[Frontiers in Bioscience 13, 833-841, January 1, 2008]

Bone tissue engineering and repair by gene therapy

Volker M. Betz[1], Oliver B. Betz[1], Mitchel B. Harris[1], Mark S. Vrahas[1], Christopher H. Evans[1]

[1]Center for Molecular Orthopaedics, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA

TABLE OF CONTENTS

1. ABSTRACT

Many clinical conditions require the stimulation of bone growth. The use of recombinant bone morphogenetic proteins does not provide a satisfying solution to these conditions due to delivery problems and high cost. Gene therapy has emerged as a very promising approach for bone repair that overcomes limitations of protein-based therapy. Several preclinical studies have shown that gene transfer technology has the ability to deliver osteogenic molecules to precise anatomical locations at therapeutic levels for sustained periods of time. Both *in-vivo* and *ex-vivo* transduction of cells can induce bone formation at ectopic and orthotopic sites. Genetic engineering of adult stem cells from various sources with osteogenic genes has led to enhanced fracture repair, spinal fusion and rapid healing of bone defects in animal models. This review describes current viral and non-viral gene therapy strategies for bone tissue engineering and repair including recent work from the author's laboratory. In addition, the article discusses the potential of gene-enhanced tissue engineering to enter widespread clinical use.

2. INTRODUCTION

There is a pressing clinical need for reliable, effective, and expeditious bone regeneration strategies. Delayed fracture healing and bone loss associated with trauma, revision joint arthroplasty, tumor resection and pseudarthrosis of the spine remain challenges for the surgeon. Autografting has become the gold standard of repair for osseous defects (1), but this technique exposes patients to additional surgical procedures and the amounts of bone available for autografting are limited (2). Of the 500,000 bone-grafting procedures that are performed in the United States of America annually, 50% are related to spinal fusion (3). 25% percent of these patients complain about donor-site pain for up to 2 years after surgery and failure to achieve solid bony union occurs in up to 30% of patients (4, 5). Therefore, there is great interest in identifying alternative approaches to stimulate bone formation and regeneration.

Osteoinductive cytokines have been under investigation since Urist demonstrated the osteoinductive capacity of demineralized bone matrix (6). The Bone

Morphogenetic Protein (BMP) family of cytokines has been extensively studied, with 20 members already identified (7, 8). BMP-2 and BMP7- have been approved for clinical use and constitute novel tools of treating non-union fractures and spinal fusion (9, 10). Using recombinant human BMP-2 (rhBMP-2) for the treatment of open tibial fractures resulted in faster healing, 44% reduced risk of failure to heal and less infections (11). When used for spinal fusion, rhBMP-2 led to a higher fusion rate than autograft (10). For posterolateral lumbar arthrodesis rhBMP-7 proved to be more effective and safer than autograft (12). When rhBMP-7 protein was used for the treatment of long-bone fractures the results were comparable to bone grafting (5).

These clinical data confirm the great potential of molecular therapy for human bone tissue engineering. However, the application of these recombinant proteins has been impeded by delivery problems. Such protein-based therapies may not be optimal due to the short protein half-life and the poor retention of the protein in the defect site (13). The doses of recombinant protein required to accelerate healing are significantly higher than the levels expressed during normal bone repair (14). Such large amounts of recombinant proteins are expensive to produce. Gene transfer can overcome these limitations and provide a cost-effective solution. Delivery of genes encoding osteogenic growth factors can provide high, sustained concentrations of these factors locally for extended periods of time (15, 16). Moreover, endogenously synthesized proteins may have greater biological effectiveness than their exogenous, recombinant counterparts (17, 18).

Bone formation has been studied rigorously and is known to be a well-orchestrated process in which osteogenic factors play a major role together with osteoprogenitor cells. There is great interest in developing gene therapy strategies to enhance bone repair because the potential for sustained production of these osteogenic factors may enable osteoprogenitor cells to respond in a more robust fashion (19). Several gene therapy approaches have been successful in preclinical studies and are described in this review.

For clinical use, new bone repair and regeneration technologies must be cost-effective. In response to this, our group is attempting to develop novel, expedited strategies for bone repair that avoid cell isolation and long-term culture. Our novel approaches are discussed and compared to existing gene therapy methods in terms of their potential for translation into a clinical setting.

3. GENE DELIVERY VECTORS

Vectors are defined as vehicles for the delivery of genes to host cells. To achieve expression, DNA must enter the cell nucleus and either integrate into the chromosomes of the host cells or remain separate as an episome. DNA gets transcribed into mRNA, mRNA is then transported outside the nucleus, to the ribosomes, where the translation into proteins occurs. Consequently, the host cells become factories producing bioactive molecules that induce bone growth. For delivery of genetic material into cells, viral vectors (transduction) and non-viral vectors (transfection) can be used.

Viral vectors currently represent the most efficient method of gene delivery since they have highly evolved mechanisms to introduce DNA into cells. In viruses that are utilized for gene-enhanced bone tissue engineering pathogenic genes are removed and replaced by osteogenic genes. Scientists take advantage of the virus' natural tendency to enter cells and integrate its genetic material into the nucleus. A wide variety of viral constructs have been investigated for gene therapy, with the most common including adenovirus, retrovirus, lentivirus and adeno-associated virus (20).

Adenoviral vectors (Ad) are very attractive gene delivery vehicles because they can be purified at high titers and can infect a broad range of cell types. In many experiments adenoviral gene transfer has led to high level gene expression and induction of bone formation (19, 21, 22). For tissue repair, adenovirus has many advantages since it is easy to prepare, non-integrating and able to infect cells *in situ* with high transduction efficiency. Cells transduced with first generation adenovirus vectors *in vivo* typically express transgenes at high levels for 2-3 weeks, after which time expression quickly falls with complete loss of expression by about 6 weeks (23, 24). This may be an ideal expression profile for the healing of bone fractures.

Retroviral and lentiviral vectors integrate their genetic material into the host cell genome and provide sustained, long-term gene expression. These vectors are interesting candidates for gene therapy approaches because of their low immunogenity. The use of retrovirus and lentivirus as delivery vehicles for osteogenic genes may be an appropriate stragety for bone repair applications requiring long-term expression such as the treatment of very large segmental defects after severe trauma (25). To avoid unregulated overexpression and bone overproduction, inducible expression systems have been developed (26, 27). These systems contain an inducible promoter that can be activated or inactivated by exogenous chemical agents (e.g. tetracycline) to regulate expression of the osteogenic gene.

