Gene and Cell-Based BMP-2 and -6 Gene Therapy

For Equine Bone Regeneration

Dissertation

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By

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Abstract

Fracture is still a life-threatening disorder in horses, but current treatment options have not provided satisfying outcomes for complicated and catastrophic fractures in horses. Cell-mediated and direct gene therapies have provided numerous promising results in recent years and may become as practical alternative solutions for the potential treatments of equine bone repair and regeneration. We demonstrated that sufficient gene transfer could be achieved by using an adeno-viral (Ad) vector in equine cells. High vector dosages could be used in equine cells because of relative resistance to cytotoxicity in these cells compared to a human cell line. We evaluated the healing of equine metatarsal osteotomies and ostectomies in response to delayed percutaneous injection of adeno-viral bone morphogenetic protein-2 (Ad-BMP2), Ad-BMP6, or beta-galactosidase protein vector control (Ad-LacZ) administered 14 days after surgery. Radiographic and quantitative computed tomographic (qCT) assessment of bone formation indicated greater and earlier mineralized callus in the bone defects injected with Ad-BMP2 or Ad-BMP6. Peak torque to failure and torsional stiffness were greater in osteotomies treated with Ad-BMP2 than Ad-BMP6, and both Ad-BMP-2 and Ad-BMP6 treated osteotomies were greater than Ad-LacZ or untreated osteotomies. Gene expression of ostectomy
mineralized callus 8 weeks after surgery indicated upregulation of genes related to osteogenesis compared to intact metatarsal bone. These results demonstrated a greater relative potency of Ad-BMP2 over Ad-BMP6 in accelerating osteotomy healing. We also evaluated the healing of equine metacarpal/metatarsal osteotomies in response to the percutaneous injection of autologous dermal fibroblasts (DFb) genetically engineered to secrete BMP2 or demonstrate green fluorescent protein (GFP) gene expression administered 14 days after surgery. Radiographic assessment of bone formation indicated greater and earlier healing of bone defects treated with DFb with BMP2 gene augmentation. The qCT and biomechanical testing revealed greater mineralized callus and torsional strength of DFb-BMP2 treated bone defects. On the histologic evaluation, the bone defects with DFb-BMP2 implantation had greater formation of mature cartilage and bone nodules within the osteotomy gap and greater mineralization activity on osteotomy edges. In addition, we compared the DFb-mediated and direct adenoviral vector delivery of BMP2 for relative efficacy in bone regeneration. Equine rib drill defects were treated by percutaneous injection of either DFb-BMP2 or Ad-BMP2 vector. At week 6, both of the DFb-BMP2- and Ad-BMP2-treated rib defects had greater bone filling volume and mineral density, with DFb-BMP2 inducing greater bone volume and maturity in cortical bone aspect of the defect than Ad-BMP2. The transplantation of DFb alone induced modest bone formation. Increased mineral density and bone turnover were evident in the cortical and cancellous bone directly adjacent to the healing drill defects treated with either DFb-BMP2 or Ad-BMP2. These results demonstrated an efficacy and feasibility of DFb-mediated BMP2 therapy to accelerate the equine bone healing.
Additionally, we demonstrated the safety of BMP2 gene therapy for articular fracture, because the direct intra-articular administrations of Ad-BMP2 did not cause of mineralization or ossification of articular cartilage and synovium tissues. In concert, both cell-mediated and direct BMP2 gene therapy may be considered as a potential treatment for various types of fractures and bone defects.
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Chapter 1:

Cell-Mediated and Direct Gene Therapy for Bone Regeneration

1.1 Summary

Current treatment options have not provided satisfying outcomes for complicated and catastrophic fractures in horses. Bone repair and regeneration are good candidates for treatment with gene therapy, because a transient production of osteoinductive growth factors may induce clinical relevant degree of therapeutic response. Direct gene therapy has shown to be effective for in vitro bone formation and in vivo healing acceleration by using various viral and nonviral gene delivery vectors. Cell-mediated gene therapy has also been successfully performed by using wide range of carrier cells. These cell-mediated and direct gene therapies have provided numerous promising results in recent years and may become as practical alternative solutions for the potential treatments of equine bone repair and regeneration.

1.2 Background

Musculoskeletal disorders are the most important health problems\(^1\) and the most common cause of performance morbidity in horses.\(^2\) The incidence of lameness has been
estimated at 8.5 to 13.7 cases per 100 horses annually and, in a recent survey, the equine veterinarians rated the musculoskeletal problems in the most need of further research. The risk of catastrophic injuries in Thoroughbreds racehorses, most of which involve fractures, has been reported as 1.4 horses per 1,000 starts in Kentucky, 1.7 per 1,000 starts in California, and 0.8 per 1,000 starts in the United Kingdom. Reportedly, the catastrophic fractures have resulted in the 45% career ending and 40% mortality rate in horses. Fracture is still a life-threatening disorder in horses, unlike in people and small animals, because horses with fractures must bear heavy loads immediately following the injury or surgical repair. The normal physiological rate of bone healing often cannot meet the requirements for such a rapid repair, and failure or delays of bone union, and subsequent complications such as contralateral limb disorders, are common in horses. Equine bone, in general, has small windows of opportunities to restore natural integrity and this characteristic makes horses particularly vulnerable to bone failure. Surgical techniques of internal and external fixations have improved over the past decades in both implant size and in established techniques of repair. Even with these improvements, however, equine bone could particularly benefit from molecular therapeutic technologies for an acceleration and enhancement of fracture healing. Newer innovations, such as the use of growth factor proteins, genes and cell vectors are anticipated to have special importance for equine bone repair and regeneration. This chapter reviews the bone biology and the recent and future prospects of cell-mediated and direct gene therapy for bone disorders.
1.3 Bone biology

Bone structure and turnover

Equine bones can be distinguished, similarly to other species, as tubular long bones (Figure 1.1A), sesamoid bones (e.g., proximal sesamoid, navicular), short bones (e.g., pastern, carpal and tarsal), flat bones (e.g., skull, scapula, sternum), and irregular bones (e.g., vertebral column). Growing long bone is divided into regions separated by the physeal plate that is responsible for the longitudinal growth of bones through endochondral ossification (Figure 1.1A and 1.1B). The medullary cavity contains woven cancellous bone (a source for bone grafting from the sternum, tibia and ileum), and bone marrow (a source for hematopoiesis and mesenchymal stem cells). Periosteal and endosteal surfaces are lined with osteogenic cells that can be rapidly mobilized to produce greater bone during growth (modeling) and training (remodeling)\(^1\) (Figure 1.1C). The general process of remodeling of dense equine cortical bone is by classical activation of a bone resorption front of osteoclasts within the Harversian canal followed by a trail of osteoblasts laying down new bone that is termed a cutting cone (Figure 1.1D). In woven bone and flat bone, a similar but surface activation of osteoclasts followed by osteoblast activity occurs (Figure 1.1E). The coupling and order of bone resorption and subsequently bone deposition is a requirement for bone turnover and natural bone healing, both slowing the process and necessitating coordination of events. Disruption of any phase of this process can interfere with bone processing. Each of these processes can be targets (structurally,
cellularly and molecularly) to accelerate or interrupt bone turnover, resulting in more or less bone as desired and is a strategy in current approaches to modulate bone repair.

Figure 1.1 – Bone structure and natural turnover; (A): macrostructure of long bone, (B): microstructure of growth plate, (C): microstructure of metaphysis, (D): schematic image of haversian remodeling, and (E): schematic image of endosteal remodeling.
Bone cellular and matrix components

Cellular components of bones include principally three cell types; osteoblasts, osteocytes, and osteoclasts. Osteoblasts are the bone-forming cells designed for the production of collagen, noncollagenous proteins, and various growth factors critical in the upregulation of bone formation. These cells are regulated in autocrine and paracrine mechanisms by growth and endocrine factors, whose receptors can be found on the osteoblasts. Osteoblasts align, form gap junctions, and function on the bone surface (Fig 1.1C and 1.1D). Osteoblasts are derived from bone marrow stromal stem cells (endosteum) or connective tissue mesenchymal stem cells (periosteum). These stem cells can be captured and driven down osteogenic lineages by genetic engineering or the local tissue environment to augment bone repair and turnover. Bone formation by osteoblasts is initiated by a rapid production of collagen resulting a thick osteoid steam, followed by a decrease of collagen synthesis and an increase in the mineralization rate. This time lag (osteoid maturation period) occurs as inhibitors of mineralization (eg, matrix Gla protein) is removed and is required for the osteoid modification to support mineralization. Hydroxyapatite crystals grow in clusters, fill the spaces between and within the collagen fibers, and completely calcify the matrix. In the fast-growing bones such as woven bone and growth plate cartilage, the matrix vesicles loaded with alkaline phosphatases are deposited from the cytoplasmic processes of the chondrocytes or osteoblasts and
contribute to rapidly increase a local mineral concentration (e.g., Ca\(^{2+}\) and PO\(_4^{3-}\)), induce their precipitation, and hydroxyapatite crystal formation.\(^{17}\)

Osteocytes are mature osteoblasts. Only 10\% of osteoblasts become embedded in the bone matrix and differentiate into osteocytes, the rest remain on the bone surface and can be reactivated in vivo by mechanical stimulation.\(^{18}\) Osteocytes contact other osteocytes in a network of canaliculi throughout the bone matrix (Figure 1.1C). Extracellular fluid can flow through this network, regulate the exchange of mineral ions, and relay mechanical stimuli by hydraulic pressure changes and piezoelectric effects. Even though the metabolic activity of osteocytes is generally low they respond to tissue strain and influence bone remodeling activity by recruiting osteoclasts.\(^{19}\) In vitro, mechanical stimulation of osteocytes increased calcium flux and prostaglandin production by osteocytes, processes known to be coupled to bone resorption.\(^{20}\)

Osteoclasts are giant multinucleated cells designed to secrete lysosomal enzymes and resorb bone. They are found within a Howship’s lacuna on the surface of bone or at the apex of the Haversian remodeling system (Figure 1.1D and 1.1E). The osteoblast can mediate osteoclastogenesis and regulation of this process is used to control bone resorption is diseases such as osteoporosis.\(^{21,22}\) Osteoclast inhibitors, such as osteoprotegerin and bisphosphonates are being studied to enhance the amount of bone in horses and other species.\(^{23,24}\) In a microstructural level, attachment of the osteoclast to the bone surface is essential for bone resorption. Lysosomal enzymes secreted into the extracellular bone-resorbing space are sealed off until they reach a sufficiently high local concentration. Osteoclasts also secrete several metalloproteinases including MMP-9.
(gelatinase) and MMP-13 (collagenase) to facilitate bone matrix and collagen digestion as well as osteoclast migration. After a cycle of bone resorption, the osteoclast can either undergo apoptosis or return back to resting phase (Figure 1.1E).

Normal and abnormal bone healing

Healing of bone is a highly complex regenerative process that is essentially a recapitulation of bone development. Rapid progression of these processes requires a stable microenvironment, recruitment of progenitor cells, proliferation and differentiation of various types of cell, and regulation of osteoinductive signaling molecules. Normal bone development is an important process to understand, not only for the treatment of growing bone (eg, physeal injury), but also to recapitulate bone healing. Bones develop either directly from connective tissue by intramembranous ossification (Figure 1.1E) or replacement by osseous tissue, termed endochondral ossification (Figure 1.1B). Intramembranous ossification lacks a cartilaginous phase and is seen most commonly in the flat bones of the skull and on woven medullary cancellous bone. The osteoblasts enhance the synthesis process by producing more matrix as they are calcified, and some portion of these cells are trapped in the tissue and become osteocytes. This bone formation results in mature lamellar bone. Most of the skeleton is formed by endochondral ossification during growth, but repair of bone gaps, unstable bone, or subchondral bone often goes through this process. Acceleration of the endochondral ossification process is a strategy to promote bone repair and a fundamental principle in use of BMPs.
Bone is the only tissue in the body that can regenerate and regain the original mechanical strength, but this requires a supportive microenvironment, including a vascular supply and sufficient osteoprogenitor cells. Optimal bone healing requires low strains (<2%) and angulations (<0.5 degree). Normal bone healing is generated by either the processes of primary or secondary healing, although both events can concurrently occur in a single repair site. Primary bone healing is characterized by the lack of substantial callus formation and requires both minimum strain (<2%) and extremely small intrafragmentally gap (direct contact or <100 µm). It is uncommonly achieved in horses, except possibly in stable repair of nondisplaced fractures, such as condylar or slab fractures with direct contact and no gap. Secondary bone healing is characterized by callus formation and observed at fracture sites with motion or intrafragmentary gaps. Repair begins with bleeding, clot formation, angiogenesis to various degrees, and fibroplasia (inflammatory phase). The degree of stability and vascularity at the bone fragments defines the microenvironment and is the primary determining factor for the mode of osteogenesis. In conditions of vascularity and stability, new woven bone can be formed directly from osteogenic mesenchymal cells from the periosteum or in the fibrous callus, the degree of stability determining the size of the bridging callus. In conditions of poor vascularity and instability, chondroid metaplasia occurs and a bony bridge develops through the slower process of endochondral ossification.

Despite the improved surgical techniques in past decade, many complications may develop during the bone healing process such as infection, implant loosening, and fracture instability. All of these problems can lead to abnormal bone healing which are
categorized as delayed union, nonunion, malunion, and pseudoarthrosis. Delayed union precedes nonunion which can be divided into viable or non-viable nonunion. Many factors may cause the development of delayed or nonunion, including infection, poor reduction, inappropriate fixation, poor immobilization, impaired blood supply, and missing bone fragments. Infection itself can delay bone union as the result of osteolysis and implant loosening. In addition, local tissue infection and inflammatory response change the pH, enzyme releases, and proteolytic compounds, which delay bone healing due to the poor neovascularization and tissue necrosis. Inadequate re-alignment of bone fragments, technical errors of surgical reconstruction, and improper post-operative confinement or immobilization can result in excessive motion in the microenvironment at fracture sites. Bone has a limited tolerance of micro-movement and an excessive strain has been shown to inhibit differentiation of osteoprogenitor cells. Sufficient blood supply is essential for secondary bone healing and subsequent bone union. Because the equine lower limb has a limited coverage by musculature, poor blood supply at the fracture sites commonly occurs in various types of equine bone injuries.

1.4 Osteoinductive growth factors

Use of osteoinductive growth factors for the purpose of improving bone healing has been established over decades of experimental use and has been applied in animals and human clinical patients. Growth factors are defined as certain types of signaling molecules that induce cell differentiation and tissue regeneration. Increased expressions of endogenous growth factors associated with osteogenesis or bone formation have been
identified and their functions have been studied. This includes bone morphogenetic protein (BMP),\textsuperscript{32} LIM mineralization protein (LMP),\textsuperscript{33} transforming growth factor-beta (TGF-\textbeta{}),\textsuperscript{34} insulin-like growth factor (IGF),\textsuperscript{35} platelet-derived growth factor (PDGF),\textsuperscript{36} and vascular endothelial growth factor (VEGF).\textsuperscript{37} Of these, an induction of bone formation or enhancement of bone healing in experimental models has been demonstrated with application of exogenous recombinant human (rh) proteins such as rhBMP2,\textsuperscript{38} rhBMP4,\textsuperscript{39} rhBMP7 [osteogenic protein-1 (OP-1)],\textsuperscript{40} rhTGF-\textbeta{},\textsuperscript{41} and rhPDGF.\textsuperscript{42} Use of rhIGF has also improved bone healing when it was combined with rhTGF-\textbeta{}.\textsuperscript{43}

Since its discovery by Urist,\textsuperscript{44} many attempts have been made by using BMPs to heal or regenerate bone. Bone morphogenetic proteins are part of the TGF-\textbeta{} superfamily, whose members are related by the degrees of sequence similarity but that possess various biological functions.\textsuperscript{45} An in vivo osteoinductive capacity has been proven in a number of BMPs such as BMP2, 4, 6, 7, and 9,\textsuperscript{46,47} which has been demonstrated to initiate the cascade of endochondral ossification.\textsuperscript{48} BMPs appeared to act as the local signals that induce commitment of mesenchymal stem cells resident in bone marrow into osteoprogenitor cells and osteoblasts, including in horses.\textsuperscript{49,50} It has been stated that BMPs are the most promising osteogenic proteins to enhance bone healing due to their potency and biologic tolerance.

Clinical application of rhBMPs has been reported in human and veterinary hospitals for the treatment of various osseous disorders. In human clinical trials, the rhBMP2 and rhBMP7 have been applied for tibial or femoral nonunion with favorable
Outcomes. In veterinary fields, rhBMP2 has been clinically used for small animal patients. Femoral nonunion in a dog treated with rhBMP2 showed a rapid callus formation as early as at 2 weeks and successful bony union at 8 weeks after treatment. Nonunion of radius/femur/tibia in 8 small animal cases treated with rhBMP2 showed complete gap filling with new bone formation and satisfactory bony bridging in 7 cases (3/3 dogs and 4/5 cats) within 6 months. Inter-carpal joint fusion in 10 dogs utilized with rhBMP2 showed successful bony bridging within 4 months for all patients. In addition, rhBMP2 delivered in collagen carrier has shown accelerate ulnar or tibial osteotomies in dogs. Moreover, rhBMP2 has shown to accelerate and enhance the healing of equine second and fourth metatarsal bone osteotomies and osteotomies at 12 weeks. In this study, the bone defects treated with rhBMP2 had greater radiographic score and improved physical strength compared to the bone defects treated with autogenous cancellous bone grafts. Although a future prospective study with large number of clinical cases is warranted, it has been demonstrated that usage of rhBMPs can augment bone healing in horses.

The relatively new osteoinductive growth factor, LMPs has a direct association with osteoblastic differentiation and appeared to be a positive regulator of bone formation. LMPs contain the LIM domain including Lin-11, Isl-1, and Mec-3 homeodomain proteins which act as protein-protein binding interaction to bind specific recognition sequences. Unlike BMPs, which are extracellular signaling molecule and effect through cell surface receptors, LMPs are intracellular proteins and the therapeutic usage requires gene transfer of their cDNA into host cells (in vivo gene therapy) or
application of transfected carrier cells (ex vivo gene therapy). LMP gene transfer can induce synthesis of multiple BMPs such as BMP2, 4, 6, 7, as well as TGF-β. Production of multiple osteogenetic growth factors by administration of single therapeutic molecule, by which the osteoinductive signaling is being amplified, may be the largest advantage in use of LMP. Secreted different types of BMPs can be mixed and potentially formed into heterodimeric BMPs which may have more potent osteoinductivity than homodimeric BMPs. Preliminary usage of hLMP has not yet reached clinical acceptance, but the preceding studies clearly showed the effectiveness of LMP for bone healing enhancement and indicate the promising future application of this growth factor.

1.5 Direct gene therapy

Application of recombinant protein of osteoinductive growth factors can be challenging in adult horses. Large doses of rhBMPs may be required not only due to the size of the bones but also the fact that human proteins have appeared to be more effective in laboratory animals (rat, rabbit) than large animals (sheep, goat, and dog). Since BMPs have a very high (>90%) interspecies homology, this phenomenon may be explained by the difference in post-translational modification and the formation of tertiary structures. In addition, carrier matrix can inhibit fracture healing and may not be cost-effective for clinical use in large animal species. For these reasons, alternative strategies of growth factor delivery should be considered in horses.

Gene therapy has a great potential as a method for application of growth factors in the acceleration and enhancement of bone healing. This is because gene therapy can
induce a higher local concentration of therapeutic molecules for a longer time period compared to protein delivery,\textsuperscript{64} and manufacture of gene therapy products is likely much less expensive than recombinant protein.\textsuperscript{65} Also, gene therapy can produce a more biologically active form of the growth factor since the molecules are synthesized by autologous cells in situ.\textsuperscript{66} Genes of growth factors can be delivered to bone by viral vectors (adenovirus [Ad], retrovirus [Retro], lentivirus [Lenti], adeno-associated virus [AAV]) or nonviral vectors (electroporation, gene activated matrix, liposomal vectors, and sonoporation). Currently, viral vectors are preferred for gene therapy due to the high transduction efficiency, although nonviral methods may be more economical and have less immunogenecity.\textsuperscript{67} Table 1.1 summarizes the recent studies of the gene therapy for bone formation and regenerations.\textsuperscript{68-88}

\textit{Viral gene delivery vectors}

Acceleration and enhancement of bone healing by direct injection of the Ad vectors encoding BMP genes has been demonstrated in numerous rodent experimental models. Adenovirus has a number of advantages including the ability to generate high viral titers, its high transduction efficiency, and its ability to transduce both dividing and non-dividing cells.\textsuperscript{89} Three reports of rabbit or rat femur defects treated with Ad-BMP2 showed a faster callus formation on radiographs, earlier ossification on histology, and stronger bony union on bending mechanical tests, compared to untreated or vector controls.\textsuperscript{70,73,75} One of these works\textsuperscript{73} showed that delayed Ad-BMP2 injection induced superior bone healing than the injection made in earlier time points potentially due to the
higher cellularity in the injection site, greater vector confinement by solid granulation tissue, and increased expression of the adenovirus receptor.\textsuperscript{90}

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Table 1.1 – Literatures of direct gene therapy for bone formation and regeneration. BMP: bone morphogenetic protein; Ad: adenovirus; AAV: adeno-associated virus; Retro: retrovirus, pBMP: plasmid of BMP; GAM: gene-activated matrix.
In addition, several studies utilized Ad-BMP6 for acceleration of rabbit ulnar osteotomies\textsuperscript{78} or rabbit spine fusion.\textsuperscript{79} Relative osteoinductivity among different BMPs has been studied by few groups using adenoviral gene transfer. For an ectopic bone formation in athymic rats, Ad-BMP4 and Ad-BMP6 induced greater efficacy than Ad-BMP2.\textsuperscript{68} Also, two studies demonstrated the superior ectopic bone formation by Ad-BMP6 and Ad-BMP9 compared to various BMP gene transfers including Ad-BMP2 and Ad-BMP7.\textsuperscript{71,72} In contrast to Ad-vector, recombinant AAV has gained much scientific attention as having a superior safety profile, because it is non-pathogenic, immunologically inert virus, and can induce sustained transgene expression.\textsuperscript{91} Also, AAV has the unique property of its ability to maintain infectivity when dehydrated onto the implantable surface.\textsuperscript{89} The ectopic and orthotopic bone formations have been induced by AAV-BMP2,\textsuperscript{76,77} whereas co-injection of low-dose Ad-BMP2 with AAV-BMP2 resulted in the superior bone formation probably due to the E1 and E4 genes of adenovirus facilitating the second strand synthesis of AAV vectors.\textsuperscript{76} Direct gene transfer of LMP, although its effect was less extensively studied, has shown to promote the healing of rat femoral defects using retroviral vectors.\textsuperscript{80}

Nonviral gene delivery vectors

Various nonviral gene delivery systems can be useful in gene therapies for tissue regeneration.\textsuperscript{92} In an electroporation technology, negatively charged DNA can enter the
cytoplasm through the small temporary pores on the cell membrane generated by the electric pulses. The electroporation-mediated transfers of BMP2, BMP4, and BMP7 plasmids have shown to induce ectopic bone formation. In this technique, simultaneous administration of BMP2 and BMP7 showed greater bone formation than either gene alone. Gene-activated matrix (GAM) is a tissue-engineered construct that holds DNA in situ until the host cells migrate into the scaffold; thus, the embedded plasmids would be gradually released. By using this method, BMP4 gene has been delivered to enhance the bone regeneration in rat cranial defects. In addition, GAM-mediated BMP2 gene delivery has shown to improve the bone healing in rat segmental tibial defects. Moreover, liposomal vector is another nonviral gene delivery technology which has been utilized to deliver BMP2 genes for bone regeneration in several pig models. Furthermore, novel sonoporation method, in which ultrasound-mediated acoustic cavitation can increase the cell membrane permeability, has been tested to facilitate an intracellular uptake of BMP2 plasmids and induce an ectopic bone formation.

