouse liver hepatocytes by up to 900-fold. Unfortunately, this protocol required high doses of gamma radiation, which significantly limits its clinical utility.

Recently, we have developed a method for light-activated gene transduction (LAGT), which utilizes ultraviolet light (UV) irradiation to induce the gene transduction of rAAV in fibroblast-like synoviocytes (FLS),²⁶ and articular chondrocytes.³⁶ In contrast to gamma irradiation or other cell-damaging agents, we found that as little as 30 J/m² of UV irradiation causes a significant increase in gene transduction.

In order to extend our knowledge of LAGT and its effect on HuMSCs, we designed this study to determine the safety, efficiency and predictability of this procedure. We found that, at doses below the cytotoxic threshold, UV treatment significantly induced gene transduction of rAAV-eGFP. This effect was noted as early as 12 h after exposure, and lasted over 21 days. The full potency of LAGT was maintained more than 8 h after irradiation, and only a 10-min exposure to rAAV after UV irradiation was required to achieve the full LAGT effect. Moreover, the production of secreted TGFβ1 following infection of HuMSCs with rAAV-TGFβ1-IRES-eGFP was significantly induced by LAGT. These results indicate that LAGT of rAAV vectors is a promising and clinically feasible procedure to facilitate gene therapy with HuMSCs.

Results

Sensitivity of HuMSCs to UV irradiation

In order to determine the cytotoxic effect of UV irradiation on HuMSCs, cultures were exposed to various doses of UV light (Figure 1a and b). Afterwards, the number of dead cells was determined at subsequent time points by modified trypan blue exclusion. On day 2, significant levels of cytotoxicity were observed at energies of 100 J/m² and higher. However, 100 J/m² of UV light did not cause further damage at day 3, while 1000 J/m² of UV light caused further cell death over time. These results are consistent with data obtained in experiments with primary human articular chondrocytes.³⁶ They also indicated that the level of UV exposure

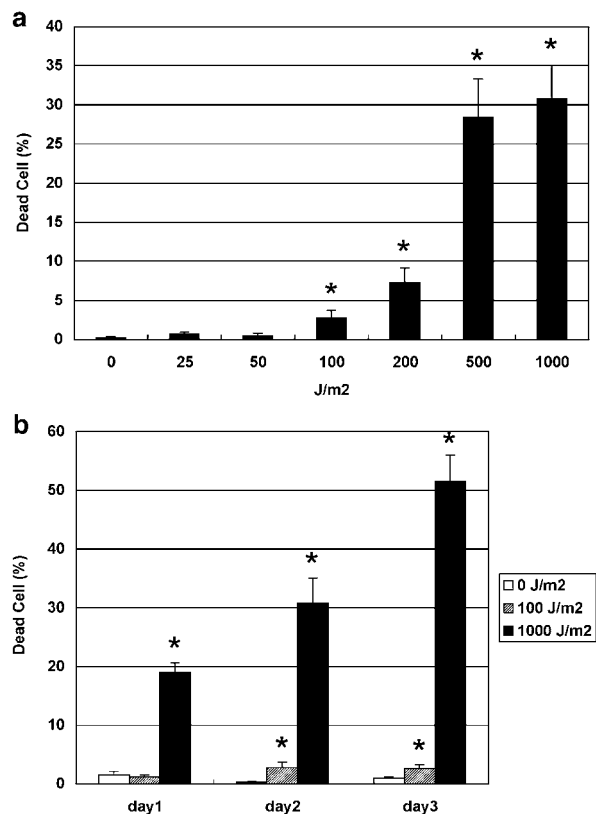


Figure 1 The cytotoxic threshold of HuMSCs to UV irradiation. HuMSCs were grown in monolayer cultures as described in Materials and methods, exposed to the indicated energies of UV, and stained with 0.2% trypan blue 48 h later (a) or at indicated time periods (b). Values are the mean \pm s.e.m. percentage of trypan blue positive cells of four independent wells. The results shown are representative of three independent experiments (* $P < 0.05$ versus unexposed control).

predicted to induce LAGT is well below the cytotoxic threshold.

Another potential adverse event that could be caused by LAGT is inhibitory effects on the differentiation potential of the HuMSCs. To address this, we examined HuMSCs chondrogenesis and osteogenesis *in vitro*. HuMSCs were differentiated under chondrogenic conditions after exposure to UV and infection with rAAV-eGFP and analyzed for upregulation of *type II collagen* mRNA levels for up to 3 weeks (Figure 2a). We also found that UV irradiation does not prevent osteogenic differentiation of HuMSCs, as evidenced by bone nodule formation (Figure 2b). Furthermore, we found that LAGT can induce *eGFP* expression in an energy-dependent manner without affecting chondrogenic (Figure 3a) or osteoblastic (Figure 3b) differentiation. Thus, we find that 200 J/m² of UV represents the safety threshold for LAGT with HuMSCs.