Adeno-associated virus (AAV) has a superior safety profile. It is non-pathogenic, non-immunogenic and the recombinant virus does not integrate into the host cell genome. Successful application of adeno-associated virus technology to bone repair is just beginning to be realized (27). Once production of this vector is simplified this gene delivery vehicle may be a promising candidate for gene-enhanced bone tissue engineering.

Non-viral vectors have been intensively studied during the last decade and typically consist of plasmid DNA alone or in combination with a carrier. Non-viral gene delivery vectors are safe, easy to prepare and cost-effective. However, transfection efficiency is low and gene expression is only transient. Electroporation has been applied to transfer osteogenic genes and induce bone formation (28). This technology uses electric pulses to

transport DNA into host cells and holds promise for orthopaedic gene therapy. The encapsulation of DNA within liposomes is another strategy to transfer osteogenic genes to cells without the help of a virus. Efficiency is lower compared to viral delivery and expression is brief but bone formation has occurred after liposomal gene transfer (29). A third strategy that has been used for non-viral delivery of osteogenic genes is the use of gene-activated matrices (GAM). Gene-activated matrices are biomaterials that incorporate and slowly release DNA. Some success has been achieved with these matrices and improved chemical methods are currently being developed in order to improve DNA delivery (30, 31).

4. TARGET GENES

Molecular biologists have identified numerous bioactive factors that induce or support bone regeneration including bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF-beta), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), LIM mineralization protein-1 (LMP-1), vascular endothelial growth factor (VEGF) and the constitutively active form of the activin receptor-like kinase-2 (caAlk2). All of these biological factors have been investigated for their potential use in bone tissue engineering and repair.

BMPs are the most widely studied osteogenic growth factors and have proven to be very potent inducers of ectopic and orthotopic bone formation. Most gene therapies for bone repair have been conducted using BMP genes. BMPs bind to transmembrane receptors to initiate signaling cascades which induce osteogenesis through autocrine and paracrine signaling. A recent study compared the osteogenic activity of 14 BMPs (BMP-2 to BMP-15) and showed that BMP-2, -6 and -9 are the most potent inducers of osteoblast differentiation of mesenchymal stem cells (32). The BMPs were delivered by adenoviral gene transfer and their osteogenic effect was evaluated *in vitro* using three different cell types. Another study compared the potential of these 14 BMPs to induce ectopic bone formation *in vivo* (33). Again, the BMPs were delivered by gene transfer using adenovirus and a superiority of BMP-2, -6, -7 and -9 was found.

TGF-betas have regulatory effects during skeletal development and fracture repair by stimulating osteoid formation and osteoblast proliferation. Investigations into the differential temporal expression of members of the TGF-beta superfamily during murine fracture healing have shown that TGF-beta1 remains high throughout the fracture healing process and TGF-beta2 and TGF-beta3 expression peak during chondrogenesis (34). Several *in vivo* studies have demonstrated that TGF-betas enhance callus formation and mechanical strength compared to untreated fractures (35, 36).

IGFs are known to enhance collagen synthesis and cell proliferation and stimulate osteoblast chemotaxis (37, 38). When administered systemically IGF-1 treatment accelerated bone healing *in vivo* (39). Local delivery of IGF-1 led to bone repair in a sheep model (40). Synergistic osteoinductive effects are postulated for the combinations of IGF-1 / TGF-β and IGF-1 / BMP-7 (41, 42).

FGFs enhance migration, proliferation and differentiation of osteoprogenitor cells (43). Basic FGF has been reported to increase the amount of callus and stimulate fracture repair in nonhuman primates (44). Local application of basic FGF resulted in healing of segmental bone defects in rabbits (45). In a clinical trial, recombinant human FGF-2 repaired osteotomies in humans (46).

LMP-1 is a novel intracellular LIM domain protein, which initiates membranous bone formation *in vitro* and *in vivo* (47). Unlike BMPs, which are extracellular proteins that act through cell surface receptors, LMP-1 is thought to be an intracellular signaling molecule that is directly involved in osteoblast differentiation. Thus, therapeutic use of LMP-1 requires gene transfer of its cDNA. LMP-1 overexpression led to BMP-2, -4, -6 and -7 expression *in vitro* (48). *In vivo,* LMP-1 gene transfer proved to be an attractive new treatment modality for spine fusion (49).

VEGF is a key component in bone formation. Angiogenesis mediated by VEGF is important for the coupling of cartilage resorption and mineralized bone formation during endochondral ossification in bone development (50). In addition to interacting with certain humoral factors that regulate bone homeostasis, VEGF can interact synergistically with osteogenic proteins, such as BMP-2 or BMP-4, to promote bone formation and bone healing by enhancing cell recruitment, prolonging cell survival, and increasing angiogenesis (51, 52). These effects of VEGF lead to enhanced cartilage formation, accelerated resorption, and improved bone formation (51, 52).

caAlk2 is a receptor that mediates BMP signaling. Overexpression of this receptor generates signals similar to BMP and induces chondrogenesis and endochondral bone formation (53). Low levels of *in vivo* expression are required to induce significant bone formation. caAlk2 signals cannot be blocked by endogenous BMP antagonists like noggin and chordin which makes caAlk2 an interesting tool for gene-enhanced bone engineering (54).

5. *IN-VIVO* GENE THERAPY

In-vivo gene delivery involves directly delivering the gene into an anatomic site by transducing or transfecting local cells. The advantage of this approach is that it requires only one step and if an off-the-shelf product could be developed, it would be very popular with surgeons. In addition, morbidity associated with the harvesting of autologous cells is avoided.

Gene-activated matrices (GAM) have been used to deliver naked DNA to bone defects. Fang *et al.* implanted GAM made of bovine tracheal collagen carrying BMP-4 plasmids into segmental bone defects in rats (30). Bridging was seen after nine weeks. Another group was

recently able to improve the efficiency of GAM by chemical modification using calcium precipitates (31). This modified matrix led to improved bone defect healing using less plasmid. Despite these promising results, GAMs have not lived up to their early promise, partly because transfection is still relatively inefficient and levels of transgene expression are low. In our experience, virally mediated gene transfer is needed to provoke robust healing responses in experimental animals.