1.6 Cell-mediated gene therapy

Direct delivery of viral or nonviral vectors to treat fractures and bony disorders will require large volumes of purified agents and the potential risks of failure of vector containment, systemic immune reaction to viral vector, and seeding of nontarget tissues with vectors. Due to these concerns, recent studies have focused on indirect delivery of growth factor genes via various cells-mediated therapies. Tissue engineering studies on
cell-mediated gene therapy for orthopedic disorders have demonstrated that the availability of cellular components of bone in local tissue is essential for successful bone healing, and various types of carrier cells can serve as this source of osteoprogenitor cells. For this purpose, pluripotent stem cells isolated from various tissues have shown promising results; however, several types of non-stem cells have also been used as gene delivery cells for an ex vivo gene therapy for bony disorders.

To induce a robust bone response, recent molecular studies have focused on the gene augmentation of stem cells with osteoinductive growth factors in a strategy known as ex vivo gene therapy. In contrast to direct application of therapeutic gene delivery vectors (in vivo gene therapy), ex vivo gene therapy can achieve high transduction efficiency, allow clinicians to choose specific cell types for the gene delivery vehicles, and increase the population of progenitor cells in local tissues. When viral vectors are used for gene transfer, ex vivo strategies may be a safer application by preventing direct contact between vectors and host cells which can minimize an inflammatory response in the local tissues. It may also be helpful to avoid host immune reaction, as occurs with in vivo viral vector injection, so that repeated gene therapy with multiple cell transplantations may be possible. In addition, the stem cells have dual autocrine and paracrine responses, and can only secrete an osteoinductive protein (eg, BMPs) but also respond and directly contribute to bone healing.\textsuperscript{95} For these reasons, cell-mediated gene therapy has been considered a primary approach in bone repair and regeneration.\textsuperscript{89} Table 1.2 summarizes the recent literatures regarding the cell-mediated gene therapy for bone formation and regenerations.\textsuperscript{59,77,96-133}
Bone marrow-derived stem/stromal cells

Stem cells are groups of cell lines that possess the potential to differentiate into multiple tissue types and can be categorized by their breadth of pluripotency. The embryonic stem (ES) cells originate from the inner cell mass of the blastocyst stage embryo and are able to differentiate into virtually any kind of tissue (totipotent capacity). Stem cells from adults have a more limited range of differentiation lineage (pluripotent capacity) and have been commonly isolated from bone marrow, muscle, and fat tissues. Adult stem cells are preferable for therapeutic purposes in musculoskeletal repair, including bone regeneration, than ES cells because they are more easily directed to mesenchymal tissue types and have less tumorigenicity.\(^{134}\) The adult stem cells that has received the most interest for bone healing, are the bone marrow derived-mesenchymal stem cells (BMD-MSC) due to their accessibility and favorable differentiating potential. BMD-MSC have shown to differentiate into various mesenchymal lineages including bone, cartilage, adipose, muscle, and tendon,\(^{135}\) including equine BMD-MSC.\(^{50}\) Human BMDMSC have been directly transplanted into bone defects and induced successful bone union.\(^{136}\) In addition, the clinical trial of bone nonunions treated with percutaneous injection of un-cultured bone marrow also demonstrated effective bone induction.\(^{137}\)
<table>
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<tr>
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**Table 1.2 - Literatures of cell-mediated gene therapy for bone formation and regeneration.** BMD-MSC: bone marrow-derived mesenchymal stem cell; BM: bone marrow; ADSC: adipose-derived stem cell; AD: adipose-derived; PLA: processed liposuspirate; MDSC: muscle-derived stem cell; DFb: dermal fibroblast; BMP: bone morphogenetic protein; Ad: adeno-virus; Retro: retrovirus; Lenti: lentivirus; VEGF: vascular endothelial growth factor; LMP: LIM mineralization protein; Runx2: Runt-related transcription factor 2; N/A: not applied; Dox: doxycycline; Tet: tetracycline.
Bone formation and regeneration have been demonstrated by gene augmented BMD-MSC and BM stromal cells in various models. Murine radial defects treated with BMP2 gene augmented MSCs produced significantly more bone formation than direct use of rhBMP-2. In addition, the engineered BMD-MSC may be capable to produce bone in a more organized manner. Several studies reported that the bone healing by BMP2-expressing stem cells has the continuous regeneration between the original defect edges and the newly formed bones, whereas rhBMP2 or BMP2 gene treatments resulted in the formation of diffuse osseous foci with no continuity to the original bones. This phenomenon may indicate that stem cells are capable to localize and orient themselves to specific sites after transplantation.

A large number of studies have reported that osteogenic potential of MSC is greatly enhanced by genetic engineering using osteoinductive growth factors such as BMP2, BMP4, BMP9 and combination of BMP4 and VEGF. Adenoviral gene delivery of BMP2 has been used for ex vivo gene therapy to improve the bone healing in several rodent models using BMD-MSC or BM stromal cells. One of these works demonstrated that Ad-BMP2 gene transfer induced more rapid healing in rat mandible defects compared to the liposomal BMP2 plasmid transfer. Adenoviral vectors have also been utilized for BMP9 gene transfer to BMD-MSC for an ectopic bone formation in athymic rats and BMP7 gene transfer to BM stromal cells for an acceleration of femoral segmental defect healing in goat. In addition, AAV-mediated
BMP2 gene delivery to BMD-MSC has shown to improved the mice calvarial defect healing in which bone formation was controlled by Dox-regulation system.\textsuperscript{77} In addition, sustained BMP2 gene transduction was achieved by using lentiviral vectors in BM stromal cells to induce the bone formation and regeneration in various rodent models including rat femoral defect healing,\textsuperscript{102} rat segmental femoral defect healing,\textsuperscript{103} and rat spine fusion.\textsuperscript{104} Furthermore, prolonged BMP2 expression associated with lentiviral gene transfer tended to produce greater quality of bone repair compared to adenoviral BMP2 gene transfer.\textsuperscript{102} These encouraging results suggest that BMD-MSC or BM stromal cell-mediated BMP gene therapy has a great potential in the treatment of orthopedic conditions.

\textit{Adipose-derived stem/stromal cells}

Compared with BMD-MSC, adipose-derived stem cells (ADSC) or AD stromal cells are easier to isolate, have a relatively lower risk of donor site morbidity, and are available in large populations.\textsuperscript{113} These fat tissue-derived cells can exhibit stable growth and sufficient cell proliferation\textsuperscript{144} and have been successfully differentiated in vitro toward osteogenic, adipogenic, myogenic, and chondrogenic pathways using established factors.\textsuperscript{145,146} Therefore, these cells are a promising substrate for the clinical application of bone tissue regeneration.

Several recent studies have applied ADSC or AD stromal cells in rodent models to induce ectopic bone formations by adenoviral gene transfers of BMP2,\textsuperscript{112} BMP7,\textsuperscript{109} and Runt-related transcription factor 2 (Runx2).\textsuperscript{111} Also, Ad-BMP2 gene delivery has
shown to be efficient to improve bone repair in ADSC-mediated therapy for rat femoral defect healing\textsuperscript{108} and AD stromal cell-mediated therapy for canine ulnar defect healing.\textsuperscript{113} In addition, ADSC was transduced with BMP7 gene by AAV vectors and induce an ectopic bone formation.\textsuperscript{110} The relative osteoinductive ability between the stem cells sourced from fat and bone marrow tissues has not been comprehensively elucidated; however, one study reported that ADSC and BMD-MSC with Ad-BMP2 gene deliveries showed the comparable efficacy in rat spine fusion.\textsuperscript{116}

It is known that liposuction aspirates contain multipotent cells, processed lipoaspirate (PAL) cells, and an average of 45% of these cells can have osteogenic potentials.\textsuperscript{145} Two previous works have demonstrated the successful ectopic bone formations by PAL cells with Ad-BMP2 gene transfer.\textsuperscript{114,115} Future studies are warranted to further elucidate the exact mechanism and fate of PAL cells in the formation of osteogenic tissue in vivo.\textsuperscript{115} Because use of PAL cells would eliminate the need for costly and lengthy tissue culture expansion and minimize the time length between the initial harvest and reimplantation,\textsuperscript{114} it may be considered as a great alternative source of stem cells. Therefore, PAL cell-mediated BMP gene therapy may offer the highly time- and cost-efficient treatment for various bony injuries and disorders.

*Muscle-derived stem cells*

Skeletal muscle represents another abundant and easily accessible tissue as a source of carrier cells for ex vivo gene therapy. Several groups have identified a population of muscle-derived stem cells (MDSC) in skeletal muscles\textsuperscript{147} which shown to
undergo multilineage differentiation into bone, and cartilage, neuron, endothelial, and hematopoietic tissues.\textsuperscript{148-150} Unlike BMD-MSC, muscle-derived inducible osteoprogenitors do not express osteogenic markers until exposed to BMP\textsuperscript{2};\textsuperscript{151,152} therefore, the MDSC should be used in combination with osteoinductive factors to achieve full osteogenic potential.

Adenoviral BMP\textsuperscript{2} and retroviral BMP\textsuperscript{4} gene transfer was applied to MDSC to induce the ectopic bone formations.\textsuperscript{117,121} These MDSC genetically engineered to secrete BMP\textsuperscript{2} or BMP\textsuperscript{4} have also improved the bone healing of the skull and long bone defects.\textsuperscript{118-120,122} Interestingly, male MDSC induced the greater ectopic bone formation by BMP\textsuperscript{4} gene transduction compared to female MDSC in mice; thus, MDSC-mediated therapies in male individuals may require less cells or less BMP\textsuperscript{4} than female individuals.\textsuperscript{120} In addition, the co-implantation of MDSC expressing BMP\textsuperscript{4} and VEGF showed superior bone regeneration than either gene alone, if applied an optimal dosage,\textsuperscript{143} and an administration of VEGF antagonist inhibited the bone formation by BMP\textsuperscript{2} gene transfer.\textsuperscript{123} These finding highlighted the synergistic interaction between VEGF-induced angiogenesis and BMP-induced osteogenesis. Moreover, by using MDSC-mediated gene delivery, the amount of bone formation has shown to be controlled by Tet-regulation of BMP\textsuperscript{4} gene expression\textsuperscript{121} or Noggin, specific BMP antagonist.\textsuperscript{122} Investigations into the clinical applications of MDSC-mediated ex vivo BMP gene delivery are ongoing for the purposes of bone repair and regeneration.
Dermal fibroblasts

Skin is one of the most readily available and easily accessible tissues in the body. Dermal fibroblasts (DFb) isolated from dermis tissue are attractive carrier cells for ex vivo gene therapy, because they can be harvested by minimally invasive and less painful procedure compared to bone marrow or fat tissue, requires less fastidious culture techniques than stem cells, and its rapid cell expansion could allow an extremely large number of autologous cell transplantation. Both fibroblasts and osteoblasts are mesenchymal origin, and osteoblasts have been express as a “sophisticated fibroblasts”. In fact, the DFb implanted in orthotopic site appeared to be differentiated into osteoblasts and embedded within the mineralized bone matrix as osteocytes. In addition, the previous study of rabbit radial fracture model showed that the radioactively-labeled DFb were aggregated in the bony callus and formed osteocytes.

Several previous studies have demonstrated the osteogenic differentiation of DFb by converting DFb into bone-forming cells with the transductions of various osteogenic genes such as BMP2, BMP7, Runx2, and LMP3. These genetically modified DFb can provide a great potential for bone healing enhancement and have been applied to induce an acceleration of the mice calvarial defect healing with Ad-BMP2 gene transfer and the rat cranial and femoral defects with Ad-BMP7 gene transfer. One of these studies reported that the implantations of DFb genetically modified to secrete BMP2 were more osteoinductive than DFb with Runx2 gene transduction. In addition, the BMP2-expressing DFb induced comparable ectopic bone formation compared to BMP2-expressing BMD-MSC in a mouse model. Autologous
implantation of LMP3 gene-augmented DFb was also utilized to induce the ectopic bone formation in paravertebral muscles and improve the bone healing in mandibular critical size defects.\textsuperscript{129} The successful bone formation and regeneration in various experimental models provides the evidences that DFb could be considered as an alternative cell source for ex vivo gene therapy.

Recently, DFb has shown to preserve a great plasticity and reprogramming capacity, where DFb can be derived into induced pluripotent stem cells with defined genes such as Oct3/4, Sox2, Klf4, and c-Myc.\textsuperscript{156,157} These induced pluripotent stem (iPS) cells were identical to ES cells in morphology, surface antigens, and epigenetic status of pluripotent cell-specific genes, and telomerase activity.\textsuperscript{156} Reportedly, iPS cells can be differentiated into multiple pathways including neurons, cardiomyocytes, hematopoeitic cells, as well as osteocyte-like cells.\textsuperscript{158-161} In the future, iPS cells that are converted from DFb may be induced toward an osteogenic differentiation and applied to the treatment of various orthopedic disorders.\textsuperscript{161}

\textit{Blood cells}

The use of buffy coat cells from venous blood as a gene carrier for ex vivo gene therapy is an interesting concept. Adenoviral delivery of LMP1 gene into the buffy-coat cells resulted in efficient secretions of BMP2, BMP6, and TGF-β1 proteins.\textsuperscript{59,131} Also, transplantation of the LMP1 gene augmented buffy coat cells has successfully induced an ectopic bone formation\textsuperscript{59} and spine fusion in rabbits.\textsuperscript{131} Because a large proportion of
buffy coat cells do not have any differentiation capacity, further works are necessary to understand the mechanism of buffy coat cell-mediated gene therapy for bone formation.

Umbilical cord blood is known to contain multipotent cells and can serve as an alternative source of MSC. The umbilical cord blood-derived MSC has an osteogenic potential, and was administered in combination with beta-tricalcium phosphate to promote an acceleration of radial defect healing in dogs. In addition, umbilical cord blood-derived MSC may be capable to evade the host immune rejection, as they have been considered as immune-privileged cells due to their surface characteristics. Therefore, an implantation of umbilical cord blood-derived MSC could have a great potential for allogenic cell-mediated gene therapy for bone regeneration.

Recent report has described the presence of skeletal stem cells in circulating blood, and the osteogenic potential of the blood-borne cells was proven by an in vivo transplantation assay. To feasibly apply these cells for bone tissue engineering, the heterogenous population of mononuclear cells was harvested from whole blood and induced to differentiate into osteoblastlike cells in vitro. Also, these peripheral blood mononuclear cells were administered with osteoinductive supplements which shown to improve the bone healing in rat calvarial defects. These findings have important implications for confirming the osteoinductive potential of stem cells in peripheral blood and for developing innovative cell-based therapeutic strategies to improve bone healing.
1.7 **Key aspects for clinical application**

The most important aspect of direct gene therapy for future clinical application is its safety. Although virus-mediated gene delivery for bone formation showed the promising results, the issues of safety have prevented this approach from being adapted for human use since the death of Jesse Gelsinger by adenoviral gene therapy and the occurrence of lymphoma in several patients by retroviral gene therapy. The gene transduction by nonviral vectors generally has low efficiency limiting its use for in vivo bone repair and regeneration, despite the fact that nonviral vectors are technically undemanding to prepare and scale up. The majority of researches in the field of orthopedic gene therapy are targeting the nonlethal conditions in human including nonunion or delayed-union fractures, segmental bone defects, and osteoporosis. Therefore, the safety of gene delivery system is of paramount importance, and the viral and nonviral vectors used must have minimum to no risks to patients’ health. On the other hand, certain types of fractures are life-threatening diseases in horses and some leeway may be allowed to give a chance of bone repair and regeneration (eg, in vivo administration of adenoviral vectors), although the safety issues still need to be properly addressed in a process of developing therapeutic strategies.

One of the main problems associated with cell-mediated gene therapy is a need of prolonged periods of in vitro culture for cell expansion. The expensive and time consuming process not only makes the ex vivo gene therapy unaffordable for some clinical patients but also may forbid the cell implantation at the most optimal timing. One proposed solution is an immunoisolation technique in which MSC can be isolated from
bone marrow by an interaction of certain surface antigen using a specific antibody. Such isolated MSC was shown to induce ectopic bone formation without in vitro cell culture.\textsuperscript{171} Therefore, use of this technology may skip the cell culture procedure in laboratory and enable the rapid isolation and implantation of stem cells on the patient side within a matter of hours.

Controlling transgene expression may be a potential problem in the orthopedic gene therapy, because an overabundance of bone formation is undesired. A number of groups have used the exogenous regulations of BMP gene expression (eg, tetracycline or doxycycline-regulation systems) to control the ectopic bone formation by BMP4-expressing MSC\textsuperscript{121} and mice calvarial defect healing by BMP2-expressing MSC.\textsuperscript{77} Although these systems can be beneficial to prevent an excessive bone formation by cell-mediated gene therapy, the efficiency and reliability of such exogenous gene regulation has not been tested in large animal models. It has been demonstrated that a short-term expression of the BMP2 is sufficient to irreversibly induce bone formation by MSC suggesting that a stable genetic modification of stem cells is not required for cell-mediated BMP therapy for bone regeneration.\textsuperscript{172} This supports an extensive usage of Ad-vectors inducing transient BMP gene transduction in a large number of experimental works (Table 2). Therefore, the necessity of tightly controlled BMP gene expression in the gene therapy is uncertain, especially for the treatments of life-risking equine catastrophic fractures.

Another important aspect of cell-mediated therapy is a stem cell tumorigenicity which can represent the key obstacle to the safe clinical use of stem cells. In general,
greater the pluripotency and self-renewal properties that a stem cell possesses, invariably the higher the probability it will cause tumors.\textsuperscript{173} Reportedly, stem cells have shown to become tumorigenic by an extended time in culture.\textsuperscript{174,175} Also, viral vectors used for genetic modification of stem cells are potentially hazardous due to the risks of insertional mutagenesis.\textsuperscript{176} The most practical approach to safe regenerative medicine would be to differentiate stem cells into the desired progenitors and kill residual stem cells. This can be achieved either by an introduction of stem cell specific suicide gene\textsuperscript{177} or use of killer antibodies against the surface antigens of undifferentiated stem cells.\textsuperscript{178} Future studies are warranted to elucidate if the cell-mediated gene therapy can cause of osseous tumor at the bone healing site where the residual undifferentiated stem cells are administered.
Chapter 2:
Relative permissiveness and cytotoxicity of equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells to adenoviral gene delivery

2.1 Summary
Objective of this study is to evaluate cell permissiveness and cytotoxicity to a recombinant adenoviral (Ad) vector and a modified Ad vector in gene delivery using equine chondrocytes, synovial cells, and adult bone marrow derived mesenchymal stem cells (BMD-MSCs). Equine chondrocytes, synovial cells, and BMD-MSCs, and human carcinoma (HeLa) cells were cultured from 15 adult horses and infected with an E-1 deficient Ad vector encoding the β-galactosidase gene (Ad-LacZ), green fluorescent protein gene (Ad-GFP), or GFP in a modified E-1 deficient vector containing the Arg-Gly-Asp capsid peptide insertion (Ad-RGD-GFP) at 6 multiplicities of infection. The %transduced cells (Ad-LacZ, Ad-GFP, Ad-RGD-GFP), cell viability (Ad-LacZ), and total and transduced cell counts (Ad-LacZ) were assessed at Days 2 and 7. Cell permissiveness to Ad vector was significantly different among all the cell types with HeLa > equine BMD-MSCs > equine chondrocytes > equine synovial cells with lower transduction efficiency at lower vector dosages. Morphologic signs of cytotoxicity were
evident in HeLa cells but not in equine cells. Total transduced cell counts decreased in all cell types by Day 7, except equine BMD-MSCs. Transduction efficiency was not significantly changed between Ad-GFP and Ad-RGD-GFP. This study demonstrated that sufficient gene transfer could be achieved by using an Ad vector in equine cells, particularly with high vector dosage. High vector dosages could be used in equine cells because of relative resistance to cytotoxicity in these cells compared to HeLa cells. BMD-MSCs may be a preferential cell target for equine gene therapy due to greater permissiveness and sustained transgene expression. Improved transduction efficiency in vivo may not be anticipated by use of the RGD peptide insertion.

2.2 Introduction

Gene therapy has been used experimentally in orthopedics for fracture repair,\textsuperscript{1-5} spine fusion,\textsuperscript{6-10} and treatment of arthritis,\textsuperscript{11-18} and specifically in horses, for treatment of osteoarthritis.\textsuperscript{19} Introduction of a gene into resident cells to achieve local therapeutic protein expression can be advantageous over direct injection of a recombinant protein, because it can be cost-effective and produce a sustained and higher local tissue concentration of molecular therapeutic.\textsuperscript{5,10,18} Administration of desirable genes can be performed by a number of vector systems, one of which is a replication deficient adenovirus (Ad) derived from the human Ad (serotype 5). Recombinant Ad vectors are attractive gene transfer vehicles due to the remarkable transduction efficiency, relatively easy preparation of high-titer purified virus, and its ability to infect both dividing and nondividing cells.\textsuperscript{3,5,10,11,18} The shorter duration of transgene expression by Ad of
weeks, compared to years in some other viral vectors, can appropriately augment healing of musculoskeletal tissues without persistence of the genes.

