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An injectable method for noninvasive spine fusion

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Abstract

BACKGROUND CONTEXT—Bone morphogenetic proteins (BMPs) induce bone formation but are difficult to localize, and subsequent diffusion from the site of interest and short half-life reduce the efficacy of the protein. Currently, spine fusion requires stripping, decortications of the transverse processes, and an autograft harvest procedure. Even in combination with BMPs, clinical spinal fusion has a high failure rate, presumably because of difficulties in localizing sufficient levels of BMP.

PURPOSE—The goal was to achieve reliable spine fusion through a single injection of a cell-based gene therapy system without the need for any surgical intervention.

STUDY DESIGN—Eighty-seven immunodeficient (n=44) and immune-competent (n=43) mice were injected along the paraspinal musculature to achieve rapid induction of heterotopic ossification (HO) and ultimately spinal arthrodesis.

METHODS—Immunodeficient and immune-competent mice were injected with fibroblasts, transduced with an adenoviral vector to express BMP2, along the paraspinal musculature. Bone formation was evaluated via radiographs, microcomputed tomography, and biomechanical analysis.

RESULTS—New bridging bone between the vertebrae and the fusion to adjacent skeletal bone was obtained as early as 2 weeks. Reduction in spine flexion-extension also occurred as early as 2 weeks after injection of the gene therapy system, with greater than 90% fusion by 4 weeks in all animals regardless of their genetic background.

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CONCLUSIONS—Injection of our cell-based system into the paraspinal musculature induces spinal fusion that is dependent neither on the cell type nor on the immune status. These studies are the first to harness HO in an immune-competent model as a noninvasive injectable system for clinically relevant spinal fusion and may one day impact human spinal arthrodesis.

Keywords

Gene therapy; Spine fusion; Heterotopic ossification; BMP2; Spinal arthrodesis

Introduction

Of the more than one million bone grafts performed worldwide annually, 50% involve spinal fusions, and of these patients, 25% complain of donor site pain from the autograft harvest for up to 2 years postoperatively [1]. These complications have driven the search for and subsequent use of alternatives. This has led to the growing use of bone morphogenetic proteins (BMPs), which have long been demonstrated to help induce bone formation [2–4].

Recombinant human BMP2 is Food and Drug Administration approved for use on collagen sponges for the treatment of open long bone fractures and in metal cages for spinal fusion. Without the sponge or cage, the BMP2 cannot be localized and tends to diffuse from the desired site, reducing its efficacy and leading to adverse effects, such as edema, ectopic bone formation, and bone resorption in the graft area [5]. Because BMPs are so rapidly diffused, large quantities of the protein are required, making the procedure very expensive [3]. Furthermore, although the use of recombinant human BMP2 for spinal fusion may negate the need for an additional surgical procedure to harvest autograft bone, the method still necessitates an operation that, with the inclusion of the sponge or cage, introduces a permanent foreign object into the body [4]. Posterolateral spinal fusion also requires decortication of the transverse processes of the vertebrae targeted for fusion, stripping of the paraspinal musculature from bone, and a fairly long operative time [1,6]. Beyond the pain associated with decortication and stripping are often other complications. Stripping the musculature compromises the stability afforded by these muscles, disrupts the blood supply to both bone and muscle, and promotes scar formation. In the clinical arena, even with addition of the powerful morphogen BMP2, there is still a considerable clinical failure rate [5], causing researchers to search for better methods to deliver higher doses.

Recently, Shore et al. [7] demonstrated that a mutation in the BMP2 receptor was responsible for the observed heterotopic ossification (HO) in the genetic disease fibrodysplasia ossificans progressiva. Soft tissues in individuals with this genetic disorder are replaced with heterotopic bone. The HO can rapidly form within a few days and even replace skeletal bone if it becomes weight bearing by fusing to existing bone in such a manner that stress shielding initiates resorption. It readily fuses to the skeletal bone and often leads to ankylosis of the joints. Harnessing this capacity in a targeted controlled manner would potentially facilitate regeneration and repair solutions for skeletal bones.

Gene therapy approaches hold much promise in spinal fusion applications by delivering locally high levels of BMP2 to elicit robust targeted HO; however, the efficient transduction of cells poses a problem for many of the currently tested systems and results in low BMP2 expression [8]. In many such spinal fusion studies, this problem is then exacerbated by inclusion of a collagen sponge or other bio-material that rapidly binds the BMP2, again reducing its effectiveness. For instance, although adenoviral vectors producing BMP2 (AdBMP2) have been used to elicit spinal fusion in rats, the transduced cells were surgically implanted with collagen sponges or demineralized bone matrix after decortication of lumbar

transverse processes [9]. Such inclusion of a biomaterial and invasive decortication procedures cause inflammation, which potentially weakens bone healing [5].