Recombinant adenovirus and retrovirus carrying cDNA encoding bone morphogenetic protein (BMP)-2, -6 or -9 have been evaluated in several different types of osseous lesions. Our group achieved enhanced bone repair when adenovirus carrying the BMP-2 gene (Ad.BMP-2) was injected into critical sized femoral defects in rats and rabbits (21, 55). Although there is concern over possible spread of the adenovirus from its site of application, studies in the rabbit model suggest that, when injected into a critical sized segmental bone defect, the transgene is expressed mostly by muscle surrounding the defect, with very little expression elsewhere in the body (56). In other studies, direct administration of Ad.BMP-2 or Ad.BMP-9 led to regeneration of critical sized mandibular defects in rats (57). Ad.BMP-2 also enhanced bone formation in conjunction with distraction osteogenesis in a rat, mandibular defect (58). Bertone *et al.* accelerated bone repair in a rabbit ulnar osteotomy model by injection of Ad.BMP-6 (59). Direct Ad.BMP-6 gene therapy was also shown to promote spine fusion in New Zealand white rabbits (60). Zhu *et al.* studied the effect of combined administration of Ad.BMP-2 and Ad.BMP-7 in a rat spinal fusion model (61). The combination of these two vectors resulted in significantly more bone formation than the use of a single vector. Rundle *et al.* used a retroviral vector encoding BMP-4 (62). Direct injection of this vector into a fracture site accelerated healing in a rat model.

In a recent study, a novel, unconventional adeno-associated virus based *in-vivo* approach for bone allograft healing was presented (63). Recombinant AAV that expresses the angiogenic molecule vascular endothelial growth factor (VEGF) and the receptor activator of the nuclear factor kappa B (NFkappaB) ligand was freeze-dried onto the surface of femoral allografts. This treatment stimulated vascular invasion and remodeled the dead bone into live cortical bone. In another experiment of the same group, allograft bone coated with freeze-dried AAV encoding caAlk2 was implanted into femoral defects in mice (54). BMP signals delivered via AAV-caAlk2 coating induced endochondral bone formation directly on the surface of the allograft.

All of these studies demonstrated that, by direct *in-vivo* gene delivery, bone induction and repair can be elicited precisely at specific anatomic sites in the body. Although the idea of repairing bone by direct *in-vivo* delivery of vector is appealing due to its simplicity and its potential for lower cost, there are obstacles to overcome before this technology can be translated into a clinical setting. Problems are the difficulty of targeting specific cells and the risk of inducing an immune-response to the vector. These challenges are currently addressed by developing vectors with enhanced tropism and less immunogenity (64, 65).

6. EX-VIVO GENE THERAPY

Ex-vivo gene therapy approaches avoid safety problems associated with *in-vivo* gene delivery because transfer of target genes occurs outside of the body. Specific cellular vehicles can be selected, genetically engineered and implanted within a bone lesion. Transduction and transfection efficiencies are increased since it can be performed *in vitro* under controlled conditions. The potential of this cell-based gene delivery strategy for bone repair has been explored using a wide variety of cell types, including cells derived from bone marrow, muscle, and fat tissue.

Lieberman and associates harvested bone marrow cells from rats, expanded them in tissue culture, transduced the cells with an Ad.BMP-2 vector and then implanted them in a critical-sized segmental defect (19). Interestingly, compared to rhBMP-2 treatment, histology revealed a more robust pattern of the newly formed bone in defects when treated with genetically engineered BMP-2 expressing cells. The authors hypothesized this could be due to a more continuous and physiological release of growth factor by engineered cells compared to the release kinetics from the demineralized bone matrix used as a protein carrier. Peterson *et al.* used the same strategy to study spine fusion in a rat model (66). 100% of the spines were fused using BMP-2 transduced bone marrow cells. In contrast, bone marrow cells that were not transduced did not induce fusion of the spine. Others have worked on non-viral gene transfer approaches using bone marrow cells. Park *et al.*, for example, have achieved bone regeneration in a rat mandibular bone defect model using liposome mediated transfection of bone marrow cells (29). Another group reported success with electroporation (67). In this study, nucleofected bone marrow derived cells were used to form bone ectopically. It remains to be seen if this exciting non-viral gene delivery technology can induce bone growth at orthotopic sites and elicit robust bone repair.

Muscle tissue represents an alternative source of adult stem cells. Huard and colleagues have demonstrated that genetically engineered muscle derived cells differentiate towards the osteogenic lineage and induce bone formation in a variety of experimental models. In one study, muscle derived stem cells were transduced with adenovirus carrying the BMP-2 gene and implanted in skull defects in mice (68). Full closure of the defects was seen as early as four weeks after treatment. In another study retrovirally BMP-4 transduced muscle derived stem cells induced ectopic and orthotopic bone formation (69). It has also been shown that functional healing can be achieved when muscle derived stem cells that express BMP-4 are implanted within a 7 mm critical-sized segmental bone defect in rats (70). Femora were tested biomechanically and proved to be almost as stable as intact, contralateral femora. In another experiment, to enhance the bone healing process, Huard's group transduced muscle derived stem cells to

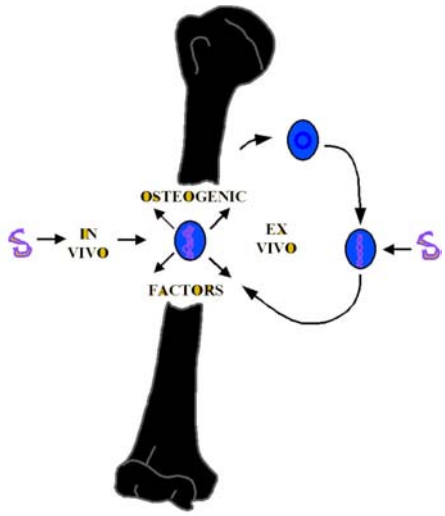

Figure 1. Expedited *ex-vivo* gene therapy for bone repair. Radiographs of 5mm rat femoral defects 8 weeks after surgery: untreated control defect (A), defects treated with unmodified muscle grafts (B) or unmodified fat grafts (C).

