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Direct Percutaneous Gene Delivery to Enhance Healing of Segmental Bone Defects

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Investigation performed at the Center for Molecular Orthopaedics and the Orthopaedic Biomechanics Laboratory, Harvard Medical School, and the Department of Orthopaedic Surgery, Boston University School of Medicine, Boston, Massachusetts

Background: Healing of segmental bone defects can be induced experimentally with genetically modified osteoprogenitor cells, an ex vivo strategy that requires two operative interventions and substantial cost. Direct transfer of osteogenic genes offers an alternative, clinically expeditious, cost-effective approach. We evaluated its potential in a well-established, critical-size, rat femoral defect model.

Methods: A critical-size defect was created in the right femur of forty-eight skeletally mature Sprague-Dawley rats. After twenty-four hours, each defect received a single, intralesional, percutaneous injection of adenovirus carrying bone morphogenetic protein-2 (Ad.BMP-2) or luciferase cDNA (Ad.luc) or it remained untreated. Healing was monitored with weekly radiographs. At eight weeks, the rats were killed and the femora were evaluated with dual-energy x-ray absorptiometry, micro-computed tomography, histological analysis, histomorphometry, and torsional mechanical testing.

Results: Radiographically, 75% of the Ad.BMP-2-treated femora showed osseous union. Bone mineral content was similar between the Ad.BMP-2-treated femora (0.045 ± 0.020 g) and the contralateral, intact femora (0.047 ± 0.003 g). Histologically, 50% of the Ad.BMP-2-treated defects were bridged by lamellar, trabecular bone; the other 50% contained islands of cartilage. The control (Ad.luc-treated) defects were filled with fibrous tissue. Histomorphometry demonstrated a large difference in osteogenesis between the Ad.BMP-2 group (mean bone area, 3.25 ± 0.67 mm$^2$) and the controls (mean bone area, 0.65 ± 0.67 mm$^2$). By eight weeks, the Ad.BMP-2-treated femora had approximately one-fourth of the strength (mean, 0.07 ± 0.04 Nm) and stiffness (mean, 0.5 ± 0.4 Nm/rad) of the contralateral femora (0.3 ± 0.08 Nm and 2.0 ± 0.5 Nm/rad, respectively).

Conclusions: A single, percutaneous, intralesional injection of Ad.BMP-2 induces healing of critical-size femoral bone defects in rats within eight weeks. At this time, the repair tissue is predominantly trabecular bone, has normal bone mineral content, and has gained mechanical strength.

Clinical Relevance: Direct administration of adenovirus carrying BMP-2 could provide a straightforward and cost-effective treatment for large osseous defects with adequate surrounding soft-tissue support. This local in vivo gene-therapy approach avoids the cost and complexity of ex vivo methods that require artificial scaffolds and autologous cell culture.
application, large bolus doses are required to induce bone-healing. Under these conditions, the release of the growth factors is not uniform over time. Instead, there is an initial rapid efflux that saturates the surrounding tissue with supra-physiological concentrations of growth factor and leads to systemic exposure. Subsequent release, although slower, provides much lower, suboptimal concentrations. Another drawback of using recombinant proteins is their high cost.

Advances in gene transfer technology provide the opportunity to overcome such problems. By delivering complementary DNAs (cDNAs) that encode osteogenic proteins, rather than delivering the proteins themselves, it is possible to achieve a sustained, local presence of the growth factor at efficacious concentrations with minimal exposure of nontarget sites. Moreover, unlike recombinant protein, the growth factor synthesized in situ as a result of gene transfer undergoes authentic posttranslational processing and is presented to the cell or synthesiz ed in situ as a result of gene transfer undergoes authentic posttranslational processing and is presented to the cell-to-cell interactions in a natural, cell-based manner. This may explain why endogenously synthesized proteins often show a greater biological effectiveness than do exogenously applied recombinant proteins.

In general terms, cDNAs can be transferred to osseous lesions by in vivo or ex vivo methods. Most investigators have used the ex vivo approach pioneered by Lieberman et al. In these experiments, cDNAs encoding BMP-2, BMP-4, BMP-7, or BMP-7 were transferred to monolayer cultures of osteoprogenitor cells derived from bone marrow, muscle, skin, or fat. The genetically modified cells were then seeded onto scaffolds and further incubated in vitro, and the cell-seeded scaffolds were implanted into experimental osseous defects in laboratory animals. While biologically successful in animal models, the ex vivo approach is complex and would be a costly and inefficient option in the clinical setting.