Here, we percutaneously deliver cells expressing high levels of BMP2 to launch HO at a targeted location, with the goal of fusing two or more vertebrae within the lumbar spine. We deliver these BMP2-transduced cells without a carrier via injection into the paraspinal musculature. Of the studies using AdBMP2 to elicit spinal fusion in rodents [9–11] (reviewed in Yoon et al. [12]), none have taken into account the fact that without prior manipulation of rodent cells, adenoviral vectors can only minimally elicit bone formation, if at all [13]. We demonstrated this conclusively in previous studies showing that when murine cells were transduced with AdBMP2 at the same multiplicity of infection with and without the aid of a lipid polyamine (GeneJammer; Stratagene, Inc., La Jolla, CA, USA), only those treated with this compound were able to elicit bone formation in vivo. This is because of the fact that the receptors for human adenovirus are present only on human cells. In many murine cell lines, such as NIH3T3, there is no adenovirus receptor at all [14]; therefore, without using special compounds to allow attachment and internalization of the adenovirus, the transduction is exceedingly poor in the rodent cells [15]. As a result, immune-deficient models must be used to achieve the desired bone formation [16]. When using immune-competent animals, lentiviral vectors outperform adenoviral vectors [11] in spite of the fact that the number of genomes that enter the nucleus and produce BMP2 is almost 100 times higher in the latter. In our previous studies, we were the first to demonstrate the ability to harness the production capacity of the adenoviral BMP2 vector in rodents [13,17], and we apply it here in a spinal fusion model. Hence, ours is the first spine fusion model using an adenoviral vector in an immune-competent host that shows the true potential of this powerful vector. In addition, because the virus replicates episomally, meaning that it is not incorporated into the host chromosome nor passed onto daughter cells like lentiviral vectors are, the adenovirus is not perpetuated in vivo and thus has a better safety profile. Adenovirus is also safer than lentiviral vectors because adenoviruses induce a strong immune response and transduced cells are cleared within 4 to 5 days [17]. Because adenoviral vectors are considered safer, it is more likely that of the gene therapy approaches, the ones using adenoviral vectors will make it into the clinical arena. Thus, any adenoviral system capable of eliciting a bone formation response within this 4- to 5-day time frame has a potential clinical impact in humans.

Materials and methods

Cell culture

Human diploid fetal lung fibroblasts (MRC-5) and murine osteoblasts (MC3T3-E1) were obtained from the American Type Culture Collection (Manassas, VA, USA) and propagated in a humidified incubator at 37°C and 5% CO₂ in α -minimum essential medium (Sigma, St. Louis, MO, USA) and Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1000 U/L penicillin, 100 mg/L streptomycin, and 0.25 μ g/mL amphotericin B (Invitrogen by Life Technologies, Gaithersburg, MD, USA), as previously described [17]. Murine stromal cells (W20-17; a gift from Genetics Institute, Cambridge, MA, USA) were propagated and maintained as described by Thies et al. [18].

Adenoviruses

The construction, propagation, and purification of the adenoviral vectors used in this study were previously described in detail [13,19]. Briefly, replication-defective adenoviruses were constructed with deletions of the E1 and E3 viral genes by in vivo homologous recombination in 293 cells. The vectors used were a first generation human type 5

adenovirus (Ad) constructed to contain complementary DNAs for human BMP2 (AdBMP2) or no transgene (AdEmpty) in the E1 region of the viral genome [19]. During purification, virus particles (VPs) were quantitated by the plaque-forming unit assay, in which each plaque signifies infection of one cell by one virus. For the viruses AdBMP2 and AdEmpty, the VP:plaque-forming unit ratios were 1:83 and 1:111, respectively, and the viruses were confirmed to be negative for replication-competent adenovirus.

Cell transduction

Cells from the murine cell line MC3T3-E1 (1×10^6) were transduced with a BMP2 adenoviral vector or control (AdBMP2 or AdEmpty, respectively) at a viral concentration of 5,000 VP/cell with 1.2% GeneJammer, as previously described [13]. Briefly, GeneJammer was added at 3% to α -minimum essential medium without supplements and incubated for 10 minutes at room temperature. Adenoviral vectors, either AdBMP2 or AdEmpty, were then added at the aforementioned concentrations, and the mixture was further incubated for 10 minutes at room temperature. This virus solution was then diluted with supplemented α -minimum essential medium to achieve 1.2% GeneJammer per volume. The resulting transduction solution was used to coat the MC3T3-E1 monolayer with the minimum volume necessary to wet the cells, which were then incubated in a humidified incubator with 5% CO₂ at 37°C for 4 hours. After 4 hours, the transduction solution was diluted with supplemented medium at an amount appropriate for cell culture and replaced in the incubator overnight.

MRC-5 human fibroblasts (1×10^6) were transduced as previously described with a BMP2 adenoviral vector or control, AdBMP2 or AdEmpty, respectively [19]. Briefly, virus was added at a viral concentration of 2,500 VP/cell to fresh supplemented Dulbecco's Modified Eagle's Medium and incubated with cells in a humidified incubator with 5% CO₂ at 37°C overnight.