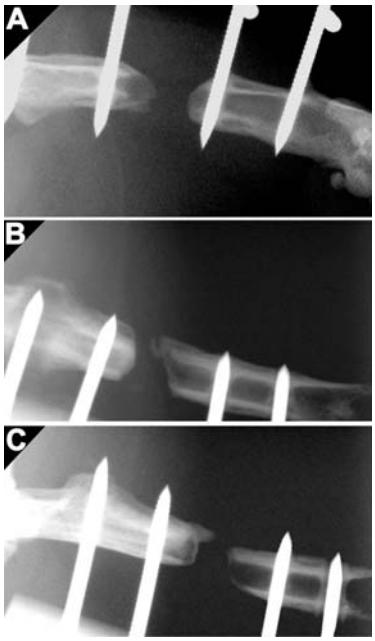

Figure 2. Expedited *ex-vivo* gene therapy for bone repair. Radiographs of 5mm rat femoral defects: Defect treated with Ad.BMP-2 activated muscle grafts at 2 weeks (A) and 8 weeks (B) after surgery and implantation. Defect treated with Ad.BMP-2 activated fat grafts at 2 weeks (C) and 8 weeks (D) after surgery and implantation.

express either human BMP-4 or VEGF and co-implanted these cells *in vivo* (51). VEGF significantly improved the efficacy of BMP-4-elicited bone formation and regeneration by enhancing angiogenesis. Administration of growth factor combinations is an interesting strategy for enhanced stimulation of bone formation. In further studies, the potential of different growth factor combinations for bone repair should be investigated.

Fat tissue has recently been discovered as an attractive source of stem cells that show potential as agents to be used in bone tissue engineering (71, 72). Human adipose tissue derived stem cells have the ability to differentiate into osteogenic cells *in vitro* when exposed to BMPs (73). Implantation of human fat derived cells overexpressing BMP-2 led to ectopic bone formation in mice (74). Peterson *et al.* healed critical size femoral defects in nude rats by implantation of Ad.BMP-2 transduced mesenchymal stem cells isolated from human adipose tissue (75). Multipotent fat stem cells can be isolated from liposuction aspirates or from the infrapatellar fat pad of the knee. Adipose tissue represents a very appealing source of cells useful for tissue engineering since there is minimal donor site morbidity and a high number of stem cells can be obtained (76).

These impressive pre-clinical data demonstrate the great potential of *ex-vivo* gene therapy for bone tissue engineering and repair. However, the disadvantage of this approach is that it requires the isolation and *ex vivo* culture of autologous cells which makes this treatment modality cumbersome, time-consuming and expensive. For these reasons, our laboratory focuses on alternative gene therapy strategies, like the expedited *ex-vivo* gene therapy approach.

7. EXPEDITED EX-VIVO GENE THERAPY

We have developed an expedited *ex-vivo* gene therapy approach that does not require isolation and long-term culture of cells. It is an attempt to simplify gene-based bone repair without making compromises in terms of safety. The idea is to harvest autologous tissue fragments from a patient and transfer osteogenic genes directly to these tissue grafts without extracting and expanding cells.

In our experiments, treatment of fat and muscle fragments with Ad.GFP efficiently transduced cells on the surface of the tissue. Under *in vitro* conditions, marker gene expression persisted for several weeks. Use of an Ad.BMP-2 vector led to high levels of BMP-2 expression and induction of alkaline phosphatase and other markers of osteogenesis within the tissue fragments. In a preliminary *in vivo* study, we explored BMP-2 gene activated autologous fat and muscle grafts as endogenous, regenerative, osteoinductive structures. Tissue grafts were harvested from rats and transduced using adenovirus. After transduction, the activated grafts were washed to remove free virus and then implanted into a 5 mm critical size defect in the rat femur. Control defects remained untreated or received unmodified tissue grafts.

Figures 1 to 4 show the results of this pilot experiment. At 2 weeks after implantation of BMP-2 gene activated grafts, there was already an osteogenic response within the lesion, and at 8 weeks complete union of the bone ends had occurred, as visualized by radiography (Figure 2). In contrast, femora that remained untreated or received unmodified tissue grafts did not heal (Figure 1). Histologically, femora treated with Ad.BMP-2 activated tissue grafts showed complete union and formation of a

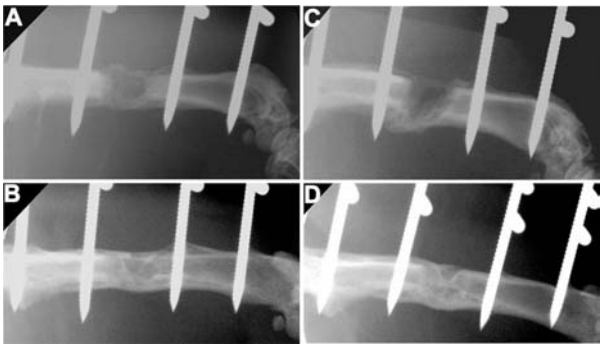

Figure 3. Expedited *ex-vivo* gene therapy for bone repair. Histological sections from mid-defect regions of 5mm rat femoral defects treated with unmodified muscle grafts (A) or unmodified fat grafts (B) 8 weeks after surgery and implantation. The slides were stained with safranin O-fast green (magnification x 10).

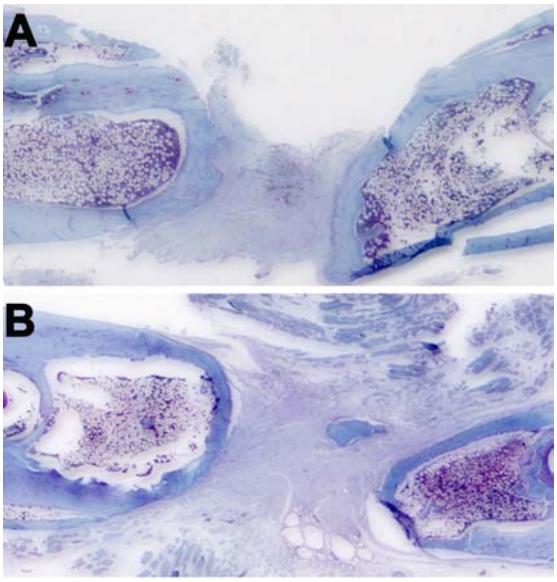

Figure 4. Expedited *ex-vivo* gene therapy for bone repair. Histological sections from mid-defect regions of 5mm rat femoral defects treated with Ad.BMP-2 activated muscle grafts (A) or Ad.BMP-2 activated fat grafts (B) 8 weeks after surgery and implantation. The slides were stained with safranin O-fast green (magnification x 12).

neo-cortex at 8 weeks after surgery (Figure 4). Control defects treated with unmodified fat or unmodified muscle tissue did not heal (Figure 3).