UV light induces rAAV-eGFP gene transduction in HuMSCs

To evaluate the effects of UV light on rAAV-mediated gene transduction in HuMSCs *in vitro*, the relative levels of *eGFP* gene expression were determined 3 days after UV light and rAAV-eGFP exposure by image analysis (Figure 4a and b). On day 3, LAGT caused a significant induction of *eGFP* expression at energies of 50 J/m² and

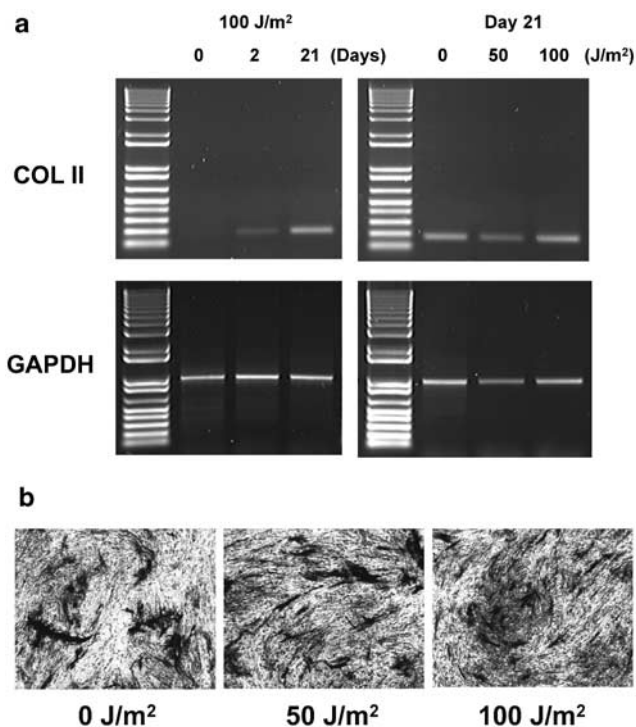


Figure 2 UV effects on HuMSCs chondrogenic and osteogenic differentiation. HuMSCs were grown in monolayer cultures and treated with the indicated energy of UV irradiation. For chondrogenesis, the cells were resuspended and grown as pellet cultures as described in Materials and methods. Then total RNA was extracted at the indicated time points and analyzed for type II collagen (COLII) and GAPDH expression by RT-PCR (a). For osteogenesis, the HuMSCs were cultured for 21 days in ascorbate, β -glycerolphosphate and dexamethasone and ALP-stained to identify the presence of ALP activities, which were equally present in all cultures (b).

higher in cells infected with both 100 and 1000 MOI. The greatest LAGT effect was obtained at 200 J/m². Also of note is the fact that no difference in transduction efficiency was obtained by increasing the MOI over 100, indicating that HuMSCs are readily infected by rAAV vectors, but require some adjuvant to be competent for efficient transduction.

Kinetics and persistence of LAGT in HuMSCs

Since eGFP is a very stable protein and its detection at late time points could be the result of synthesis at a much earlier time, we investigated to determine when the vector actually expresses the gene of interest. To do this, we performed real-time quantitative PCR using eGFP-specific primers to evaluate the kinetics of LAGT and determine how long the effect can be maintained in HuMSCs. As shown in Figure 5a, the LAGT effect can be detected as early as 12 h after treatment and reaches its peak at 24 h after exposure to UV and rAAV.

To see how long the LAGT effect lasts, we cultured the HuMSCs for 21 days after infection and determined eGFP mRNA expression levels by RT-PCR. Figure 5b shows that the UV-exposed cells still have a significant increase in eGFP expression 21 days after treatment.