BMP2 quantification

Bone morphogenetic protein-2 expression was evaluated for MC3T3 and MRC-5 cells transduced with AdBMP2 or AdEmpty using enzyme-linked immunosorbent assays and alkaline phosphatase assays. Culture supernatant from transduced cells were collected 72 hours after adenovirus transduction and assayed with a BMP2 Quantikine enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN, USA) to measure BMP2 expression. Transduced cells were cocultured with W20-17 cells, with the transduced cells in the 0.4- μ m pore polycarbonate membrane six-well transwell inserts (Corning Inc., Lowell, MA, USA) and the W20-17 cells in the wells of six-well plates. After 72 hours, W20-17 cells were assayed for alkaline phosphatase activity using a chemiluminescence procedure [20]. Three freeze-thaw cycles were performed in a 100- μ M/cm² concentration of 25 mM Tris-HCl (pH 8.0) and 0.5% Triton X-100 to extract cellular alkaline phosphatase. This alkaline phosphatase activity was then measured by adding a ready-to-use CSPD (Tropix; Applied Biosystems, Foster City, CA, USA) substrate with Sapphire-II enhancer (Tropix; Applied Biosystems, Foster City, CA, USA) to the samples. After a 2-second delay, the light output from each sample was integrated for 10 seconds with a luminometer (TD-20/20; Turner BioSystems, Sunnyvale, CA, USA). Alkaline phosphatase levels were recorded in relative luminescence units and normalized to protein content with the bicinchoninic acid assay, using bovine serum albumin to derive a standard curve. Bone morphogenetic protein-2 levels and functional activity were found to be similar to previously published results per cell number and virus dose [8].

Spinal fusion

Female nonobese diabetic severe combined immunodeficiency (NOD/SCID; n=44) and C57BL/6 (n=43) mice (8–12 weeks old; Charles River Laboratories, Wilmington, MA, USA) were maintained in accordance to Baylor College of Medicine Institutional Animal Care and Use Committee protocols. Fig. 1 depicts an overall schematic of the process. Each mouse strain was separated into two major groups, animals receiving control-transduced cells or animals receiving BMP2-transduced cells; NOD/SCID animals received AdBMP2- (n=35) or AdEmpty-transduced cells (n=9), whereas C57BL/6 mice received AdBMP2- (n=32) or AdEmpty-transduced cells (n=11). Animals receiving AdBMP2-transduced cells were further divided into groups to be harvested at 2, 4, and 6 weeks (for C57BL/6, n=8, 4, and 20, respectively; for NOD/SCID, n=11, 12, and 12, respectively). All animals receiving AdEmpty-transduced cells were harvested at 6 weeks. Before paraspinous injections, the back of each mouse was prepared and a limited portion of the skin was incised to reveal the paraspinous muscles. Although the injection could have been conducted without opening the skin, the incision was performed to ensure appropriate placement of the transduced cells. Transduced cells were collected for injection after removal from tissue culture plates with trypsin and resuspension in phosphate-buffered serum at a concentration of 5×10^6 cells per 100 μL of phosphate-buffered serum and then delivered by intramuscular injection into the right paraspinous muscles along the length of the spine (Fig. 2). The placement of the needle was performed manually. The needle was positioned within the longissimus muscle, 1- to 2-mm distant from the lamina and spinous process. Multiple deliveries of approximately 10 μL of cell suspension were injected at about 2 or 3 mm intervals along the spinal segment targeted by advancing the needle without completely withdrawing it. Cells were not injected with the needle in contact with the bone. A total of 50 μL of volume was injected for each animal. After 2, 4, and 6 weeks, mice were sacrificed, and the spines with attendant musculature were removed and fixed at room temperature overnight in 4% formaldehyde solution (VWR, Sugar Land, TX, USA).

Microcomputed tomography analysis

All harvested intact spines (N=87) were scanned at 14- μm resolution with a commercial microcomputed tomography system (GE Locus SP; GE Healthcare, London, Ontario, Canada). Three-dimensional reconstructions of the spine and any mineralized tissue in the surrounding muscle were created at 29- μm resolution to visualize endochondral mineralized tissues. A volume of interest was defined for each specimen, and a threshold was chosen to exclude any nonmineralized tissue. The total volume of endochondral bone was then measured (eXplore MicroView, v. 2.0; GE Healthcare, London, Ontario, Canada), and preexisting bone from the spines of the animals was excluded from mineralized tissue measurements.

Biomechanical testing

After microcomputed tomography, formaldehyde-fixed spines from each group (n=64) were encased in alginate to obtain flexion and extension radiographs. Spines with attendant soft tissue from the C57BL/6 mice, AdEmpty (6 weeks, n=8), AdBMP2 (2 weeks, n=8; 4 weeks, n=4; and 6 weeks, n=8), and NOD/SCID mice (n=9, each group) were suspended in an in-house mold. Alginate powder was combined in an equal volume to water (30 mL of each) and mixed until smooth. The alginate was poured into the mold and allowed to solidify such that no portion of the spine protruded from the solidified alginate. Solidified alginate blocks were placed in an in-house spring loaded clamp with rigid 110° arcs (Fig. 3). Radiographs were taken of molds in flexion and extension orientations for each spine. These digitized radiographs were used to quantify intervertebral motion with Food and Drug Administration–approved software (KIMAX QMA; Medical Metrics, Inc., Houston, TX, USA) that has been validated for the clinical assessment of spinal fusion by measuring

relative intervertebral motion. The software compares radiographs in extension and flexion, detects the concomitant intervertebral rotation and translation, and has been demonstrated to report the movement with an accuracy better than 0.5° for rotation and 0.5 mm for translation between adjacent vertebrae [21,22]. Intervertebral motion was measured at each vertebral level. Preliminary data [23] demonstrated that the normal mouse spine undergoes an average of 5° of intervertebral motion using this test. Spines were considered fused when adjacent vertebrae did not exhibit rotation beyond 1.5° , which represents a 70% reduction in motion.