8. THE POTENTIAL OF GENE-ENHANCED BONE REPAIR STRATEGIES FOR CLINICAL USE

Protein-based therapy has found acceptance among surgeons. Human recombinant BMP-2 and BMP-7 have been approved for clinical use and there is no doubt that, in the near future, molecular biologists will bring more biologicals to the orthopaedic field. We believe that gene therapy is the next logical step to follow protein therapy. Gene transfer technology offers a sophisticated solution for delivery problems experienced with recombinant protein therapy.

Patient safety must, however, take priority, since these new molecular therapies will be used to improve the patient's quality of life and not to cure life-threatening illness. Tissue engineering strategies must also be cost-effective in order to draw interest from commercial entities. Our expedited *ex-vivo* gene therapy approach seems to fulfill these requirements and solve problems associated with current cell-based tissue engineering methods. With this new technology, vector is not directly introduced into the body and no time-consuming and costly cell expansion is necessary. The technology may have the potential to be applied in a one-step surgical procedure.

In contrast, conventional *ex-vivo* gene therapy requires expensive and time-consuming isolation and culture of autologous cells and the use of two invasive clinical procedures. The single biggest problem lies with the cost and complexity of undertaking autologous cell culture in a GMP (good manufacturing practice) facility. Not only is the facility itself expensive to create and maintain, but the culture media, sera and other necessities of cell culture are unavoidable and very costly. The need for separate procedures to harvest primary autologous tissue and then to implant the engineered product creates a second economic and logistical burden.

In-vivo gene therapy is another expedited, one-step approach that has the potential for lower cost. There is significant interest in developing an *in-vivo* gene therapy approach for bone repair because it would be technically simple to use in the operating room. Once vectors are available, that allow specific cell-targeting *in-vivo* and display attractive safety profiles, *in-vivo* gene therapy will have to be considered as a straightforward bone repair strategy with great potential for translation into a clinical setting.

9. PERSPECTIVE

Gene-based technologies for bone repair will gain a central place in the field of bone tissue engineering. There is a growing need for improved biological solutions to create and regenerate bone and pre-clinical data have shown that gene therapy may be the answer. However, to enter widespread clinical use, a technology must be not only scientifically sound but also cost-effective and well suited to clinical application. Now, that we have proven effectiveness of gene therapy for bone repair in a large variety of animal models, we have to focus on the development of expedited approaches. Once such expedited approaches prove to be safe, we will be able to translate the technology from bench to bedside.

10. ACKNOWLEDGMENT

Results from our laboratory discussed here were funded by the NIH (grant AR050243).

