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Ex Vivo Transfer of the Hoxc-8-interacting Domain of Smad1 by a Tropism-Modified Adenoviral Vector Results in Efficient Bone Formation in a Rabbit Model of Spinal Fusion

Joanne T. Douglas, PhD^{1,2,3}, Angel A. Rivera, PhD¹, Gray R. Lyons, BS⁴, Patricia F. Lott, MS⁵, Dezhi Wang, MD⁵, Majd Zayzafoon, MD, PhD^{3,4}, Gene P. Siegal, MD, PhD^{2,3,4}, Xu Cao, PhD^{3,4}, and Steven M. Theiss, MD^{3,5}

¹Division of Human Gene Therapy, Departments of Medicine, Obstetrics & Gynecology, Pathology and Surgery, University of Alabama at Birmingham, Birmingham, Alabama, USA

²The Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama, USA

³The Center for Metabolic Bone Disease, University of Alabama at Birmingham, Birmingham, Alabama, USA

⁴Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama, USA

⁵Division of Orthopedic Surgery, Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama, USA

Abstract

Study Design—Ex vivo gene transfer for spinal fusion.

Objective—This study aimed to evaluate ex vivo transfer of the nuclear-localized Hoxc-8-interacting domain of Smad1 (termed Smad1C) to rabbit bone marrow stromal cells (BMSCs) by a tropism-modified human adenovirus serotype 5 (Ad5) vector as a novel therapeutic approach for spinal fusion.

Summary of Background Data—Novel approaches are needed to improve the success of bone union after spinal fusion. One such approach is the ex vivo transfer of a gene encoding an osteoinductive factor to BMSCs which are subsequently reimplanted into the host. We have previously shown that heterologous expression of the Hoxc-interacting domain of Smad1 in the nuclei of osteoblast precursor cells is able to stimulate the expression of genes related to osteoblast differentiation and induce osteogenesis in vivo. Gene delivery vehicles based on human Ad5 are well-suited for gene transfer for spinal fusion because they can mediate high-level, short-term gene expression. However, Ad5-based vectors with native tropism poorly transduce BMSCs, necessitating the use of vectors with modified tropism to achieve efficient gene transfer.

Methods—The gene encoding Smad1C was transferred to rabbit BMSCs by an Ad5 vector with native tropism or a vector retargeted to α_v integrins, which are abundantly expressed on rabbit BMSCs. Transduced BMSCs were maintained in osteoblastic differentiation medium for thirty days. Alkaline phosphatase activity was determined and cells stained for calcium deposition. As positive controls for osteogenesis, we employed Ad5 vectors expressing bone morphogenetic protein 2. As negative controls, BMSCs were mock-transduced or transduced with an Ad5 vector expressing β -

galactosidase. In an immunocompetent rabbit model of spinal fusion, transduced BMSCs were coated onto absorbable gelatin sponge and implanted between decorticated transverse processes L6 and L7 of 8-week-old female New Zealand white rabbits. Animals were sacrificed 4 weeks post-implantation of the sponges, the fusion masses harvested and the area of new bone quantified using image analysis software.

Results—The Smad1C-expressing tropism-modified Ad5 vector mediated a significantly higher level of alkaline phosphatase activity and calcium deposition in transduced rabbit BMSCs than all other vectors. The rabbit BMSCs transduced ex vivo with the Smad1C-expressing tropism-modified Ad5 vector mediated a greater amount of new bone formation than BMSCs transduced with any other vector

Conclusion—Delivery of the Smad1C gene construct to BMSCs by an αv integrin-targeted Ad5 vector shows promise for spinal fusion and other applications requiring the formation of new bone in vivo.

Keywords

adenoviral vector; bone marrow stromal cell; gene transfer; rabbit; spinal fusion

INTRODUCTION

Spinal fusion is a relatively common orthopedic surgical procedure, with more than 250,000 performed annually in the United States for a variety of spine pathologies.¹ This constitutes half of all bone graft procedures.¹ However, failure to achieve a solid bony union can occur in up to 35% of patients, often necessitating further surgery.² As a result, various strategies have been employed to improve the fusion rate. These strategies include the use of external or internal fixation, postoperative electrical stimulation or requiring patients to stop concurrent use of substances such as nicotine that are known to inhibit bone formation. In addition, alternative graft materials have also been investigated, including allografts, ceramics and coralline hydroxyapatite. However, the nonunion rate remains unacceptably high and is a significant clinical problem.^{3–6} Hence there is a need for novel approaches to improve the success of bone union after spinal fusion.