Ad vector has been successfully and popularly used for delivery of desirable genes both in vitro equine cells and tissue cultures and in vivo in the horse. Key processes of Ad entry into target cells are the initial recognition of coxsackie adenovirus receptor (CAR) and subsequent internalization via binding of the Arg-Gly-Asp (RGD) sequence on the penton base to \(\alpha_5\beta_3\) and \(\alpha_4\beta_3\) integrins. One of the factors, and the initial required step, that determines cell permissiveness to Ad vector is the amount of receptor available on the cell surface. Variable transduction by Ad vector can occur and has been limiting in a number of human cell types with low-intensity of the receptors. It is known that Ad vector can infect even non-permissive cells when virus is applied at very high multiplicities of infection (MOI), but this can be associated with severe cytotoxicity. Therefore, it is relevant to evaluate specific cell types, in regards to species and tissue of origin, as to their permissiveness and sensitivity to toxicity for Ad vectors. Chondrocytes and synovial cells are the targeted cell types for local gene therapy to joints for osteoarthritis or rheumatoid arthritis. Although in vivo transduction by Ad vector has been reported in equine chondrocytes and synovial cells, relative permissiveness between these articular cell types has not been reported.

Stem cells are a focus of cell-based molecular therapy and tissue-engineering studies. Even though adult stem cells have a more limited range of differentiation lineages than the embryonic stem cells, they can be more easily directed to specific tissue types and are considered safer for transplantation due to lesser proliferation capacity and
Bone marrow derived mesenchymal stem cells (BMD-MSCs) have a pluripotent potential to differentiate into many mesenchymal lineages such as muscle, bone, cartilage, tendon, and ligament, and can be promising carrier cells for ex vivo gene transfer methods. Localized delivery of cells transduced by Ad vector has proven successful to induce efficient tissue concentration of the therapeutic protein and reduce host immune response that may achieve safe and repeated gene therapy applications. Because BMD-MSCs can be acquired from horses by relatively simple techniques, equine gene therapies may select in vivo or ex vivo application options.

Various modifications of Ad vectors have been made to increase transduction efficiency in certain cell types with low permissiveness to virus vectors. This includes an alteration of receptor usage by fiber pseudotyping, use of bifunctional antibodies connecting the Ad vector to receptor other than CAR, and insertion of heterologous sequences into the fiber knob. It has been demonstrated that an insertion of the RGD motif into the HI loop of the Ad fiber can allow the vectors to use the \( \alpha_v \beta_3 \) or \( \alpha_v \beta_5 \) integrin as an alternative receptor resulting in an increase of transduction efficiency in CAR-deficient cells. By using this strategy, an enhanced infectivity was achieved in human tumor cells. We selected this Ad-RGD vector to investigate whether an application of a low titer of Ad vector can result in successful gene transfer into equine cells. If successful, an efficient therapeutic response could be achieved with the minimum local adverse effect on the surrounding bone or articular structures in addition to the low risk of systemic viral biodistribution into other organs.
Our long term goal was to define the permissiveness of various cell types to Ad vector-mediated gene transduction and evaluate modified Ad vectors that might more efficiently transduce poorly permissive cell types. Transduction efficiency to specific cell types may be enhanced by modification of the Ad vector or selection of alternate vectors for certain articular cell types if a relative permissiveness can be characterized. Concomitantly, cytotoxicity has not been compared in equine cell types infected with various dosages of Ad vector required for efficient transgene expression. Definition of these relationships will serve to aid vector selection and dosage for certain equine cell types.

The specific objective of this study was to compare the permissiveness and cytotoxicity of two modified serotype 5 Ad vectors, E-1deleted (Ad) and E-1A defective Ad with a modified capsid containing an RGD peptide insert (Ad-RGD), for gene delivery to equine chondrocytes, synovial cells, and BMD-MSCs. Our hypotheses were that variable transduction efficiency would be identified among the three cell types, high viral titers would induce variable cytotoxic effect and diminish cell viability, and that Ad-RGD would increase transduction efficiency, particularly of cell types with lower permissiveness to Ad vector gene delivery. The results of this study would characterize the equine cell type tropism for Ad vector and possibly identify a modified vector (Ad-RGD) with improved efficiency for certain cell types. This information would direct the selection of vector for equine studies and assist in establishing preferable gene therapy protocols in horses for certain cells and uses.
2.3 Methods

Study design

Three equine cell types were harvested from 15 horses, 5 different horses for each cell type (chondrocytes, synovial cells, and BMD-MSCs) and cultured in monolayer in duplicate. HeLa cells (human malignant cervical carcinoma of Henrietta Lacks; ATCC, Rockville, MD) were used as a reference cell type. Permissiveness of each cell type to Ad vectors (Ad and Ad-RGD) encoding the cDNA of β-galactosidase (Ad-LacZ) or green fluorescent protein (Ad-GFP and Ad-RGD-GFP) were compared by quantifying transduction efficiency (the percent of cells with positive gene expression) at 2 and 7 days after infection. Cytotoxicity of Ad vector on each cell type was evaluated using Ad vector at six multiplicities of infections [(MOI) range, 0 to 100] by histopathologic scoring for cell morphology, trypan blue stain for cell viability, and hemocytometer counting for cell numbers.

Horses

All fifteen horses that were included in the study were normal on physical and lameness examination (age range 2 to 15 years; median age 5). The 10 horses that provided articular cartilage (n=5) and synovium (n=5) had tarsocrural joints that were palpably, visibly, and radiographically normal prior to euthanasia and joint tissues that were grossly normal at necropsy. Horses that provided bone marrow (n=5) were normal on physical examination and hematology and had palpably and visibly normal sternabrae.
Tissue harvest and cell preparation

Articular cartilage and synovium were harvested from the tarsocrural joint. Following the aseptic preparation of the joint, articular cartilage was harvested as 1 to 2 mm full-thickness slices from the trochlear ridges of the talus. Villus synovium was harvested and dissected from underlying adventitia, fat or joint capsule using a dissecting microscope. Chondrocytes and synovial cells were isolated by digestion with collagenase and cultured in a monolayer as described elsewhere.\textsuperscript{51} BMD-MSCs were obtained by standard bone marrow aspiration from the sternum as follows. Immediately following euthanasia, horses were positioned in dorsal recumbency, the skin aseptically prepared, the skin and pectoral muscles dissected, and the ventral aspect of sternum exposed. A bone marrow aspiration needle (MD Tech Inc., Gainesville, FL) was inserted into the vertebral body from the ventro-lateral/medial aspect of the sternum and bone marrow was aspirated into heparin-flushed (Heparin sodium [American Pharmaceutical Partners Inc, Schaumburg, IL]: 1,000 USP units/ml) 12 ml sterile syringe. The procedure was repeated until a minimum of 10 ml of bone marrow was collected. Primary BMD-MSCs were isolated by centrifugation and cultured in a monolayer as described elsewhere.\textsuperscript{46} BMD-MSCs were confirmed pluripotent by culture in controlled osteogenic, chondrogenic, and adipogenic media cocktails containing dexamethasone with ascorbate, rhTGF-\(\beta\)1, and dexamethasone with insulin and indomethacin, respectively.\textsuperscript{5} HeLa cells were chosen as the reference cell type and purchased from the American Type Culture Collection (ATCC, Rockville, MD). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with L-glutamine (300 \(\mu\)g/ml),
penicillin (30 µg/ml), streptomycin (30 µg/ml), and 10% fetal bovine serum, at 37°C under a 5% CO₂ atmosphere.

*Generation of Ad vectors*

Three different types of Ad vectors including a recombinant, replication deficient, E-1 deleted, Ad vector encoding cDNA of β-galactosidase (Ad-LacZ), and a recombinant, replication deficient, E-1A defective Ad vector encoding cDNA of GFP without (Ad-GFP) or with an insertion of RGD peptide into the HI loop of the fiber knob domain (Ad-RGD-GFP) were generated as previously described.\(^{37}\) Briefly, the cDNA of β-galactosidase or GFP was subcloned into an expression vector and co-transfected with a plasmid containing the 9-36 map units of E1 defective human adenovirus-5 into human embryonic kidney 293 (HEK293) cells (ATCC, Rockville, MD). The resulting Ad vectors were named Ad-LacZ and Ad-GFP respectively. Another plasmid containing a gene of GFP and additional RGD sequences in the HI loop was constructed by homologous recombination and similarly transfected into HEK293 cells. The resulting Ad vector was named Ad-RGD-GFP (Sidney Kimmel Cancer Center, San Diego, CA). All three Ad vectors were amplified in HEK293 cells and purified by three rounds of cesium chloride centrifugation following salt removal by dialysis in a sucrose-containing buffer. The infectious unit (IU) of the Ad vectors was determined by the BD Adeno-X Rapid Titer kits (Corning Inc., Corning, NY). All Ad vector preparations were stored at -80°C in Gey’s balanced salt solution (Gibco, Grand Island, NY).
**Cell permissiveness to Ad vector**

The three types of equine cells and HeLa cells were expanded to confluence and used at low passage number (2 to 5 passages in equine cells and 20 to 22 passages in HeLa cells), and a final cell suspension was placed in 48-well plates (Sigma Chemical Co, St Louis, MO) at a density of 10,000 cells per well. At 24-hours after the final seeding (Day 0), DMEM was changed to contain Ad-LacZ at 0 (control), 1, 5, 10, 50, and 100 MOI (IU per cell). At each MOI, equine cells seeded in duplicate wells for each of the 5 horses and HeLa cells seeded in five replicate wells were infected. DMEM volume was controlled at 500 µl for all wells. In the 100 MOI assigned wells, for instance, the 500 µl of DMEM contained 1 million IU of Ad-LacZ [(100 IU per cell) x (10,000 cells per well)]. The final seeding and viral application were performed at the same time for all cell types, and Ad vectors were thawed just before the infection.

The %transduced cells by the Ad vector was assessed at Days 2 and 7 using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining (Corning Inc., Corning, NY) and expressed as a percent of total cells counted in five fields of 25 µm x 25 µm area per well under 200X magnification. If the cell population was low in certain MOI groups, more than five fields were used to obtain minimum 1,000 total cell counts per well for accurate calculation. The equine cells’ values in duplicate wells were averaged.

**Evaluation of Ad cytotoxicity**

At Days 2 and 7, morphology scores were assigned for each culture well at 200X magnification as follows: 0 = less than 10% of cells appeared rounded with minimum
signs of cell detachment from bottom surface of the culture wells; 1 = 11 to 25% of cells appeared rounded with mild signs of cell detachment; 2 = 26 to 50% of cells appeared rounded with moderate signs of cell detachment; 3 = 51 to 75% of cells appeared rounded with severe signs of cell detachment; 4 = 76 to 100% of cell appeared rounded and cell population was devastated by very severe cell detachment. The median score of three representative fields was used for each culture well.

At Days 2 and 7, the cells in monolayer cultures were harvested by removing media from the well and adding 125 µl of EDTA-trypsin to each well. Following five minutes incubation at room temperature, trypsin was neutralized by adding 125 µl of DMEM in each well and cells were harvested by gentle pipetting (250 µl total volume). The 10 µl of harvested cells were mixed with 90 µl of trypan blue solution (10X staining dilution) and placed in duplicate in a hemacytometer (Hausser Scientific, Horsham, PA). Following the one minute incubation at room temperature, total cell number and dead cell number were counted within eighteen of 1 mm x 1 mm fields (0.1 mm in depth) in the hemacytometer at 40X magnification. Total cell number per well was calculated as follows; (Total cell number per well ) = [the mean cell counts of the hematocytometer eighteen fields (= cell number per 0.1 µl)] x [10 (=100:10 µl staining dilution)] x [2500 (=250/0.1 µl volume ratio)]. If the cell population was low in certain MOI groups, the 2X (100:50 µl) or 1.11X (100:90 µl) staining dilutions with five minutes incubation were used to obtain minimum 30 total cell counts per eighteen hematocytometer fields for accurate calculation. Cell viability was calculated by dead cell number divided by total cell number within all eighteen fields and expressed as percent.
Evaluation of sustained transgene expression for Ad vector

Total number of transduced cells in each culture well was estimated as follows;

\[(\text{Total transduced cells per well}) = (\text{Total cell number per well}) \times (\% \text{viability}) \times (\% \text{transduced cells})\].

The calculation was made for each horse using the percent viability and percent transduction in each MOI group at Day 2 and 7.

Comparison between Ad vectors with and without RGD peptide insertion

Equine cells and HeLa cells were similarly seeded in different 48 well plates (10,000 cells per well). At 24-hour after the final seeding (Day 0), medium was changed by 500 µl DMEM containing Ad-GFP or Ad-RGD-GFP at 0 (control), 1, 5, 10, 50, and 100 MOI in a volume-control manner as described above. The % transduced cells was assessed using a fluorescent microscope at Days 2 and 7 by the calculation of number of GFP positive cells divided by the number of total cells counted within the five fields of 25 µm x 25 µm area per well under 200X magnification and expressed as a percent.

Statistical methods

All data were analyzed by using a statistical software program (SAS Institute Inc, Cary, NC). P <0.05 was considered statistically significant for all analyses. Distribution of objective data (continuous variables) were assessed by Proc Univariate model using subset of normality tests (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests), and all objective data were normally distributed (P>0.15).
Objective data were analyzed by multivariate ANOVA with *Proc Mixed* model using cell types, MOIs, time points, horse, and two types of Ad vectors as explanatory variables. Horse was treated as a random variable, and the repeated measures were considered nested within time points and horse. Horse was not a significant explanatory variable for all analyses. For the explanatory variables with significant overall p-values, subsequent multiple comparisons were made among four cell types, among six MOI groups, between Day 2 and 7, and between two Ad vectors (Ad-GFP and Ad-RGD-GFP). Subjective morphology scores data (categorical variables) were analyzed by multivariate ANOVA with *Proc Genmod* model using cell types, MOIs, time points, and horse as explanatory variables. For the explanatory variables with significant overall p-values, subsequent multiple comparisons were similarly made among four cell types, among six MOI groups, and between Day 2 and 7. To clarify the relation between two parts of our experiments, the %transduced cells calculated by Ad-LacZ and Ad-GFP were also compared by multivariate ANOVA using cell types, MOIs, horse, and Ad vector types as explanatory variables.

### 2.4 Results

*Transduction efficiency for Ad vector*

HeLa cells had significantly greater transduction efficiency than the primary equine cell isolates (P<0.01; Figs 2.1 to 2.3). BMD-MSCs had a significantly greater transduction efficiency than chondrocytes and synovial cells (P<0.01; Figs 2.1 to 2.3). Chondrocytes had a significantly greater transduction efficiency than synovial cells.
(P<0.01; Figs 2.1 to 2.3). Therefore, the significant order of tropism of Ad vector for these cell types was HeLa > equine BMD-MSCs > equine chondrocytes > equine synovial cells.

**Figure 2.1** – Transduction efficiency in HeLa cells, equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs). Mean ± SD of the %transduced cells at Day 2 (top) and 7 (bottom). Four cell types were transfected with adenoviral vector encoding marker gene (Ad-LacZ) using 6 different multiplicities of infection (MOI) and %transduced cells was assessed using X-gal staining at Day 2 and 7. Among the four cell types at each MOI, different letters are significantly different (P<0.05). Among the five MOIs at each cell type, different symbols are significantly different (P<0.05).
For all four cell types, %transduced cells was significantly declined between Day 2 and 7 (P<0.01; Figs 2.1 to 2.3) which were two- to seven-fold decreases at most MOIs (eg, from 83% to 11% in chondrocytes at 100 MOI). Transduction efficiency calculated by Ad-LacZ and Ad-GFP were not significantly different for any cell types in any MOIs at both time points.

**Figure 2.2** – X-gal staining at Day 2 in HeLa cells, equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs) transfected with adenoviral vector encoding marker gene (Ad-LacZ) at 1, 10, and 100 multiplicities of infection (MOI). The %transduced cells (percentage of blue cells) was decreased from HeLa cells to BMD-MSCs to chondrocytes to synovial cells and with lower MOIs.
Cytotoxicity

HeLa cells were significantly more sensitive to Ad vector dosage than all three equine cell types. For HeLa cells, the total cell number per well was significantly lower
compared to all three equine cells (P<0.01) and at higher MOIs (P<0.01). Specifically, HeLa cells’ number per well was significantly decreased compared to control (0 MOI) even at 1 MOI at both Day 2 and 7 (Table 2.1). For all three equine cells, total cell number per well was not significantly decreased from control in any MOIs at both Day 2 and 7, except synovial cells in 100 MOI at Day 2 (Table 2.1). Overall, total cell numbers per well were significantly greater at Day 7 than Day 2 (P<0.01) representing cell proliferation (Table 1; Figs 2.2 and 2.3).

For HeLa cells, the %viability was significantly lower compared to all three equine cells (P<0.01), at higher MOIs (P<0.01), and between Day 2 and 7 (P<0.01). Specifically, HeLa cells’ %viability was significantly decreased compared to control (0 MOI) even at 1 MOI at both Day 2 and 7 (Table 2.1). For all three equine cells, the %viability was not significantly decreased from control in any MOIs at both Day 2 and 7 (Table 2.1).

For HeLa cells, the morphology scores were significantly higher compared to all three equine cells (P<0.01), at higher MOIs (P<0.01), and between Day 2 and 7 (P<0.05). Specifically, HeLa cells’ morphology scores were significantly higher compared to control for 100 MOI group at Day 2 and 10, 50, 100 MOI groups at Day 7 (Table 2.1). For all three equine cells, the morphologic signs of cytotoxicity were not observed in any MOI or at both Day 2 and 7 (Table 2.1).
**Table 2.1** – Cytotoxicity parameters in equine chondocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs).

* Different significantly (P<0.05) from the control wells (0 MOI) within each cell type.

MOI (multiplicity of infection) = number of infectious viral particles per cell

SD = standard deviation

Morphology scores: 0 = less than 10% of cells appeared rounded with minimum signs of cell detachment from bottom surface of the culture wells; 1 = 11 to 25% of cells appeared rounded with mild signs of cell detachment; 2 = 26 to 50% of cells appeared rounded with moderate signs of cell detachment; 3 = 51 to 75% of cells appeared rounded with severe signs of cell detachment; 4 = 76 to 100% of cell appeared rounded and cell population was devastated by very severe cell detachment.
Figure 2.4 – Duration of transgene expression in HeLa cells, equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs). Mean ± SD of the total transduced cells per well at Day 2 (gray bars) and 7 (black bars) in HeLa cells, equine chondrocytes, synovial cells, and BMD-MSCs transfected with adenoviral vector encoding marker gene (Ad-LacZ) at 1, 5, 10, 50, and 100 multiplicities of infection (MOI). Total numbers of transfected cells per well were calculated by [total cell counts per well] x [%viability] x [%transduced cells]. *Differs significantly (P<0.05) between Day 2 and 7.
Figure 2.5 – Transduction efficiency of Ad-GFP and Ad-RGD-GFP vectors. Mean ± SD of the %transduced cells in HeLa cells, equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs) with Ad-GFP at Day 2 (solid diamond), Ad-RGD-GFP at Day 2 (solid square), Ad-GFP at Day 7 (open diamond), and Ad-RGD-GFP at Day 7 (open square). Four cell types were transfected with adenoviral vectors encoding marker gene without (Ad-GFP) and with (Ad-RGD-GFP) modification of RGD-peptide insertion at 1, 5, 10, 50, and 100 multiplicities of infection (MOI). For all cell types and at both Days 2 and 7, there is no significant difference between two Ad vectors.
Figure 2.6 – Fluorescent microscopy of Ad-GFP and Ad-RGD-GFP gene transduction. Fluorescent microscopy of HeLa cells, equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells (BMD-MSCs) transfected with adenoviral vectors encoding marker gene without (Ad-GFP) and with (Ad-RGD-GFP) modification of RGD-peptide insertion at 100 multiplicities of infection (MOI) at Days 2 and 7. For all cell types and at both Days 2 and 7, %transduced cells were not significantly different between Ad-GFP and Ad-RGD-GFP. Similar cytotoxicity of HeLa cells was seen at the high MOIs as with Ad-LacZ.
Sustained transgene expression

For three cell types (chondrocytes, synovial cells and HeLa cells), the total number of transduced cells per well significantly declined between Day 2 and 7 (P<0.01) which were two- to ten-fold decreased at most MOIs (eg, 6.6 to 1.2 million cells in chondrocytes at 100 MOI; Fig 2.4). The total number of transduced cells of BMD-MSCs remained similar between Day 2 and 7, whereas there were no significant difference in the total number of transfected cells between Day 2 and 7 for all MOIs (Fig 2.4).

Comparison between Ad vectors with and without RGD peptide insertion

In all four cell types, transduction efficiency was not significantly different between the Ad-GFP and Ad-RGD-GFP vectors at any MOI (Figs 2.5 and 2.6). As seen previously with Ad-LacZ, transduction efficiency decreased between Day 2 and 7 (P<0.01; Fig 2.5) and HeLa cells showed signs of cytotoxicity at high MOIs (Fig 2.6).

2.5 Discussion

To the authors’ knowledge, our study is the first to show a significant cell type tropism of Ad vector in equine cells relevant to equine orthopedic gene therapy. Additionally, our study documented a relative resistance of these primary equine cell isolates to the cytotoxic effects of Ad, compared to a standard human cell line, including at high dosages of vector. Based on these findings, Ad vector should be effective at transducing equine chondrocytes, synovial cells, and BMD-MSCs, but differential cell type transduction should be anticipated, particularly at low MOIs. Synovial cells will be
the most difficult to transduce. This may be relevant to intra-articular injection of vector, intra-articular fracture injection or use of co-culture systems as gene transfer may not occur equitably across the available cell types resulting in differential tissue expression of therapeutic agent, particularly at lower MOIs. Further studies would be necessary to confirm this effect in vivo.

Our study demonstrated that Ad vector was generally efficient at gene transduction for all three equine cell types approaching 100% for HeLa cells and equine BMDMSCs, 80% for equine chondrocytes and 60% for equine synovial cells at the highest MOI. Cytotoxicity was not evident in BMD-MSCs and chondrocytes and negligible for synovial cells. Synovial cells with 100 MOI Ad infections showed lower cell population, but the decline of cell viability was not statistically significant. This may indicate that the high dosage of Ad vector primarily induced an interference of the synovial cells’ proliferation more than cell death. HeLa cells had the highest transduction efficiency but also had more cell death and cytopathologic effects (CPE) even at lower MOI (Figs 2.1 to 2.3). The occurrence of a higher cytotoxicity in HeLa cells maybe explained by greater viral replication in human cells as compared to equine cell lines. Small quantities of wild type human Ad-5 in viral preparations may replicate within human cells more readily than equine cells. In additional studies performed to investigate the possible cause of our findings, we confirmed and quantified the presence of wild-type Ad-5 in our vector preparations by performing six serial passages of cell lysate onto our equine cells and HeLa cells. The development of microscopic CPE on cells indicated wild-type virus replication which would amplify with each passage because only wild-
type Ad-5, not E-1deficient Ad vector, can replicate in any of these cells. Cytopathologic effects (CPE) from replicating virus did develop in HeLa cells in which the amount of CPE gradually increased with every passage. A subsequent dilutional titering study looking for CPE on HeLa cells estimated the Ad-5 titer at approximately 0.28% of Ad-LacZ vector titer. Presence of CPE, and therefore replicating Ad-5, was not observed in any equine cells even after six passages. We postulated that human cell lines may naturally contain factors that facilitate human virus replication that does not occur in equine cell lines. Presence of replication-competent virus in recombinant vector preparations can not be completely eliminated through the Ad vector generation process. Our study uniquely identified the difference in CPE formation between the human and equine cells with Ad vector gene delivery, although further study is necessary to elucidate its mechanism. The potential adverse effect of Ad vector may be relevant in gene therapy applications in humans. The fact that viral replication was not observed in equine cells may enhance the safety of Ad vector used in horses or other animals.

