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THORACOSCOPIC INTRADISCAL SPINE FUSION USING A MINIMALLY INVASIVE GENE-THERAPY TECHNIQUE

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Background: Gene therapy has been utilized to achieve posterior intertransverse process fusion in rodents. To our knowledge, however, no one has previously reported on the use of this technique to achieve anterior spinal fusion in mammals. The purpose of the present study was to determine if a gene-therapy technique can be utilized to achieve anterior intradiscal fusion in pigs with use of minimally invasive techniques.

Methods: Mesenchymal stem cells were isolated from each of three pigs, expanded in culture, and transduced with an adenovirus carrying either the gene for bone morphogenetic protein-2 (Adv-BMP2) or the control gene, β -galactosidase (Adv- β gal). In vitro, assays were performed to detect BMP-2 expression as well as protein markers of bone formation. In vivo, four thoracic disc spaces in each of three pigs were injected thoracoscopically with cells after 1 cm³ of the disc had been removed. In each of the three pigs, two discs were injected with autologous mesenchymal stem cells transduced with Adv-BMP2, the third disc was injected with cells transduced with Adv- β gal (control 1), and the fourth disc served as the sham-operated control (control 2). The three animals were killed six weeks after the implantation. Computerized tomographic scanning was performed on two of the specimens, and histological examination was performed on all specimens. The computerized tomographic scans and histological examinations were then interpreted in a blinded fashion.

Results: In the in vitro study, a human BMP-2 protein band was detected in the medium of Adv-BMP2-transduced stem cells but not in that of the control cells. The Adv-BMP2-transduced stem cells were associated with a fivefold increase in alkaline phosphatase activity compared with the controls as well as with matrix mineralization and increased protein expression of type-I collagen, osteopontin, and bone sialoprotein. In the in vivo study, radiographic examination demonstrated anterior spinal fusion in all six disc spaces that had been treated with implantation of Adv-BMP2-transduced stem cells. In contrast, the six control disc spaces had little or no intervening bone. Histological examination demonstrated bridging bone from end plate to end plate in all six disc spaces that had been treated with implantation of Adv-BMP2-transduced stem cells. The six control disc spaces had no bridging bone.

Conclusion: The Adv-BMP2-transduced mesenchymal stem cells produced BMP-2 protein. Further, the cells differentiated into osteoblasts and induced anterior spinal fusion in six of six disc spaces in this pig model. Although many technical and practical challenges remain, the results of the present study suggest that it may eventually be possible to use similar techniques to achieve anterior spinal fusion in humans.

A number of experiments have suggested that bone morphogenetic protein (BMP) may be used to enhance bone fusion in the posterior part of the spine and perhaps even to act as a bone substitute^{1,2}. In a number of recent studies, gene-therapy techniques have been used as an alternative to BMP to achieve bone formation in various animal models³⁻⁵. To date, we are aware of only a few studies in which either BMP or gene therapy has been used to achieve anterior spinal fusion without the use of any bone or metallic im-

plants^{6,7}. The current approach to anterior spinal fusion involves removal of the disc followed by placement of bone in the intradiscal space. The use of autograft carries with it the morbidity of a second procedure, whereas the use of allograft may be associated with a decreased fusion rate. The purpose of the present study was to determine if implantation of mesenchymal stem cells that have been transduced with an adenovirus carrying a gene for BMP-2 could induce spinal fusion in pigs with use of minimally invasive means.

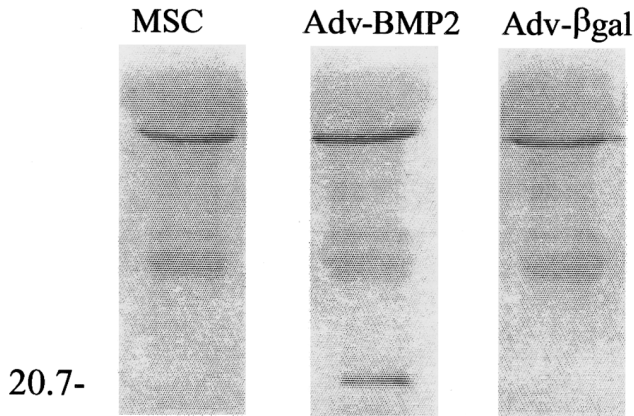


Fig. 1
Western blot analysis was performed for the detection of secreted BMP-2. The specific band at 20.7 kDa was detected only in the conditioned medium of cells transduced with Adv-BMP2. MSC = mesenchymal stem cells.