Histologic analysis

After biomechanical testing, four fixed spines from each group and time point ($n=32$) were isolated for histologic analysis as previously described [24] to confirm that any apparent mineralized bone observed on the radiographs was true osteoid and that it had integrated at these tentative points of fusion. The spines and adjacent tissues were decalcified in hydrochloric acid, processed, and embedded into a single paraffin block, where serial sections were then cut at a thickness of 5 μm . Every fifth section was stained with hematoxylin and eosin and observed under light microscopy to identify the tentative points of fusion. Representative photomicrographs ($2\times$ and $4\times$) of samples from each model were taken 2, 4, and 6 weeks after induction of HO. To further confirm the vertebral fusion, a subset of spines ($n=9$; one from each group and time point, two from NOD/SCID at 6 weeks) were immersed in bleach for approximately 1 hour, which removed all soft tissues. Midway through this process, nylon wire was threaded up the spinal canal to maintain the relative position of the vertebrae.

Statistical analysis

Statistical analysis was performed as described previously [17]. Briefly, all data were taken in triplicate and reported as mean and standard deviation. A Student *t* test with a 95% confidence interval ($p<.05$) was performed between the control and each experimental condition.

Results

Radiologic analysis of bone formation

In all animals receiving AdBMP2-transduced cells, heterotopic bone formation occurred along the injection site adjacent to the spine, with greater than 90% of all animal spines showing bridging and fusing to the skeletal bone (Fig. 4F–L) by 4 weeks (NOD/SCID: 89% and C57BL/6 mice: 100%). Two-dimensional microcomputed tomography images show cross sections through the injected area (Fig. 4A–D, M–P). The radiographs and three-dimensional reconstructions demonstrate that both the immune-incompetent system, NOD/SCID mice receiving human cells transduced with AdBMP2 (Fig. 4I–P), and the immune-competent system, C57BL/6 mice receiving AdBMP2-transduced allogeneic murine cells (Fig. 4A–H), appear to produce similar bone within 4 to 6 weeks. In particular, the newly formed heterotopic bone appears to have integrated into the vertebral cortical bone (Fig. 4B–D, O–P). These points of fusion appear to be in the laminae region of the vertebra, with most of the fusions encompassing the entire spinous and transverse processes, suggesting robust significant fusion. This new bone appears to be remodeled with a contiguous cortical bone exterior. Although only 44% of the NOD/SCID spines harvested at 2 weeks were fused by this time point, unfused spines from this group showed potential points of fusion: Fig. 4N shows an early time point in which the heterotopic bone, although extensive, has not yet fused into the vertebrae. Interestingly, we did not see a similar failure to fuse in wild-type mouse models—at all time points, each animal injected with AdBMP2-transduced cells had developed a fused spine. In other words, in the C57BL/6 mice, the fusion rate was 100%: as

seen in Fig. 4B, even at the same early 2-week time point, the substantial bone has fused to the adjacent vertebra. Despite the 100% fusion, there are some areas in the C57BL/6 animals in which not all of the newly formed bone has fused with the spine: in Fig. 4C, some of the heterotopic bone appears to have formed slightly distal to the vertebral bodies. At no time was bone formation observed within the spinal canal. In no cases was bone formation or bridging observed in the spines receiving the AdEmpty-transduced cells.

Biomechanical analysis to confirm reduced motion of the spine

Comparisons of harvested spine radiographs subjected to 110° of flexion and extension revealed reduced intervertebral motion in animals receiving AdBMP2 (Fig. 3D,E). Spines were considered fused when the KIMAX QMA software, which is used to report clinical fusions [21,22], indicated that relative intervertebral rotation between adjacent vertebrae was reduced by 70% of normal (Table). When analyzed for relative intervertebral rotation and translation, in no cases did tissues receiving AdEmpty-transduced cells show a reduction in motion or spine stiffening. Conversely, in NOD/SCID animals that received the human cells transduced with AdBMP2, approximately 44% of the spines at 2 weeks and 89% of those at 4 and 6 weeks had reduced movement, consistent with fusion of at least one level. Interestingly, in the C57BL/6 group receiving murine AdBMP2-transduced cells, 100% of the spines at all time points consistently showed a reduction in motion correlating with fusion.