Our study was unable to demonstrate a significant improvement in gene transfer in our cells by using the RGD modified Ad vector. This may reflect the relatively high efficiency of the unmodified Ad vector to enter these cells. Receptor-mediated transfer may have been maximized, such that additional binding of Ad to the integrin cell surface receptor could not induce a detectable increase in subsequent gene transcription. The small, but significant increase in transduction produced at very high MOIs may occur by vector entry using other receptor-independent entry mechanisms, such as endocytosis, pinocytosis or phagocytosis. In such cases, transduction efficiency should not be
altered by integrin receptor binding of vector with RGD. It is less likely that failure of this vector to improve transduction efficiency was due to lack of integrin receptors on at least the chondrocytes and BMD-MSCs.\textsuperscript{54} Instead, it is predictable that an intensity of primary Ad receptor, CAR, was high on the cell surface of HeLa and equine cells in our study. Therefore, an availability of the secondary Ad-cell attachment pathway through the integrin receptor did not produce a detectable increase in transduction efficiency. Recent studies have shown that incorporation of RGD peptide into Ad or adeno-associated-virus (AAV) vectors can augment the efficiency of transgene expression in certain types of cells that have low expression of CAR.\textsuperscript{48,49,55,56} In comparison, AAV vector has less infectivity to some articular tissues than Ad and may be significantly improved by this modification as an alternative vector for equine gene therapy.\textsuperscript{57,58} The cytotoxicity of RGD-modified vector (Fig 2.6) was not quantified in our study, but it appeared to be similar level compared to Ad-LacZ (Fig 2.2 and 2.3). It should be emphasized that MOIs in our study were determined by the calculation from infectious unit assay, not due to the rather crude estimation from optometric particle counts. For this reason, the transduction efficiencies in Ad-LacZ (Fig 2.1) and Ad-GFP (Fig 2.5) were approximately equal with no statistically significant differences. Thus, the cytotoxic effects by means of an amount of viral infection should be comparable between different types of Ad vectors.

BMD-MSCs are excellent candidates for ex vivo equine gene therapy using Ad vector. First, our results showed that BMD-MSCs had significantly higher transduction efficiency by Ad vector than the other two equine cell types evaluated in this study.
(chondrocytes and synovial cells). Second, BMD-MSCs did not appear to be sensitive to cytotoxicity associated with Ad infection and had sustained gene expression for at least 7 days. The total number of transduced BMD-MSCs was similar between Day 2 and 7 despite the total cell population per culture well increasing and resulting in a decrease in percentage of transduced cells. Because of the lack of gene integration into host genome by Ad vector, presumably only one of the daughter cells would contain the initially transfected gene following the single cell division. Hence, our results suggest that the portion of BMD-MSCs that initially transfected by Ad vector were able to maintain transgene expression in longer time periods compared to other cell types. Ex vivo gene delivery can be utilized to achieve high transduction efficiency, allow clinicians to select specific cells as the delivery carrier, increase the local tissue cellularity, and may contribute safer application when it prevents direct contact between Ad vector and host cells. Ready access to BMD-MSCs makes these cells a promising gene delivery vehicle for ex vivo gene therapy. The fact that BMD-MSCs are osteogenic precursors makes them promising candidates for use in bone healing.\(^3^5\) Moreover, BMD-MSCs may further enhance fracture healing by dual autocrine and paracrine responses, because they are not only able to secrete an osteoinductive protein (eg, bone morphogenetic protein; BMP) but also respond to it.\(^3^5\) For these reasons, genetically modified BMD-MSCs have been demonstrated to have sufficient potency to enhance bone formation in various animal models.\(^2^,3^4,40-42,59,60\)

Equine chondrocytes had greater permissiveness to Ad vector than synovial cells in our study, which is similar to AAV vector in these equine cell types.\(^5^4\) This lower
transduction of synovial cells may be explained by a relatively low intensity of cell surface receptor. The primary Ad receptor, CAR, is undetectable in human synovial cells\textsuperscript{20} and very low in murine synovial cells.\textsuperscript{61} Equine chondrocytes can be readily transduced by Ad vector in vitro, but may be limited in vivo, because Ad vector is generally not able to penetrate the dense extra-cellular matrix surrounding articular chondrocytes and genetically modify them sufficiently to induce a clinically relevant response.\textsuperscript{62,63} Conversely, although synovial cells may be less permissive to Ad vector, sufficient transgene expression has been obtained with Ad vector to be clinically relevant. Synovial lining has a high surface area and direct contact with the joint space and secreted therapeutic proteins induced by the augmented genes can subsequently diffuse into other intra-articular tissues such as cartilage.\textsuperscript{63} Therapeutic gene can be efficiently transduced to synovial cells by Ad vector resulting in high intra-articular transgene expression from the arthritic synovium.\textsuperscript{64-67} Genetically modified cells injected into joints engrafted almost entirely in the synovial lining and subsequently secreted gene products.\textsuperscript{68}

Percentage of transduced cells by Ad vector decreased between 2 and 7 days after infection (Figs 2.1 to 2.3) which was likely due to the synergism of increasing total cell number (Fig 2.4) and decreasing transduced cell number (Fig 2.6). This decline in transgene expression, however, may not be a limiting factor of in vivo gene therapy application for equine musculoskeletal disorders. Ad as a vector for bone augmented genes (eg, BMP) was sufficient to accelerate the process of bone healing.\textsuperscript{69} Ad vectors typically do not integrate the transgene into the host DNA, resulting a decline of
transgene expression over weeks to months.\textsuperscript{11,14,20,21} Specifically, chondrocytes used for ex vivo gene delivery persisted as graft cells at 3-8\% of resident cells at four weeks.\textsuperscript{21,70} Genetic modification of chondrocytes transplanted in vivo can have sustained gene expression at biologically relevant transgene influence for two to four weeks.\textsuperscript{11,14,21,25} Therefore, although only small portions of cells maintain transgene expression, this may be sufficient for the production of therapeutic proteins at a clinically effective concentration. This relatively rapid loss of gene expression has been reported with Ad vector and was seen in our study as a decline in experimental cells within 7 days (except equine BMD-MSCs). In our in vitro model this reflects lack of integration of the Ad vector with the host cell genome with less effect of host immune reaction to the vector. In vivo gene expression in horses is anticipated to last 30-60 days.\textsuperscript{19}

In summary, our study demonstrated a variable permissiveness of equine cell types to Ad vector in the order of BMD-MSCs, chondrocytes, and synovial cells from most to least permissive. Transgene expression persisted at least 7 days in equine BMD-MSCs but waned in equine chondrocytes and synovial cells. Cytotoxicity of Ad vector on equine cell types was minimal and exposed cells readily proliferated in number between Day 2 and 7. The addition of an RGD peptide did not significantly increase the efficiency of Ad vector transduction.
Chapter 3:

Osteogenic gene regulation and relative acceleration of healing by adenoviral-mediated transfer of human BMP-2 or -6 in equine osteotomy and ostectomy models

3.1 Summary

This study evaluated healing of equine metatarsal osteotomies and ostectomies in response to percutaneous injection of adenoviral (Ad) bone morphogenetic protein (BMP)-2, Ad-BMP-6 or beta-galactosidase protein vector control (Ad-LacZ) administered 14 days after surgery. Radiographic and quantitative computed tomographic assessment of bone formation indicated greater and earlier mineralized callus in both the osteotomies and ostectomies of the metatarsi injected with Ad-BMP-2 or Ad-BMP-6. Peak torque to failure and torsional stiffness were greater in osteotomies treated with Ad-BMP-2 than Ad-BMP-6, and both Ad-BMP-2 and Ad-BMP-6 treated osteotomies were greater than Ad-LacZ or untreated osteotomies. Gene expression of ostectomy mineralized callus 8 weeks after surgery indicated upregulation of genes related to osteogenesis compared to intact metatarsal bone. Expression of transforming growth factor beta-1, cathepsin H, and gelsolin-like capping protein were greater in Ad-BMP-2 and Ad-BMP-6 treated callus compared to Ad-LacZ treated or untreated callus. Evidence
of tissue biodistribution of adenovirus in distant organs was not identified by quantitative PCR, despite increased serum anti-adenoviral vector antibody. This study demonstrated a greater relative potency of Ad-BMP-2 over Ad-BMP-6 in accelerating osteotomy healing when administered in this regimen, although both genes were effective at increasing bone at both osteotomy and ostectomy sites.

3.2 Introduction

Bone morphogenetic proteins (BMPs) are the most studied osteogenic growth factors to induce bone formation. Use of recombinant human (rh) BMP in patients to enhance spine fusion and fracture healing has been successful. Effective bone formation has been demonstrated by the use of the adenoviral delivery of BMP-2, BMP-6, BMP-7, and BMP-9. However, few studies have evaluated the comparative osteoinductive potencies between BMP genes. BMP-2 and -4 genes were found to be comparable. BMP-4 and -6 genes were found to have different mechanisms of bone formation. BMP-6 genes showed more mature in vivo ectopic bone formation within murine muscle than BMP-2 genes, but BMP-2 genes showed more robust in vitro osteogenesis in equine bone marrow stem cells than BMP-6 genes. The effect of BMP-6 on bone formation has received recent research interest, but its in vivo osteoinductive efficacy at bone healing sites relative to the BMP-2 gene has not been reported. Additionally, despite the comprehensive comparisons of bone formation by endogenous rhBMPs, the relative osteoinductivity among exogenous BMPs administered by gene delivery is only lightly studied. In gene delivery, protein biologic effect may vary due to
inherent gene function as well as post-transcriptional modification and dual paracrine and autocrine effects.

Efficacy of BMP gene application may largely vary depending upon bone healing environments, possibly more than protein application, due to the differences in targeted cell types to produce exogenous BMP, mechanical load, and defect size. Mechanism and rate of bone formation has shown to be effected by gap size and loading.\textsuperscript{13} Bone inductions by BMP-2 and -6 genes may involve different mechanisms.\textsuperscript{9,14} Whereas BMP-2 gene may provoke bone formation through endochondral ossification as occurs in fracture gaps, the bone formation induced by BMP-6 gene may undergo direct, intramembranous ossification.\textsuperscript{9} Ad-BMP-6 injection into murine quadriceps induced bone at the ligament insertions.\textsuperscript{5} Different type of osteogenic growth factors may be optimal in different in vivo atmosphere, because the cell availability, loading mode, or presence of co-regulators can be substantially different, for instance, between gap healing and appositional bone healing. Therefore, comparison of osteoinductive capacity between BMP-2 and -6 in different healing environments may be warranted to optimize a choice of BMPs based on the type of injuries.

An equine model offers additional information to the efficacy studies on BMPs published in mice, rats, and rabbits. An efficacy of the certain therapeutic agent can be compared at multiple types of fracture models within the same individual to reduce animal variation. Optimal choice of BMPs depending upon fracture types can therefore be studied. In addition, the high tolerance to morbidity in rodents may overestimate the functionally recovery of the fractured limbs, compared to larger animals or humans.
Moreover, because many rodent species used in fracture research have been immuno-
incompetent with suppressed local and systemic host immune or inflammatory responses,
the result is influenced by an exaggerated ability to resolve differences among genes that
may not be clinically relevant in people. In this regard, a previous study indicated that the
efficacy of BMP gene therapy in small animal experiments cannot simply be transferred
to large animal models.\textsuperscript{15}

Profiling gene expression in bone healing sites can provide an opportunity to
understand normal repair process of osseous tissue and evaluate the effects of various
treatment strategies. Novel, robust microarray technology has been used to evaluate the
large scale of gene expression for normal bone healing,\textsuperscript{16} nonunions,\textsuperscript{17} and rhBMP-2-
induced bone formation in muscles,\textsuperscript{18} but not at the sites of fracture healing enhanced by
BMPs. Bone formation is a complex series of transcription processes even in natural bone
repair sites. Microarray analysis may offer a valuable tool to not only comprehensively
assess the signaling processes during osteogenesis but also identify the specific genes
contributing an acceleration of fracture healing. If upregulation of certain signaling
molecules were linked with the process of genetically enhanced bone healing, they may
be further studied as the candidates for future therapeutic applications. In addition, large-
scale gene expression analysis may explain the differences in osteoinductivity among
types of BMPs by events occurring at the molecular level.

Our study was the first to compare the relative potencies and gene expression
changes of hBMP-2 and -6 genes on the acceleration of bone healing in gap and nongap
fracture models using immune-competent large animals. We hypothesized that Ad-BMP-
2 and Ad-BMP-6 gene therapy can accelerate fracture healing in an equine model and Ad-BMP-2 may be superior in a gap healing model.

3.3 Methods

Study design

Twelve skeletally mature horses (weight 405 to 505 kg) had surgically created bilateral fourth metatarsal (Mt4) 1mm transverse osteotomies and bilateral second metatarsal (Mt2) 1cm gap ostectomies. One randomly assigned hindlimb in each horse was treated with adenoviral vector encoding hBMP-2 gene (Ad-BMP-2; n=6 limbs) or hBMP-6 gene (Ad-BMP-6; n=6), and the contralateral limbs were assigned to either untreated (n=6) or treated with adenoviral vector encoding bacterial β-galactasidase gene (Ad-LacZ; n=6) (Table 3.1). All adenoviral constructs were injected two weeks after surgery. Six weeks following the injection, the horses were euthanized and Mt4 and Mt2 specimens were harvested. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University. Efficacy was assessed by weekly radiographs, quantitative computed tomography, mechanical testing, histology, and gene expression analysis. Safety of gene delivery was assessed by physical examination, peripheral venous blood hemogram, serum adenovirus titer neutralization assay, and quantitative PCR for the CMV promoter regions of the adenovirus in multiple organ tissues.
### Table 3.1 – Limb assignments and outcome measurements in 12 horses used.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Randomly-assigned treatment hindlimb</th>
<th>Contralateral hindlimb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horse</td>
<td>Treatment</td>
</tr>
<tr>
<td>#01</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#02</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
<tr>
<td>#03</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#04</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
<tr>
<td>#05</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#06</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
<tr>
<td>#07</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#08</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
<tr>
<td>#09</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#10</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
<tr>
<td>#11</td>
<td>Ad-BMP-2</td>
<td>Mt4 osteotomy</td>
</tr>
<tr>
<td>#12</td>
<td>Ad-BMP-2</td>
<td>Mt2 osteotomy</td>
</tr>
</tbody>
</table>


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**Generation of adenoviral vector construct**

E-1 defective Adenovirus-5 preparations were generated using hBMP-2 and hBMP-6 cDNA under the control of the cytomegalovirus (CMV) promoter as previously reported.\(^5\,\,^{11}\) Particle titers were determined by optical density at 260nm and diluted to a concentration of \(1 \times 10^{12}\) /ml in phosphate buffered saline. Presence of transgene expression was verified.\(^{11}\)
Surgical procedure

Following general anesthesia and aseptic preparation of the hindlimb, a nitrogen-driven oscillating bone saw was used to create a 1mm slit defect in the proximal Mt4 and 1cm gap defect in the distal Mt2 on both hindlimbs (Fig 3.1). Antibiotics were

Figure 3.1 – Schematic image of transverse osteotomy on the fourth metatarsal bone (Mt4; lateral side) and gap ostectomy on the second metatarsal bone (Mt2; medial side).
administered for one day following surgery (procaine penicillin, IM, 22000 IU/kg and gentamicin, IV, 6.6 mg/kg); an anti-inflammatory drug (phenylbutazone, PO, 4.4 mg/kg) was administered for three days following surgery. Two weeks after surgery, under general anesthesia and aseptic preparation, the randomly assigned osteotomy and ostectomy sites were treated by percutaneous fluoroscopic-guided needle injections of 5 x 10^{11} adenoviral particles in a 500 µl volume directly into the osteotomy and ostectomy.

Physical examinations and blood test

Physical examination, lameness at the walk, circumference of the metatarsal region at the levels of the surgery sites, and pain-free range of joint motion for the tarsocrural and metatarsophalangeal joints, was performed weekly. Swelling and drainage at the surgery sites were scored (0 – none, 1 – minimum, 2 – mild, 3 – moderate, 4 – marked). Peripheral hemogram and serum chemistry was performed before and every two weeks after surgery.

Radiographic evaluation

Radiographs of Mt4 and Mt2 were obtained weekly for both hindlimbs. Using the dorsolateral-plantaromedial-oblique views, external callus area at the Mt4 osteotomy site was estimated by multiplying the length and width of the callus measured on the plantarolateral surface of the Mt4 (mm²).^{5,19} Using the dorsomedial-plantarolateral-oblique views, mineralization area at the Mt2 ostectomy site was estimated by multiplying the length and width of the mineralized tissue within the gap of Mt2 (mm²).
Quantitative computed tomography

Six weeks following the adenoviral injection, horses were sedated with xylazine hydrochloride (IV, 1 mg/kg) and euthanized with by lethal intravenous overdose of pentobarbital (Beuthanasia, IV, 2.2 mg/kg). Immediately after the euthanasia, both hindlimbs were dissected at the level of tarsometatarsal joint. Quantitative computed tomography (QCT) was performed in transverse sections at 1mm intervals to evaluate the bone formation and soft tissue mineralization using potassium phosphate standards. All transverse slices across the Mt4 slits and Mt2 gaps were used to calculate the total callus volume in each defect. The three central transverse slices were used to calculate the mineral density in each defect. After standardization, the Hounsfield unit density values were converted to ash density.\(^\text{20}\)

Biomechanical testing

Both ends of the Mt4 osteotomy specimens were dissected and embedded in square molds using polymethylmethacrylate at 1.5cm distant from defects to both sides (i.e., 3cm lengths of bones were exposed) and tested clockwise quasi-statically to failure in torsion (1.5°/s) using a servohydraulic materials testing system. Left limbs’ Mt4 were tested distal end up and right limbs’ Mt4 tested proximal end up to ensure similar directional rotation for both sides. The testing was stopped when the specimen broke, or when 90° of rotation was reached, whichever occurred first. The torque-rotation data were used to compute the maximum torque, torsional stiffness, and energy absorbed to
failure. To compare the data with characteristics of intact bones, six Mt4 specimens were collected from six adult horses (three from right and three from left hindlimbs), who were euthanized for reasons unrelated to either hindlimbs, and mechanical testing was performed in the same manner.

**Serum neutralizing antibody assay**

Peripheral serum samples were collected from each horse before surgery, before the adenoviral injections, and 2, 4, and 6 weeks after the injections for the neutralizing antibody (NAb) titer assay. Titers were determined by analyzing the ability of serum antibody to inhibit Ad-LacZ infection (1x10⁴ particles/cell) on HeLa cells (5x10³ cells/well) at 48 hours. By applying 2-fold dilution series of sample serum, the NAb titer was calculated as the highest serum dilution inhibiting Ad-LacZ transduction by >50%.

**Histologic evaluation**

Histologic evaluation was performed for all Mt4 and the two representative Mt2 specimens per group. The 2 of 6 Mt2 specimens with the median mineralized tissue sizes on week 5 radiographs were selected in each treatment group. Remaining Mt2 were processed for gene expression analysis. The calcified bone specimens were dehydrated in alcohol, embedded in methylmethacrylate, cut into 10μm sections in the sagittal plane, and stained with Masson’s Trichrome. The bone specimens were evaluated subjectively for the presence, extent, maturity, and porosity of external callus and bony bridging.
Gene expression analyses

Gene expression analysis was performed using RNA samples from the gap tissue and from 4 intact Mt2 bone specimens collected from four additional normal adult horses for intact bone comparisons. Total RNA was extracted by TRIzol (Invitrogen Life Technologies) from the homogenized bone tissues using established protocol. The 5μg of RNA were prepared and processed for gene expression analysis with an equine-specific microarray (Custom Equine GeneChip®, Affymetrix, Inc., Santa Clara, CA). The names and functions of genes were annotated by the commercially available software (BlastN, TimeLogic Corp, Carlsbad, CA; National Council Biotechnology Information [NCBI] Web site).

Biolocalization of adenoviral vectors

Genomic DNA was extracted from whole blood using QIAamp Blood Kit and from tissue samples such as Liver, Kidney, Spleen, Lung, and Ovary, using QIAamp Mini Kit (Qiagen®, Valencia, CA). Quantitative polymerase chain reaction (PCR) was performed using primers that amplify CMV sequences, which was detected by the internal fluorogenic probe labeled with FAM reporter dye, and ABI Prism 7000 sequence detection instrument (PE Applied Biosystems, Foster City, CA). Each of triplicate reactions contained the 10ng and 100ng of test DNA for blood and tissue samples, respectively. A standard curve was generated using plasmid containing 5×10^1 through 5×10^7 copies of CMV. DNA was also isolated from HeLa cells (3×10^6 cells in a 75cm^2
flask) with and without Ad-LacZ transduction (1x10^3 particles/cell) and included in each PCR plate as positive and negative controls.

**Statistical analysis**

Repeated-measured analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC) was used to evaluate the effects of Ad-BMP-2 and -6 on the objective data of radiography, QCT, mechanical testing, NAb titer, and histologic evaluation. Repeated variables such as treatment and time points were considered to be nested within horse, and multiple comparisons were made among the 4 treatment groups and across time points. Significant level was set at P<0.05 for these analyses. Gene expression data were analyzed with GeneChip® analysis software (dChip, Harvard University, Boston, MA) by use of hierarchal clustering method followed by 1 – Pearson correlation as the distance measurement. With Bonferroni correction, significance level was set at P<0.005 for these analyses.

### 3.4 Results

**Physical examinations and blood tests**

Physical examination, hemogram, lameness, and pain-free ranges of joint motion for the tarsocrural and metatarsophalangeal joints were not significantly changed from the baseline values in all horses (results not shown). The circumference of the metatarsal region at the level of the surgery sites, and subjective scores of local swelling and incisional drainage had no significant differences among treatment groups at any time
point (results not shown), although these parameters were significantly increased from the baseline values until three to four weeks after the surgery (one to two weeks after injection).