HuMSCs are rapidly infected by rAAV

Even though it is known that AAV infection occurs very rapidly,³⁷ with respect to the practicality of clinical *ex vivo* gene therapy, the incubation time needed to achieve

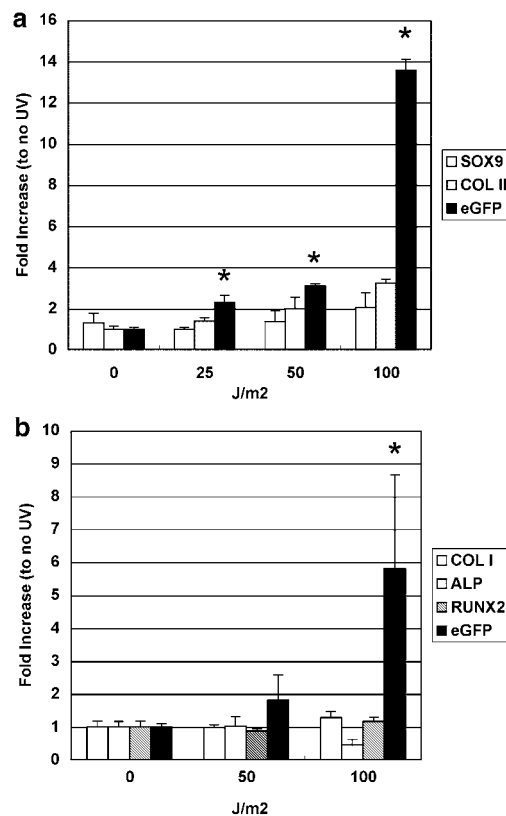


Figure 3 UV effects on eGFP expression in HuMSCs through chondrogenic and osteogenic differentiation. HuMSCs were treated with UV irradiation and rAAV-eGFP in monolayer cultures and then grown under chondrogenic (a) or osteogenic (b) conditions as described in Figure 2. Total RNA was quantitatively analyzed for SOX9, type II collagen and eGFP (a), or type I collagen (COLI), alkaline phosphatase (ALP), Cbfa-1/RUNX2 (RUNX2) and eGFP (b) expression by real-time PCR. The results shown are representative of three independent experiments (* $P < 0.05$ versus non-UV treated control).

maximal rAAV transduction needs to be formally established. In order to answer this question, we treated HuMSCs with UV light and exposed cells to rAAV-eGFP for various time periods before removing the free virus from the culture. The transduction efficiency in these cultures was then determined after 3 days by fluorescent microscopy (Figure 6a and b). Remarkably, only a 10-min exposure to rAAV is required to achieve the maximal LAGT effect, as greater incubation periods failed to produce a significant effect on transduction. Interestingly, without UV, it took 20 min to induce the maximal amount of gene induction (Figure 6a). This result may indicate that UV light can accelerate the gene induction or the uptake of rAAV.

The LAGT effect is maintained for 8 h after UV exposure

Another empirical property of LAGT that is important to consider is how long after UV exposure can cells be infected with rAAV and receive the effects of LAGT. To address this question, rAAV-eGFP was administered to HuMSCs at various time points after UV exposure, cultured for 3 days, and evaluated for transduction by fluorescence microscopy. Figure 7 shows that the LAGT effect was lost within 24 h after UV exposure, but more