Materials and Methods

Generation of Recombinant Adenoviral Vector Carrying the Human BMP-2 or β -Galactosidase Gene

The recombinant adenovirus carrying the human BMP-2 gene (Adv-BMP2) was constructed in our laboratory as previously described^{14,5,8}. Adenovirus carrying the β -galactosidase gene (Adv- β gal) served as a control in our experiments and was generated in the same fashion with use of the gene encoding β -galactosidase in place of the BMP-2 gene.

Harvest and Culture of Pig Bone Marrow Mesenchymal Stem Cells

Pig ribs were harvested with use of sterile surgical techniques, and mesenchymal stem cells were isolated from the rib marrow. Three adult male pigs were used for these experiments. These animals weighed 56, 65, and 68 kg at the time of the first operation. The animals were five to six months old at the time of the rib harvest and seven to eight months old at the time of the thoracoscopic surgery. Approximately 8 cm of each rib was resected and placed in sterile phosphate-buffered saline solution. The pigs were kept alive for the second part of the study, in which the cells were im-

planted thoracoscopically into the disc spaces. The mesenchymal stem cell suspension was prepared as previously reported⁸.

Cell Cultures

Pig bone-marrow stromal cells were isolated from the ribs as described previously^{5,9}. Briefly, the ribs were cleaned of any extraneous tissue and then were split. Bone-marrow cells were obtained by gently flushing the marrow cavity with α -minimum essential medium Eagle (α MEM). The cells were purified through a Histopaque-1077 density gradient and were allowed to adhere to culture flasks for one week. Nonadherent cells were removed, and adherent bone-marrow stromal cells, which represented relatively immature preosteoblastic cells^{5,9}, were allowed to grow to confluency in α MEM supplemented with 10% heat-inactivated fetal bovine serum (growth medium).

Recombinant Adenovirus Transduction of Pig Mesenchymal Stem Cells

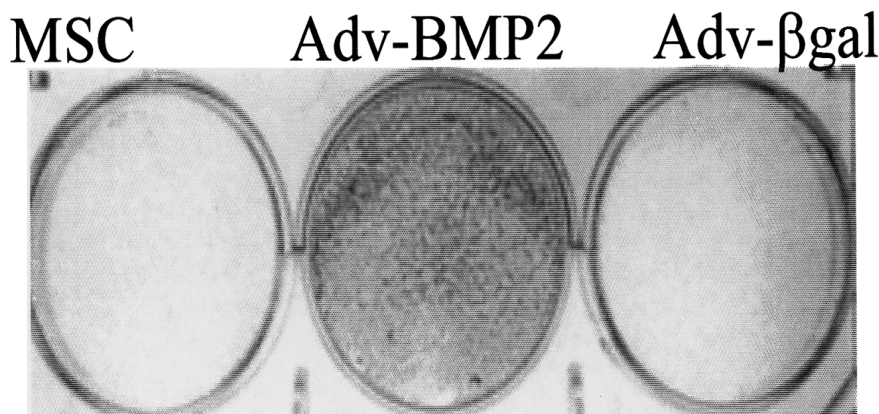
We subcultured the cells three times to increase the number of cells. When the third subculture produced enough cells to cover 90% of the culture dish, the cells were divided into thirds. One third each was incubated with ADV-BMP2 or ADV- β gal at fifty plaque formation units (pfu)/cell. The final third was left alone in culture overnight. After removal of transduction medium, the cells were further cultured in the growth medium for seven days, with a medium change on the third or fourth day. The cells were harvested for analysis of bone-matrix proteins or were resuspended in a collagen solution at 3×10^6 cells/mL for autologous implantation at the site of anterior spinal arthrodesis. The conditioned medium was also harvested for examination of BMP-2 secretion.

Biochemical Assays

Immunostaining was performed to detect the presence of BMP-2 in the cell layer, and Western blot analysis was performed to detect secreted human BMP-2^{5,8}. Molecular weight markers were utilized to confirm the identification of the bone-matrix proteins.

Thoracoscopic Implantation of Cells into Pig Disc Spaces

Three or four thoracoscopic ports were used for each animal.



Von
Kossa

Fig. 2
Von Kossa staining demonstrated matrix mineralization in only the Adv-BMP2-transduced cells. MSC = mesenchymal stem cells.