**Figure 3.2** – A time course collage of Mt4 transverse osteotomy healing (left) and Mt2 gap ostectomy healing (right) from 1 to 5 weeks after adenoviral injection. Ad-BMP-2 and -6 treated bones showed greater bone formation at earlier time points.
Radiographic evaluation

For both Mt4 osteotomy and Mt2 ostectomy, rapid bone formation and a greater area of mineralized callus was observed radiographically within two weeks after injection (four weeks after surgery) in the Ad-BMP-2 and -6 treated bone defects compared to the Ad-LacZ treated or untreated defects (Fig 3.2 and 3.3). For Mt4 osteotomy model, the Ad-BMP-2 treated bone defects had a significantly greater mineralized callus than Ad-BMP-6 treated defects at two weeks after injection (Fig 3.3; Left).

Quantitative computed tomography

For both Mt4 osteotomy and Mt2 ostectomy models, the volume and ash density of mineralized callus were significantly greater in the Ad-BMP-2 and -6 treated bone
defects compared to the Ad-LacZ treated or untreated defects (Table 3.2). For both models, there was no significant difference in the bone volume or density between the Ad-LacZ treated and untreated bone defects (Table 3.2). On the soft tissue scans, there was no evidence of ectopic mineralization or bone formation in the surrounding soft tissues including superficial and deep digital flexor tendons, suspensory ligament, and interosseous ligament.

<table>
<thead>
<tr>
<th></th>
<th>Bone volume (mm$^3$)</th>
<th>Mineral density (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mt4 osteotomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>192.7 ± 16.0</td>
<td>379.2 ± 31.0</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>234.5 ± 41.4</td>
<td>396.1 ± 32.7</td>
</tr>
<tr>
<td>Ad-BMP-2</td>
<td>429.8 ± 56.6</td>
<td>702.7 ± 31.3</td>
</tr>
<tr>
<td>Ad-BMP-6</td>
<td>334.8 ± 56.2</td>
<td>511.1 ± 39.7</td>
</tr>
<tr>
<td><strong>Mt2 ostectomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>7.7 ± 1.6</td>
<td>248.8 ± 15.0</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>20.2 ± 7.1</td>
<td>362.4 ± 41.2</td>
</tr>
<tr>
<td>Ad-BMP-2</td>
<td>57.0 ± 29.6</td>
<td>458.1 ± 43.8</td>
</tr>
<tr>
<td>Ad-BMP-6</td>
<td>95.5 ± 43.6</td>
<td>555.4 ± 39.6</td>
</tr>
</tbody>
</table>

Table 3.2 – Effect of Ad-BMP-2 and -6 on properties of the mineralized callus, assessed by peripheral quantitative computed tomography (mean ± SD).
*Significantly greater than untreated and Ad-LacZ treated bone defects (p < 0.03).

Biomechanical testing

For the Mt4 osteotomy, all parameters of biomechanical strength (peak failure torque, torsional stiffness, and energy absorbed to failure) were significantly greater in
the Ad-BMP-2 and -6 treated bone defects compared to the Ad-LacZ treated or untreated defects and not significantly different than the intact bones (Table 3.3). Ad-BMP-2 treated Mt4 bone defects had a significantly greater failure torque and torsional stiffness compared to Ad-BMP-6 treated defects (Table 3.3).

<table>
<thead>
<tr>
<th></th>
<th>N-m</th>
<th>% intact bone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Failure torque</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.92 ± 0.25</td>
<td>34.3 ***</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>1.16 ± 0.31</td>
<td>43.4 ***</td>
</tr>
<tr>
<td>Ad-BMP-2</td>
<td>2.17 ± 0.35</td>
<td><em>,</em>* 81.0</td>
</tr>
<tr>
<td>Ad-BMP-6</td>
<td>1.75 ± 0.19</td>
<td>* 65.2</td>
</tr>
<tr>
<td>N-m/deg (X100)</td>
<td></td>
<td></td>
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<tr>
<td>Untreated</td>
<td>4.28 ± 1.33</td>
<td>29.0 ***</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>6.68 ± 2.43</td>
<td>45.3 ***</td>
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<tr>
<td>Ad-BMP-2</td>
<td>17.71 ± 5.02</td>
<td><em>,</em>* 120.0</td>
</tr>
<tr>
<td>Ad-BMP-6</td>
<td>12.47 ± 2.53</td>
<td>* 84.5</td>
</tr>
<tr>
<td><strong>Torsional stiffness</strong></td>
<td></td>
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<tr>
<td>Energy absorbed to failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.47 ± 3.29</td>
<td>35.6 ***</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>14.50 ± 4.70</td>
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<tr>
<td>Ad-BMP-2</td>
<td>25.00 ± 3.71</td>
<td>* 77.5</td>
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<tr>
<td>Ad-BMP-6</td>
<td>34.41 ± 9.38</td>
<td>* 106.7</td>
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</tbody>
</table>

Table 3.3 – Effect of Ad-BMP-2 and -6 on torsional biomechanics (mean ± SD).
*Significantly greater than untreated and Ad-LacZ treated osteotomies (p < 0.02)
**Significantly greater than Ad-BMP-6 treated osteotomies (p < 0.02)
***Significantly lower than intact Mt4 values (p < 0.04)
Serum neutralizing antibody assay

Serum NAb titer at 2, 4, and 6 weeks after injection was significantly increased from the baseline in all horses. Median and range of NAb titers were 1.5 [1-4], 48 [2-256], 48 [8-256], and 48 [16-256] for baseline, week 2, 4, and 6, respectively. There was no significant difference in serum NAb titer between the unilaterally and bilaterally injected horses at any time point. Serum NAb titer did not appear to be correlated with any outcome measurements.

Histologic evaluation

For Mt4 osteotomy, Ad-BMP-2 and -6 treated bone defects appeared similar to each other and had greater and more mature formation of external callus and bony bridging compared to Ad-LacZ treated or untreated defects (Fig 3.4). The callus in the Ad-BMP-2 and -6 treated bone defects were interpreted to be more mature than in the Ad-LacZ treated and untreated defects because they contained greater amount of lamellar bone compared with woven bone. The cut edges in Ad-BMP-2 and -6 groups had greater bone formation area and less bone resorption area compared to Ad-LacZ treated or untreated defects. For Mt2 ostectomy, Ad-BMP-2 and -6 treated bone defects appeared to have greater new bone formation within the ostectomy gaps (Fig 3.4).
Gene expression analysis

Of the 3,098 genes on the species specific microarray, a mean of 62.9±3.9%, 63.1±3.5%, 63.9±4.9%, and 63.3±5.7% were expressed in the intact, untreated, Ad-BMP-2, and Ad-BMP-6 treated ostectomy callus, respectively, indicating consistent overall gene expression in the tissues. Genes associated with endochondral ossification and osteogenesis, such as transforming growth factor beta-1 (TGFB1), cathepsin H, and
gelsolin-like capping protein, were upregulated in Ad-BMP-2 and -6 treated ostectomy callus compared to intact bone. Other genes, such as type II collagen (COL2A1), cartilage oligomeric matrix protein (COMP), and matrix metalloproteinase-9, were upregulated in all ostectomy calluses regardless of adenovirus injection, compared to intact bone. In contrast, the expression of two genes, collagen type III alpha-1 and dominant negative helix-loop-helix protein, were downregulated in all ostectomy calluses, regardless of adenoviral injection, compared to intact bone. A few genes associated with an immune response, such as immunoglobulin G light chain (IGLG) and immunoglobulin mu heavy chain constant chain (IGHM), were upregulated in Ad-BMP-2 and -6 treated ostectomy callus, but not in untreated ostectomy callus, compared to intact bone, suggestive of a persistent immune response to Ad vector. Additional immune-related genes had altered expressions in all ostectomy calluses regardless of adenovirus injection compared to intact bone, such as T-cell receptor, CCAAT/enhancer binding protein, beta-defensin-1, chemokine receptor-4, FK506 binding protein-5, chemokine ligand-12, and interferon gamma inducible protein-30, suggesting a role in mineralized callus formation and bone healing. Most of the genes related to inflammation and cell proliferation were upregulated in all ostectomy calluses and not changed by adenoviral exposure, including CD68 protein, myristoylated alanine-rich protein kinase, lymphocyte cytosolic protein-1, galectin-8, glia maturation factor-gamma, ferritin-L, and chimerin-1. In contrast, a subset of inflammatory and cell growth genes, such as CCAAT/enhancer binding protein, nuclear factor of kappa light polypeptide enhancer, thioredoxin interacting protein, gelsolin, serpin peptidase inhibitor, glutamate-ammonia ligase, phosphatidic acid
phosphatases, and manganese superoxide dismutase, were downregulated in all osteotomy callus compared to intact bone, and not altered by adenoviral exposure. Genes that were up- or downregulated among groups are listed in the supplemental data (Appendix A).

Biolocalization of adenoviral vectors

Promoter DNA in the CMV portion of the Ad vector was not detected in any untreated osteotomy callus and peripheral blood samples at any time point, or any of five organs evaluated such as Liver, Kidney, Spleen, Lung, and Ovary. The CMV promoter sequences (CMV copy #; mean ± SD) were detected in osteotomy callus treated with Ad-LacZ (4.4x10^2 ± 2.1x10^2), Ad-BMP-2 (2.6x10^4 ± 1.2x10^4), and Ad-BMP-6 (2.6x10^4 ± 1.0x10^4). The CMV copy # was greater in mineralized callus with Ad-BMP-2 (p=0.07) and -6 (p=0.14) compared to Ad-LacZ treated mineralized callus.

3.5 Discussion

Single delayed injection of adenovirus encoding BMP-2 or -6 genes was beneficial to accelerate fracture healing. Our study showed that Ad-BMP-2 and Ad-BMP-6 treated bone had greater callus formation, higher bone density and regained greater structural strength earlier than untreated bone. Ad-BMP-2 was superior to Ad-BMP-6 in accelerating callus formation and in regaining biomechanical strength of the bone when administered in this dosing regimen.
We chose in our study to deliver the viral vectors 14 days after bone injury and this may have enhanced the benefits seen with Ad-BMP delivery. In a rat femur bone gap, significantly greater bone formed after delayed Ad-BMP-2 delivery into mature healing tissue compared to delivery at surgery.\(^{22}\) The lack of solid granulation tissue and low population of targeted osteoprogenitor cells may also explain why the Ad-BMP-2 injected into cortical drill defects created in sheep iliac crest at the time of surgery did not induce sufficient bone formation.\(^{15}\) In addition, delayed injection may coincide with peak expression of adenovirus receptor on immature osteoblasts and support more efficient adenoviral gene deliver.\(^{23}\) Injection of Ad-BMP-2 at the time of surgery can, however, effectively enhance bone healing in rodent models,\(^{3,4}\) even if the outcome is less than optimal. It has also been noted that Ad-BMP-6 vector delivered into an ulna osteotomy in rabbits resulted in undesirable migration of the vector and heterotopic bone formation.\(^{5}\) In a clinical setting, delayed application of viral vector into a well-structured granulation tissue may retain the therapeutic genes and target a larger number of host cells for more efficient transduction. This strategy also would permit flexibility in the timing of injection for patients and resolution of initial acute traumatic bruising and inflammation at the fracture site.

To our knowledge, our study is the first to compare osteoinductive effects of hBMP-2 and -6 genes using the same fracture models and viral dosage. Ad-BMP-2 was superior to Ad-BMP6 in rapidity of bone formation and bone biomechanical strength in the osteotomy. Similarly, in vitro, Ad-BMP-2 induced more robust osteogenic differentiation of equine bone marrow derived mesenchymal stem cells compared to Ad-
BMP-6. Our results using a large animal model showed greater potency of Ad-BMP2 in healing an osteotomy and similar potency between Ad-BMP-2 and Ad-BMP-6 in healing a bone gap. The most effectual time of treatment has been studied rhBMP-2 but not for hBMP-6 protein/gene. In addition, Ad-BMP-6 had greater osteoinductive potency compared to Ad-BMP-2 in the nude rat muscle pouch. The further study is warranted to determine the optimal timing and sites of different BMPs applications.

Genes related to endochondral ossification and osteogenesis including TGFB1, COL2A1, and COMP, remained upregulated 6 weeks after ostectomy in the Ad-BMP-2 and Ad-BMP-6 groups compared to the untreated ostectomy callus or intact bone. TGFB1 has been known as one of the most important osteogenic growth factor during fracture healing, and its sustained upregulation at six weeks after hBMP-2 gene application may represent a signaling mechanism of action of enhanced Ad-BMP bone formation. COL2A1 is one of the major markers of extracellular matrix synthesis during endochondral ossification; therefore, its upregulation has been identified not only during normal bone healing but also in the sites of bone formation induced by recombinant hBMP-2. The increased gene expression of COMP was likely a result of chondrogenesis by hBMP-2 gene transduction, although COMP and BMP-2 might work synergistically.

When adenovirus is used as the gene delivery vehicle, adverse effects become a large safety concern and need to be addressed prior to clinical application. According to our results, an injection of adenoviral vector (5x10 particles) into the mature granulation tissue did not produce signs of systemic viral infection (e.g., fever, depression,
or elevation of WBC) or local adverse effects (eg, swelling, heat, or incisional discharge). Systemic biodistribution of adenovirus was not evident in the distant organs despite the increased serum antibody titer. Ad-BMP treated bone defects retained greater adenovirus copies within bone repair sites. Possibly mineralization impounds the cells and prevents migration. Gene expression analysis revealed that the upregulation of most of the inflammation-related genes occurred in all groups and were increased compared to intact bone. This suggests that the local inflammation was related to the healing process. Two genes associated with immune responses, IGLG and IGHM, were upregulated in adenoviral treated ostectomy callus indicating an immune response to viral vectors.

Fracture models in equine metatarsal bones were created with a simple surgical technique and minimal soft tissue destruction, and did not cause noticeable pain, discomfort, or gait impairment, because equine Mt4 and Mt2 are semi-retrogressed, minimally weight-bearing bones. In addition, its superficial location made a percutaneous gene delivery accurate and uncomplicated. Due to the presence of thick interosseous ligament between proximal aspects of equine third and fourth metatarsal bones, the Mt4 osteotomies provides the relatively stable, uncomplicated, and appositional bone healing models without use of a fixator. Conversely, the distal aspects of equine Mt2 have no rigid articulation and are persistently impinged by suspensory ligaments making the Mt2 ostectomies considered as the unstable, demanding, and critical size gap healing model. Therefore, Mt4 and Mt2 fracture models permitted a comparison of BMP osteoinductivity in multiple healing environments with different complexity levels within same animals. Moreover, the large size of equine bone specimens made a mechanical
testing constant and uncomplicated and facilitated a collection of sufficient amount of callus tissue for gene expression analysis. Furthermore, fracture research using equine models would also be considerably beneficial to horse racing industry and equine orthopedics field due to the high mobility in large animal species with long bone fractures.

In summary, our results demonstrated that delayed percutaneous delivery of adenoviral vector encoding BMP genes can enhance fracture healing without the risks of local and systemic adverse effects. BMP-2 gene therapy appeared to be superior to BMP-6 by providing greater and stronger bone callus in earlier time points. Gene expression profiles of BMP treated bone callus indicated increased signaling for osteogenesis through TGFB1.
Chapter 4: 
Dermal fibroblast-mediated BMP2 therapy to accelerate bone healing in an equine osteotomy model

4.1 Summary

This study evaluated healing of equine metacarpal/metatarsal osteotomies in response to percutaneous injection of autologous dermal fibroblasts (DFb) genetically engineered to secrete bone morphogenetic protein-2 (BMP2) or demonstrate green fluorescent protein (GFP) gene expression administered 14 days after surgery. Radiographic assessment of bone formation indicated greater and earlier healing of bone defects treated with DFb with BMP2 gene augmentation. Quantitative computed tomography and biomechanical testing revealed greater mineralized callus and torsional strength of DFb-BMP2 treated bone defects. On the histologic evaluation, the bone defects with DFb-BMP2 implantation had greater formation of mature cartilage and bone nodules within the osteotomy gap and greater mineralization activity on osteotomy edges. Autologous DFb were successfully isolated in high numbers by a skin biopsy, rapidly expanded without fastidious culture techniques, permissive to adenoviral vectors, and efficient at in vitro BMP2 protein production and BMP2-induced osteogenic
differentiation. This study demonstrated an efficacy and feasibility of DFb-mediated BMP2 therapy to accelerate the healing of osteotomies. Skin cell-mediated BMP2 therapy may be considered as a potential treatment for various types of fractures and bone defects.

4.2 Introduction

Transplantation of genetically engineered autologous cells, known as cell-mediated gene therapy or ex vivo gene therapy, has a great potential for the treatment of orthopedic disorders to accelerate bone healing and restore the loss of bony structure and function. Cell-mediated delivery of an osteogenic gene has the advantages of controlling cell transduction efficiency compared to direct gene delivery, increasing a cellularity of the recipient injury site, permitting surgeon selection of cell type for implantation, and capitalizing on an autocrine and paracrine effect in which the transplanted cells not only differentiate into desired cell types but also secrete osteogenic growth factor into the treatment site. Pluripotent stem cells such as embryonic stem cells (ESC) or adult mesenchymal stem cells (MSC) have been extensively studied for cell-mediated therapy for osteogenic gene augmentation. However, use of ESC involves an ethical issue and potential risk of adverse immune reaction by allogenic implantation, and application of MSC requires an invasive biopsy procedure as the cells are commonly isolated from bone marrow or adipose tissue. In addition, stem cells have shown to require fastidious culture conditions during cell expansion in order to maintain their pluripotentiality and to grow to high numbers without morbidity.
While ESC and MSC are strong candidates for cell-mediated gene therapy, dermal fibroblasts (DFb) have received scientific attention as a carrier cell because they can be easily isolated by a relatively less painful harvest technique with less risk of infection or donor site morbidity. In recent years, the plasticity and reprogramming capacity of DFb has been shown by deriving DFb into induced pluripotent stem cells with defined genes such as Oct3/4, Sox2, Klf4, and c-Myc. Such DFb-derived pluripotent cells have been differentiated into several functional phenotypes including neurons, cardiomyocytes and hematopoietic cells. In addition, DFb have been demonstrated to undergo osteogenic differentiation and convert to bone-forming cells with a single osteogenic gene transduction, including bone morphogenetic protein-2 (BMP2), BMP7, Runt-related gene-2, and Lim mineralization protein-3. Autologous implantation of DFb with osteogenic gene augmentation has also shown to induce ectopic bone formation and contribute to the bone healing in rodent models based on radiography, computed tomography, and histology. These encouraging results support additional work to investigate skin cell-mediated gene delivery, improvement of mechanical strength of repaired bone, and efficacy for bone healing in larger animal species to demonstrate a clinical relevance of enhanced healing in people. The efficacy of molecular therapy in small animal experiments may not straightforwardly transfer to large animal models. Use of a large animal model will also evaluate the practicality in cost- and time-efficiency for cell transplantation using large numbers of genetically engineered cells in an immunocompetent adult animal.
We have previously shown that the delayed percutaneous delivery of adenoviral vectors encoding BMP2 gene, as well as BMP6 gene, into the equine osteotomy model induced an acceleration of bone healing and upregulation of genes associated with osteogenesis or endochondral ossification.\textsuperscript{15,16} We have also demonstrated acceleration of bone formation by a stem cell-mediated BMP2 therapy administered by delayed injection into an articular fracture in immunoincompetent rats.\textsuperscript{2} We propose that skin cells transduced with BMP2 should readily expand to the larger numbers needed for large bone defects, induce similar robust and rapid bone formation as direct gene delivery and stem cell-based BMP2 delivery, and be effective in our large animal model. The delivered genetically engineered DFb should locally secrete BMP2 and serve as bone-forming cells to contribute to mineral deposition and matrix synthesis. A delayed cell injection into granulation tissue will be used to optimize the local environment for cell growth, minimize cell leakage into surrounding tissue, and accumulate the secreted growth factor within the repair site, as shown in delayed administration of adenoviral vectors encoding BMP2 and BMP6 gene.\textsuperscript{15-17} This strategy would also mimic a regimen of cell-mediated therapy for clinical use in which a surgeon could perform a skin biopsy at the first surgery and cell injection at few weeks later.

The objective of this study was to demonstrate an efficacy and feasibility of DFb-mediated BMP2 therapy to accelerate and enhance the bone healing in an equine model. We hypothesized that transplantation of DFb genetically engineered to secrete BMP2 would induce robust bone formation, more mature and dense bone healing, and greater mechanical strength of bone.
4.3 Methods

Study Design

Six skeletally mature horses (weight 445 to 550 kg) were used to isolate dermal fibroblasts (DFb) by full-thickness skin punch biopsy. After successful cell expansion, the same horses had surgically created 1-mm transverse osteotomies in bilateral fourth metacarpal (Mc4) and metatarsal bones (Mt4) (Day 0). The Mc4/Mt4 in each horse was assigned in a block design to rotate treatments among the limbs. Osteotomies were treated with percutaneous injection of autologous DFb with adenoviral transduction of bone morphogenetic protein-2 gene (DFb-BMP2; n=6 limbs), green fluorescent protein gene (DFb-GFP; n=6) as a vector control, DFb alone (DFb; n=6), or Gay’s balanced salt solution (Sigma-Aldrich, St Louis, MO) as a saline control (GBSS; n=6). All injections were made two weeks after surgery (Day 14). Six weeks following the injection (Day 56), the horses were euthanized and Mc4 and Mt4 specimens were harvested. Efficacy was assessed by weekly radiographs, quantitative computed tomography, mechanical testing, and histology. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Dermal fibroblast isolation and in vitro osteogenic differentiation

Full-thickness skin tissue was harvested using 5-mm diameter biopsy punch from the pectal region (10-12 punches per horse) from each of the six horses and cultured separately for autologous implantation. The dermal layer was dissected from the
epidermis under a microscope, and DFb were isolated by type-1 collagenase digestion (GIBCO, Grand Island, NY) and cultured in DMEM supplemented with L-glutamine (300 μg/mL), penicillin (30 μg/mL), streptomycin (30 μg/mL), and 10% fetal bovine serum at 37 ºC in a 5% CO2 atmosphere. To demonstrate in vitro gene transduction and osteogenic differentiation, DFb were seeded in triplicate wells of 48-well plates (Falcon, Franklin Lakes, NJ) at a density of 5×10^4 cells/well and, 24-hours later, transfected with adenoviral (Ad) vector encoding BMP2 gene (Ad-BMP2) or GFP gene (Ad-GFP) at 200 MOI (eg, 2×10^2 infectious unit [IFU] per cell; 1×10^7 IFU per well) (Adeno-X Rapid Titer Kit, Clontech, Mountain View, CA). Two and seven days after gene transduction, gene transduction efficiency was quantified by fluorescent microscopy, and BMP2 protein production confirmed with ELISA (R&D Systems, Mineapolis, MN). Seven days after gene transduction, osteogenic differentiation of DFb was quantified by the number of von Kossa-positive mineralized bony nodules within the well and the intensity score of alkaline phosphatase (ALP, Sigma-Aldrich) staining in three representative X100 microscopic fields per well using the following grading scheme: 0 (0% of cells in the field showing stain uptake), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%).