Histologic analysis of the spine fusion

Hematoxylin and eosin-stained slides revealed structures of mature bone in all de novo bone samples from animals receiving BMP2-transduced cells (Fig. 5). Osteoclasts, osteocytes, and tentative bone marrow elements in the heterotopic bone were observed in these slides, as well as cartilage, analogous to the growth plate structures in the normal long bone. The new heterotopic bone appeared to grow in a direction toward the skeletal bone, with the most mature bone being distant from the skeletal bone in the 2-week samples. There is substantial new bone adjacent to and fused with the more mature vertebral bone along the transverse process and laminae region of the vertebra (Fig. 5A and B). Although mature bone with tentative marrow elements was observed at 2 weeks, this structure was always distal to the vertebral bone (data not shown), suggesting that the original HO started de novo in the muscle and grew toward the vertebrae to encompass the existing bone.

At the 4-week time point (Fig. 5C and D), the heterotopic bone displays a much more mature morphology and a cellular process appears to be rapidly removing the mature cortex of the skeletal bone at the point of fusion. This large number of cells involved in this process has resulted in a moth-eaten appearance of the mature cortical bone of the vertebra during its apparent removal and replacement by the maturing heterotopic bone (Fig. 5G). The origin of the large number of cells is unclear. At the 6-week time point, the cortical boundary is completely remodeled (Fig. 5E and F) with the newly formed and original bone contiguous as one integrated structure with a well-defined cortex and trabecular interior, which houses the bone marrow. At this point, the only evidence distinguishing the newly formed HO is the presence of substantial amounts of adipose tissue, which is found within heterotopic regions in contrast to the mature marrow within the vertebra (Fig. 5F). All cases (Fig. 5) show what appears to be fusion with the transverse process within the laminae region of the vertebra, which was the target region for fusion. Depending on the depth of the histology section, more or less of this region was involved in the fusion site. In many cases, the fusion actually encompassed both the spinous process through the laminae to the transverse process. In all tissues analyzed, the HO appeared to grow into the vertebral body, which itself did not appear to undergo growth, and there was no evidence of new bone formation within the spinal canal, similar to radiologic findings.

Functional demonstration of fusion

The nine spines with soft tissues bleached away confirmed fusion in all specimens displaying biomechanical constraint during mechanical testing. A representative 6-week spine shows that five vertebrae of the lumbar spine are remodeled into a single structure (Fig. 6, Top). In cases that did not meet our biomechanical criteria for fusion (several NOD/SCID animals at 2 weeks) in that intervertebral motion did not appear to be constrained after induction of bone formation, there was heterotopic bone that was not integrated with the vertebrae but rather formed individual bones, confirming our biomechanical findings.

Soft tissue removal and induced scoliosis demonstrate the fusion of the spine (Fig. 6, Top, Bottom Right). Representative radiographs show a distinct curvature of the spine toward the area of new bone formation and tentative fusion in animals receiving AdBMP2-transduced cells (Fig. 6, Bottom Right). This scoliosis has occurred in 6-month-old growing mice in both immune-competent and incompetent strains. This was observed in a large number of animals with hetero-topic bone and tentative fusion but absent in animals that received the control cells (Fig. 6, Bottom Left).

Discussion

To determine if our cell-based gene therapy system induces spine fusion through intramuscular injection without invasive surgery or additional carriers, we established two different murine models for testing. We previously characterized these models and found the HO in the mouse quadriceps muscle to be similar [17]. In this study, we applied this cell-based gene therapy system to the paraspinal musculature in the region of the vertebral laminae to determine whether heterotopic bone formation could be targeted to this location; form bridging bone between two or more skeletal vertebrae; and ultimately fuse the spine. In this approach, either AdBMP2-transduced cells or AdEmpty-transduced cells are delivered to the paraspinal musculature of a mouse through a simple injection at points adjacent to the levels of desired fusion. With this system, HO is generated rapidly, fused, and remodeled into two or more of the adjacent vertebrae, reducing spine motion. The fusion appeared to be rapid, at a time scale of 2 weeks, and the new bone was limited in size and scale to regions of muscle that received the cells.

Fusion of both the heterotopic bone to the skeletal bone and the resultant bridging of two vertebrae were rapidly achieved through simple injection. Within 2 weeks, 44% to 100% of the spines in the two different murine models were considered fused by all criteria—radiologic, histologic, and biomechanical. In samples tested 4 to 6 weeks after induction, greater than 90% of all mice had achieved spine fusion in the two models and noticeable scoliosis was observed radiologically in the animals' spines, suggesting that the fusion could restrain the spine, even during continued growth. Our system is the first approach reported in the literature to achieve clinically relevant spinal fusion in an animal model within 2 weeks through a single intramuscular injection. Although it can be argued that several other systems achieve this goal in various animal models [25–28], each of these studies fall short of a clinically relevant regimen. In a study by Hasharoni et al. [25], the authors inject recombinant human BMP2-producing mesenchymal stem cells (MSCs) into the paraspinal muscles of immune-deficient mice. Although this is indeed a percutaneous approach, it is not a clinically relevant percutaneous approach in that this study neither reports the percentage of mice that achieve fusion nor performs biomechanical testing of the fusion. In addition, the assumption is that MSCs are required in addition to their production of BMP2.