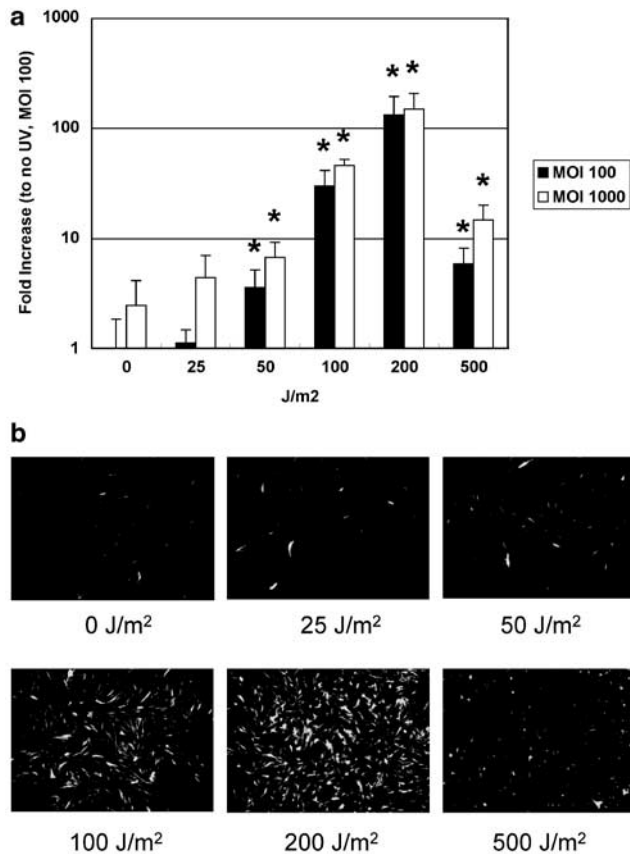


Figure 4 The effects of LAGT in HuMSCs infected with rAAV-eGFP. HuMSCs were grown in monolayer cultures, exposed to the indicated energy of UV, and infected with rAAV-eGFP. After 3 days of culture, the transduction efficiency was determined by image analysis as described in Materials and methods (a). Values are the mean fold increase in eGFP positive area compared to non-UV exposed cultures \pm s.e.m. of four independent wells. The results shown are representative of three independent experiments (* $P < 0.05$ versus unexposed controls in MOI 100 and 1000 wells, respectively). Representative photomicrographs of the cultures infected at 100 MOI are shown (b).

than 50% of the effect was still maintained up to 8 h after UV exposure.

LAGT induces TGF β 1 production by HuMSCs

TGF β 1 is known to be a potent regulator of HuMSC differentiation. As such it is an obvious candidate for *ex vivo* gene therapy. To examine if LAGT can induce secreted target gene production from HuMSCs, we treated the cells with various energies of UV light and rAAV-TGF β 1-IRES-eGFP, cultured them for 3 days, and measured the amount of TGF β 1 protein in the supernatants by ELISA (Figure 8). While the UV light treatment did not have an effect on the endogenous TGF β 1 production by HuMSCs at the noncytotoxic energies (< 500 J/m²), the UV light stimulated a significant energy-dependent increase of more than 120 and 220 pg/ml in TGF β 1 protein production at 100 and 200 J/m², respectively. As the cells not exposed to UV produced merely 45 pg/ml of TGF β 1 protein over rAAV-LacZ infected cells, UV irradiation caused a five-fold increase of TGF β 1 protein in HuMSCs. Infection of rAAV-LacZ did not cause significant difference in the production of TGF β 1 protein (data not shown). This

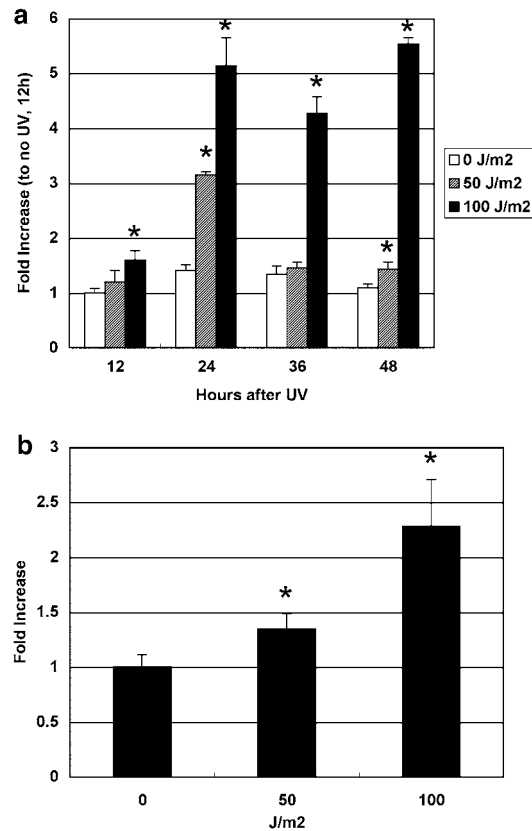


Figure 5 The kinetics of LAGT in HuMSCs infected with rAAV-eGFP. HuMSCs were grown in monolayer cultures and exposed to UV irradiation at energy of 0, 50 or 100 J/m². Then they were infected with rAAV-eGFP at 1000 MOI, and total RNA was collected at the indicated time (a), or 21 days after infection (b), and then subjected to real-time PCR analysis for eGFP expression. The results shown are representative of three independent experiments (* $P < 0.05$ versus non-UV treated control).

result leads us to conclude that UV light can significantly induce secreted gene product expression by rAAV in HuMSCs.