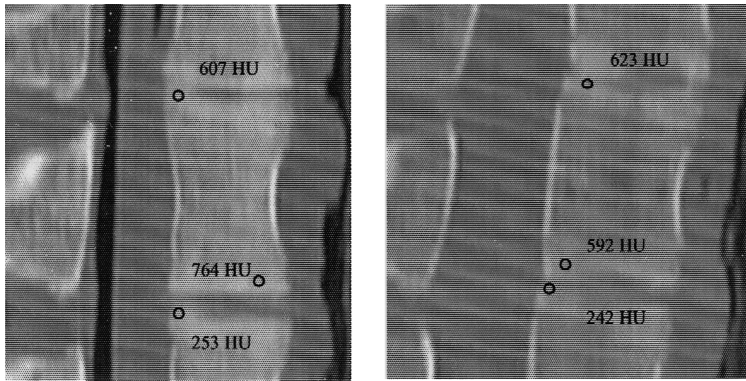


Fig 1

Fig 2

Fig. 3

Sagittal reformation of computed tomographic images of the spines of two pigs. Bone formation was clearly visualized in the fused (upper) disc spaces but not in the control (lower) disc spaces. The attenuation values in the fused disc spaces were higher than those in the control disc spaces, quantitatively confirming bone formation.

Under thoracoscopic guidance, the left anterolateral aspect of each disc space was identified and the overlying parietal pleura was incised. With use of a combination of sharp and blunt thoracoscopic instruments, alternating disc spaces were entered. A minimal amount (1 cm^3) of disc material was removed to provide a space for cell implantation. Under thoracoscopic guidance, cells transduced with either the control (β -gal) or the experimental (BMP-2) adenovirus were injected into the disc space with use of an 18-gauge spinal needle inserted through the thoracoscopic ports. Each disc space received approximately 5×10^7 transduced autologous mesenchymal stem cells. Care was taken to avoid cross-contamination of either the disc space or the overlying tissue. In each of the three pigs, two discs were injected with the BMP-2 experimental cells, one disc was injected with the β -gal control cells (control 1), and one disc served as the sham-operated control (control 2). The pigs remained healthy until they were killed at six weeks. There were no obvious deleterious effects from the adenoviral vectors. The spines were harvested six weeks after the autologous implantation of mesenchymal stem cells.

Plain Radiography

After the spine had been harvested from the first pig, a plain radiograph was made. Because we had difficulty ascertaining

whether a fusion had occurred, we elected to proceed directly to histological examination of this specimen. After consulting with our radiologist, we elected to forego plain radiographs and used computerized tomographic scans for the next two pigs.

Computed Tomography

The spines harvested from the other two pigs were evaluated with computed tomography. Reformatted images of the computed tomographic scans were evaluated qualitatively for the presence of intradiscal bone formation. In addition, we measured the x-ray attenuation of the computed tomography scans (in Hounsfield units) to quantify bone formation by placing circular regions of interest over the disc spaces and the cortical end plates. Because bone reduces the intensity of the x-ray beam far more than soft tissue does, the computed tomography attenuation value increases with bone formation. The computed tomographic scans were interpreted by a radiologist who was blinded to the protocol of cell injection.

Twelve measurements were made for each pig, with three measurements (two from the disc and one from the end plate) being made for each of the four discs (the two treated with BMP-2, the one treated with β -gal, and the one treated with the sham operation).

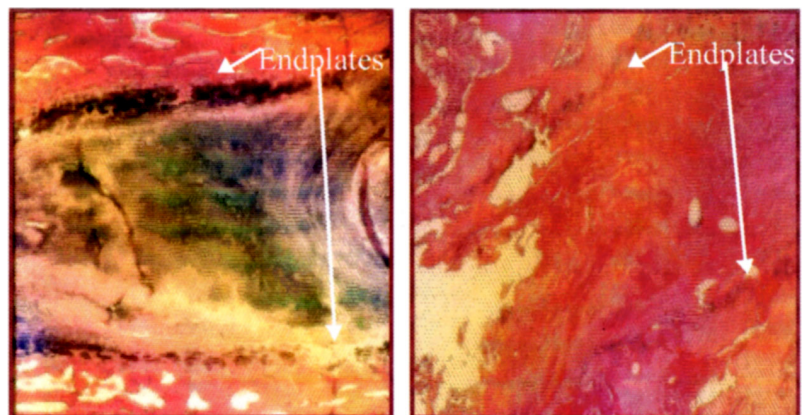


Fig. 4

Photomicrographs demonstrating bone from one end plate to the next in the Adv-BMP2-treated disc (right) but not in the control disc (left) (hematoxylin and eosin).