Several osteoinductive growth factors that can stimulate bone formation have been identified, including members of the family of bone morphogenetic proteins (BMPs).⁷ Recombinant BMPs have been shown to reliably induce bone formation in numerous animal models.⁸ In fact, recombinant human BMP2 and BMP7 are currently approved for clinical use in select spine procedures.^{9–11} In addition, other BMPs are being evaluated in human clinical trials.^{11, 12} However, the use of recombinant osteoinductive proteins to augment bone formation suffers from a number of limitations. In this regard, studies in humans have revealed a need for unexpectedly higher doses of recombinant BMPs than those needed in animals, resulting in an increased cost that could prove prohibitive.^{9, 13} Moreover, BMPs are soluble and containment of the protein in an appropriate carrier for sufficient time is difficult. Therefore, administration of a single dose of recombinant BMP may not induce a sufficient osteogenic signal in human patients.^{14, 15}

In contrast to the delivery of recombinant proteins, the local delivery of a gene encoding an osteoinductive factor affords the potential to express the factor at a specific anatomic site and at a physiologic dose for a sustained period of time to achieve enhanced bone formation.^{14, 15} To date, most gene transfer approaches to bone formation have involved delivery of BMP genes. Both in vivo¹⁶ and ex vivo^{17–21} strategies to transfer BMP genes have been shown to induce bone formation in animal models. The ex vivo approach involves the harvesting of cells from the recipient, their transduction with a gene encoding an osteoinductive factor and their

subsequent reimplantation into the appropriate anatomic site. Although it is more labor-intensive than direct in vivo transfer, this ex vivo approach permits gene delivery selectively to target cells. While a number of cell types have been employed in ex vivo gene transfer approaches for bone formation, bone marrow-derived stromal cells (BMSCs) offer the advantages of easy harvest and purification. Moreover, the transduced BMSCs will secrete diffusible osteoinductive proteins as part of their maturation process, which can locally affect other cells that did not receive the gene, thereby increasing the efficiency of the bone repair response.

The intracellular signaling initiated by the binding of BMPs to BMP type I receptors on the surface of target cells is mediated by direct phosphorylation of Smad1.^{22, 23} Phosphorylated Smad1 associates with Smad4 and the complex translocates to the nucleus where it recruits DNA-binding proteins to activate genes that induce osteoblast differentiation and bone formation. We have previously shown that heterologous expression of a domain of Smad1 termed Smad1C (amino acid residues 145–278) in the nuclei of osteoblast precursor cells is able to stimulate the expression of genes related to osteoblast differentiation, by interacting with the homeodomain transcription factor Hoxc-8 and dislodging it from its DNA binding element.^{24, 25} Moreover, Smad1C mimics BMPs in the induction of osteogenesis in vivo.²⁶ We hypothesize that expression of a nuclear-localized form of Smad1C in BMSCs would afford advantages over the expression of a single BMP protein, by more closely mimicking the natural sequence of events involved in osteoinduction and thereby controlling bone formation more efficiently.

Gene transfer for spinal fusion mandates a vector capable of mediating high level gene expression in the target BMSCs for a period of days or weeks to initiate bone formation. Sustained expression of the osteoinductive factors is not required and might in fact be deleterious. An ideal vector for this purpose is based on human adenovirus serotype ⁵ (Ad5). The safety of Ad5-based vectors derives in part from the natural biology of the virus, which is non-oncogenic, and is further enhanced by the deletion of the E1 region of the viral genome, which renders the vector replication-defective and capable of propagation only in specially designed complementing cell lines.²⁷

However, previous studies have shown that Ad5 vectors only poorly transduce BMSCs from a number of species, including human, mouse and rat,^{19, 20} due to a low level of expression of the primary cellular receptor, the coxsackievirus and adenovirus receptor (CAR^{28, 29}), that binds the carboxy-terminal knob domain of the Ad5 fiber protein.³⁰ The inefficient transduction of CAR-deficient BMSCs by Ad5 vectors restricts the therapeutic efficacy of the vectors. For example, inefficient transduction of rabbit BMSCs by an Ad5 vector carrying the human BMP2 gene results in limited efficacy of bone formation in a rabbit model of spinal fusion.¹⁷ To address this problem, we and others have previously demonstrated that the efficacy of Ad5 vectors can be enhanced by modifying the fiber proteins to allow efficient transduction of CAR-deficient target cells via a CAR-independent pathway.³¹

In this report, we describe the employment of a tropism-modified Ad5 vector to transfer the Smad1C gene to rabbit BMSCs, followed by the evaluation of the osteogenic potential of the transduced BMSCs in vitro and in an immunocompetent rabbit model of spinal fusion.³²

MATERIALS AND METHODS

Adenoviral Vectors

All recombinant adenoviral vectors employed in this study were first generation, E1-, E3deleted Ad5 vectors expressing the transgene under the control of the cytomegalovirus (CMV) immediate-early promoter. A gene construct encoding amino acid (aa) residues 125–

278 of Smad1, designated Smad1C, fused with cDNA encoding a nuclear localization signal at the 3' end and a FLAG epitope tag at the 5' end,²⁴ was first subcloned in place of the Ad5 E1 viral region downstream of the CMV promoter of pShuttleCMV33 to create pShuttleCMV-Smad1C. The construct was then subjected to homologous recombination in the *E. coli* strain BJ5183 with plasmid pAdEasy1.33 which contains the E3-deleted Ad5 backbone with wild-type fibers, or with plasmid pVK503C.E3,³⁴ which contains the E3-deleted Ad5 backbone with RGD-modified fibers. The recombinant plasmids were used to transfect mammalian 293 cells, and homogeneous vectors, designated Ad5-Smad1C and Ad5-Smad1C-RGD, respectively, were amplified in these cells.