Discussion

Recognition of the broad growth and differentiation potential of MSCs and the facility in obtaining and expanding these cells has suggested several classes of clinical applications.^{1,2,38,39} The most obvious clinical application of MSCs involves repairing or regenerating damaged, localized defects in cartilage and bone. The first strategy to be heavily investigated involved the isolation of HuMSCs from bone marrow of a patient, followed by *ex vivo* expansion and reimplantation into poorly healing bone or cartilage.² A number of attempts have been made to use osteoblasts or chondrocytes for these situations, but the supply of normal cells and their *ex vivo* potential for expansion is limited. Furthermore, an appropriate repair may require the need to use different types of cells, which could only be achieved by transdifferentiation of stem cells. Therefore, MSCs that can differentiate into varying mature cells depending on different circumstance are an attractive alternative. To this end, recent clinical studies suggesting that systemic transplantation of marrow-derived MSCs as a source of

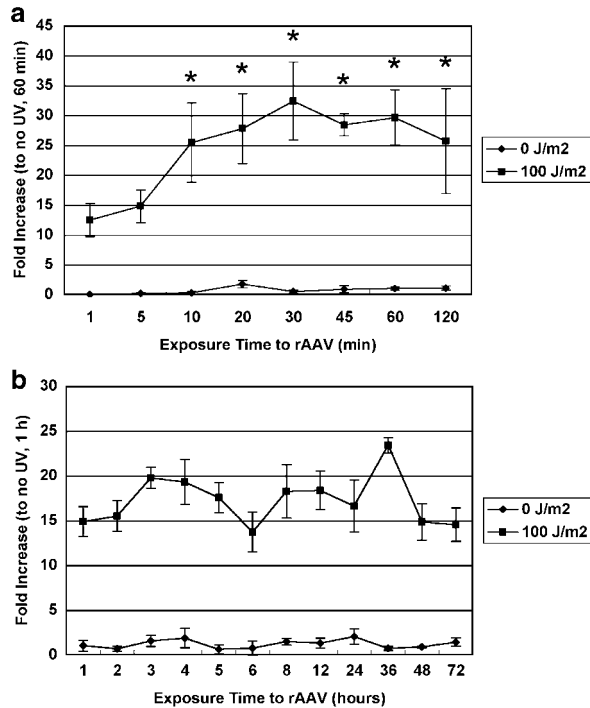


Figure 6 The kinetics of rAAV infection of HuMSCs required for LAGT. HuMSCs were grown in monolayer cultures, treated with and without UV, and infected with rAAV-eGFP at 1000 MOI. After the indicated time, the cultures were washed twice with fresh media and incubated for 3 more days. At this time the transduction efficiency was determined by image analysis as described in Figure 4. Values are the mean \pm s.e.m. of the integrated intensity values of four independent wells, expressed as the fold increase over the 60 min infection-non-UV treated control. The results shown from short-term infections (a) and long-term infections (b) are representative of three independent experiments.

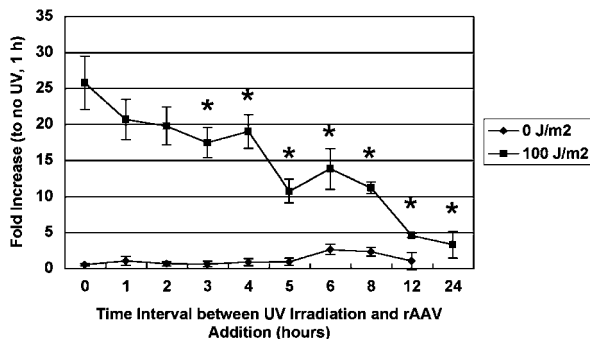


Figure 7 The persistence of the LAGT effect after UV exposure in HuMSCs. HuMSCs were grown in monolayer cultures, exposed to 100 J/m² of UV and cultured without rAAV-eGFP. The rAAV-eGFP was then administered to cell cultures at the indicated time and the cells were cultured for 3 more days. The transduction efficiencies were then determined by image analysis described above (* $P < 0.05$ versus immediate rAAV addition control).

osteoblasts may be useful for the correction of osteogenesis imperfecta^{11,12,40} or in conjunction with a bone marrow transplantation to speed engraftment.¹⁴

Toward the augmentation of HuMSCs for skeletal repair, several groups have described the use of recombinant adenovirus or retrovirus expressing bone morphogenetic proteins (BMPs) with MSCs to stimulate bone formation in a variety of systems.^{20,21,23,24} While