11. REFERENCES

1. U. Kneser, D. J. Schaefer, E. Polykandriotis & R. E. Horch: Tissue engineering of bone: the reconstructive surgeon's point of view. *J Cell Mol Med* 10, 7-19 (2006)
2. N. R. Colterjohn & D. A. Bednar: Procurement of bone graft from the iliac crest. An operative approach with decreased morbidity. *J Bone Joint Surg Am* 79, 756-759 (1997)
3. R. W. Bucholz: Nonallograft osteoconductive bone graft substitutes. *Clin Orthop Relat Res* 44-52 (2002)
4. J. C. Steinmann & H. N. Herkowitz: Pseudarthrosis of the spine. *Clin Orthop Relat Res* 80-90 (1992)
5. S. D. Boden: The ABCs of BMPs. *Orthop Nurs* 24, 49-52; quiz 53-44 (2005)
6. M. R. Urist: Bone: formation by autoinduction. *Science* 150, 893-899 (1965)
7. P. De Biase & R. Capanna: Clinical applications of BMPs. *Injury* 36 Suppl 3, S43-46 (2005)
8. E. Carlisle & J. S. Fischgrund: Bone morphogenetic proteins for spinal fusion. *Spine J* 5, 240S-249S (2005)
9. G. E. Friedlaender, C. R. Perry, J. D. Cole, S. D. Cook, G. Cierny, G. F. Muschler, G. A. Zych, J. H. Calhoun, A. J. LaForte & S. Yin: Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. *J Bone Joint Surg Am* 83-A Suppl 1, S151-158 (2001)
10. S. D. Boden, T. A. Zdeblick, H. S. Sandhu & S. E. Heim: The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. *Spine* 25, 376-381 (2000)
11. S. Govender, C. Csimma, H. K. Genant, A. Valentin-Opran, Y. Amit, R. Arbel, H. Aro, D. Atar, M. Bishay, M. G. Borner, P. Chiron, P. Choong, J. Cinats, B. Courtenay, R. Feibel, B. Geulette, C. Gravel, N. Haas, M. Raschke, E. Hammacher, D. van der Velde, P. Hardy, M. Holt, C. Josten, R. L. Ketterl, B. Lindeque, G. Lob, H. Mathevon, G. McCoy, D. Marsh, R. Miller, E. Munting, S. Oevre, L. Nordsletten, A. Patel, A. Pohl, W. Rennie, P. Reynders, P. M. Rommens, J. Rondia, W. C. Rossouw, P. J. Daneel, S. Ruff, A. Ruter, S. Santavirta, T. A. Schildhauer, C. Gekle, R. Schnettler, D. Segal, H. Seiler, R. B. Snowdowne, J. Stapert, G. Taglang, R. Verdonk, L. Vogels, A. Weckbach, A. Wentzensen & T. Wisniewski: Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J Bone Joint Surg Am* 84-A, 2123-2134 (2002)
12. A. R. Vaccaro, D. G. Anderson, T. Patel, J. Fischgrund, E. Truumees, H. N. Herkowitz, F. Phillips, A. Hilibrand, T. J. Albert, T. Wetzel & J. A. McCulloch: Comparison of OP-1 Putty (rhBMP-7) to iliac crest autograft for posterolateral lumbar arthrodesis: a minimum 2-year follow-up pilot study. *Spine* 30, 2709-2716 (2005)
13. R. Talwar, L. Di Silvio, F. J. Hughes & G. N. King: Effects of carrier release kinetics on bone morphogenetic protein-2-induced periodontal regeneration *in vivo*. *J Clin Periodontol* 28, 340-347 (2001)
14. H. Uludag, T. Gao, T. J. Porter, W. Friess & J. M. Wozney: Delivery systems for BMPs: factors contributing to protein retention at an application site. *J Bone Joint Surg Am* 83, 128-135 (2001)
15. C. H. Evans, S. C. Ghivizzani & P. D. Robbins: The 2003 Nicolas Andry Award. Orthopaedic gene therapy. *Clin Orthop Relat Res* 316-329 (2004)
16. A. A. Scaduto & J. R. Lieberman: Gene therapy for osteoinduction. *Orthop Clin North Am* 30, 625-633 (1999)
17. C. H. Evans & P. D. Robbins: Possible orthopaedic applications of gene therapy. *J Bone Joint Surg Am* 77, 1103-1114 (1995)
18. S. S. Makarov, J. C. Olsen, W. N. Johnston, S. K. Anderle, R. R. Brown, A. S. Baldwin, Jr., J. S. Haskill & J. H. Schwab: Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA. *Proc Natl Acad Sci U S A* 93, 402-406 (1996)
19. J. R. Lieberman, A. Daluiski, S. Stevenson, L. Wu, P. McAllister, Y. P. Lee, J. M. Kabo, G. A. Finerman, A. J. Berk & O. N. Witte: The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. *J Bone Joint Surg Am* 81, 905-917 (1999)
20. T. J. Oligino, Q. Yao, S. C. Ghivizzani & P. Robbins: Vector systems for gene transfer to joints. *Clin Orthop Rel Res* 17-30 (2000)
21. O. B. Betz, V. M. Betz, A. Nazarian, C. G. Pilapil, M. S. Vrahas, M. L. Bouxsein, L. C. Gerstenfeld, T. A. Einhorn & C. H. Evans: Direct percutaneous gene delivery to enhance healing of segmental bone defects. *J Bone Joint Surg Am* 88, 355-365 (2006)
22. D. S. Musgrave, P. Bosch, S. Ghivizzani, P. D. Robbins, C. H. Evans & J. Huard: Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone* 24, 541-547 (1999)
23. H. Cao, D. R. Koehler & J. Hu: Adenoviral vectors for gene replacement therapy. *Viral Immunol* 17, 327-333 (2004)
24. I. M. Verma & N. Somia: Gene therapy -- promises, problems and prospects. *Nature* 389, 239-242 (1997)
25. O. Sugiyama, D. S. An, S. P. Kung, B. T. Feeley, S. Gamradt, N. Q. Liu, I. S. Chen & J. R. Lieberman: Lentivirus-mediated gene transfer induces long-term transgene expression of BMP-2 *in vitro* and new bone formation *in vivo*. *Mol Ther* 11, 390-398 (2005)
26. H. Peng, A. Usas, B. Gearhart, B. Young, A. Olshanski & J. Huard: Development of a self-inactivating tet-on retroviral vector expressing bone morphogenetic protein 4 to achieve regulated bone formation. *Mol Ther* 9, 885-894 (2004)
27. Y. Gafni, G. Pelled, Y. Zilberman, G. Turgeman, F. Apparailly, H. Yotvat, E. Galun, Z. Gazit, C. Jorgensen & D. Gazit: Gene therapy platform for bone regeneration using an exogenously regulated, AAV-2-based gene expression system. *Mol Ther* 9, 587-595 (2004)
28. M. Kawai, K. Bessho, S. Kaihara, J. Sonobe, K. Oda, T. Iizuka & H. Maruyama: Ectopic bone formation by human bone morphogenetic protein-2 gene transfer to skeletal muscle using transcutaneous electroporation. *Hum Gene Ther* 14, 1547-1556 (2003)
29. J. Park, J. Ries, K. Gelse, F. Kloss, K. von der Mark, J. Wiltfang, F. W. Neukam & H. Schneider: Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. *Gene Ther* 10, 1089-1098 (2003)