The control vectors Ad5-BMP2 and Ad5-BMP2-RGD, which carry the full-length rat BMP2 gene, were constructed in a similar fashion. Full-length BMP-2 is a 396 aa glycosylated polypeptide with a ¹⁹ aa signal sequence, a 263 aa pro-region and a 114 aa mature region. The mature segment has a predicted molecular weight of ¹⁴ kDa but is actually ¹⁸ kDa and is assumed to be glycosylated. The functional form of the molecule consists of two disulfide-linked mature chains. There is 100% amino acid identity in the mature region of the human, mouse and rat proteins.

Control vectors Ad5-βgal and Ad5-βgal-RGD, which carry the β-galactosidase reporter gene subcloned from the commercial expression vector pSV-β-Galactosidase (Promega, Madison, WI), were also generated.

All recombinant vectors were propagated on the permissive 293 cell line and purified by two rounds of cesium chloride density centrifugation. To determine the viral particle concentration, the virus was diluted in 10 mM Tris (pH 8.0), 1 mM EDTA, 0.1% (w/v) SDS, incubated at 56 °C for 10 min, and the absorbance at 260 nm was measured. Under these conditions, an absorbance of 1 corresponds to 1.1×10^{12} particles/ml.³⁵

Cell Culture

BMSCs were purified from the bone marrow of New Zealand white rabbits. Rabbits were anesthetized with a combination of intramuscular ketamine (100 mg/kg) and xylazine (10 mg/kg), and BMSCs harvested from the femoral intramedullary canal by percutaneously inserting an 18-gauge needle between the femoral condyles and aspirating 3 ml of marrow in a heparinized syringe. Sterile PBS was added to a total volume of 20 ml, and the cells dispersed into an even suspension in a 50-ml conical tube. Twenty milliliters of lymphocyte separation medium (density 1.077 g/ml; Mediatech, Herndon, VA) were underlaid and the gradient centrifuged at 1800 rpm for 15 min at 4 °C. The white band of cells was then transferred into a fresh 50-ml conical tube, washed with 40 ml PBS and centrifuged at 1800 rpm for 10 min at 4 °C. The wash buffer was aspirated and 5 ml of ACK erythrocyte lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA) were added to lyse any red blood cells. The cells were incubated for 5 min at room temperature before adding 20 ml α-minimal essential medium (αMEM) + 10% fetal calf serum (FCS) and then centrifuging at 1800 rpm for 10 min at 4 °C. The cell pellet was resuspended in α-MEM supplemented with 10 % FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), and the dispersed cells were transferred to a 75-cm² flask and propagated for up to twelve passages.

HeLa cells, U-118 MG glioma cells³⁴ and MDA-MB-435 breast cancer cells³⁶ were obtained from the American Type Culture Collection (Manassas, VA). Human ovarian surface epithelial (HOSE) cells³⁷ were obtained from Dr Theresa Strong (University of Alabama at Birmingham (UAB), Birmingham, AL), with permission of the originator of this cell line, Dr Andrew Berchuck (Duke University Medical Center, Durham, NC). 293 cells³⁸ were purchased from Microbix (Toronto, Ontario, Canada). All cell lines were maintained in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12) supplemented

with 10 % FCS, penicillin (100 units/ml) and streptomycin (100 µg/ml). FCS was purchased from Sigma (St Louis, MO) and media and supplements were from Mediatech. All cells were propagated at 37°C in a humidified 5% CO₂ atmosphere.

Flow Cytometry to Determine CAR Levels

Expression of CAR on the cell surface was detected as previously described,³⁹ using recombinant Ad5 fiber knob (with a 6xHis tag at the N-terminal)⁴⁰ as the primary probe. Flow cytometry was performed in the UAB Arthritis and Musculoskeletal Center Flow Cytometry Core Facility on a FACSCalibur machine using Cell Quest FACS analysis software (Becton Dickinson, Franklin Lakes, NJ). Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction to Determine av Integrin Levels