In vivo approaches offer modalities that are less expensive, simpler, quicker, and far less invasive. These approaches are compatible with the use of recombinant, first-generation adenovirus vectors that are straightforward to produce and very efficient. Most investigators, however, have eschewed the use of such vectors in the present context because of concerns about their immunogenicity. Whereas the intramuscular administration of recombinant human BMP-2 induces ectopic bone formation in rodents, ectopic bone formation does not occur when adenovirus vectors carrying BMP-2 cDNA (Ad.BMP-2) are used for delivery, unless the recipients are immunodeficient or immunosuppressed. Nevertheless, there are preliminary data suggesting that an in vivo, adenovirus-based strategy for delivering osteogenic genes locally to osseous defects may successfully promote the formation of new bone.

In the present study, we subjected this possibility to rigorous evaluation in a well-characterized segmental defect model in rats.

Materials and Methods

Study Design

5-mm, critical-size midfemoral defect was created in the right femur of each of forty-eight male Sprague-Dawley rats (weight, 400 to 425 g) and was stabilized by an external fixator. The rats were assigned to one of three groups: twenty-four rats received human BMP-2 cDNA in an adenoviral vector (Ad.BMP-2), twelve rats received adenovirus carrying the luciferase marker gene (Ad.luc), and twelve rats had no treatment of the defect. Twenty-four hours after the surgery to create the defect, adenoviral vectors (4 x 10⁸ plaque-forming units [pfu]) were injected percutaneously into the defects of the first two animal groups. Bone-healing was monitored with weekly radiographs. All animals were killed eight weeks after the surgery, the femora were harvested, and healing was evaluated with dual-energy x-ray absorptiometry, micro-computed tomography, histological analysis, histomorphometry, and torsional mechanical testing.

Vector Production

Serotype 5, E1, E3-deleted, first-generation adenoviral vectors Ad.BMP-2 and Ad.luc were constructed by Cre-lox recombination as described in the literature. The transgenes were cloned into the E1 domain, with expression driven by the human cytomegalovirus early promoter. Recombinant adenoviruses were propagated in 293/Cre8 cells. High-titer preparations were generated by amplification in 293 cells, purification on cesium-chloride gradients, and dialysis against 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 10 mM MgCl₂, and 4% sucrose buffer. Viral titers were estimated as 10¹² to 10¹³ particles/mL by optical density and 10¹⁰ to 10¹¹ pfu/mL by standard plaque assay. This vector has been described previously.

Operative Procedure

An established critical-size femoral defect rat model was used in this study. All operative procedures were approved by our institutional standing committee on animals. Adult, male Sprague-Dawley rats weighing 400 to 425 g were placed under general anesthesia by the administration of isoflurane with a small-animal vaporizer. The animals then received intramuscular injections of 20 mg/kg of cefazolin (antibiotic) and 0.08 mg/kg of buprenorphine (analgesic) into the left thigh. The right hindlimb of each animal was shaved and was disinfected with povidone iodine. Subsequently, the animals were placed in a sterile field and covered with a surgical drape so that only the prepared limb was exposed. An incision was made in the postero-lateral aspect of the right thigh. The lateral intermuscular septum with respect to the femur was dissected to expose the diaphysis of the femur. The periosteum was removed from the anterolateral aspect of the femur, and a fixator template was placed and was fastened to the femur with two sterile cable ties. Care was taken to affix the template to allow the pins to be placed in the central portion of the femoral shaft, reducing the likelihood of fracture due to pin placement. Once the template was securely placed, the proximal hole was drilled with use of a drill guide and a sterile 0.9-mm drill bit. The pin (a 1.1-mm threaded Kirschner wire) was then secured in the femur. The distal pin was placed in the same fashion, with special care taken to place it, and all subsequent pins, parallel to the proximal pin. The remaining two pins were placed in the same fashion. The
template was then removed, and the skin was pulled over the pins. Small incisions were made to allow the pins to penetrate the skin. The fixator was then secured as close to the skin as possible without risking skin ulcerations.