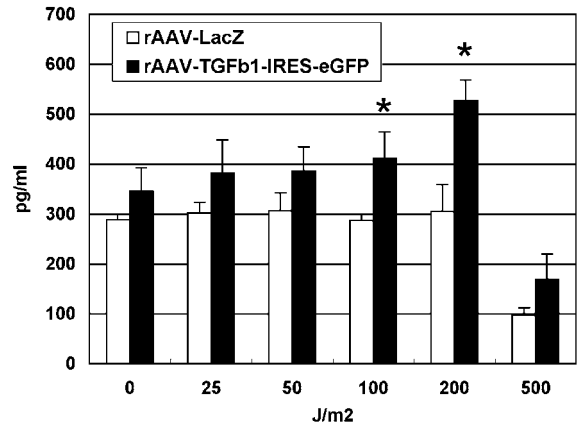


Figure 8 LAGT of TGFβ1 in HuMSCs. HuMSCs were grown in monolayer cultures, treated with the indicated energy of UV and infected with rAAV-TGFβ1-IRES-eGFP (1×10^6 particles/cell) or rAAV-LacZ (1000 MOI). After 3 days of culture, the concentration of TGFβ1 in the supernatant was determined by ELISA as described in Materials and methods. The data from four independent wells are given as the mean \pm s.e.m. for each group. The results shown are representative of three independent experiments (* $P < 0.05$ versus rAAV-LacZ-infected controls).

these studies formally demonstrate the potential of combination MSC-gene therapy, serious concerns regarding the safety of these vectors beg for alternative approaches.

Based on promising preclinical data, several clinical trials to evaluate the safety and efficacy of rAAV vectors have been performed.⁴¹ Although the total number of patients who have received rAAV gene therapy is small, the trials indicate that this is the safest viral vector currently available. The data also suggest that the transient transgene expression that is readily achievable with rAAV might be adequate for reparative gene therapy. However, the low fidelity of transduction most recently observed in stem cells,^{29,30} highlights the need for an adjuvant procedure to overcome this obstacle.

Previously, we reported the effects of LAGT in FLS²⁶ and in articular chondrocytes.³⁶ In both cell types, LAGT induced about a seven-fold increase in transduction efficiency as determined by fluorescence-activated cell scanning (FACS) analysis of transgene expression. Due to the limited supply of HuMSCs, we were unable to perform similar FACS analyses here. However, the 150-fold effect of LAGT observed via fluorescent image analysis (Figure 4) and the five-fold increase over no-UV controls in mRNA expression using real-time quantitative PCR (Figure 5a) clearly demonstrate the potential of this technique in HuMSCs.

If LAGT is to be clinically useful, its kinetics has to be consistent with medical procedures. To investigate this, we determine four critical variables: (i) the time it takes to achieve the peak LAGT effect (Figure 5a), (ii) the duration of the LAGT effect (Figure 5b), (iii) the infection period required to achieve the maximum LAGT effect (Figure 6), and (iv) the maximum time interval between UV exposure and infection that still permits the full LAGT effect (Figure 7). Consistent with prior studies,^{37,42} we find that infection of HuMSCs by rAAV is very rapid and is essentially complete within 10 min. Also consistent with studies that looked at AAV replication post-UV exposure,⁴³ here we show that LAGT in HuMSCs peaks

at 24 h. Our findings that the LAGT effect is maintained over 21 days after UV exposure used, and that the rAAV vector can be added to the HuMSCs up to 3 h after UV exposure and still achieve that maximal LAGT effect are also clinically favorable aspects to this procedure.

One major limitation to the current LAGT procedure, which utilizes short-wavelength UVC (ie 254 nm), is the narrow window between the UV dose that induces rAAV transduction (~ 200 J/m²) and the cytotoxic dose (~ 500 J/m²). Furthermore, even though we did not see significant cytotoxicity at UV doses < 200 J/m², it is still possible that this radiation could induce DNA mutations that would be undesirable. Thus, our future direction is to develop LAGT with a laser that delivers high-wavelength UVA (ie 325 nm). Preliminary results with this system indicate that the LAGT-cytotoxicity window is somewhere between 500–10 000 J/m². Studies to quantify DNA mutations in cells exposed to this radiation are ongoing.

The molecular mechanism of LAGT remains largely unknown and controversial. Russell *et al*⁴⁴ showed that rAAV transduction frequency of S phase cells is about 200 times that of non-S phase cells, while Miao *et al*⁴⁵ reported that cell cycling does not influence rAAV transduction in hepatocytes. In other studies that focused on DNA damaging agents, investigators have shown that the increased transduction is proportional to the appearance of double-stranded replicative forms or circular intermediates of rAAV.^{34,46} Based on these reports and our previous studies, we believe that second-strand synthesis is the most crucial step in rAAV-mediated gene transduction and is dependent on a yet-to-be-determined host DNA repair enzyme. Consequently, we believe that UV irradiation is the most useful way of inducing this process, and that once we have established this procedure, the low transduction efficiency of rAAV vectors will be overcome, making it suitable for human gene therapy. Furthermore, understanding this process can help to identify adjunctive factors to the UV irradiation, which may lessen the energy of UV and the titers of rAAV required for transduction.