Total RNA was extracted from semiconfluent cell cultures using an RNeasy mini RNA extraction kit (Qiagen) according to the manufacturer's protocol. Five hundred nanograms of total RNA were then employed as the template for one-step semiquantitative RT-PCR using a Qiagen OneStep RT-PCR kit according to the manufacturer's protocol. Briefly, reverse transcription using oligo-dT primers with Omniscript and Sensiscript reverse transcriptases was performed for 30 min at 50 °C. The reaction was then heated to 95 °C for 15 min to inactivate the reverse transcriptases and activate HotStarTaq DNA polymerase. Thirty cycles of PCR were performed at 94 °C for 60 sec, 54 °C for 30 sec and 72 °C for 60 sec, using primers designed to amplify a 526-bp fragment of both human and rabbit av integrin cDNA (forward primer: 5' ACTTCTTGGTGGTCTGGTAGC-3'; reverse primer: 5'-CTATGGCACTGCCAAACCGTGC-3'). A 450-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as a control, using primers 5'-CAACTACATGGTTTACATGTTCCAA-3' and 5'GCCAGTGGACTCCACGACGT-3'. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Ad-mediated Gene Transfer to BMSCs

Monolayers of 70% confluent rabbit BMSCs were transduced by adding Ad vectors diluted in 100 µl a-MEM + 2% FCS and incubating for 1 h at room temperature. The medium containing unbound vectors was aspirated, 100 µl a-MEM + 10% FCS added and the cells incubated at 37 °C.

Immunohistochemistry to Detect Ad-mediated Transfer of Smad1C

Forty-eight hours post-transduction, rabbit BMSCs were permeabilized with ice-cold 100% methanol. Expression of nuclear-localized Smad1C with a FLAG epitope tag was confirmed by immunohistochemical analysis using an anti-FLAG monoclonal antibody (clone M2; Sigma) followed by an alkaline phosphatase-conjugated rabbit secondary antibody (Jackson ImmunoResearch Laboratories). BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; Sigma) was employed as the chromogenic substrate. Mayer's hematoxylin solution (Sigma) was used to counterstain the cells.

Assays for Osteogenic Differentiation in Vitro

Following transduction of rabbit BMSCs by the Ad vectors, medium containing unbound vectors was aspirated and the cells incubated at 37 °C in osteoblastic differentiation medium, aMEM supplemented with 10 % FCS, 5 mM glycerol 2- phosphate, 10 mM ascorbic acid, 1 × 10⁻⁸ M dexamethasone, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Alkaline phosphatase activity was measured 30 days post-transduction. BMSCs were washed with PBS, 100 µl 0.05% (v/v) Triton X-100 (Sigma) were added and the cells lysed by three

cycles of freezing at -70°C followed by thawing at 37°C . Alkaline phosphatase activity in the cell lysate was determined using p-nitrophenol phosphate as the substrate in alkaline buffer (5% (v/v) amino methyl propanol, 100 mM MgCl_2 , pH 10.3). The reaction mixture was incubated at 37°C for 1 h and absorbance at 405 nm was then measured. The background level of alkaline phosphatase activity in unstimulated rabbit BMSCs maintained for 30 days in growth medium was subtracted from the readings. The protein concentration of the lysates was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) to permit normalization of the data.

Expression of alkaline phosphatase was also visualized by staining transduced rabbit BMSCs grown in osteoblastic differentiation medium. Thirty days post-transduction, cells were washed with cold PBS, fixed in 2% (v/v) paraformaldehyde in PBS for 15 min, and stored at 4°C in 100 mM cacodylic acid buffer (pH 7.4). Cells were then incubated at 37°C with freshly prepared alkaline phosphatase substrate solution (100 mM Tris-maleate buffer (pH 8.4), 2.8% (v/v) N,N-dimethyl formamide, 1 mg/ml Fast Red TR, and 0.5 mg/ml naphthol AS-MX phosphate). The reaction was terminated after 30 min by removal of the substrate solution and washing with 100 mM cacodylic acid buffer.⁴¹ Mineralization was then assessed by von Kossa staining of the same cells (5 min in 3% AgNO_3).⁴² All chemicals were purchased from Sigma unless otherwise indicated.

Formation of New Bone in a Rabbit Model of Spinal Fusion

Following transduction of rabbit BMSCs by the Ad vectors, 105 transduced BMSCs were coated onto $2 \times 2 \times 1\text{-cm}^3$ blocks of absorbable gelatin sponge (Surgifoam™; distributed by Johnson & Johnson Wound Management, Somerville NJ; manufactured by Ferrosan, Soeborg, Denmark) and incubated for 12 h at 37°C in osteoblastic differentiation medium to allow the cells to adsorb to the matrix.

Spinal fusions³² were performed on female New Zealand white rabbits, each weighing 4 to 4.5 kg (Myrtle's Rabbitry, Thompsons Station, TN). All animal studies were approved by the UAB Institutional Animal Care and Use Committee. For the spine fusion procedure, rabbits were intubated and anesthetized with isoflurane. A longitudinal incision was made over the lower lumbar spine and the transverse processes of vertebrae L6 and L7 were exposed bilaterally through a paraspinous muscle splitting approach. The transverse processes were then decorticated with an electric burr (Stryker Instruments, Kalamazoo, MI) and the absorbable gelatin sponge carriers loaded with transduced BMSCs were implanted between and in contact with the decorticated transverse processes bilaterally. Two rabbits were treated per group, giving a total of four fusion masses per group. Animals were sacrificed 4 weeks post-implantation of the gelatin sponges and the fusion masses were harvested.