A 5-mm osteotomy was then performed with use of a sterile, round dental burr attached to a dental handpiece (AEU-10C/SS Power-Tip; Aseptico, Woodinville, Washington). After completion of the osteotomy, the site was copiously irrigated with cefazolin solution. The fascia was closed with use of 3-0 gut, creating a tight muscle chamber around the defect. The skin incision was closed with use of 4-0 silk and then cleaned with Betadine (povidone iodine). The animals were given a 0.10 mg/kg intramuscular injection of buprenorphine for additional analgesia. During the subsequent two days, each animal received a 20 mg/kg intramuscular injection of cefazolin once per day and a 0.10 mg/kg intramuscular injection of buprenorphine twice per day.

The virus was administered with the rat under general anesthesia and in accordance with National Institutes of Health Biosafety Level-2 (NIH BL-2) guidelines twenty-four hours after the surgery. Forty µL of viral suspension (4 × 10⁸ plaque-forming units) appropriately diluted in phosphate-buffered solution, was drawn into an airtight 50-µL Hamilton syringe and administered with a single injection. To ensure accurate injection, a 2-in (5.1-cm), 22-gauge needle was attached to the barrel of the syringe and inserted into a channel engineered in the external fixator so that the tip of the needle entered the exact center of the defect. The rats were housed in accordance with NIH BL-2 guidelines for twenty-four hours after gene transfer. The animals were killed fifty-six days after treatment. Both femora of each animal were harvested and either immediately frozen for biomechanical testing, dual-energy x-ray absorptiometry, and micro-computed tomography or placed in 3-0 gut, creating a tight muscle chamber around the defect zone and pristine bone. By assessing the total bone mineral content rather than the area bone mineral density, we were able to minimize errors associated with positioning.

Radiographic Evaluation
Bone-healing was monitored weekly with radiographs made with a digital dental x-ray unit (Heliodent DS; Sirona, Bensheim, Germany). While under general anesthesia, the rats were placed in a ventral position and the hindlimbs were laterally rotated. The digital radiographs were examined by two blinded reviewers who assigned the findings to one of three categories: bone ends united (yes or no), bone formation within the defect without union (yes or no), or no noticeable bone formation within the defect.

Micro-Computed Tomography
The segmental defect region of six rats in each group was scanned with use of a desktop micro-tomographic imaging system (µCT40; Scanco Medical, Bassersdorf, Switzerland) equipped with a 10-mm focal-spot microfocus x-ray tube. The entire defect region was scanned with use of a 34-µm slice increment, requiring approximately 200 micro-computed tomography slices per specimen. Images were reconstructed and filtered, and a threshold was created as previously described, with the threshold determined after pilot scans on several specimens.

Dual-Energy X-Ray Absorptiometry
The bone mineral content of the defect region of six rats in each group was assessed with peripheral dual-energy x-ray absorptiometry (PIXimus 2, GE Lunar; GE Healthcare, Madison, Wisconsin). Specimens were placed on a lucite block to simulate soft tissue during scanning. The scans were acquired with use of the small-animal high-resolution mode. The region of interest for the measurement of the total bone mineral content was between the inner two pins of the fixator, which included the defect zone and pristine bone. By assessing the total bone mineral content rather than the area bone mineral density, we were able to minimize errors associated with positioning.

Histological Analysis
Tissue samples from five rats in the Ad.luc group and from thirteen rats in the Ad.BMP-2 group were fixed in ice-cold 4% paraformaldehyde for three days and were then decalcified in 14% EDTA for up to two weeks. Pins were then removed from the bones before embedding and sectioning. Fixed and decalcified tissues were dehydrated in graded ethanol up to 100%, transferred to xylene, and embedded in paraffin. Five-micro-meter paraffin sections were placed on slides coated with poly-L-lysine, were dried overnight, and were evaluated immediately or were stored at 4°C. Alternate sections were stained with hematoxylin and eosin or safranin O-fast green.