Surgical procedure and autologous cell implantation

Osteotomy surgeries were performed using the same six horses after the successful DFb isolation. Following general anesthesia, aseptic preparation of the lateral metacarpal/tarsal area, and a creation of 1-cm skin incision, a nitrogen-driven oscillating bone saw was used to create a 1-mm transverse defect in the proximal Mc4 and Mt4 (Day
The cut was made 7-cm distal to the palpable proximal aspect of Mc4 and 9-cm distal from the palpable proximal aspect of Mt4, to make the cut surface of the bone approximately of equal area. Antibiotics were administered for one day following surgery (procaine penicillin, IM, 22000 IU/kg and gentamicin, IV, 6.6 mg/kg); the anti-inflammatory drug (phenylbutazone, PO, 4.4 mg/kg) was administered for three days following surgery.

One week after the surgery (Day 7), DFb were seeded in 18 large culture flasks (75-cm²; 3×10⁶ cells per flask). On Day 12, 2 days before injection, the DFb (80-90% confluent) were treated with Ad-BMP2 (DFb-BMP2; 6 flasks), Ad-GFP (DFb-GFP; 6 flasks), or untreated (DFb; 6 flasks). The total cell number at this time point was estimated at 1×10⁷ DFb per flask based on a pilot trial and hemacytometer counting (Hausser Scientific, Horsham, PA). The Ad-BMP2/GFP were administered at 200 MOI; therefore, 2×10⁹ IFU of vector were administered in each flask.

Two weeks after the surgery (Day 14), 48 hours after DFb gene transduction, cells were harvested by trypsinization (GIBCO), counted by hemacytometer, centrifuged, and resuspended into a 500 µl total volume with GBSS containing 5×10⁷ cells for each of DFb, DFb-GFP, and DFb-BMP2. All the cell injections were completed within 60 to 90 minutes after the trypsinization. Horses were placed under general anesthesia and the assigned osteotomies were treated by percutaneous fluoroscopic-guided needle injection of 5×10⁷ cells of DFb-BMP2, DFb-GFP, DFb, or GBSS in a 500 µl volume directly into the osteotomy gaps.
For fluorescent labeling of bone mineralization activity, calcein (Sigma-Aldrich) was administered at a rate of 20 mg/kg, dissolved in 2% sodium bicarbonate solution (Abbott Laboratories, North Chicago, IL) intravenously at three and four weeks after the DFb injection (Day 35 and 42, respectively).

Radiographic evaluation

Radiographs of Mc4 and Mt4 were obtained weekly after the osteotomy surgery using digital radiography system (EDR 3, Eklin Medical Systems, Santa Clara, CA). Using the dorsolateral-palmaro/plantar-medial-oblique views, external callus area at the osteotomy site was estimated by multiplying the length and width of the callus measured on the caudolateral surface of the Mc4/Mt4 (mm²).

Quantitative computed tomography

Six weeks following the cell injection (Day 56), horses were sedated with xylazine hydrochloride (IV, 1 mg/kg) and euthanized with by lethal intravenous overdose of pentobarbital (Beuthanasia, IV, 2.2 mg/kg). Immediately after the euthanasia, all four limbs were dissected at the level of carpometacarpal or tarsometatarsal joint. Quantitative computed tomography (QCT) (Picker PQS Helical CT Scanner, Philips Medical Systems, N.A., Bothell, WA) was performed in transverse sections at 1-mm intervals to evaluate the bone formation and soft tissue mineralization using potassium phosphate standards and an image analysis software (Mimics, Materialise, Ann Arbor, MI). The three central transverse slices within the osteotomy gap were used to calculate the cubic volume and
mineral density of bony bridging. All transverse slices above and below the osteotomy gap containing the external callus were used to calculate the cubic volume and mineral density of callus. The volume of bony bridging and external callus was standardized by the volume of adjacent Mc4/Mt4 and expressed as a percentage.

**Biomechanical testing**

Both ends of the Mc4/Mt4 osteotomy specimens were dissected and embedded in square molds using polymethylmethacrylate at 1.5-cm distant from defects to both sides (i.e., 3cm lengths of bones were exposed) and tested clockwise quasi-statically to failure in torsion (1.5º/s) using a servohydraulic materials testing system (Bionix 858, MTS Systems, Eden Prairie, MN). Left limbs’ Mc4/Mt4 were tested distal end up and right limbs’ Mc4/Mt4 tested proximal end up to ensure similar directional rotation for both sides. The testing was stopped when the specimen broke, or when 90º of rotation was reached, whichever occurred first. The torque-rotation data were used to compute the maximum torque, torsional stiffness, and energy absorbed to failure. The value of each mechanical parameters were standardized by the mean value of GBSS treated Mc4 (n=3) or Mt4 (n=3) and expressed as percentage.

**Histologic evaluation**

The calcified Mc4/Mt4 specimens were dehydrated in alcohol, embedded in methylmethacrylate, cut into 10-µm sections in the sagittal plane, and stained with Masson’s Trichrome. The bone specimens were evaluated semi-quantitatively for the
composition of osteotomy gap tissue (eg, bone, cartilage, or fibrous tissue) and mineralization activity (calcein-labeled tissue) of osteotomy gap tissue (% area) and osteotomy edges (% length).

Statistical analysis

Repeated-measure analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC) was used to evaluate the effects of DFb-mediated BMP2 therapy with the post-test multiple comparisons between the treatment groups using Proc Mixed statistical models for continuous outcomes (i.e., von Kossa bony nodule count, radiography, QCT, mechanical testing, and histologic data) and Genmod statistical models for categorical outcomes (i.e., ALP stain uptake score). Repeated variables were considered to be nested within horse, and the distribution of data was assessed by use of a subset of normality tests (eg, the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests). Additional ANOVA was run for all outcomes by adding an indicator variable for Mc4/ Mt4 to validate an appropriateness of comparing data between Mc4 and Mt4. Significant level was set at P<0.05 for all analyses.

4.4 Results

In vitro gene transduction and osteogenic differentiation of DFb

The DFb from all 6 horses showed sufficient GFP gene transduction of >95% at 2 days after gene transduction. BMP2 secreted protein production into the media of DFb-BMP2 was 2831 ± 324 pg/mL (mean ± SE) and 4788 ± 398 pg/mL at 2 and 7 days after
gene transduction, respectively. The DFb and DFb-GFP secreted no detectable BMP2 protein. The von Kossa staining showed efficient mineralized nodule formation of DFb-BMP2 with nodule counts of 34.6 ± 2.5 per well (mean ± SE) at 7 days after gene transduction ( ). The DFb and DFb-GFP formed no bony nodules. ALP stain score of DFb-BMP2 (median 4; range 2-4) was greater (P<0.001) than DFb (median 0; range 0-1) or DFb-GFP (median 1; range 0-2) at 7 days after gene transduction.

**Radiographic evaluation**

Rapid bone formation and a greater area of mineralized callus was observed radiographically within two weeks after injection (four weeks after surgery) in the DFb-BMP2 treated bone defects (Figure 4.1). The size of mineralized callus in the DFb-BMP2 treated bone defects was greater than the GBSS or DFb-GFP treated defects at 2 through 5 weeks after the DFb injection (P<0.05) (Table 4.1).

Data comparison between Mc4 and Mt4 was considered appropriate, because the indicator variable of Mc4/Mt4 was not significant factor (P>0.39) for any outcome including the data of the radiographic evaluation, quantitative computed tomography, biomechanical testing, and histologic evaluation. The normal distribution of data were confirmed by the normality tests (P>0.21) for all outcomes as well.
Figure 4.1 – A time course collage of Mc4/Mt4 osteotomy healing from 1 to 5 weeks after cell or GBSS injections. The DFb-BMP2 treated bones showed greater bone formation at earlier time points.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBSS</td>
<td>8.5 ± 4.4</td>
<td>30.1 ± 5.7</td>
<td>46.0 ± 8.3</td>
<td>65.0 ± 19.2</td>
<td>66.6 ± 17.9</td>
</tr>
<tr>
<td>DFb</td>
<td>8.4 ± 6.4</td>
<td>39.1 ± 14.5</td>
<td>60.5 ± 23.4</td>
<td>86.4 ± 30.7</td>
<td>73.17 ± 20.9</td>
</tr>
<tr>
<td>DFb-GFP</td>
<td>7.0 ± 3.9</td>
<td>34.2 ± 11.2</td>
<td>48.9 ± 15.5</td>
<td>61.2 ± 12.1</td>
<td>65.1 ± 12.5</td>
</tr>
<tr>
<td>DFb-BMP2</td>
<td>8.6 ± 3.6</td>
<td>54.6 ± 9.1*</td>
<td>73.0 ± 21.9*</td>
<td>109.7 ± 24.0†</td>
<td>110.5 ± 23.4†</td>
</tr>
</tbody>
</table>

**Table 4.1** – Effect of DFb-mediated BMP2 therapy on mineralized callus size on radiography (mm²: Mean ± SE) from 1 to 5 weeks after the injection.

*Significant difference from GBSS treated bone defects (P<0.04).
†Significant difference from GBSS and DFb-GFP treated bone defects (P<0.05).

*Quantitative computed tomography*

The volume of mineralization within the osteotomy gaps was greater in the DFb-BMP2 treated bone defects compared to the GBSS, DFb, and DFb-GFP treated defects (P<0.001; Figure 4.2 and Table 4.2). The volume of external callus around the osteotomy site was greater in the DFb-BMP2 treated bone defects compared to the GBSS and DFb-GFP treated defects (P<0.02; Figure 4.2 and Table 4.2). The mineral density of gap tissue and external callus was not significantly different among the treatment groups (Table 4.2).
Figure 4.2 – Cross-sectional (left) and three-dimensional computed tomographic images of Mc4/Mt4 osteotomy healing at 6 weeks after cell or GBSS injections. The DFb-BMP2 treated bones showed greater bone volume and mineral density of the bony bridging and external callus.
### Table 4.2 – Effect of DFb-mediated BMP2 therapy on properties of the bony bridging and mineralized callus, assessed by peripheral quantitative computed tomography (Mean ± SE).

*Significant difference from all other treatments (P<0.001).
†Significant difference from GBSS and DFb-GFP treated bone defects (P<0.02).

<table>
<thead>
<tr>
<th>Region</th>
<th>Treatment</th>
<th>Bone Volume (% Adjacent Mc4/Mt4)</th>
<th>Mineral density (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bony bridging</td>
<td>GBSS</td>
<td>71.6 ± 11.9</td>
<td>497 ± 25</td>
</tr>
<tr>
<td></td>
<td>DFb</td>
<td>81.7 ± 12.0</td>
<td>515 ± 18</td>
</tr>
<tr>
<td></td>
<td>DFb-GFP</td>
<td>53.4 ± 12.0</td>
<td>505 ± 31</td>
</tr>
<tr>
<td></td>
<td>DFb-BMP2</td>
<td>171.9 ± 21.8*</td>
<td>549 ± 27</td>
</tr>
<tr>
<td>External callus</td>
<td>GBSS</td>
<td>84.5 ± 11.3</td>
<td>593 ± 22</td>
</tr>
<tr>
<td></td>
<td>DFb</td>
<td>95.0 ± 17.5</td>
<td>594 ± 17</td>
</tr>
<tr>
<td></td>
<td>DFb-GFP</td>
<td>81.5 ± 8.8</td>
<td>590 ± 19</td>
</tr>
<tr>
<td></td>
<td>DFb-BMP2</td>
<td>119.0 ± 13.7†</td>
<td>577 ± 11</td>
</tr>
</tbody>
</table>

Bone volume: The cubic volume of bony bridging and external callus was standardized by the volume of adjacent Mc4 or Mt4 and expressed as percentage.

**Biomechanical testing**

The energy absorbed to failure was greater in the DFb-BMP2 treated bone defects compared to the GBSS, DFb, and DFb-GFP treated bone defects (P<0.001; Table 4.3). The peak failure torque was greater in the DFb-BMP2 treated bone defects compared to the DFb and DFb-GFP treated bone defects (P<0.01; Table 4.3). The torsional stiffness was greater in the DFb-BMP2 treated bone defects compared to the DFb-GFP treated bone defects (P=0.02; Table 4.2). The torsional stiffness was less in the DFb-GFP treated bone defects compared to the GBSS treated bone defects (P=0.02; Table 4.3).
### Table 4.3 – Effect of DFb-mediated BMP2 therapy on torsional biomechanics (Mean ± SE).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Failure torque (%GBSS)</th>
<th>Torsional stiffness (%GBSS)</th>
<th>Energy absorbed to failure (%GBSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBSS</td>
<td>100.0 ± 19.8</td>
<td>100.0 ± 11.8</td>
<td>100.0 ± 33.5</td>
</tr>
<tr>
<td>DFb</td>
<td>69.1 ± 17.0</td>
<td>74.8 ± 21.9</td>
<td>96.4 ± 44.9</td>
</tr>
<tr>
<td>DFb-GFP</td>
<td>58.6 ± 5.3</td>
<td>46.5 ± 10.6†</td>
<td>108.4 ± 30.4</td>
</tr>
<tr>
<td>DFb-BMP2</td>
<td>125.8 ± 29.6*</td>
<td>156.6 ± 71.2†</td>
<td>171.0 ± 18.4‡</td>
</tr>
</tbody>
</table>

*Significant difference from DFb and DFb-GFP treated bone defects (P<0.01)
†Significant difference from DFb-GFP treated bone defects (P=0.02)
‡Significant difference from all other treatments (P<0.001).
¶Significant difference from GBSS treated bone defects (P=0.02).

%GBSS: Data were standardized by the mean value of GBSS treated Mc4 (n=3) or Mt4 (n=3) and expressed as percentage.

### Histologic evaluation

The osteotomy gap tissue in the DFb-BMP2 treated bone defects was composed of a greater amount of bone and cartilage and less fibrous tissue compared to the GBSS, DFb, or DFb-GFP treated bone defects (P<0.04; Figure 4.3 and Table 4.4), and had greater area of calcein-positive mineralization activity compared to the GBSS treated bone defects (P=0.01; Figure 4.3 and Table 4.4). The osteotomy edge in the DFb-BMP2 treated bone defects had greater length of calcein-positive mineralization activity compared to the GBSS and DFb-GFP treated bone defects (P<0.03; Figure 4.3 and Table 4.4). Also, the bone defects treated with DFb and DFb-GFP had greater amount of...
bone/cartilage in the osteotomy gap tissue (P<0.03; Figure 4.3 and Table 4.4) and increased mineralization activity in the osteotomy gap tissue and edge (P<0.03; Figure 4.3 and Table 4.4) compared to the GBSS treated bone defects.

Figure 4.3 – Masson’s trichrome staining of Mc4/Mt4 osteotomy healing. The DFb-BMP2 treated bone defects showed greater amount of nodular bone and cartilage in the osteotomy gap tissue (top and middle) and greater bone mineralization activity in the osteotomy gap tissue and edge (middle and bottom).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Osteotomy gap tissue composition (%)</th>
<th>Osteotomy gap bone activity (%)</th>
<th>Osteotomy edge bone activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nodular bone</td>
<td>Cartilage</td>
<td>Fibrous tissue</td>
</tr>
<tr>
<td>GBSS</td>
<td>3 ± 1</td>
<td>20 ± 2</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>DFb</td>
<td>7 ± 2*</td>
<td>26 ± 5</td>
<td>67 ± 6*</td>
</tr>
<tr>
<td>DFb-GFP</td>
<td>5 ± 1*</td>
<td>27 ± 2*</td>
<td>69 ± 2*</td>
</tr>
<tr>
<td>DFb-BMP2</td>
<td>11 ± 2†</td>
<td>40 ± 3‡</td>
<td>49 ± 3‡</td>
</tr>
</tbody>
</table>

*Significant difference from GBSS treated bone defects (P<0.03)
†Significant difference from GBSS and DFb-GFP treated bone defects (P<0.04)
‡Significant difference from all other treatments (P<0.01)

%Bone activity: The composition of calcein-labeled active bone formation in the total bone area (Osteotomy gap) or length (Osteotomy edge).

4.5 Discussion

Single percutaneous injection of autologous DFb genetically engineered to secrete BMP2 was beneficial to accelerate bone healing in an adult large animal. In this study, the DFb-BMP2 treated bone defects had earlier and greater callus formation, regained greater structural strength, and induced more cartilage and nodular bone formation than saline control. An autologous DFb implantation without gene augmentation also produced more mineralized callus formation, but the transduction of the osteogenic BMP2 gene was shown to be advantageous and provoke greater amount of endochondral
ossification within the bone healing site, and to stimulate mineralization of adjacent bone. Our study also demonstrated the feasibility of DFB-mediated gene therapy, in which sufficiently rapid growth and cell expansion rate enabled the transplantation of substantial number of autologous cells in short time period.

Our results showed that the osteotomy gap tissues treated with DFB-BMP2 were composed of a greater amount of mature cartilage and nodular bone formation compared to control or DFB without BMP2 gene transduction. This is compatible with an enhanced process of endochondral ossification. The secreted BMP2 might induce recruitment of native osteoprogenitor cells, promote chondrogenic differentiation of resident and recruited cells, and facilitate subsequent bone/cartilage tissue composition, because BMP2 has shown to play a key role during an endochondral ossification and promote chondrogenesis in MSC. It is unclear whether or not the DFB that we injected were actually converted to chondrocytes. Authors’ are unaware of any study reporting a direct derivation of DFB into chondrocytes by BMP2 gene transduction. However, DFB have been shown to undergo chondrogenesis in optimal conditions such as a three-dimensional scaffold with chondrogenic signaling molecules. The osteotomy gap may contain adequate host cell-produced morphogenetic and micro-environmental factors to differentiate the transplanted DFB into a chondrogenic phenotype programmed for endochondral ossification. Also, the previous studies reported that DFB can form a cartilageous lacuna structure when co-cultured with chondrocytes or be directed to chondrogenic differentiation when treated by demineralized bone powder. It is thus possible that at least a sub-population of DFB transplanted in our study could contribute
to cartilage formation by an interaction with recipient site chondrocytes or signaling molecules. Similar phenomena have been reported in stem cell research, where the culture medium from the primary chondrocyte was re-used onto the MSC monolayer inducing efficient chondrogenesis. However, it is unclear if the efficacy of DFb-BMP2 is primarily due to the increased BMP2 level within the osteotomy sites or an integration of BMP2-expressing DFb making a direct contribution for cartilage/bone formation. Future study using an in vivo cell tracking technique to verify integration of the transplanted DFb within the newly formed bone is warranted.

The study reported here suggested that a cell-mediated BMP2 therapy may not be superior to the acceleration of equine bone healing compared to a direct gene delivery from our previous published study, where the cell/vector injections (2 weeks after surgery) and euthanasia (6 weeks after the injections) were performed in the same timing as this study. In terms of bone volume, the DFb-BMP2 treated bone defects had 2.4 times greater size of bone formation than GBSS treated defects (Table 4.1), whereas the Ad-BMP2 treated bone defects had 2.2 times greater size of bone formation than untreated defects. In terms of mechanical strength, however, the DFb-BMP2 treated bone defects had only 1.3 to 1.7 times greater strength than GBSS treated defects (Table 4.2), whereas the Ad-BMP2 treated bone defects had 2.4 to 4.1 times greater strength than untreated defects. This suggested that the osteotomy callus from direct Ad-BMP2 injection was stronger than DFb-BMP2 which would correlate with the robust diffuse bony bridging between the osteotomy edges seen histologically in that study compared to the DFb-BMP2 treated bone defects with mature cartilage and nodular bone formation.
(endochondral ossification) within the osteotomy (Figure 4.3). These results may imply that the transplantation of genetically modified DFb in this study induced some mineral deposition and endochondral ossification, but less woven/lamellar bone than seen histologically with direct gene delivery at the 6 week time period after injection explaining the lower mechanical characteristics at the same time point. It might be anticipated that the DFb-BMP2 would progress to full boney union through the endochondral ossification process. Although BMP2 is a key factor for chondrocyte maturation during endochondral ossification, multiple signaling molecules from osteoprogenitor cells such as BMP-2, 4, 6, and 7 are involved in the apoptosis of hypertrophic chondrocytes.\textsuperscript{24} Osteoblastic precursor cells have also been shown to play an important role in osteoclastogenesis of macrophage-monocyte lineage by the receptor activator of nuclear factor kappa B ligand (RANKL),\textsuperscript{25} but BMP2 alone was unable to induce osteoclast formation by inhibiting RANKL expression.\textsuperscript{26} Therefore, the DFb-BMP2 may require an interaction with host osteoprogenitor cells to facilitate an efficient cartilage-to-bone conversion during the late physiologic event of endochondral ossification. Since the Mc4/Mt4 bone used for this study is primarily a cortical bone environment with some marrow cavity, this long bone environment may not be the optimal bone environment for cell-mediated BMP2 bone formation. Future study may be warranted to determine if a cell-mediated BMP2 therapy could accelerate bone healing more effectively within a cancellous bone environment where a rich source of osteoprogenitor cells is readily available to interact with the transplanted cells and BMP2. Also, bone healing may need to be monitored longer than 6 weeks, because the
transplantation of DFb-BMP2 in this study did not achieve a complete bone union in the time length. However, the improved biomechanical strength shown in DFb-BMP2 group could still be beneficial to reduce the micromotion in the bone healing site and decrease the risk of delayed/non-union formation.