We have, however, shown repeatedly that stem cells are not necessary, and that any cell that produces a high level of BMP2 will suffice [8,13,17]. This enables us to qualify cells for our purposes and use them without concern about their stem cell nature. The use of BMP2-

producing MSCs also raises questions as to their ultimate fate because they are not destined for immune destruction like our virally transduced cells. In an attempt to avoid the use of such transduced MSCs, Sheyn et al. [26] used transfection techniques to incorporate the BMP6 gene for spinal fusion in immune-deficient mice. This approach failed to achieve complete success because of their admittedly low efficiency of transduction of the ovine cells. We have also found that injection of BMP2-producing cells ultimately induces the production of BMP6 (on Day 2) and BMP7 (on Days 5 and 6) as assessed by microarray analysis (Davis et al., 2007, unpublished). Therefore, it is probably not necessary, and may in fact be detrimental, to use different types of BMPs because administration may not occur at the proper time nor place.

Reported time scales for achieving spinal fusion in various animal models using ex vivo gene therapy approaches, in which virally transduced cells are injected, range between 6 weeks and 3 months; using in vivo gene therapy approaches, in which the virus itself is injected, spinal fusion typically proceeds between 4 and 12 weeks [2,29]. Nevertheless, despite the reported successes using these approaches, such results were tempered with difficulties in isolating stem cell populations to use as delivery cells and problems with low-gene transduction efficiencies [2,29,30]. The work presented here circumvents these issues. Surprisingly, we observed 100% fusion in the immune-competent wild-type mice within 2 weeks of induction, yet only 44% fusion at 2 weeks in the immune-deficient mice. This difference in success rate may be attributable to a learning curve as the small scale of the mouse spine made injections more difficult. On analysis of the three-dimensional reconstructions, it appeared that some of the new bone was slightly distal to the vertebra in the NOD/SCID immune-incompetent mice that failed to fuse, whereas it was more closely associated with the vertebra in the wild type. Previous comparisons of our immunocompetent and immunodeficient models show that they function almost identically in production of heterotopic bone [1]. Therefore, it is unlikely that this difference is linked to the immunodeficiency, but rather that as we progressed, we improved our ability to place the cells proximal to the vertebra within the paraspinal musculature, indicating that placement may be critical to the eventual fusion.

These results suggest that heterotopic bone can be rapidly induced by delivering locally high levels of BMP2. This is not surprising because recombinant BMP2 is currently used clinically; however, the rapid 2-week time frame has not previously been reported (see Mussano et al. [4] for review). This may be a direct result of our ability to produce high levels of BMP2 for a prolonged period of time. This process may be explained by taking cues again from the human genetic disease fibrodysplasia ossificans progressiva, in which HO can readily occur within 1 week; this disease is caused by a mutation in a BMP receptor that leads to constitutive activity but can still be further activated through addition of BMP [31]. Thus, physiological doses of BMP2 normally released after trauma effectively become high doses, leading to rapid HO at the local injury site. During adenovirus transduction, multiple VPs enter the cell with large amounts of vector DNA effectively delivered to the nucleus. Because of its episomal (extrachromosomal) nature, the vector DNA is present at high numbers, driving high-level expression of BMP2.

Therefore, one of the things separating adenovirus from other gene therapy vectors is the high level of transgene expression that can be achieved after efficient transduction. As long as the virus can efficiently infect the specific cell types [13], this system can be used to produce these high doses of BMP. Thus, we have developed this as a cell-based gene therapy system rather than a direct approach, to circumvent potential problems with inefficient uptake of the adenovirus, problems that prohibit production of the BMP2 levels necessary for achieving rapid fusion [8]. This is perhaps why our approach is so extremely effective at making rapid targeted bone. Furthermore, by prior transduction of the cells with

the virus, no free adenovirus is delivered to the animal, minimizing adverse effects of the virus on other tissues.

In addition to forming bone rapidly, this study is the first to demonstrate the ability of HO to form clinically relevant bridging bone and fuse adjacent vertebral bone without contribution from the skeletal bones. Both current clinical approaches using recombinant BMP2 and other gene therapy approaches [1] require exposure of the vertebra and decortication to induce bone growth and ultimately fusion of the skeletal bone to heterotopic bone. Often, autologous bone graft is harvested to use in place of ectopic bone, which requires an additional extensive surgical procedure. Here, through both histologic analysis and biomechanical analysis, we demonstrate the ability of the heterotopic bone to fuse into skeletal bone without prior exposure and decortication.

Furthermore, all spines receiving the AdBMP2-transduced cells appeared to have extensive heterotopic bone formation on radiologic analysis. In no cases did we observe any ossification in the spinal canal. In the animals receiving AdEmpty-transduced cells, there was no ossification whatsoever. The results suggest that the heterotopic bone formation is targetable to a discrete location. On radiologic analysis of samples taken 2, 4, and 6 weeks after initial injection of the AdBMP2-transduced cells, heterotopic bone was found between the transverse process adjacent to the paraspinal musculature receiving the cells and laminae. This heterotopic bone eventually encompassed these structures, fusing the spine. In many cases, we observed significant fusion of the entire laminae, from the tip of the spinous process through the entire transverse process. There was some variation in the amount of vertebral bone involved in each fusion, but this did not seem to affect the overall arthrodesis itself. Furthermore, because the transduced cells can target the location of bone formation, perhaps the variation observed could also be a result of placement of these cells within the spinous musculature of the mice. Regions of soft tissue adjacent to corresponding distal structures of the same vertebra were not involved in any bone formation and appeared normal in these radiographs.