Control

Adv-BMP2

Histological Evaluation

Following the radiographic or computed tomographic examination, the specimens were fixed in neutral formalin. The experimental disc spaces were isolated and treated with Decalcifier I (Surgipath Medical Industries, Richmond, Illinois) overnight. The samples were then treated with standard procedures for embedding, incision, and staining with hematoxylin.

Results

Mesenchymal stem cells transduced with Adv-BMP2 were capable of overexpressing BMP-2 protein seven days after transduction. BMP-2 expression was detectable in >80% of these cells on immunostaining. Cells transduced with Adv-BMP2 secreted human BMP-2 into the conditioned medium, whereas no BMP-2 secretion was detected in the conditioned medium of cells transduced with Adv- β gal or that of untreated cells (Fig. 1). Moreover, the BMP-2 in the conditioned medium of cells transduced with Adv-BMP2 had a molecular weight of 21 kDa, consistent with the size of mature BMP-2. The alkaline phosphatase activity of cells transduced with Adv-BMP2 showed a dramatic increase compared with the activity of control cells. Seven days af-

ter transduction, Adv-BMP2-transduced cells had as much as five times the alkaline phosphatase activity of control cells.

The level of type-I collagen in Adv-BMP2-transduced cells was greatly increased compared with the levels in Adv- β gal-transduced cells and untreated cells. In addition, osteopontin and bone sialoprotein expression was increased in Adv-BMP2-transduced cells. Matrix mineralization occurred in Adv-BMP2-transduced cells by eighteen days after transduction, whereas the matrices of the Adv- β gal-transduced cells and the untransduced cells remained unmineralized (Fig. 2).

Computed Tomographic Assessment

Sagittally reconstructed computed tomographic images clearly demonstrated increased bone formation in the disc spaces injected with Adv-BMP2-transduced cells compared with the control disc spaces (Fig. 3). The attenuation measurements in these disc spaces were much higher than those in the control disc spaces and were close to those in the area of the cortical end plates. The mean value of the attenuation was 562 Hounsfield units (HU) for the experimental discs compared with 186 HU for the controls ($p < 0.001$). The disc alignment was neutral.