Our data suggested that autologous transplantation of naïve DFb, devoid of osteogenic modification, may modestly accelerate bone healing, but to a lesser degree than when genetically engineered to secrete BMP2. Bone defects treated by DFb without BMP2 gene augmentation had a significantly greater composition of bone and cartilage (Table 4.3) and slightly higher bone volume and mineral density (Table 4.1) compared to the saline control group; but, the DFb-BMP2 treated bone defects had even greater cartilage/bone formation (Table 4.3) and mechanical strength (Table 4.2) over both the DFb and DFb-GFP groups. On the radiographs, the DFb-BMP2 group also showed an increased bone formation as early as 2 weeks after the injection (Appendix B), although this measurement only reflected the size of the bone formation on the palmar/plantar-lateral aspect of Mc4/Mt4 and therefore might not represent the total volume. These results suggested that placing DFb into a bone repair site alone may support bone healing, but an introduction of the BMP2 gene was advantageous for greater bone formation and accelerated return of biomechanical properties. Our large animal experiment corroborates an in vivo rabbit stem cell study describing that an orthotopic transplantation of osteogenically differentiated MSC resulted in much more extensive bone formation than the transplantation of undifferentiated MSC. However, it is not entirely clear why the DFb-GFP-treated bone defects had a greater composition of bone and cartilage (Table
4.3) but lower stiffness (Table 4.2) compared to control. The DFb group also presented a similar trend. The histomorphometry only showed the smaller percentage of fibrous tissue composition in DFb and DFb-GFP group than GBSS (Table 4.3), but not total volume; therefore, the possibility of unmodified fibroblasts-induced fibrosis causing weakness cannot be ruled out. Also, the GFP gene expression or adenoviral vectors remained on the cell surface could induce an inflammatory or innate immune reaction and diminish the efficacy. Moreover, it is possible that the DFb without BMP2 gene could not establish a rigid integration between the gap healing tissue and osteotomy edges, despite the increased bone and cartilage formation in the middle of osteotomy gap. In contrast, the GBSS group might have a less cartilageous healing tissue but tightly connected to the osteotomy edges. The interface between the gap tissue and osteotomy edges appeared to be the weakest link and, in the torsional mechanical testing, almost all bone specimens broke at this region, not in the middle of the osteotomy gaps. Hence, the DFb- and DFb-GFP-treated bone defects might start failing at this interface at lower torque than the GBSS group resulting in diminished torsional stiffness.

In the study presented here, the bone forming activity on the osteotomy edges were significantly increased in the DFb-BMP2 treated bone defects (Table 4.3). This may be a result of a paracrine effect of the secreted BMP2 by the transplanted cells provoking a bony mineralization in neighboring osseous tissue. Previously, a paracrine signal by BMP2-expressing cells has been shown to play an essential role in stimulating osteoblastic activity and mineralization in the recipient site.28,29 In our study, an integration between the osteotomy edges and gap filling tissue may be solidified by an
effect of secreted BMP2 making greater stiffness and toughness in the bone defects treated with DFb-BMP2 (Table 4.2).

Our study demonstrated the feasibility of skin cell-mediated gene therapy for bone regeneration in a clinically relevant large animal model. Isolation of DFb by dermal tissue biopsy was uncomplicated and consistent. Expansion of DFb was rapid and undemanding using simple culture techniques. In this study, the 150 million cells (50 million DFb times 3 cell-injection sites) were successfully cultured within 18-24 days using the 10-12 of 5-mm diameter dermis tissues by skin punch biopsy. Cell-based therapy with large cell numbers may be practically applicable in a clinical setting by performing a skin biopsy at an initial treatment and injecting DFb at 2 to 3 weeks later. Future study would be necessary to directly compare the efficacy of stem cell- and skin cell-mediated BMP2 delivery to determine if the utility and applicability of DFb therapy could compensate for the greater differentiation capacity of the stem cells. In our study, a transplantation of 50 million DFb-BMP2 induced the 381-mm³ volume of mineralized tissue within the osteotomy gap (172% of adjacent Mc4/Mt4 volume; Table 4.1). This appeared to be a relevant cell dosage, based on a previous report introducing a mathematical model to estimate that approximately 70 million osteoblasts would be needed for a 1,000-mm³ volume of bone formation.  

Currently, recombinant human BMP2 is most commonly used for the treatments of tibial non-union in human orthopedics. 31,32 Our results are encouraging to show the significant acceleration of bone healing in equine Mc4/Mt4 bones which are much larger than other types of fracture models in rodents. Also, the distal one third of tibia where
non-union fractures are frequently occurred is surrounded by lesser musculature than its proximal region; therefore, an innovative treatment in an injectable form such as cell-mediated therapy may be easily applied percutaneously without additional invasive surgical procedure. The equine Mc4/Mt4 osteotomy models were created with a simple surgical technique and minimal soft tissue destruction, did not cause noticeable pain or discomfort, were treated by simple percutaneous injection of therapeutic agents, permitted a comparison of multiple treatments within the same animal, and its large bone size made mechanical testing consistant and uncomplicated. Because it was unknown if the bone healing rate was similar between the Mc4 and Mt4 defects, the data standardization was performed by generalizing the outcome with the adjacent Mc4/Mt4 (Table 4.1) or the GBSS-treated bone defects (Table 4.2). The statistical analysis also validated the data comparison between Mc4 and Mt4 was suitable, because the site of osteotomy (Mc4 v.s. Mt4) had no significant effect on any outcome.

In summary, our study demonstrated that a percutaneous DFb-mediated BMP2 therapy was beneficial to accelerate bone healing. In this study, an autologous DFb implantation alone promoted modest healing without an increase in mechanical properties at this time point, but the BMP2 gene transduction had superior bone healing obtained through endochondral ossification within the repair site and bony mineralization in the neighboring osseous tissue.
Chapter 5:

Comparative efficacy of dermal fibroblast-mediated and direct adenoviral bone morphogenetic protein-2 gene therapy for bone regeneration in an equine rib model

5.1 Summary

Cell-mediated and direct adenoviral vector gene therapies can induce bone regeneration, including dermal fibroblasts (DFb). We compared two effective therapies, DFb-mediated and direct adenoviral vector delivery of bone morphogenetic protein-2 (BMP2), for relative efficacy in bone regeneration. Equine rib drill defects were treated by percutaneous injection of either DFb-BMP2 or an adenoviral (Ad)-BMP2 vector. At week 6, both of the DFb-BMP2- and Ad-BMP2-treated rib defects had greater bone filling volume and mineral density, with DFb-BMP2 inducing greater bone volume and maturity in cortical bone aspect of the defect than Ad-BMP2. The transplantation of DFb alone induced modest bone formation. Increased mineral density and bone turnover were evident in the cortical and cancellous bone directly adjacent to the healing drill defects treated with either DFb-BMP2 or Ad-BMP2. Using our cell/vector dosage and model, BMP2, whether delivered by DFb-vector or direct adenoviral vector, induced greater and robust bone regeneration. DFb-mediated BMP2 therapy promoted greater cortical bone
regeneration than direct gene delivery, possibly due to an increased cellularity of the bone healing site. BMP2 delivery, regardless of gene delivery method, increased mineral density of neighboring bone which may be beneficial clinically in repairing or weak bone.

5.2 Introduction

Regenerative strategies for inferior bone repair are a growing need in aging people in which our immediate population demands include slow healing fractures (estimated 1 million patients annually), spine fusions (300,000 surgeries and $18 billion annually), and osteoporotic fractures (704,000 cases annually) [www.boneandjointdecade.org]. Currently, pharmaceuticals (i.e. osteoclast inhibitors) and bioactive proteins (i.e. bone morphogenetic protein-2 [BMP2]) are recognized advancements in bone repair; however, the use of living cell vectors as regenerative treatment for bony disorders offers the advantages of molecular engineering to release paracrine and autocrine bioactive factors, direct integration of the cells into the regenerative process, and a broad diversity of medical applications. While direct BMP2 gene delivery has shown to promote bone regeneration, relative potency between the gene- and cell-mediated BMP2 deliveries is unknown.

Although embryonic and adult mesenchymal stem cells have been extensively studied for cell-mediated gene therapy, other cell sources, such as dermal fibroblasts (DFb) may be an alternative candidate due to their excellent reprogramming capacity. In fact, DFb have been differentiated into bone-forming cells by the transduction of osteogenic genes including BMP2, and such genetically-modified DFb have been
autologously transplanted to induce ectopic bone formation$^{14,15}$ and promote bone healing in rodent models.$^{12,13,15}$ In contrast to the use of stem cells, the skin cell-mediated therapy is attractive for clinical use because of the relatively less painful harvest technique, low risk of donor site infection or morbidity, less fastidious culture procedure, and rapid high cell yield.$^{12-15}$

The authors’ laboratory has demonstrated in separate studies that DFb-mediated and direct adenoviral BMP2 gene therapy$^7$ significantly accelerated cortical long-bone healing in an equine metacarpal/tarsal model, with DFb functioning through the process of endochondral ossification and direct Ad-BMP2 delivery functioning through direct bone formation. The study reported here was designed to use drill hole defects within equine ribs to directly compare the efficacies of these two strategies that were successful in long bone. Unlike other animal models such as slit osteotomy and critical-size gap ostectomy,$^{16-18}$ the rib drill defect model provides a well-contained area for percutaneous injection, a well-confined filling defect to quantify bone formation with high resolution, both cortical and cancellous bone healing environments, and permits multiple bone defects per animal for appropriate control groups within the same animal. In addition, the rib drill defect model with a rich cancellous bone environment may be a more receptive environment to exhibit differences in these two methods of BMP2 gene delivery mechanisms, because of the potentially greater numbers of osteoprogenitor cells as receptive targets for paracrine molecular signals of BMP2.

The goal of our study was to use an equine rib drill defect model to evaluate the relative efficacy of DFb-mediated BMP2 and Ad-BMP2 gene delivery to promote bone
regeneration. We compared the effects of cell-mediated and direct BMP2 gene delivery using a percutaneous injection of DFb or viral vectors with and without BMP2 gene transduction. In this model, we successfully demonstrated that an autologous transplantation of BMP2-expressing DFb and direct Ad-BMP2 induced bone regeneration, and DFb-BMP2 vectors were more potent than a direct vector injection for cortical bone regeneration. Cell- and gene-mediated BMP2 delivery showed equivalent increased bone regeneration in the cancellous bone region and increased bone density in adjacent osseous tissues.

5.3 Results (Note: Methods section begins on page XXX)

*In vitro gene transduction and osteogenic differentiation*

Equine DFb were shown to be permissive to adenoviral gene transduction, efficiently induced BMP2 protein, and successfully differentiated into bone forming cells in vitro. In six horses, DFb isolated from skin punch biopsy were successfully transfected by adenoviral vector (Ad-) encoding green fluorescent protein (GFP) or BMP2 gene at 200 MOI to achieve >80% transduction efficiency (Figure 5.1a and 5.1b) and efficient BMP2 protein production. In the Von Kossa staining performed at 7 days after gene transduction, BMP2-expressing DFb showed mineralized nodule formation (Figure 5.1d and 5.1f) and positive alkaline phosphatase stain (Figure 5.1e and 5.1g). There was no nodule formation in DFb-GFP and DFb alone (Figure 5.1f) and significantly greater numbers of DFb-BMP2 were positive on the ALP staining than DFb-GFP and DFb alone (Figure 5.1g).
Figure 5.1 – In vitro gene transduction and osteogenic differentiation of equine dermal fibroblast (DFb). Equine dermal fibroblasts (DFb) were seeded in 48-well plates at a density of $5 \times 10^4$ cells/well and, 24-hours later, the monolayer cells were transfected with adenoviral (Ad) vector encoding bone morphogenetic protein-2 gene (Ad-BMP2) or green fluorescent protein gene (Ad-GFP) at 200 MOI. Successful transgene GFP expression was achieved within 2 days (a) with 84% efficiency (b). Efficient BMP2 production in the culture medium was confirmed by ELISA with the peak protein secretion at 7 days after gene transduction (c). The Von Kossa staining at day 7 demonstrated the mineralized bony nodule formations in DFb-BMP2, but DFb and DFb-GFP showed no nodule formations (d,f) (*P=0.001). The alkaline phosphatase (ALP) staining at day 7 demonstrated the significantly greater ALP uptake in DFb-BMP2 compared to DFb and DFb-GFP ($^{\#}$P=0.007) (e,g). Data were expressed as Mean + SEM.
Isolation and culture of bone marrow-derived mesenchymal stem cells (BMD-MSCs) were also attempted as an alternative cell source. Unfortunately, the culture and cell expansion of BMD-MSCs were inconsistent. Bone marrow aspirations were attempted for 15 times from the six horses to isolate BMD-MSCs. Of these, no cells were growed over 3 weeks in 4 occasions, bacterial/fungal contaminations occurred in 3 occasions, and BMD-MSCs were appeared to be died off in 6 occasions between the 6-8 passages of the cells. Therefore, only 3 of 6 horses provided the sufficient numbers of BMD-MSCs to perform the autologous cell implantations. Also, the rates of cell growth and expansion were substantially varied among the horses making impractical to standardize the cell passages at the time of implantation. For these reasons, DFb was selected as an alternative cell source for the cell-mediated BMP2 gene delivery for this project.

Clinical assessment

Equine rib drill defects were treated by subcutaneous injection of Gey’s balanced salt solution (GBSS: control), Ad-GFP, Ad-BMP2, DFb alone, DFb-GFP, and DFb-BMP2 (Figure 5.2a). All injections were made at 2 weeks after the rib drilling, and all rib specimens were harvested at 6 weeks after the injections (Figure 5.2b). During this period, all six horses showed good incisional and surgical site healing, and there were no
differences in healing of skin, edema and inflammation in subcutaneous or muscular layers between the treatment groups.

Figure 5.2 – Experimental models and assignments (a) and protocol (b) of the in vivo study. The rib drill defects were used to demonstrate an acceleration of bone healing by dermal fibroblast (DFb)-based bone morphogenetic protein-2 (BMP2) delivery and the ilium drill defects were used for an in vivo tracking of transplanted DFb. By using six healthy adult horses, the 8 mm diameter 10-12 mm deep drill hole defects were created bilaterally in the tenth or eleventh ribs (three holes per rib, six holes per horse) and, 2 weeks later, treated by percutaneous injections of Gey’s balanced salt solution (GBSS: saline control, n=6 defects), adenoviral vector encoding green fluorescent protein (GFP) gene (Ad-GFP: 5x10^9 IFU/defect, n=6), adenoviral vector encoding BMP2 gene (Ad-BMP2, n=6), DFb with no genetic modification (DFb: 5x10^7 cells/defect, n=6), DFb with GFP gene transduction (DFb-GFP, n=6), and DFb with BMP2 gene transduction (DFb-BMP2, n=6). For fluorescent labeling of bone mineralization activity, calcein was administered intravenously at three and four weeks after the cell/vector injections. The 8 mm diameter 10-12 mm drill holes were also created in the tuber coxae region of ilium (two holes per side, four holes per horse) at day 40 and, 2 weeks later which was 2 days before euthanasia, treated by percutaneous injections of GBSSS (n=6), DFb (n=6), DFb-GFP (n=6), and DFb-BMP2. All six horses were euthanized at 6 weeks after the cell/vector injection and the rib specimens and ilium drill hole tissues were harvested for analyses.
**Computed tomography**

Quantitative computed tomography (qCT) at 6 weeks after injection revealed an induction of bone regeneration in equine rib drill defects by direct gene delivery and DFb-mediated BMP2 therapy (Figure 5.3a). In the cortical filling zone, the bone volume was significantly greater in Ad-BMP2, DFb-GFP, and DFb-BMP2 groups (Figure 5.3c), and the cortical mineral density was significantly greater in Ad-BMP2 group (Figure 5.3d). In the medullary filling zone, the bone volume was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.3e). Bone density was not changed in any group (Figure 5.3f). In the adjacent cortex zone, the mineral density was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.3g). In the adjacent medullary zone (Figure 5.3b), the mineral density was significantly greater in DFb-BMP2 group (Figure 5.3h).

Of the twelve ribs used in six horses (three holes per rib, thirty six total holes), five ribs were fractured at one of the three drill holes. The treatment assignments of these five fractured drill holes were GBSS (2), Ad-GFP (2), and Ad-BMP2 (1), and none of the drill holes with DFb injections were fractured regardless the GFP/BMP2 gene transduction. Thus, the risk of fractures in the DFb-injected defects (zero out of eighteen defects) was significantly lower than the non-DFb-injected defects (five out of eighteen defects) (P=0.045). Due to the fracture confounding our bone regeneration outcomes, these five fractured drill defects were not included in qCT and histologic evaluation and treated as missing data points in data analysis. Despite this data exclusion, the computed powers were greater than 0.8 in all outcomes of qCT and histology.
Figure 5.3 – Computed tomographic (CT) images and quantitative analysis of bone regeneration in equine rib drill defect model. Six skeletally mature horses had surgically created three drill hole defects in the tenth or eleventh ribs bilaterally (three defects per rib; six rib defects per horse). Two weeks after surgery, the six defects were treated with ultrasound-guided percutaneous injections of autologous dermal fibroblasts (DFb) following BMP2 gene transduction (DFb-BMP2; n=6 defects), GFP gene transduction (DFb-GFP; n=6), DFb alone (DFb; n=6), Ad-BMP2 (n=6), Ad-GFP (n=6), or Gay’s balanced salt solution (GBSS; n=6). Quantitative CT of the ribs were performed at 6 weeks after injections. (a) Three-dimensional images of the representative rib drill hole defects in each treatment group (Scale bars: 1cm). (b) Schematic images of the four regions evaluated by quantitative CT. In the cortical filling zone, the bone volume was significantly greater in Ad-BMP2, DFb-GFP, and DFb-BMP2 groups (c), and the cortical mineral density was significantly greater in Ad-BMP2 group (d). In the medullary filling zone, the bone volume was significantly greater in Ad-BMP2 and DFb-BMP2 groups (e). Bone density was not changed in any group (f). In the adjacent cortex zone, the mineral density was significantly greater in Ad-BMP2 and DFb-BMP2 groups (g). In the adjacent medullary zone, the mineral density was significantly greater in DFb-BMP2 group (h). Data were expressed as Mean ± SEM. abc-Different letters differ significantly (P<0.05). NS: No significant differences among groups.
Histologic evaluation

Histomorphometry at 6 weeks after treatments revealed denser and more mature bone formation and greater mineralizing activity in equine rib drill defects by direct gene delivery and DFb-mediated BMP2 therapy (Figure 5.4 and 5.5). In the cortical filling zone (Figure 5.4a), the %porosity and %mineralizing area was significantly greater in DFb-BMP2 group (Figure 5.4b and 5.4c), and the %lamellar bone composition was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.4d). In the medullary filling zone (Figure 5.4e), the %porosity was significantly greater in Ad-BMP2, DFb, DFb-GFP, and DFb-BMP2 groups (Figure 5.4f), the %mineralizing area was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.4g), and the %lamellar bone composition was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.4h). In the adjacent cortex zone (Figure 5.5a), the mineral apposition rate, osteon filling period, and osteon activation frequency was significantly greater in Ad-BMP2 and DFb-BMP2 groups (Figure 5.5b, 5.5c, and 5.5d). In the adjacent medullary zone (Figure 5.5e), the mineral apposition rate was significantly greater in Ad-BMP2, DFb, and DFb-BMP2 groups (Figure 5.5f), the %mineralizing surface was significantly greater in Ad-BMP2, DFb, DFb-GFP, and DFb-BMP2 groups (Figure 5.5g), and the surface activation frequency was significantly greater in Ad-BMP2, DFb, and DFb-BMP2 groups (Figure 5.5g).
Figure 5.4 – Histologic evaluation of the cortical and medullary bone filling regions of the rib drill hole defects. Six skeletally mature horses had surgically created three drill hole defects in the tenth or eleventh ribs bilaterally (three defects per rib; six rib defects per horse). Two weeks after surgery, the six defects were treated with ultrasound-guided percutaneous injections of autologous dermal fibroblasts (DFb) following BMP2 gene transduction (DFb-BMP2; n=6 defects), GFP gene transduction (DFb-GFP; n=6), DFb alone (DFb; n=6), Ad-BMP2 (n=6), Ad-GFP (n=6), or Gay’s balanced salt solution (GBSS; n=6). Histologic evaluations were performed at 6 weeks after injections. In the cortical filling zone (a) (Scale bars: 0.5mm), the %porosity and %mineralizing area was significantly greater in DFb-BMP2 group (b,c), and the %lamellar bone composition was significantly greater in Ad-BMP2 and DFb-BMP2 groups (d). In the medullary filling zone (e) (Scale bars: 0.5mm), the %porosity was significantly greater in Ad-BMP2, DFb, DFb-GFP, and DFb-BMP2 groups (f), the %mineralizing area was significantly greater in Ad-BMP2 and DFb-BMP2 groups (g), and the %lamellar bone composition was significantly greater in Ad-BMP2 and DFb-BMP2 groups (h). Data were expressed as Mean + SEM. Different letters differ significantly (P<0.05).
Figure 5.5 – Histologic evaluation of the adjacent cortex and medulla regions of the rib drill hole defects. Six skeletally mature horses had surgically created three drill hole defects in the tenth or eleventh ribs bilaterally (three defects per rib; six rib defects per horse). Two weeks after surgery, the six defects were treated with ultrasound-guided percutaneous injections of autologous dermal fibroblasts (DFb) following BMP2 gene transduction (DFb-BMP2; n=6 defects), GFP gene transduction (DFb-GFP; n=6), DFb alone (DFb; n=6), Ad-BMP2 (n=6), Ad-GFP (n=6), or Gay’s balanced salt solution (GBSS; n=6). Histologic evaluations were performed at 6 weeks after injections. In the adjacent cortex zone (a) (Scale bars: 0.5mm), the mineral apposition rate, osteon filling period, and osteon activation frequency was significantly greater in Ad-BMP2 and DFb-BMP2 groups (b,c,d). In the adjacent medullary zone (e) (Scale bars: 0.5mm), the mineral apposition rate was significantly greater in Ad-BMP2, DFb, and DFb-BMP2 groups (f), the %mineralizing surface was significantly greater in Ad-BMP2, DFb-GFP, and DFb-BMP2 groups (g), and the surface activation frequency was significantly greater in Ad-BMP2, DFb, and DFb-BMP2 groups (h). Data were expressed as Mean ± SEM. abc Different letters differ significantly (P<0.05).
In vivo tracking of transplanted DFb

Equine ilium drill defects were treated by subcutaneous injection of GBSS, DFb alone, DFb-GFP, and DFb-BMP2 (Figure 5.2). An in vivo tracking of transplanted DFb was successful by using the drill hole tissues harvested 2 days after injection. There were
significant BMP2 gene upregulation in the drill hole tissues with DFb-BMP2 injection, but there were no significant GFP gene upregulation in the drill hole tissues with DFb-GFP injection (Figure 5.6a). The drill hole tissues were digested and seeded in culture dishes. The BMP2 protein concentration in culture medium was significantly higher in DFb-BMP2 group than GBSS, DFb, or DFb-GFP groups (Figure 5.6b), and the concentrations were less than the detection limit in all of GBSS, DFb, and DFb-GFP-treated drill hole tissues and the 3/6 of DFb-BMP2-treated tissues (Figure 5.6b).