Histomorphometry
Histomorphometry was carried out, as previously described, on histological slides of specimens from five rats in the Ad.luc group and thirteen rats in the Ad.BMP-2 group. Bone was consecutively sectioned until all four cortices were observable in the plane of the sections. Serial sections were then made, and three serial 5-µm sections were taken at three equidistant increments from the center of the defect from each bone. The sections were stained with either safranin O-fast green or hematoxylin and eosin. Each of these sections was photographed with an Olympus BX51 light microscope (Olympus, Melville, New York) attached to a digital camera. With the callus placed on a horizontal plane in the center of the field, each photograph was made at 2.0× magnification and was downloaded onto Image-Pro Plus software (version 4.1.0.0 for Windows; Media Cybernetics, Silver Spring, Maryland). An area of interest, from which the histomorphometric measures were generated, was created by loading a uniform box (2.48 × 3.48 mm) onto the photograph. The area of interest was defined by placement of the box edge at a fixed increment (1.49 mm) from the proximal pinhole that was the closest to the osteotomy site and centering the area of tissue-healing within the box. The bone was then outlined within the uniform area of interest, with exclusion of any muscle, periosteum, or other soft tissue. With use of a color-match
program, the total area of the cartilage (red) and bone (green) was quantified with use of a filter range of 573.921 µm to 5.73921e + 03 µm. Areas of unstained space and areas stained dark blue to purple that included marrow elements and adipose tissues were quantified in the combined category of “other.” Specimen means were calculated for the individual bones and then were used to create group means, standard deviations, and standard errors for each animal group.

**Mechanical Testing**
Following all noninvasive imaging, seven specimens in each group were tested to failure in torsion. Both ends of each specimen were embedded in polymethylmethacrylate to provide an appropriate and reproducible gripping interface with the testing module. Specimens were tested to failure under regular deformation control and at a constant deformation rate of 5 rad/min. Angular deformation and applied load data were acquired at 10 Hz. The torque and rotation data were used to calculate the torsional stiffness and strength of the healing defect.

**Statistical Methods**
The histomorphometric data were examined for significance with use of the Student t test for unequal variance (p < 0.001). The significance of the dual-energy x-ray absorptiometry data

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**TABLE I Radiographic Findings Eight Weeks After Surgery**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>United Bone</th>
<th>Bone Formation, No Union</th>
<th>No Bone Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad.BMP-2 (n = 24)</td>
<td>18</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ad.luc (n = 12)</td>
<td>0</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Untreated (n = 12)</td>
<td>1</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

Fig. 1
Representative radiographic images of segmental bone defects after direct injection of adenoviral vectors encoding human BMP-2 (Ad.BMP-2) or luciferase cDNA (Ad.luc). Most defects that had been treated with Ad.BMP-2 displayed bone formation within the defect by four weeks (A) and complete union by eight weeks (C). Control defects treated with Ad.luc (and untreated defects) did not display appreciable signs of healing within this time (B and D).
and the radiographic data was determined with use of the Mann-Whitney nonparametric test \((p < 0.05)\).

**Results**

**Safety**

The administration of Ad.BMP-2 or Ad.luc to the rats produced no obvious adverse effects during the eight weeks of the experiment.

**Radiographic Evaluation**

Eighteen (75%) of the twenty-four animals treated with Ad.BMP-2 showed united bone eight weeks after surgery (Fig. 1 and Table I). Another two animals showed bone formation in the defect without union, and four animals showed no relevant bone formation in the defect. Only one defect in the rats with the untreated defect and none in the rats treated with Ad.luc had any evidence of osseous union. An additional five defects (three treated with Ad.luc and two untreated defects) displayed bone formation but no union, whereas eighteen defects (nine in the Ad.luc group and nine in the untreated group) showed no relevant bone formation at eight weeks. The difference between the Ad.BMP-2 group and both control groups was significant \((p < 0.001)\).

The four rats that were not operated on and that received an injection of 40 \(\mu\)L of vector into the quadriceps muscle showed no radiographic evidence of bone formation at the site of the intramuscular vector injection.

**Micro-Computed Tomography**

The micro-computed tomography evaluation of the Ad.BMP-2-treated defects confirmed osseous union (Fig. 2, A). In contrast, the defects treated with Ad.luc (Fig. 2, B) and the untreated defects showed no relevant bone formation and no union.