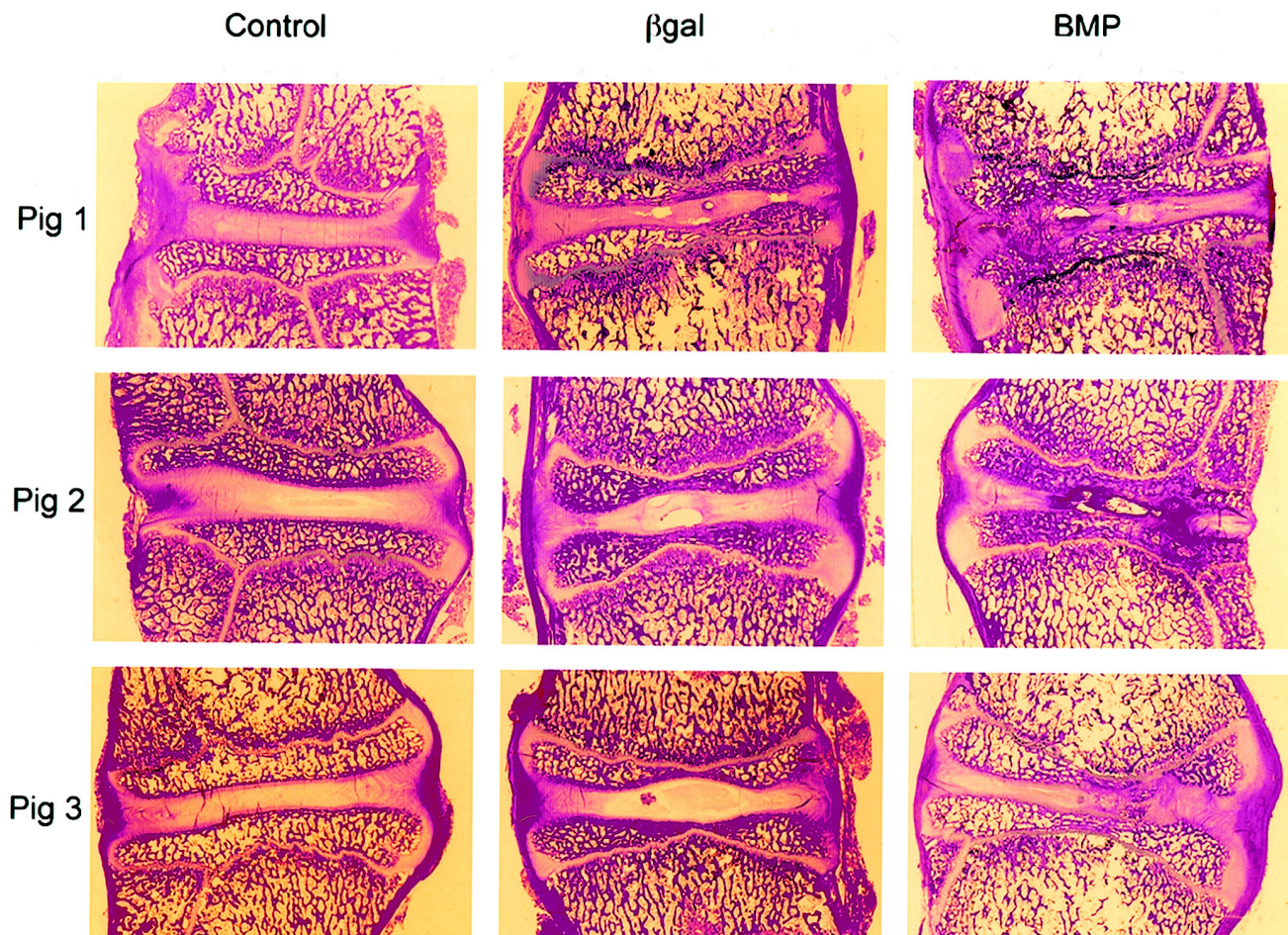


Fig. 5

Low-power photomicrographs of the control, Adv- β gal-treated, and Adv-BMP2-treated discs for the three pigs. Note that only the Adv-BMP2-treated discs demonstrate bone formation.