In summary, we demonstrated that UV light is able to induce gene expression of rAAV in HuMSCs, efficiently and safely without jeopardizing their differentiation potential. The kinetics of LAGT makes it very practical for use in combination rAAV-HuMSC gene therapy either *ex vivo* or *in vivo*. Future studies to evaluate LAGT in animal models of cartilage and bone repair with rAAV vectors that express chondrogenic and osteogenic genes are warranted.

Materials and methods

Preparation of rAAV-eGFP and rAAV-TGF β 1-IRES-eGFP

The rAAV-eGFP vector, which was obtained directly from the Gene Core Facility of the University of North Carolina, contains the gene for enhanced green fluorescent protein (eGFP) under the transcriptional control of the TRUFR promoter. The viral particles were generated using the adenovirus-free system in which the transfer vector was cotransfected with pXX2 and pXX6 plasmids into 293T cells.⁴⁷ The rAAV-eGFP was titered on human embryonic kidney 293 cells, as we have done pre-

viously,⁴⁸ in order to determine a relative concentration of infectious units, which was $\sim 1.2 \times 10^9$ /ml. As primary HuMSCs are more difficult to transduce, we found that the effective viral titer in the HuMSC was ~ 10 times lower than the viral titer in 293 cell culture.

A rAAV vector coexpressing TGF β 1 with eGFP via an internal ribosome entry sequence (IRES) was prepared by generating a transfer vector (pAAV-TGF β 1-IRES-eGFP) via multiple subcloning steps. First, the porcine TGF β 1 cDNA fragment was generated by digestion of pPK9a-porcine TGF β 1 (a generous gift from Dr AH Greenberg, Manitoba Institute of Cell Biology, Winnipeg, Canada) with *Bgl*III and ligated into the *Bgl*III site in the multiple cloning site of pSub 201 (a gift from Dr RJ Samulski, University of North Carolina, Chapel Hill, NC, USA). Then, the OPG cDNA in pAAV-OPG-IRES-eGFP48 was replaced with the TGF β 1 cDNA in pSub201-TGF β 1 by subcloning into the *Not*I and *Eco*RI sites to generate rAAV-TGF β 1-IRES-eGFP. Extensive restriction digests and double-stranded cDNA sequencing of the TGF β 1 cDNA were performed to confirm the authenticity of the vector. A 0.5-mg quantity of purified pAAV-TGF β 1-IRES-eGFP was sent to the Gene Core Facility, University of North Carolina at Chapel Hill, NC, which prepared the purified rAAV-TGF β 1-IRES-eGFP with use of a helper-virus-free method as described above. The resulting rAAV-TGF β 1-IRES-eGFP was titered by dot blot in order to determine the concentration of virus particles, which was approximately 4×10^{12} /ml.

Isolation and culture of HuMSCs

To isolate HuMSCs, 2- to 10-ml of bone marrow aspirates were obtained from the iliac crests of patients during spinal surgery after informed consent under a protocol approved by an Institutional Review Board at the University of Rochester Medical Center. The HuMSCs were then cultured as previously described.¹⁸ Briefly, the heparinized aspirated bone marrow (10 ml) was mixed with equal volume of phosphate-buffered saline (PBS) into 50 ml conical centrifuge tube. A volume of 6 ml Lymphocyte Separation Medium (Mediatech, Inc., Herndon, VA, USA) was slowly layered underneath the aspirate/PBS mixture and centrifuged at $900 \times g$ for 30 min at 25°C. After removing the upper layer, the mononuclear cell layer was transferred to a new centrifuge tube and washed with PBS by centrifuging at $900 \times g$ for 5 min twice. The pelleted mononuclear cells were resuspended in complete culture medium (DMEM, Invitrogen; 10% FBS, Invitrogen; 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate, Invitrogen) and plated in a 75 cm² culture flask. After 24 h, nonadherent cells were discarded, and adherent cells were incubated for 5–7 days, harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, and replated in a couple of 150 cm² flasks. After 10–14 days, the cells (passage 1) were harvested with trypsin/EDTA, suspended at 1×10^6 cells/ml in 10% DMSO and 20% FBS, and frozen in 1-ml aliquots in liquid nitrogen. To expand a culture, a frozen vial of HuMSCs was thawed, plated in a 75 cm² culture flask, and incubated for 3–4 days. The cells were harvested and diluted for further expansion (passage 2). In this study, we used passage 5–15 cells derived from five females.