30. J. Fang, Y. Y. Zhu, E. Smiley, J. Bonadio, J. P. Rouleau, S. A. Goldstein, L. K. McCauley, B. L. Davidson & B. J. Roessler: Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *PNAS* 93, 5753-5758 (1996)
31. M. Endo, S. Kuroda, H. Kondo, Y. Maruoka, K. Ohya & S. Kasugai: Bone regeneration by modified gene-activated matrix: effectiveness in segmental tibial defects in rats. *Tissue Eng* 12, 489-497 (2006)
32. H. Cheng, W. Jiang, F. M. Phillips, R. C. Haydon, Y. Peng, L. Zhou, H. H. Luu, N. An, B. Breyer, P. Vanichakarn, J. P. Szatkowski, J. Y. Park & T. C. He: Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs).[erratum appears in J Bone Joint Surg Am. 2004 Jan;86-A(1):141]. *J Bone & Joint Surg Am* 85, 1544-1552 (2003)
33. Q. Kang, M. H. Sun, H. Cheng, Y. Peng, A. G. Montag, A. T. Deyrup, W. Jiang, H. H. Luu, J. Luo, J. P. Szatkowski, P. Vanichakarn, J. Y. Park, Y. Li, R. C. Haydon & T. C. He: Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther* 11, 1312-1320 (2004)
34. T. J. Cho, L. C. Gerstenfeld & T. A. Einhorn: Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res* 17, 513-520 (2002)
35. M. Lind, B. Schumacker, K. Soballe, J. Keller, F. Melsen & C. Bunger: Transforming growth factor-beta enhances fracture healing in rabbit tibiae. *Acta Orthop Scan* 64, 553-556 (1993)
36. H. M. Nielsen, T. T. Andreassen, T. Ledet & H. Oxlund: Local injection of TGF-beta increases the strength of tibial fractures in the rat. *Acta Orthop Scand* 65, 37-41 (1994)
37. S. N. Khan, M. P. Bostrom & J. M. Lane: Bone growth factors. *Orthop Clin North Am* 31, 375-388 (2000)
38. T. A. Linkhart, S. Mohan & D. J. Baylink: Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* 19, 1S-12S (1996)
39. S. R. Thaller, A. Dart & H. Tesluk: The effects of insulin-like growth factor-1 on critical-size calvarial defects in Sprague-Dawley rats. *Ann Plast Surg* 31, 429-433 (1993)
40. L. Meinel, E. Zoidis, J. Zapf, P. Hassa, M. O. Hottiger, J. A. Auer, R. Schneider, B. Gander, V. Luginbuehl, R. Bettschart-Wolfisberger, O. E. Illi, H. P. Merkle & B. von Rechenberg: Localized insulin-like growth factor I delivery to enhance new bone formation. *Bone* 33, 660-672 (2003)
41. G. Schmidmaier, B. Wildemann, T. Gabelein, J. Heeger, F. Kandziora, N. P. Haas & M. Raschke: Synergistic effect of IGF-I and TGF-beta1 on fracture healing in rats: single versus combined application of IGF-I and TGF-beta1. *Acta Orthop Scand* 74, 604-610 (2003)
42. L. C. Yeh, M. L. Adamo, M. S. Olson & J. C. Lee: Osteogenic protein-1 and insulin-like growth factor I synergistically stimulate rat osteoblastic cell differentiation and proliferation. *Endocrinology* 138, 4181-4190 (1997)
43. T. Nakamura, K. Hanada, M. Tamura, T. Shibanushi, H. Nigi, M. Tagawa, S. Fukumoto & T. Matsumoto: Stimulation of endosteal bone formation by systemic injections of recombinant basic fibroblast growth factor in rats. *Endocrinology* 136, 1276-1284 (1995)
44. H. Kawaguchi, K. Nakamura, Y. Tabata, Y. Ikada, I. Aoyama, J. Anzai, T. Nakamura, Y. Hiyama & M. Tamura: Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2. *J Clin Endocrin Metabol* 86, 875-880 (2001)
45. K. Inui, M. Maeda, A. Sano, K. Fujioka, Y. Yutani, A. Sakawa, Y. Yamano, Y. Kato & T. Koike: Local application of basic fibroblast growth factor minipellet induces the healing of segmental bony defects in rabbits. *Calcif Tissue Int* 63, 490-495 (1998)
46. H. Kawaguchi, S. Jingushi, T. Izumi, M. Fukunaga, T. Matsushita, T. Nakamura, K. Mizuno, T. Nakamura & K. Nakamura: Local application of recombinant human fibroblast growth factor-2 on bone repair: a dose-escalation prospective trial on patients with osteotomy. *J Orthop Res* 25, 480-487 (2007)
47. M. Viggeswarapu, S. D. Boden, Y. Liu, G. A. Hair, J. Louis-Ugbo, H. Murakami, H. S. Kim, M. T. Mayr, W. C. Hutton & L. Titus: Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation *in vitro* and *in vivo*. *J Bone Joint Surg Am* 83-A, 364-376 (2001)
48. S. T. Yoon, J. S. Park, K. S. Kim, J. Li, E. S. Attallah-Wasif, W. C. Hutton & S. D. Boden: ISSLS prize winner: LMP-1 upregulates intervertebral disc cell production of proteoglycans and BMPs *in vitro* and *in vivo*. *Spine* 29, 2603-2611 (2004)
49. S. D. Boden, L. Titus, G. Hair, Y. Liu, M. Viggeswarapu, M. S. Nanes & C. Baranowski: Lumbar spine fusion by local gene therapy with a cDNA encoding a novel osteoinductive protein (LMP-1). *Spine* 23, 2486-2492 (1998)
50. H. P. Gerber, T. H. Vu, A. M. Ryan, J. Kowalski, Z. Werb & N. Ferrara: VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5, 623-628 (1999)
51. H. Peng, V. Wright, A. Usas, B. Gearhart, H. C. Shen, J. Cummins & J. Huard: Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest* 110, 751-759 (2002)
52. H. Peng, A. Usas, A. Olshanski, A. M. Ho, B. Gearhart, G. M. Cooper & J. Huard: VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. *J Bone Min Res* 20, 2017-2027 (2005)
53. D. Zhang, E. M. Schwarz, R. N. Rosier, M. J. Zuscik, J. E. Puzas & R. J. O'Keefe: ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. *J Bone Miner Res* 18, 1593-1604 (2003)
54. M. Koefoed, H. Ito, K. Gromov, D. G. Reynolds, H. A. Awad, P. T. Rubery, M. Ulrich-Vinther, K. Soballe, R. E. Guldberg, A. S. Lin, R. J. O'Keefe, X. Zhang & E. M. Schwarz: Biological effects of rAAV-caAlk2 coating on structural allograft healing. *Mol Ther* 12, 212-218 (2005)
55. A. W. Baltzer, C. Lattermann, J. D. Whalen, P. Wooley, K. Weiss, M. Grimm, S. C. Ghivizzani, P. D. Robbins & C. H. Evans: Genetic enhancement of fracture repair: healing of an experimental segmental defect by adenoviral transfer of the BMP-2 gene. *Gene Ther* 7, 734-739 (2000)