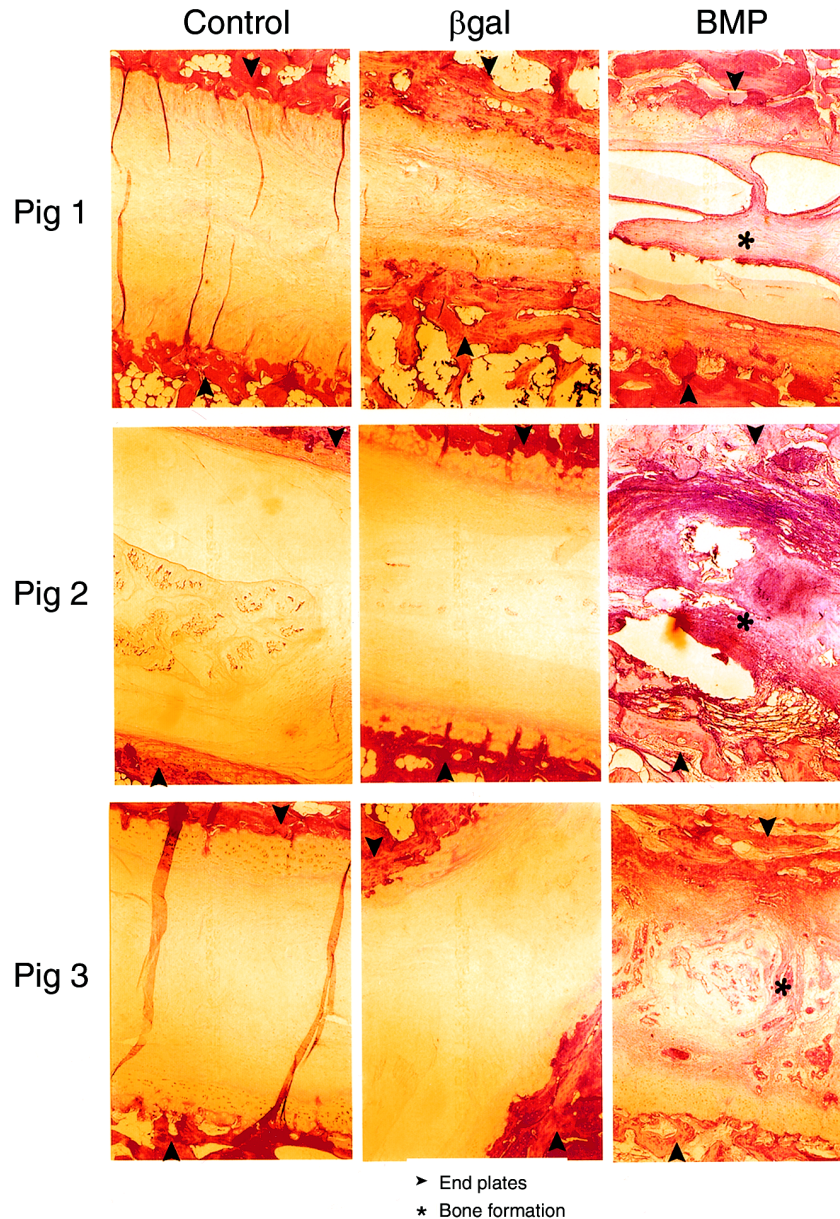


Fig. 6
Medium-power photomicrographs of the control, Adv- β gal-treated, and Adv-BMP2-treated discs for the three pigs. The arrowheads point to the end plates of each disc, and the asterisks show the newly formed bone.

Histological Assessment

Solid bone formed across all six of the disc spaces that had been treated with Adv-BMP2. The bone bridged the superior and inferior end plates of each of the disc spaces. In contrast, none of the three disc spaces that had received Adv- β gal and none of the three sham-operated disc spaces had any bone formation (Figs. 4, 5, and 6).

Discussion

In the present study of anterior spinal fusion with use of gene therapy, we used minimally invasive means and no in-

strumentation. We observed contiguous bone formation from one end plate to the adjacent end plate in all six disc spaces (two discs in each of three pigs) that had been treated with implantation of Adv-BMP2-transduced mesenchymal stem cells. There was no new-bone formation in any of the control discs that had been treated with the sham operation (three discs) or with implantation of Adv- β gal-transduced mesenchymal stem cells (three discs). These findings suggest that when mesenchymal stem cells carrying the gene for BMP-2 are placed into a minimally disrupted intradiscal space, they are capable of producing end plate-to-end plate bone formation.

The intervertebral disc is unique in that it is relatively avascular and acellular. We thought that in such an environment it might be advantageous to add mesenchymal stem cells that were capable of differentiating into an osteoblastic lineage. Previously, we performed a pilot experiment in which we disrupted the intradiscal space and injected BMP-2 (unpublished data). Because we were unable to demonstrate any new-bone formation, we abandoned this technique. In contradistinction to the findings that have been noted following injections of BMP-2 into muscle and other tissues that are cellular and vascular, we believe that our failure to demonstrate any new-bone formation might have been due to the relative avascularity and acellularity of the intradiscal space. This failure might also have been due to an inadequate dosage of drug. Muschik et al. were able to induce anterior spinal fusion with use of bovine morphogenetic protein in rabbits⁷. While other authors have achieved anterior spinal fusion with use of bone morphogenetic proteins, they also used either metallic or bone implants^{6,10,11}.

Because the current investigation was a limited pilot study to determine if our techniques could be successfully used to produce intradiscal bone, we did not make adequate provisions to perform biomechanical testing. Further experiments that will include biomechanical testing are being planned. In addition, there was no attempt to quantitate the actual amount of bone formation. We believe that because blinded radiographic and histological evaluators were able to determine easily and conclusively that end plate-to-end plate bone formation was present in the experimental discs but not in the control discs, actual quantification was unnecessary.

Whether the results that we obtained in this porcine model might be extrapolated to humans is unknown. Although the posterior intertransverse process fusion rate in the rabbit model appears to be similar to that in humans, the rate in the porcine model has never been compared with that in humans, to our knowledge. On the other hand, the porcine model has been

widely utilized for studies on endoscopic procedures because of its anatomic similarity to humans¹²⁻¹⁴. While our results suggest the eventual possibility of similar results in humans, there are numerous technical and practical hurdles that must first be overcome. Not the least of these is that the issue of safety surrounding the use of viral vectors must first be adequately addressed.

In summary, all six discs that had been treated with implantation of adenovirally transduced mesenchymal stem cells carrying the gene for BMP-2 demonstrated fusion whereas all six control discs remained unfused. Although we utilized thoracoscopic techniques to perform these operations, we believe that our techniques could be modified to achieve similar results with use of fluoroscopically controlled percutaneous injections. ■

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