Histologic Analysis of New Bone Formation

Histologic analysis of new bone formation was performed in the UAB Center for Metabolic Bone Disease Histomorphometry and Molecular Analysis Core Laboratory. Each fusion mass, including the two transverse processes, was trimmed of soft tissue and fixed in 10% neutral buffered formalin for several weeks. The specimens were decalcified for 2 weeks in 10% (w/v) ethylenediamine tetra acetic acid (EDTA) solution. After washing in running water for several hours to remove the EDTA, the fusion masses were processed on a VIP 100 Tissue Processor (Sakura, Torrance, CA), embedded in oversized molds, and cut into 5 micron sections using a Leica 2265 motorized microtome (Leica Microsystems Inc., Bannockburn, IL).

The sections were cut in the sagittal plane, through the middle of the section to include the sponge. After deparaffinization and hydration, sections were stained with hematoxylin and

eosin stains (Richard-Allan Scientific, Kalamazoo, MI). Image analysis was performed using Bioquant Image Analysis Software (R&M Biometrics, Nashville, TN). Sections were analyzed for new bone growth within the region of interest between the two transverse processes. Two sections per fusion mass were analyzed, giving a total of eight sections per group.

Statistical Analysis

Differences between groups were analyzed using the Student-Fisher t-test. $P < 0.05$ was considered statistically significant.

RESULTS

We wished to use recombinant Ad5-based vectors to achieve high-level, short-term expression of the Smad1C construct in rabbit BMSCs. Previous studies have reported that BMSCs from a number of species, including human, mouse and rat, express low levels of CAR, the primary cellular receptor that binds the knob domain of the Ad5 fiber capsid protein, and are therefore refractory to transduction by Ad5 vectors.^{19, 20} Therefore, we performed flow cytometry using recombinant Ad5 fiber knob protein to determine the level of CAR on rabbit BMSCs; CAR-positive human HeLa cells and CAR-negative U-118 MG glioma cells³⁴ were used as controls. As shown in Fig. 1A, rabbit BMSCs express only very low levels of CAR.

Since rabbit BMSCs are CAR-deficient, efficient Ad5-mediated gene transfer to these cells mandates a vector capable of transduction via a CAR-independent pathway. Previous studies have reported that BMSCs from other species, including human and rat, are positive for αv integrins.^{19, 20, 43} Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are heterodimeric cell surface receptors that bind cell adhesion molecules, including fibronectin and vitronectin, via an Arg-Gly-Asp (RGD) sequence in these matrix molecules. Moreover, these integrins also serve as secondary cellular receptors for Ad5, mediating internalization of the virus during the native pathway of infection.⁴⁴

Since there is not an antibody against rabbit αv integrins, we evaluated the expression of αv integrin mRNA in rabbit BMSCs by semi-quantitative RT-PCR using primers designed also to recognize human αv integrin DNA. As controls, we employed human MDA-MB-435 breast cancer cells and human ovarian surface epithelial (HOSE) cells, which are known to express high and low levels of αv integrins, respectively.^{36, 37} The housekeeping gene GAPDH was also subjected to semi-quantitative RT-PCR, to confirm that equivalent amounts of template were employed in each sample. As shown in Fig. 1B, a greater level of αv integrin mRNA was amplified from rabbit BMSC RNA than from the same amount of total RNA from MDA-MB435 or HOSE cells.

This finding suggested that an Ad5 vector retargeted to αv integrins would mediate higher levels of gene transfer to rabbit BMSCs than the unmodified CAR-dependent vector. To this end, we employed an Ad5 vector in which the knob domain of the fiber protein was modified by incorporation of an RGD-4C peptide into the so-called HI loop in order to retarget the vector to cellular integrins $\alpha v\beta 3$ and $\alpha v\beta 5$.³⁴ Rabbit BMSCs were transduced at multiplicities of infections (MOIs) of 10, 100, 500 and 1000 particles per cell with either an Ad5 vector with wild-type fibers, Ad5- β gal, or with the integrin-targeted Ad5 vector carrying the β -galactosidase reporter gene, Ad5- β gal-RGD. Forty-eight hours post-transduction, cells were stained with the insoluble β -galactosidase substrate, X-gal. At a given MOI, the greater number of blue cells indicated that the integrin-targeted vector mediated a higher level of gene transfer to rabbit BMSCs than the vector with wild-type fibers (data not shown).