Histologic analysis of this model suggests that the heterotopic bone grows into the skeletal bone with a somewhat organized growth plate, cortex, and tentative periosteum, similar to the vertebral bone. Initially less mature bone is observed at the junction of fusion, and depending on the model, this results in a reduction of motion by 2 weeks in approximately 44% to 90% of the animals tested. As the two bones fuse, there appears to be a cellular reaction, which is destructive to the vertebral cortex. This process allows for the replacement of the cortical boundary with mature trabecular bone and bone marrow over time. It is not surprising from these results that in all cases, the latter 4- and 6-week structures are well fused both histologically and biomechanically. Interesting to note, by 6 weeks, the old vertebral structure is gone with the only remnants of the newer heterotopic bone found in the marrow cavity. This observation suggests that adipose may form in the bone marrow cavity before the housing of true bone marrow because in all less mature heterotopic bone, we observed extensive white fat, whereas in the vertebral bone marrow, there was very little if any present. Whether this tissue plays a key role in establishment of the marrow is yet to be determined.

Ideally, on successful fusion, the newly fused heterotopic bone should restrict mobility within the spine. Therefore, biomechanical testing was performed to measure the changes in angle of the spine under force. In spines with a significant reduction in mobility, we observed well-integrated collagen fibers running contiguously through the bone, suggesting that it was a remodeled single structure, whereas those that appear as two separate structures under polarized light were not capable of reducing flexion-extension. In animals with reduced spinal mobility, we also observed scoliosis because of the arthrodesis. Because the

epiphyseal growth plates of rodents do not close until 2 years of age (the approximate life span of laboratory mice), their skeletons essentially do not stop growing [32]. The fusion of the vertebrae in essence mechanically fixed the right side of the spine, causing imbalanced growth, resulting in scoliosis. The ability to fuse the spine without surgical intervention would be a significant advancement in health care. The creation of a bony fusion by means of the percutaneous injection of a biologically active material, without extensive surgical dissection and -bony decortication, would have many clear clinical advantages. This system is quite versatile, in that any cell can be used as a delivery cell, as long as adequate transduction with adenovirus is achieved [33]. Thus qualified cell lines used in current clinical trials, such as MSCs, can be readily adapted for use in this cell-based gene therapy system, making it very feasible to introduce clinically. The rapid onset of bone in addition to the rapid clearance of the transduced cells bode well for the future therapeutic application of this system in humans. The goal of this work was to demonstrate that these gene therapy methods can be used to rapidly and reliably accomplish fusions without extensive and highly invasive surgical procedures. The location of the fusions that we demonstrate here is equivalent to the classic posterior approach to spinal fusion. Although this location for the fusion bone has ample human precedent clinically, the technique could be reasonably applied to fusions in other anatomic areas, for example, the posterolateral (intertransverse process) location. Alternate locations like the intertransverse process were not pursued in this study because getting the transduced cells to this target in these very small animals is challenging without X-ray guidance.

In this study, the fusion mass was large and induced scoliosis because of our delivery of cells only into the right paraspinal muscles. For clinical applications in humans, we envision that delivery would be balanced on both sides of the spine and BMP2 expression would be controlled by a “TET-on” (tetracycline-on) or other inducible system in which gene expression is controlled by the administration of tetracycline or other small molecule [34]. In such a system, the size of the fusion mass could be monitored, and over time, this fusion would be remodeled: we have seen remodeling of the fusion mass in the animals (and indeed in humans). We expect that the ultimate dimensions and stiffness of the fusion mass will have more to do with Wolff’s law than the dimensions of the original fusion.

The system presented herein could potentially markedly decrease the pain, blood loss, and recovery time for patients undergoing these procedures, thus significantly reducing health care and associated costs. Our viral vectors have already been used clinically in Phase I-II cancer trials to augment the expression and immunogenicity of the antigen latent membrane protein-2 and have resulted in complete tumor response of patients with relapsed lymphoma [35]. With the safety of the viral vector already established in humans, using the vector to deliver BMP2 clinically is an attainable goal. This system could potentially improve success rates of spinal fusion, thus improving the quality of health care in this arena overall. With large animal studies and clinical trials yet to be performed, such a future is a ways off, but our system is the first step toward that day.

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Fig. 1. Schematic depiction of spine fusion using the cell-based gene therapy system. Adenoviral vectors producing BMP2-transduced cells injected into the muscle rapidly recruit host cells to undergo all stages of endochondral ossification [24,36]. Arrows indicate order of steps. Within 1 week, mineralized osteoid can be detected in photomicrographs and radiographs. BMP2, bone morphogenetic protein-2.