UV irradiation of HuMSCs

HuMSCs were grown in 96- or 24-multiwell plastic plates at an initial density of 2×10^4 or 1×10^5 cells/well, respectively. At 8 h after plating, HuMSC monolayer cultures were exposed to UV irradiation (Stratalinker: Stratagene, La Jolla, CA, USA) as described before.²⁶ Briefly, cells were exposed to UV irradiation at different energy levels from 0 to 1000 J/m². The cultures were infected with 1000 MOI of rAAV-eGFP in 100 μ l of media per well for 96-well plates or 500 μ l of media per well for 24-well plates at indicated time points.

In vitro chondrogenesis

For chondrocyte differentiation, a pellet culture system was used as described before.³ Approximately, 2×10^5 cells were plated in a 6-cm² Petridish in DMEM supplemented with 10% of FBS. After 8 h, cells were exposed to UV irradiation as described above and infected at 1000 MOI of rAAV-eGFP. After 3 days, the cells were harvested, placed in a 15-ml polypropylene tube (Corning Corporation, Corning, NY, USA), and centrifuged to pellet. The pellet was cultured at 37°C in a humidified 5% CO₂/95% air in 500 μ l of serum-free media that contained high-glucose DMEM supplemented with 50 μ g/ml ascorbate-2-phosphate, 100 μ g/ml pyruvate and 50 mg/ml ITS + Premix (Becton Dickinson; 6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA, and 5.35 mg/ml linoleic acid).

In vitro osteogenesis

For osteogenic differentiation, HuMSCs were plated in a 24-multiwell plate at a density of 1×10^5 cells/well. After 8 h, cells were exposed to UV irradiation as described above and infected with 1000 MOI of rAAV-eGFP. The cells were cultured at 37°C in a humidified 5% CO₂/95% air in 500 μ l of media containing high-glucose DMEM supplemented with 20% FBS, 50 μ g/ml ascorbate-2-phosphate, 5 mM β -glycerophosphate, and 10^{-7} M dexamethasone as described before.³ For alkaline phosphatase (ALP) immunostaining, after cultured for 3 weeks, cells were washed with PBS (-) twice, fixed with 10% neutral-buffered formalin, and stained with 1-Step NBT/BCIP (Pierce, Rockford, IL, USA).

Real-time quantitative PCR assays

To examine gene expression levels, cells were cultured and total RNA was extracted using RNeasy kit (QIAGEN Inc., Valencia, CA, USA). Single-stranded cDNA was made using a reverse transcription kit (Invitrogen). PCR was performed on Rotor-Gene 2000 real-time amplification operator (Corbett Research, Mortlake, Australia) as described before.⁴⁹ The primers for eGFP, SOX9, type II collagen, Cbfa1/RUNX2, type I collagen, ALP and actin were from Invitrogen (San Diego, CA, USA). The PCR reactions contained a final concentration of 1SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 10 μ M specific primers and 2.5 ng of cDNA. The relative levels of mRNA of a specific gene were calculated using the standard curve generated from cDNA dilutions. The mean cycle threshold (Ct) values from quadruplicate measurements were used to calculate the gene expression, with normalization to actin as an internal control. Calculations of the relative level of gene expression were

conducted according to the instructions from User's Bulletin (P/N 4303859) from Applied Biosystems.

Image analysis of eGFP

To determine the expression levels of eGFP produced by rAAV-eGFP, eGFP of infected HuMSCs were visualized under a fluorescent microscope. The images were analyzed with a Macintosh G3 computer using Scion Image (Scion Corp., Frederick, MD, USA). Background density points were removed by thresholding the image, and integrated intensity values (area \times intensity) of eGFP were measured. Uninfected cells were analyzed simultaneously and it was confirmed that their values were naught.

Transduction and expression of TGF β 1

HuMSCs were plated in a 24-multiwell plate at a density of 1×10^5 cells/well and cultured for 8 h. Then cells were exposed to UV irradiation at different energy levels (0 to 500 J/m²) as described above and infected with 1×10^6 particles of rAAV-TGF β 1-IRES-eGFP/cell. After 3 days, the culture media were analyzed for TGF β 1 protein by enzyme-linked immunosorbent assay (ELISA) (R&S systems, Minneapolis, MI, USA), according to the manufacturer's instructions. Culture supernatants from uninfected and rAAV-LacZ infected HuMSCs were used as negative controls.

Statistical analysis

All data acquisition and analyses were performed blindly. Data were calculated as the mean \pm s.e.m., and the groups were compared using two-tailed analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

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