Having determined that the tropism-modified Ad5 vector achieved enhanced gene transfer to rabbit BMSCs, we constructed an Ad5-based vector, Ad5-Smad1C-RGD, carrying the

Smad1C gene construct with the RGD-4C peptide incorporated into the fiber proteins. As a control, we generated an isogenic vector, Ad5-Smad1C, with wild-type fibers. The Smad1C gene construct encoded a nuclear localization signal at the 3' end and a FLAG epitope tag at the 5' end. In order to validate expression of nuclear-localized Smad1C, rabbit BMSCs were transduced at MOIs of 10, 100, 500 and 1000 particles per cell with Ad5-Smad1C or Ad5Smad1C-RGD. Forty-eight hours post-transduction, immunohistochemistry was performed to detect the FLAG epitope tag. As expected, at a given MOI, the greater number of blue cells indicates that the integrin-targeted vector mediated a higher level of expression of nuclear-localized Smad-1C in rabbit BMSCs than the vector with wild-type fibers (Fig. 2).

We next sought to evaluate the ability of Ad5-Smad1C and Ad5-Smad1C-RGD to induce bone cell differentiation. Rabbit BMSCs were transduced at an MOI of 1000 particles per cell with Ad5-Smad1C or Ad5-Smad1C-RGD and maintained in osteoblastic differentiation medium. As positive controls for osteogenesis, we employed BMP2-expressing Ad5-based vectors with wild-type and RGD-modified fibers, designated Ad5-BMP2 and Ad5-BMP2-RGD, respectively. As a negative control, BMSCs were mock-transduced or transduced with the tropism-modified vector expressing the β -galactosidase reporter gene, Ad5- β gal-RGD. Thirty days post-transduction, the cells were lysed and assayed for activity of alkaline phosphatase, a marker of osteoblastic differentiation. Fig. 3 shows that the vector expressing β -galactosidase did not induce alkaline phosphatase levels above those seen in the untransduced rabbit BMSCs ($P > 0.05$). All vectors expressing BMP2 or Smad1C induced the rabbit BMSCs to undergo osteoblastic differentiation. The BMP2-expressing vector with integrin-targeted fibers mediated a significantly higher level of alkaline phosphatase activity in transduced rabbit BMSCs than the isogenic vector with wild-type fibers ($P < 0.005$). Similarly, the Smad1C-expressing vector with integrin-targeted fibers mediated a significantly higher level of alkaline phosphatase activity than the isogenic vector with wild-type fibers ($P < 0.0002$). Ad5-Smad1C-RGD mediated a significantly higher level of alkaline phosphatase activity than all other vectors ($P < 0.0002$).

Entry of transduced rabbit BMSCs into the osteoblastic lineage was also visualized by staining. Rabbit BMSCs were transduced at an MOI of 100, 500 or 1000 particles per cell with Ad5-Smad1C or Ad5-Smad1C-RGD and maintained in osteoblastic differentiation medium. As positive controls for osteogenesis, we employed BMP2-expressing Ad5-based vectors with wild type and RGD-modified fibers, designated Ad5-BMP2 and Ad5-BMP2-RGD, respectively. As a negative control, BMSCs were mock-transduced or transduced with the tropism-modified vector expressing β -galactosidase reporter gene, Ad5- β gal-RGD. Thirty days post-transduction, the cells were fixed and stained with the insoluble alkaline phosphatase substrate, naphthol AS-MX phosphate. Fig. 4 shows that the vector expressing β -galactosidase did not induce alkaline phosphatase levels above those seen in the untransduced rabbit MSCs. The BMP2-expressing vector with integrin-targeted fibers mediated a higher level of alkaline phosphatase activity in transduced rabbit BMSCs than the isogenic vector with wild-type fibers, as seen by the greater number of red cells visible at MOIs of 500 and 1000. Similarly, the Smad1C-expressing vector with integrin-targeted fibers mediated a higher level of alkaline phosphatase activity than the isogenic vector with wild-type fibers. Ad5-Smad1C-RGD mediated alkaline phosphatase activity at a lower MOI than all other vectors.

The same cells were then subjected to von Kossa staining to visualize deposition of calcium, another marker of osteoblastic differentiation. Fig. 5 shows that the vector expressing β -galactosidase did not induce mineralization. The BMP2-expressing vector with integrin-targeted fibers mediated a higher level of calcium deposition in transduced rabbit BMSCs than the isogenic vector with wild-type fibers, as seen by the greater number of red cells visible at MOIs of 500 and 1000. Similarly, the Smad1C-expressing vector with integrin-

targeted fibers mediated a higher level of calcium deposition than the isogenic vector with wild-type fibers.

Having demonstrated that Ad5-Smad1C-RGD could mediate enhanced osteogenic differentiation of rabbit BMSCs in vitro, we next sought to determine whether this would translate into enhanced formation of new bone in vivo. To this end, we employed the transduced BMSCs in an immunocompetent rabbit model of spinal fusion. Rabbit BMSCs were incubated for 1 h ex vivo at an MOI of 1000 particles per cell with Ad5-Smad1C or Ad5-Smad1C-RGD. As positive controls for osteogenesis, we employed BMP2-expressing Ad5-based vectors with wild-type and RGD-modified fibers, designated Ad5-BMP2 and Ad5-BMP2-RGD, respectively. As a negative control, BMSCs were transduced with the tropism-modified vector expressing β galactosidase reporter gene, Ad5- β gal-RGD. One hundred thousand transduced BMSCs were then coated onto $2 \times 2 \times 1$ -cm³ blocks of absorbable gelatin sponge and incubated for 12 h in osteoblastic differentiation medium to allow the cells to adsorb to the matrix.