**Dual-Energy X-Ray Absorptiometry**

The mean bone mineral content in the Ad.BMP-2 group \((0.045 \pm 0.020 \text{ g})\) was close to that in the contralateral, control femora \((0.047 \pm 0.003 \text{ g})\). The mean bone mineral content in the defects that received Ad.luc \((0.007 \pm 0.004 \text{ g})\) was significantly lower \((p \leq 0.001)\) (Fig. 3).
Fig. 4-A
Figs. 4-A and 4-B Representative histological sections from mid-defect regions from the Ad.BMP-2 and Ad.luc-treated groups stained with hematoxylin and eosin. **Fig. 4-A** Low-magnification images (×20).

Fig. 4-B
Higher-magnification microphotographs of tissue in the osteotomy gap shown in the boxed areas in Fig. 4-A (×100).
Histological Analysis

Representative micrographs of the tissues within the Ad.luc and Ad.BMP-2-treated defects are presented in Figures 4 and 5. The hematoxylin and eosin-stained sections (Figs. 4-A and 4-B) demonstrate the general appearance of the tissue within the osteotomy gap, whereas the safranin O-fast green-stained sections (Figs. 5-A and 5-B) demonstrate the presence of cartilage, indicative of endochondral bone formation. As can be seen in both figures, the Ad.BMP-2-transduced tissues showed well-developed thick cortical and trabecular bone in the healing defect gap. As seen in Figure 5-A, there was complete cortical bridging in some sections, with the bridging tissue fully remodeled to final dense cortical bone. In contrast, the bones from the animals that had been injected with Ad.luc showed no appreciable bone formation. In these specimens, the gap showed dense fibrotic, adipose, and, in some areas, muscle tissue filling the gap (Figs. 4-B and 5-B). It is also interesting to note that, in the absence of healing, the marrow space adjacent to the cortical ends was extensively infiltrated with adipogenic tissues, which were largely absent from the Ad-BMP-2-treated samples.

Fig. 5-A
Figs. 5-A and 5-B Representative histological sections from mid-defect regions from the Ad.BMP-2 and Ad.luc-treated groups stained with safranin O-fast green to demonstrate mature cartilage. Fig. 5-A Low-magnification images (×20). The black arrows denote the proximal edge of the hole from the pin used to secure the external fixator to the femur. The ring of reactive bone that encapsulated the pin can be easily seen. The gray arrows denote the formation of a neocortex. The boxes enclose the areas encompassed by the osteotomy gap and the area of interest that was used for histomorphometric analysis. B = bone, and C = cartilage (stained red).
Nine of the Ad.BMP-2-treated specimens were completely bridged by trabecular bone. The remaining nine specimens showed both cartilage and bone within the gap. In two of these nine specimens, small islands of cartilage remained within the osseous repair tissue. The remaining seven specimens contained a band of endochondral cartilage which, in contrast with normal fracture-healing, traversed the gap itself (Figs. 5-A and 5-B).

Histomorphometry
The tissue composition of the repair site as assessed with histomorphometric measurements is presented in Figure 6. The composition of the repair tissue was compared with that of the intact bone in the comparable mid-diaphyseal region of the contralateral femur of the same animal. There was a large difference in the formation of skeletal connective tissues between the group treated with Ad.BMP-2 (mean bone area, 3.25 ± 0.67 mm²) and the Ad.luc group (mean bone area, 0.65 ± 0.67 mm²).

It is interesting to note that the small amount of bone that formed within the gap in the control group was always seen at the edges of the osteotomy site, and never traversing the gap.

Mechanical Testing
Torsional testing was used to investigate the mechanical properties of the Ad.BMP-2-treated femora as compared with those of the intact, contralateral femora. It was not possible to evaluate the mechanical properties of the control groups (Ad.luc-treated and untreated) because they lacked sufficient structural integrity. The Ad.BMP-2-treated limbs had approximately one fourth of the strength (mean, 0.07 ± 0.04 Nm) and stiffness (mean, 0.5 ± 0.4 Nm/rad) of the contralateral, intact femora (0.3 ± 0.08 Nm and 2.0 ± 0.5 Nm/rad, respectively).