Fig. 2.

Delivery of the transduced cells into the paraspinal musculature. The needle was positioned by hand within the longissimus muscle, 1- to 2-mm distant from the lamina and spinous process. Left image shows multiple delivery sites of the transduced cells (AdBMP2 or AdEmpty) of approximately 10 μ L of cell suspension per site at 2- to 3-mm intervals along the spinal segment targeted. Multiple delivery is accomplished by advancing the needle without completely withdrawing it. (Right) Cell delivery (a total of 50 μ L per animal) was performed with the needle in the muscle, never in contact with the bone. AdBMP2, adenoviral vectors containing complementary DNAs for human bone morphogenetic protein-2; AdEmpty, adenoviral vectors with no transgene.

Fig. 3.

(A) Schematic of the in-house flexion device. After 2, 4, or 6 weeks, spines were harvested with attendant soft tissue attached, (B) encased in a block of alginate, (C) then placed in the device to induce flexion or extension at 110°, and (D and E) radiographed. (D and E) Representative images used to evaluate biomechanical function of spinal fusion. (D) Spine in extension. (E) Spine in flexion. Such images were evaluated with KIMAX QMA software (Medical Metrics, Inc., Houston, TX, USA) to determine whether adjacent vertebrae rotated more than 1.5° (30% of normal).

Fig. 4. (A–H) Radiographs at 2, 4, and 6 weeks of C57BL/6 and (I–P) NOD/SCID spines imaged after intramuscular injection into the paraspinal musculature of cells transduced with AdEmpty control virus (A, E, I, and M) or AdBMP2 (B–D, F–H, J–L, and N–P). Control animals injected with AdEmpty were scanned 6 weeks after delivery of the transduced cells (A, E, I, and M). Mice receiving the AdBMP2-transduced cells were scanned 2 weeks (B, F, J, and N), 4 weeks (C, G, K, and O), and 6 weeks (D, H, L, and P) after the initial induction of HO. Two-dimensional X-rays (A–D: C57BL/6, and M–P: NOD/SCID) show a cross section through the three-dimensional reconstructions (E–H: C57BL/6, and I–L: NOD/SCID) of tentative fusions between the HO and the vertebral bone. NOD/SCID, nonobese diabetic severe combined immunodeficiency; HO, heterotopic ossification; AdBMP2, adenoviral vectors containing complementary DNAs for human bone morphogenetic protein-2; AdEmpty, adenoviral vectors with no transgene.

Fig. 5.

Hematoxylin and eosin–stained representative photomicrographs of tentative vertebral fusion with the heterotopic bone, taken 2 (A, B: 2× and 4×, respectively), 4 (C, D: 2× and 4×, respectively), and 6 weeks (E, F: 2× and 4×, respectively) after initial injection of the AdBMP2-transduced cells. (E) A representative photomicrograph (10×) of a sample taken 4 weeks after the initial injection of AdBMP2-transduced cells. As can be seen in this sample, there are a significant number of cells associated with the boundary between the new heterotopic and the old vertebral bone (arrows). Scale bars are 2.0 mm except for G, which is 300 μm. AdBMP2, adenoviral vectors containing complementary DNAs for human bone morphogenetic protein-2.

Fig. 6.

(Top) Spine fusion was observed in bones isolated from the mouse after induction of targeted heterotopic ossification. Associated soft tissues were removed by bleaching, leaving only the bone. A wire was threaded through the spinal column, to preserve the orientation of the vertebra. Unfused vertebrae hang free; fused vertebrae remain joined and rigid. Ruler is in millimeters. Radiographs of mouse spines at 6 weeks after induction of spine fusion AdEmpty- (Bottom Left) or AdBMP2-transduced (Bottom Right) cells. (Bottom Right) The image shows obvious curvature of the spine suggesting a significant scoliosis, as compared with the normal mouse spine, shown in Bottom Left. AdBMP2, adenoviral vectors containing complementary DNAs for human bone morphogenetic protein-2; AdEmpty, adenoviral vectors with no transgene.

Table**Spinal fusion in injected animals**

Treatment *	N	Immune status[†]	Time at harvest (wk)	Animals with two or more vertebrae fused (% of group)
AdEmpty-transduced cells	9	Deficient	6	0 (0)
AdBMP2-transduced cells	9	Deficient	2	4 (44)
AdBMP2-transduced cells	9	Deficient	4	8 (89)
AdBMP2-transduced cells	9	Deficient	6	8 (89)
AdEmpty-transduced cells	8	Competent	6	0 (0)
AdBMP2-transduced cells	8	Competent	2	8 (100)
AdBMP2-transduced cells	4	Competent	4	4 (100)
AdBMP2-transduced cells	8	Competent	6	8 (100)

AdBMP2, adenoviral vectors containing complementary DNAs for human bone morphogenetic protein-2; AdEmpty, adenoviral vectors with no transgene.

* Treatment indicates type of cells injected.

[†] Immune status indicates type of animal used. Immune-deficient animals were nonobese diabetic severe combined immune-deficient mice. Immune-competent animals were C57BL/6 mice.