Spinal fusions were performed on 8-week-old female New Zealand white rabbits. A longitudinal incision was made over the lower lumbar spine and the transverse processes of vertebrae L6 and L7 were exposed bilaterally. The transverse processes were decorticated and the absorbable gelatin sponge carriers loaded with transduced BMSCs were implanted between and in contact with the transverse processes bilaterally. Animals were sacrificed 4 weeks post-implantation of the gelatin sponges and the fusion masses, including the two transverse processes, were harvested. The specimens were fixed, decalcified and cut into 5 micron sections in the sagittal plane, through the middle of the section and including the sponge. After deparaffinization and hydration, sections were stained with hematoxylin and eosin.

The area of new bone formed within the region of interest between the two transverse processes was quantified using image analysis software. As shown in Fig. 6, the area of new bone formed in rabbits that received BMSCs transduced ex vivo with Ad5-BMP2, Ad5-BMP2RGD, Ad5-Smad1C or Ad5-Smad1C-RGD was significantly greater than the area of new bone formed in rabbit that received BMSCs transduced ex vivo with the negative control vector, Ad5 β gal-RGD ($P < 0.04$ for each group compared with the negative control group). The rabbit BMSCs transduced ex vivo with the BMP2-expressing vector with integrin-targeted fibers mediated a greater amount of new bone formation than the rabbit BMSCs transduced with the isogenic vector with wild-type fibers ($P < 0.04$). Similarly, the rabbit BMSCs transduced ex vivo with the Smad1C-expressing vector with integrin-targeted fibers mediated a greater amount of new bone formation than the rabbit BMSCs transduced with the isogenic vector with wild-type fibers ($P < 0.0007$). The rabbit BMSCs transduced ex vivo with the Smad1C-expressing vector with integrin-targeted fibers mediated a greater amount of new bone formation than the rabbit BMSCs transduced with any other vector ($P < 0.02$ for each group compared with the Ad5Smad1C-RGD group). Representative histologic sections are shown in Fig. 7.

DISCUSSION

Since ex vivo gene transfer for spinal fusion mandates a vector capable of mediating high level gene expression in the target BMSCs for a period of days or weeks, we chose to employ an Ad5-based vector. We demonstrated that rabbit BMSCs express low levels of the primary cellular receptor, CAR^{28, 29}, that binds the carboxy-terminal knob domain of the Ad5 fiber protein³⁰ and are, therefore, only poorly transduced by Ad5 vectors with wild-type fibers. Similar findings have been reported in BMSCs from other species, including human, mouse and rat.^{19, 20} After demonstrating that rabbit BMSCs express abundant α v integrins, we achieved efficient gene transfer by employing a tropism-modified Ad5-based vector in which the fiber proteins are modified by incorporation into the HI loop of an RGD-4C peptide that retargets the vector to these cell surface proteins. To our knowledge, this is the first report of

enhanced gene transfer to rabbit BMSCs by a tropism-modified Ad5 vector. Previous studies have reported efficient gene transfer to rat and human BMSCs by an Ad5 vector with the same fiber modification^{20, 21}.

To date, most gene transfer approaches to bone formation, both in vivo¹⁶ and ex vivo^{17–21} have involved delivery of BMP genes. In this study, we employed the Hoxc-8-interacting domain of Smad1, designated Smad1C, as the osteoinductive factor. This is a novel approach in that Smad 1C is an intranuclear protein. We demonstrated that Ad-mediated delivery of Smad1C resulted in differentiation of transduced BMSCs into the osteoblastic lineage in vitro, as visualized by two markers of osteogenesis, expression of alkaline phosphatase and deposition of calcium. Delivery of Smad1C by the integrin-targeted Ad5 vector mediated a higher level of alkaline phosphatase activity and enhanced deposition of calcium relative to the isogenic vector with wild-type fibers. Moreover, delivery of Smad1C by the integrin-targeted Ad5 vector mediated a higher level of alkaline phosphatase activity and enhanced deposition of calcium relative to delivery of BMP2 by the integrin-targeted Ad5 vector.

After evaluating the osteogenic differentiation of rabbit BMSCs in vitro, we next employed the transduced BMSCs in an immunocompetent rabbit model of spinal fusion. The transduced BMSCs were loaded onto an absorbable gelatin sponge which was implanted between the transverse processes of vertebrae L6 and L7. Rabbits were sacrificed four weeks later. Histological analysis of sections of the fusion masses showed that the rabbit BMSCs transduced ex vivo with the Smad1C-expressing vector with integrin-targeted fibers mediated a greater amount of new bone formation than the rabbit BMSCs transduced with the isogenic vector with wild-type fibers. Moreover, the rabbit BMSCs transduced ex vivo with the Smad1C-expressing vector with integrin-targeted fibers mediated a greater amount of new bone formation than the rabbit BMSCs transduced with the BMP2-expressing vector with integrin-targeted fibers.