Discussion
This study demonstrated that direct, local delivery of an adenovirus encoding human BMP-2 cDNA can induce healing of critical-size femoral defects in immunocompetent rats. Radiographic evidence of new bone formation in responsive animals was already present at four weeks after administration of Ad.BMP-2, and it led to osseous union by eight weeks. These findings agree well with those of Lieberman et al., who used ex vivo delivery of BMP-2 cDNA with an adenovirus vector and observed radiographic union in a similar rat model by eight weeks. In our experiments, the control animals showed very little bone formation, and only one control demonstrated osseous union. The union may have resulted from incomplete removal of the periosteum during surgery. These data confirm and extend the findings of a preliminary study of rabbits.

The radiographic observations were supported by additional analyses. Dual-energy x-ray absorptiometry, for example, confirmed that bone mineral content returned to normal values within eight weeks after Ad.BMP-2 treatment. Although histological evaluation clearly showed that expression of human BMP-2 augmented repair of the defect by bone induction, 50% of the specimens showed residual cartilage traversing the defect area. This suggests that healing in response to Ad.BMP-2 occurs by means of endochondral ossification, with the cartilage appearing to form intralesionally. Alternatively, the presence of cartilage may represent the formation of a nonunion. To distinguish between these possibilities, it will be necessary to conduct time-course studies to determine the natural history of healing induced by gene transfer in this model.
The presence of residual cartilage suggests that healing is not complete by eight weeks. This conclusion is supported by the results of the mechanical testing, which revealed that the torsional strength and stiffness of the newly formed bone reached approximately one-fourth of the strength and stiffness of the intact, contralateral bone. It is also possible that the return of full mechanical strength was constrained by the continued presence of the external fixator, which effectively bypassed load-bearing across the healing defect.

Although robust osteogenesis was noted within the Ad.BMP-2-treated lesions, intramuscular injection of the same vector into the quadriceps muscle of similar animals did not lead to bone formation, presumably because of the vector’s immunogenicity. This finding suggests that the immune response to Ad.BMP-2 differs between intraosseous and intramuscular sites, and this may explain the absence of ectopic bone formation in the animals in the present study. If so, our investigation revealed an unexpected advantage of using adenovirus vectors in this context. Other, less antigenic vectors, such as aden-associated virus and retrovirus, might be more prone to induce ectopic bone formation in an immunocompromised individual.

The direct, percutaneous introduction of first-generation adenovirus vectors carrying cDNA encoding BMP-2 and, presumably, other suitable osteogenic products may provide a straightforward approach to enhancing bone-healing when soft-tissue support has not been excessively compromised. There needs to be an adequate population of viable cells within the defect for the virus to infect and thereby elicit robust, endogenous synthesis of BMP-2. Atrophic nonunions and other settings in which this condition is not satisfied may not respond to this method.

The external fixator used in this study contained a precisely machined channel that ensured accurate placement of the Ad.BMP-2 in the center of the defect. Such accuracy may not be possible in a clinical setting. Additional studies are needed to determine the degree to which healing is affected by the placement of the vector. In a study of a rabbit model in which a guidance system was not used to inject the vector, Baltzer et al. noted very high expression of the transferred cDNA in the musculature surrounding the defect. More modest expression was seen in the bone and, transiently, in the liver. None was detected in the lung, spleen, or contralateral femur. This observation suggests, in a preliminary way, that placement of the transgene is not critical. Because neither manufactured scaffolds nor ex vivo cell culture is required, the procedure should be cost-effective.

Clinical use of adenoviruses as gene therapy vectors is associated with safety concerns, which are largely related to the immunogenicity of both the virus itself and the cells transduced by the virus. Activation of the immune system curtails transgene expression and leads to severe inflammatory reactions. Although we know of one reported death of a subject who had received recombinant adenovirus, an infusion of nearly 10¹⁴ virions had been administered directly into the liver of that patient. No deaths and few adverse events have been reported in association with lesser doses administered systemically or locally in the 271 human clinical trials in which adenovirus vectors have been used. The technology described in this paper is unlikely to provoke major adverse events, as modest amounts of vector are used and they are applied locally to discrete anatomical sites. Indeed, no obvious adverse effects of vector administration were noted in the present study. These issues can be addressed in greater detail in future studies.
References