56. A. W. Baltzer, C. Lattermann, J. D. Whalen, S. Braunstein, P. D. Robbins & C. H. Evans: A gene therapy approach to accelerating bone healing. Evaluation of gene expression in a New Zealand white rabbit model. *Knee Surg Sports Traumatol Arthrosc* 7, 197-202 (1999)
57. T. D. Alden, E. J. Beres, J. S. Laurent, J. A. Engh, S. Das, S. D. London, J. A. Jane, Jr., S. B. Hudson & G. A. Helm: The use of bone morphogenetic protein gene therapy in craniofacial bone repair. *J Craniofacial Surg* 11, 24-30 (2000)
58. R. L. Ashinoff, C. L. Cetrulo, Jr., R. D. Galiano, M. Dobryansky, K. A. Bhatt, D. J. Ceradini, J. t. Michaels, J. G. McCarthy & G. C. Gurtner: Bone morphogenic protein-2 gene therapy for mandibular distraction osteogenesis. *Ann Plast Surg* 52, 585-590; discussion 591 (2004)
59. A. L. Bertone, D. D. Pittman, M. L. Bouxsein, J. Li, B. Clancy & H. J. Seeherman: Adenoviral-mediated transfer of human BMP-6 gene accelerates healing in a rabbit ulnar osteotomy model. *J Orthop Res* 22, 1261-1270 (2004)
60. J. J. Laurent, K. M. Webb, E. J. Beres, K. McGee, J. Li, B. van Rietbergen & G. A. Helm: The use of bone morphogenetic protein-6 gene therapy for percutaneous spinal fusion in rabbits. *J Neurosurg Spine* 1, 90-94 (2004)
61. W. Zhu, B. A. Rawlins, O. Boachie-Adjei, E. R. Myers, J. Arimizu, E. Choi, J. R. Lieberman, R. G. Crystal & C. Hidaka: Combined bone morphogenetic protein-2 and -7 gene transfer enhances osteoblastic differentiation and spine fusion in a rodent model. *J Bone Miner Res* 19, 2021-2032 (2004)
62. C. H. Rundle, N. Miyakoshi, Y. Kasukawa, S. T. Chen, M. H. Sheng, J. E. Wergedal, K. H. Lau & D. J. Baylink: *In vivo* bone formation in fracture repair induced by direct retroviral-based gene therapy with bone morphogenetic protein-4. *Bone* 32, 591-601 (2003)
63. H. Ito, M. Koefoed, P. Tiyapatanaputi, K. Gromov, J. J. Goater, J. Carmouche, X. Zhang, P. T. Rubery, J. Rabinowitz, R. J. Samulski, T. Nakamura, K. Soballe, R. J. O'Keefe, B. F. Boyce & E. M. Schwarz: Remodeling of cortical bone allografts mediated by adherent rAAV-RANKL and VEGF gene therapy. *Nat Med* 11, 291-297 (2005)
64. D. G. Miller, P. R. Wang, L. M. Petek, R. K. Hirata, M. S. Sands & D. W. Russell: Gene targeting *in vivo* by adeno-associated virus vectors.[see comment]. *Nat Biotechnol* 24, 1022-1026 (2006)
65. N. Maheshri, J. T. Koerber, B. K. Kaspar & D. V. Schaffer: Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol* 24, 198-204 (2006)
66. B. Peterson, R. Iglesias, J. Zhang, J. C. Wang & J. R. Lieberman: Genetically modified human derived bone marrow cells for posterolateral lumbar spine fusion in athymic rats: beyond conventional autologous bone grafting. *Spine* 30, 283-289; discussion 289-290 (2005)
67. H. Aslan, Y. Zilberman, V. Arbeli, D. Sheyn, Y. Matan, M. Liebergall, J. Z. Li, G. A. Helm, D. Gazit & Z. Gazit: Nucleofection-based *ex vivo* nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Eng* 12, 877-889 (2006)
68. J. Y. Lee, H. Peng, A. Usas, D. Musgrave, J. Cummins, D. Pelinkovic, R. Jankowski, B. Ziran, P. Robbins & J. Huard: Enhancement of bone healing based on *ex vivo* gene therapy using human muscle-derived cells expressing bone morphogenetic protein 2. *Hum Gene Ther* 13, 1201-1211 (2002)
69. V. Wright, H. Peng, A. Usas, B. Young, B. Gearhart, J. Cummins & J. Huard: BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. *Mol Ther* 6, 169-178 (2002)
70. H. C. Shen, H. Peng, A. Usas, B. Gearhart, F. H. Fu & J. Huard: Structural and functional healing of critical-size segmental bone defects by transduced muscle-derived cells expressing BMP4. *J Gene Med* 6, 984-991 (2004)
71. J. K. Fraser, I. Wulur, Z. Alfonso & M. H. Hedrick: Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* 24, 150-154 (2006)
72. B. M. Strem & M. H. Hedrick: The growing importance of fat in regenerative medicine. *Trends Biotechnol* 23, 64-66 (2005)
73. J. L. Dragoo, J. Y. Choi, J. R. Lieberman, J. Huang, P. A. Zuk, J. Zhang, M. H. Hedrick & P. Benhaim: Bone induction by BMP-2 transduced stem cells derived from human fat. *J Orthop Res* 21, 622-629 (2003)
74. J. L. Dragoo, J. R. Lieberman, R. S. Lee, D. A. Deugarte, Y. Lee, P. A. Zuk, M. H. Hedrick & P. Benhaim: Tissue-engineered bone from BMP-2-transduced stem cells derived from human fat. *Plast Reconstr Surg* 115, 1665-1673 (2005)
75. B. Peterson, J. Zhang, R. Iglesias, M. Kabo, M. Hedrick, P. Benhaim & J. R. Lieberman: Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. *Tissue Eng* 11, 120-129 (2005)
76. D. A. De Ugarte, K. Morizono, A. Elbarbary, Z. Alfonso, P. A. Zuk, M. Zhu, J. L. Dragoo, P. Ashjian, B. Thomas, P. Benhaim, I. Chen, J. Fraser & M. H. Hedrick: Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 174, 101-109 (2003)

Abbreviations: rhBMP: recombinant human bone morphogenetic protein, Ad: adenoviral vector, Ad.BMP-2: adenovirus carrying the BMP-2 gene, AAV: adeno-associated virus, GAM: gene activated matrix, TGF-beta: transforming growth factor beta, IGF: insulin-like growth factor, FGF: fibroblast growth factor, LMP: LIM mineralization protein, VEGF: vascular endothelial growth factor, caAlk: constitutively active form of the activin receptor-like kinase, NFkappaB: nuclear factor kappa B, GMP: good manufacturing practice

Key Words: Tissue Engineering, Bone, Bone Healing, Bone Regeneration, Fracture Repair, Spine Fusion, Gene Therapy, Gene Transfer, Bone Morphogenetic Protein, Review

Send correspondence to: Dr. Volker M. Betz, Center for Molecular Orthopaedics, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, BLI-152, Boston, MA 02115**,** USA**,** Tel: 617-732-8604**,** Fax: 617-730-2846**,** E-mail: vbetz@rics.bwh.harvard.edu

http://www.bioscience.org/current/vol13.htm