Delivery of the Smad1C gene construct to BMSCs by a tropism-modified Ad5 vector therefore shows promise for spinal fusion and other applications requiring the formation of new bone in vivo. Future studies will focus on optimizing the conditions that will enable this ex vivo gene transfer strategy to be implemented in the surgical setting in humans. Among criteria requiring further investigation, the incubation time, number of transduced BMSCs to be employed and the choice of matrix will all need to be better defined.

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HeLa

U-118 MG

Rabbit BMSC

Fig. 1.

1A. Determination of CAR levels by flow cytometry

Cells in suspension were incubated with recombinant His-tagged Ad5 fiber knob or PBS alone. The binding of the knob to CAR was detected by incubation with an anti-His monoclonal antibody, followed by a FITC-conjugated anti-mouse IgG, Fc-specific antibody. The cells were then analyzed by flow cytometry. The thin and thick lines show staining in the absence or presence of the Ad5 fiber knob protein, respectively. HeLa and U-118 MG cells served as CAR-positive and -negative controls, respectively. Data are representative results of analyses performed on three separate occasions.

1B. Determination of α v integrin levels by semiquantitative RT-PCR

Total RNA was extracted from rabbit BMSCs, HOSE cells and MDA-MB-435 breast cancer cells, and subjected to semiquantitative RT-PCR for α v integrin and GAPDH. Data are representative results of analyses performed on three separate occasions.

Fig. 2. Ad-mediated transfer of Smad1C

Monolayers of rabbit BMSCs were transduced with Ad5-Smad1C or Ad5-Smad1C-RGD at the indicated MOIs. Forty-eight hours post-transduction, cells were subjected to immunohistochemical analysis using a primary antibody against the FLAG tag incorporated at the N-terminus of Smad1C. BCIP/NBT was employed as the chromogenic substrate, and cells were counterstained with hematoxylin. Representative results are shown.

Fig. 3. Alkaline phosphatase activity of transduced rabbit BMSCs

Monolayers of rabbit BMSCs were transduced with the indicated vectors at an MOI of 1000 particles per cell and maintained in osteoblastic differentiation medium. Thirty days post-transduction, cells were lysed and assayed for activity of alkaline phosphatase. Data shown are means $\pm$ standard deviations of triplicate samples and are representative results of analyses performed on three separate occasions.

Fig. 4. Alkaline phosphatase activity of transduced rabbit BMSCs

Monolayers of rabbit BMSCs were transduced with the indicated vectors at an MOI of 100, 500 or 1000 particles per cell and maintained in osteoblastic differentiation medium. Thirty days post-transduction, cells were stained for activity of alkaline phosphatase. Representative results are shown.

Fig. 5. Calcium deposition by transduced rabbit BMSCs

Monolayers of rabbit BMSCs were transduced with the indicated vectors at an MOI of 100, 500 or 1000 particles per cell and maintained in osteoblastic differentiation medium. Thirty days post-transduction, cells were subjected to von Kossa staining to visualize calcium deposition. Representative results are shown.

Fig. 6. Quantification of new bone formation in vivo

Rabbit BMSCs were transduced ex vivo with the indicated vectors at an MOI of 1000 particles per cell, loaded onto blocks of absorbable gelatin sponge and incubated for 12 h in osteoblastic differentiation medium to allow the cells to adsorb to the matrix. The gelatin sponge carriers loaded with transduced BMSCs were then implanted between the transverse processes of vertebrae L6 and L7 of New Zealand white rabbits. Animals were sacrificed after 4 weeks and the fusion masses, including the two transverse processes, were harvested. The specimens were fixed, decalcified and cut into 5 micron sections in the sagittal plane, through the middle of the section and including the sponge. After deparaffinization and hydration, sections were stained with hematoxylin and eosin. The area of new bone formed within the region of interest between the two transverse processes was quantified using image analysis software. Data are the means $\pm$ standard deviations of values from two sections from four fusion masses per group.

Fig. 7. Visualization of new bone formation in vivo

Rabbit BMSCs were transduced ex vivo with the indicated vectors at an MOI of 1000 particles per cell, loaded onto blocks of absorbable gelatin sponge and incubated for 12 h in osteoblastic differentiation medium to allow the cells to adsorb to the matrix. The gelatin sponge carriers loaded with transduced BMSCs were then implanted between the transverse processes of vertebrae L6 and L7 of New Zealand white rabbits. Animals were sacrificed after 4 weeks and the fusion masses, including the two transverse processes, were harvested. The specimens were fixed, decalcified and cut into 5 micron sections in the sagittal plane, through the middle of the section and including the sponge. After deparaffinization and hydration, sections were stained with hematoxylin and eosin. Representative sections are shown.