5.4 Discussion

Skin cell-mediated gene therapy using a single percutaneous injection of DFb genetically modified to secrete BMP2 can accelerate bone healing, which was at least equally and potentially more effectively than direct BMP2 gene delivery. In our study, DFb-BMP2-treated defects had greater amount of cortical bone filling and more mature lamellar bone formation compared to the Ad-BMP2-treated defects. For the same reasons delayed direct vector injection was performed, delayed cell injection into a solid granulation tissue may be advantageous to contain the DFb in the healing site, accumulate secreted growth factors in the local region, target rich host cells by BMP2, and potentially provide a healthy environment for transplanted cells. Interestingly, our DFb-BMP2 had greater cortical bone regeneration than Ad-BMP2. Possibly, the integration of the DFb cells into a less cellular environment area may have contributed directly to bone formation.
Bone regeneration has been induced by both cell- and gene-vectors, but potency between two effective dosages and strategies have not yet been compared within same animal. The equine rib defect model in our study offered advantages for the direct comparison of bone regeneration between cell-mediated and direct gene delivery of BMP2 by qCT and histologic evaluation. Also, the rib model permitted the assessment of the effect of local BMP2 on both cortical and cancellous bone in a confined space for high resolution outcome of bone formation. In concert, our model demonstrated that cell-mediated BMP2 delivery appeared to promote bone regeneration more effectively and extensively than direct BMP2 gene delivery, at least in this model and at the particular cell/vector dosages used in this study.

Cell-mediated gene delivery method offers many advantages, because using cells as vectors can permit the manipulation and control of many steps that occur in vivo with direct delivery. First, the conditions can be controlled to optimize cell transduction efficiency, plus injecting cells increases the cellularity at the repair site. Many viral vectors, and certainly adenoviral vector, induces a local tissue inflammation, although the cell injection might also induce an inflammatory response. In addition to inflammation, activation of the innate immune response by viral vectors is well confirmed, and indeed in horses injected directly with adenoviral vector develop detectable serum antibody within weeks. Complicating the potential effectiveness of direct adenoviral vector delivery would be prior exposure to wildtype adenovirus, the consequence of which would be limited efficacy of the direct vector injection. In total,
these advantages of cell-mediated gene therapy may be responsible for the relatively greater bone regeneration capacity shown in the DFb-BMP2-treated rib drill defects.

We chose to compare dosages of cell or adenoviral vectors that had been shown in an equine model to have a comparable influence on bone repair (Ishihara et al, accepted by JOR). For DFb-BMP2-treated rib defects 50 million DFb that were transfected with $1 \times 10^{10}$ IFU of Ad-BMP2 (=200 IFU/cell) were injected, the same dosage that significantly promoted bone formation in the equine metacarpal/tarsal osteotomy model. For Ad-BMP2-treated rib defects, $5 \times 10^9$ IFU of vector was injected, the same dosage that significantly induced bone formation in the same metacarpal/tarsal osteotomy model. The %transduction and level of BMP2 upregulation may be greater in DFb-BMP2-treated drill defects than Ad-BMP2, because the vectors were applied under the optimal, controlled, serum-free culture condition at twice higher viral yield, although it was unknown. In contrast, the number of host cells transduced by Ad-BMP2 was likely larger than 50 million, because the direct vector injection was made into the mature, highly cellular granulation tissue formed by 2 weeks after the drill defects were created. It should be emphasized that relatively potency between cell and gene therapies is largely dependent upon the amount and type of cell/vector used, difference in experimental models, and timing of cell/vector injections. We selected two known methods that had shown comparable effectiveness in vivo in a large animal model to provide comparison of two potentially clinically applicable methods.

In equine metacarpal/tarsal bone, DFb-BMP2-treated osteotomies were composed of mature cartilage that did not fully convert to bridging woven bone. The authors
speculated that incomplete endochondral ossification may have been due to the limited number of osteoprogenitor cells in the dominantly cortical bone healing site. Even though BMP2 is a key factor for chondrocyte maturation during endochondral ossification, multiple signaling molecules from osteoprogenitor cells such as BMP-2, 4, 6, and 7 are involved in the apoptosis of hypertrophic chondrocytes.\textsuperscript{23} We speculate that the intra-medullarly delivered DFb-BMP2 may work synergistically with host osteoprogenitor cells present in the cancellous bone environment and efficiently complete the endochondral ossification process. The rib also has such a large population of host osteoblastic lineage that may serve as a target for the secreted BMP2 from the transplanted DFb to induce bone regeneration. For this reason, a cortical bone injury site may be treated by cancellous bone graft at the time of initial surgery so that the bone regeneration capacity of subsequent cell-mediated BMP2 gene therapy could be enhanced by cellular/molecular interaction of skin cells, BMP2, and osteoprogenitor cells.

It is important to point out that the direct BMP2 gene delivery was still an effective strategy for bone regeneration. In agreement to our previous studies,\textsuperscript{7,16} delayed vector injection (2 weeks after surgery) into the mature granulation tissue appeared to be supportive to an effective outcome, presumably by viral containment, growth factor accumulation, and gene transduction to a large receptive host cell population.\textsuperscript{7,16} An advantage of direct gene therapy is less complexity of vector preparation, as opposed to cell-mediated therapy, but can involve a risk of innate immune reaction or an increase of neutralizing antibody which may prohibit multiple vector injections in the same individual. In the clinical setting, single direct Ad-BMP2 treatment may be applied to an
orthopedic patient without the need to wait for sufficient numbers of autologous cells to be prepared for cell-mediated BMP2 therapy.

Our results indicated that placing DFb in the medullar cavity by itself may be beneficial to facilitate bone regeneration. In this study, the volume of medullary filling was greater in all DFb-treated bone defects regardless the BMP2 gene transduction (i.e., all of DFb, DFb-GFP, DFb-BMP2) compared to saline control (Figure 5.4e and 5.4f). This result may suggest that the cancellous bone environment may provide the differentiation factors and three-dimensional architecture such that DFb could sufficiently undergo osteogenic differentiation. Possibly, the transplanted DFb synthesized extracellular matrix to support the migration of host osteoprogenitor cells. Dermal fibroblasts have been shown to produce cartilage-like dense extracellular matrix when cultured in osteogenic-induction medium,24 which may serve as a biological scaffold to stimulate recruitment of osteoprogenitor cells from the surrounding cancellous bone or promote endochondral ossification. In this regard, DFb-mediated BMP2 therapy may be considered to have a dual osteoinductive and osteoconductive effect; therefore, possibly the simultaneous injection of DFb and stem cells would promote greater bone regeneration than use of an individual cell type.

Our results showed that five of eighteen drill holes with acellular injections (eg, GBSS, Ad-GFP, or Ad-BMP2) were fractured, but all eighteen drill holes with cellular injections (eg, DFb alone, DFb-GFP, or DFb-BMP2) were intact and this was a statistically significant association. This may suggest that, although transplanted DFb only modestly accelerated bone production, this may have been sufficient to physically
stabilize the bone defects and decrease the risk of fracture at early time points. Cell-mediated release of BMP2 may have served to augment this effect. These rib fractures occurred early after drilling (<3 weeks after the injection), because the callus contained calcein label. The lack of fractures in our treated defects is serendipitous evidence that an acceleration of bone regeneration can be important in vivo for superior healing. These five fractured defects were excluded for qCT and histologic evaluation reducing the sample sizes (from n=6 to n=4 or 5) in a subset of treatment groups; however, the calculated powers were greater than 0.8 in all outcomes with significant differences suggesting that our study had reliably demonstrate the treatment effects with 5% alpha-error and 20% beta-error levels in spite of the missing data points.

Direct or cell vector BMP2 delivery stimulated mineralization activity and improved bony density in the neighboring original osseous tissue. This may contribute to a greater strength of the injured bone unit, in this case of the drilled rib, resulting in less fracture. The mechanism of this increased mineralization in remote sites was unknown, but bone surface osteocytes may interact with secreted BMP2 or be directly transfected by soluble Ad-BMP2 and subsequently spread the activation signals by a canaliculi network throughout the adjacent cortex. This result supports that BMP2 delivery has relevant potential to strengthen existing bone and the entire bone unit. For instance, an infusion of DFb-BMP2 into the marrow cavity of long bones may become an innovative therapeutic strategy to stimulate the mineralization and increase the bone density for the treatments of fragile bone disorders such as osteoporosis, disuse osteomalacia, and osteogenesis imperfecta.
To the authors’ knowledge, this is the first study reporting the increased osteon/surface activation frequency by gene- or cell-mediated BMP2 delivery (Figure 5d and 5h). Activation of bone remodeling is initiated as an osteoclastogenesis, not osteoblastogenesis, and therefore has not been considered as a primary function of BMPs. In recent years, however, new roles of BMPs associated with osteoclasts have been discovered including a recruitment of monocytic precursor cells,\textsuperscript{25} stimulation of osteoclastic differentiation,\textsuperscript{26} and facilitating the transition from bone resorption to bone formation phases as coupling factors.\textsuperscript{23} In addition, BMP2 has been shown to activate hematopoietic cells of murine bone marrow resulting in an upregulation of osteoclast differentiation factor and enhanced osteoclast-like multinucleated cell formation.\textsuperscript{27} Recruitment of hematopoietic cells and conversion of these precursors into osteoclasts are the important process in the initiation phase of bone remodeling.\textsuperscript{28} Therefore, in our study, the intra-medullarly delivered BMP2 may stimulate the hematopoietic precursors present in the rib marrow cavity and activate bone turnover of the adjacent cortex and medulla. Because BMP2 may have a role to coordinate the functional connection between osteoclastic and osteoblastic activities as a coupling factor,\textsuperscript{28} the increase of bone resorption could be tightly orchestrated with subsequent bone formation ensuring an anabolic bone remodeling. One of the potential clinical applications of this aspect is bone fragility disorders such as osteoporosis, because encouraging anabolic remodeling in specific long bones may be a more physiological way to improve bone mineral density than shutting down systemic bone turnover. Use of an osteoclast-suppressor has been a principal treatment of osteoporosis to systemically reduce a rate of catabolic bone
remodeling and preserve the mineral density of long bones. Reportedly, however, prolonged use of osteoclast-suppressors in osteoporosis patients may result in bone fragility and low-energy fractures, which are likely due to an impaired microcrack repair and accumulation of microfractures.\textsuperscript{29} For these reasons, cell or direct vector BMP2 delivery may be a potential therapy for osteoporosis to stimulate an anabolic bone remodeling for microcrack repair without loss of mineral density.

In vivo cell tracking successfully identified the functional presence of our injected cells in the granulation tissue bed. We chose to harvest the ilium drill hole tissue 2 days after the DFb transplantation. Our results demonstrated that the percutaneously transplanted DFb-BMP2 were present at the recipient site, maintained BMP2 transgene expression, and local BMP2 production, at least when the cells were retrieved and recultured (Figure 5.6). The DFb-GFP-treated drill hole tissue also had higher GFP transgene expression than DFb or DFb-BMP2. The BMP2 gene transduction could be supportive for cell viability so that more number of DF-BMP2 than DFb-GFP might survive. Cultures of stem cells engineered to express BMP2 outlive the cells transduced with marker genes and appeared more robust and morphologically healthy. It is also possible that, because a significantly greater GFP expression was not detected in the DFb-GFP-treated tissues, an active bone healing environment may be more favorable for the DFb to sustain BMP2 expression than GFP expression.

In summary, we used the equine rib drill defect model to compare the effects of cell-mediated and direct BMP2 gene delivery using a percutaneous injection of DFb or viral vectors with and without BMP2 gene transduction. This study demonstrated that
both of the autologous transplantation of BMP2-expressing DFb and direct Ad-BMP2 successfully induced bone regeneration, but DFb-BMP2 was more potent for cortical bone regeneration compared to a direct vector injection. Cell- and gene-mediated BMP2 delivery showed comparable bone regeneration capacity in the cancellous bone region and improved bone density in adjacent osseous tissues.

5.5 Methods

Adenoviral vector production

Recombinant Ad vectors containing either a 1,547 base-pair open reading frame segment of human BMP-2 (Ad-BMP2) or GFP (Ad-GFP) under the control of the cytomegalovirus promoter were generated. Expression of transgenes was verified in cell culture.

Experimental design

Six skeletally mature horses (weight 445 to 550 kg) were used to isolate DFb by full-thickness skin punch biopsy. In vitro gene transduction and osteogenic differentiation were confirmed in DFb from all 6 horses. The in vivo study used the same six horses in two separate experiments; (i) rib drill hole defects for an assessment of bone regeneration, and (ii) ilium drill hole defects for an in vivo cell tracking (Figure 5.2). All procedures were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.
At day 0, the same six horses had surgically created three drill hole defects in the tenth or eleventh ribs bilaterally (three defects per rib; six rib defects per horse) (Figure 5.2a). Skin biopsy had been performed 2 weeks prior to the rib drilling (Day -14) to culture sufficient DFb for injection. For each horse, the six rib drill hole defects were treated with percutaneous injection of autologous DFb following BMP2 gene transduction (DFb-BMP2; n=6 defects), GFP gene transduction (DFb-GFP; n=6), DFb alone (DFb; n=6), Ad-BMP2 (n=6), Ad-GFP (n=6), or Gay’s balanced salt solution (GBSS; n=6) (Sigma-Aldrich, St Louis, MO). The six defects in each horse were assigned in a block design to rotate these injections. All injections were made two weeks after surgery (Day 14). Six weeks following the injection (Day 56), the horses were euthanized and rib specimens were harvested. Efficacy was assessed by quantitative computed tomography and histology.

Using the same six horses, two drill hole defects were also created bilaterally on the tuber coxae of ilium (two defects per side, four ilium defects per horse). Separate skin biopsies had been performed 2 weeks prior to the ilium drilling (Day 26) to culture sufficient DFb. For each horse, the four ilium drill hole defects were treated with percutaneous injection of DFb-BMP2 (n=6 defects), DFb-GFP (n=6), DFb (n=6), or GBSS (n=6). The four defects in each horse were assigned in a block design to rotate these injections. The drill defects were created at Day 40 of the experiment, the injections were performed at Day 54 (2 weeks after drilling), and the horses were euthanized at Day 56; therefore, the tissues within the ilium drill holes were harvested 2 days after the injection. The ilium defect tissues were processed for the PCR to detect BMP2/GFP gene
expression and the collagenase digestion to culture BMP2-secreting or GFP expressing DFb.

*Dermal fibroblast isolation and in vitro osteogenic differentiation*

Full-thickness skin tissue was harvested using a 5-mm diameter biopsy punch from the pectoral region (10-12 punches per horse) from each of the six horses. The dermal layer was dissected from the epidermis under a microscope, and DFb were isolated by type-1 collagenase digestion (GIBCO, Grand Island, NY) and cultured in DMEM supplemented with L-glutamine (300 μg/mL), penicillin (30 μg/mL), streptomycin (30 μg/mL), and 10% fetal bovine serum at 37 ºC in a 5% CO₂ atmosphere. To demonstrate in vitro gene transduction and osteogenic differentiation, DFb were seeded in triplicate wells of 48-well plates (Falcon, Franklin Lakes, NJ) at a density of 5×10⁴ cells/well and, 24-hours later, transfected with Ad-BMP2 or Ad-GFP at 200 MOI (eg, 2×10² infectious unit [IFU] per cell; 1×10⁷ IFU per well) (Adeno-X Rapid Titer Kit, Clontech, Mountain View, CA). Two and 7 days after gene transduction, gene transduction efficiency was quantified by fluorescent microscopy, and BMP2 protein production confirmed with ELISA (R&D Systems, Mineapolis, MN). Seven days after gene transduction, osteogenic differentiation of DFb was quantified by the number of von Kossa-positive mineralized bony nodules within the well and the intensity score of alkaline phosphatase (ALP, Sigma-Aldrich) staining in three representative X100 microscopic fields per well using the following grading scheme: 0 (0% of cells in the field showing stain uptake), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%).
BMD-MSCs were obtained as an alternative cell source by standard bone marrow aspiration from the sternum as follows. Horses were sedated the skin aseptically prepared, and stab incision made over cranial ventrum over the sternum. A bone marrow aspiration needle (MD Tech Inc., Gainesville, FL) was inserted into the vertebral body from the ventro-lateral/medial aspect of the sternum and bone marrow was aspirated into heparin-flushed (Heparin sodium: 1,000 USP units/ml) 12 ml sterile syringe. The procedure was repeated until a minimum of 10 ml of bone marrow was collected. Primary BMD-MSCs were isolated by centrifugation and cultured in a monolayer. BMD-MSCs were cultured in DMEM as described above.

Rib drill hole defect model

The rib drill hole defects were used to evaluate the relative efficacy of direct gene- and DFb-mediated BMP2 delivery to induce bone regeneration. Skin punch biopsy was performed at 2 weeks before the rib drilling (Day -14). At day 0, general anesthesia was induced by xylazine (Rompun; Bayer, Pittsburgh, PA; IV, 1.1 mg/kg), ketamine (Ketaset; Fort Dodge Animal Health, Overland Park, KS; IV, 2.2 mg/kg), and diazepam (Valium; Roche, Madison, WI; IV, 0.11 mg/kg), and maintained by Isoflurane (IsoFlo; Abbott, Parsippany, NJ; Infusion, 2-5%). Following aseptic preparation of the bilateral latero-ventral thorax, the 4-cm stab incisions through the skin and external and internal abdominal oblique muscles were made and a low-speed cordless electric drill was used to create 8-mm diameter 10-mm deep defects on the tenth or eleventh ribs bilaterally. Three drill holes were made in each rib at 10-cm apart (i.e., 6 rib defects per horse), and the
most ventral hole was created at 20-cm dorsal from the costochondral junction. Efforts were made to create the drill holes on the middle of the ribs. The 3 drill hole defects in each rib were created by the 3 separate stab incisions to prevent the periosteal communication between the defects, and were throughly flushed with sterile saline solution to remove all bone shards. Antibiotics were administered for one day following surgery (procaine penicillin [Crystacillin; Solvay, Marietta, GA; IM, 22000 IU/kg] and gentamicin [Gentocin; Schering, Kenilworth, NJ; IV, 6.6 mg/kg]); the anti-inflammatory drug (phenylbutazone [Butazolidin; Schering, Kenilworth, NJ; PO, 4.4 mg/kg]) was administered for three days following surgery.

One week after the surgery (Day 7), DFb were seeded in 18 large culture flasks (75-cm$^2$; 3×10$^6$ cells per flask). On Day 12, 2 days before injection, the DFb (80-90% confluent) were treated with Ad-BMP2 (DFb-BMP2; 6 flasks), Ad-GFP (DFb-GFP; 6 flasks), or untreated (DFb; 6 flasks). The total cell number at this time point was estimated at 1×10$^7$ DFb per flask based on a pilot trial and hemacytometer counting (Hausser Scientific, Horsham, PA). The Ad-BMP2/GFP were administered at 200 MOI; therefore, 2×10$^9$ IFU of vector were administered in each flask.

Two weeks after the surgery (Day 14), 48 hours after DFb gene transduction, cells were harvested by trypsinization (GIBCO), counted by hematocytometer, centrifuged, and resuspended into a 500 µl total volume with GBSS containing 5×10$^7$ cells for each of DFb, DFb-GFP, and DFb-BMP2. All the cell injections were completed within 60 to 90 minutes after the trypsinization. Also, the Ad-BMP2 and Ad-BMP2 vectors were diluted into a 500 µl total volume with GBSS containing 5×10$^9$ IFU.
Horses were placed under general anesthesia and the assigned rib defects were treated by percutaneous ultrasound-guided (Technos MPX, Esaote S.p.A, Genoa, Italy) needle injection of $5\times10^7$ cells of DFb-BMP2, DFb-GFP, DFb, or $5\times10^9$ IFU of Ad-GFP or Ad-BMP2, or GBSS in a 500 µl volume directly into the drill hole defects. For fluorescent labeling of bone mineralization activity, calcein (Sigma-Aldrich) was administered at a rate of 20 mg/kg, dissolved in 2% sodium bicarbonate solution (Abbott Laboratories, North Chicago, IL) intravenously at three and four weeks after the DFb injection (Day 35 and 42, respectively).

Quantitative computed tomography

Six weeks following the cell/vector rib injection (Day 56), horses were sedated with xylazine and euthanized with by lethal intravenous overdose of pentobarbital (Beuthanasia, IV, 2.2 mg/kg). Immediately after the euthanasia, the ribs with drill hole defects were dissected. Quantitative computed tomography (QCT) (Picker PQS Helical CT Scanner, Philips Medical Systems, N.A., Bothell, WA) was performed in the transverse plane (perpendicular to the rib axis) at 1-mm contiguous slices to evaluate the bone regeneration. The entire drill hole defect along with 1.5 cm margins dorsal and ventral were imaged. Two-dimensional images in the coronal plane (perpendicular to the drill hole axis) were created by using an image analysis software (Mimics, Materialise, Ann Arbor, MI). The coronal sections allowed visualizing the cross-sectional area of cylinder drill hole defects and using the 8-mm diameter circular region of interest (ROI) to trace the original size of the drill hole. Three central coronal slices across the cortical
zone were selected, and the volume and mineral density of cortical bone filling was calculated within the 8-mm diameter circular ROIs positioned at the center of the remaining defect. By using the same three coronal slices, the mineral density of adjacent cortex was also calculated within four of the 4-mm diameter circular ROIs concentrically positioned at 8-mm away from the center of the remaining defect. Threshold level of 600-3000 hounsfield units (HU) was used for cortical filling and adjacent cortex zones to trace ‘compact’ bone but exclude ‘spongial’ bone, and fibrous, muscle, and fat tissues (Mimics software tutorial manual, Materialise, Ann Arbor, MI). Similarly, three central coronal slices acrossed the medullar zone were selected, and the volume and mineral density of medullary bone filling was calculated within the 8-mm diameter circular ROIs positioned at the center of the remaining defect. By using same three coronal slices, the mineral density of adjacent medulla was calculated within four of the 4-mm diameter circular ROIs concentrically positioned at 8-mm away from the center of the remaining defect. Threshold level of 150-3000 HU was used for medullary filling and adjacent medulla zones to trace both ‘compact’ and ‘spongial’ bone but exclude fibrous, muscle, and fat tissues (Mimics software tutorial manual, Materialise, Ann Arbor, MI). All of the HU values were converted to mineral density using potassium phosphate standards (CT Calibration Phantom, Mindways Software, Inc., San Francisco, CA).

**Histologic evaluation**

The calcified rib specimens were dehydrated in alcohol, embedded in methylmethacrylate, cut into 10-μm sections in the transverse plane perpendicular to the
longitudinal axis at the center of the drill hole, and stained with Masson’s Trichrome. The rib specimens were evaluated semi-quantitatively for 4 regions: cortical filling zone, medullary filling zone, adjacent cortex, and adjacent medulla (Figure 5.2).

The cortical filling zones were evaluated for the porosity (% unfilled area), maturity (% composition of immature woven, mature lamellar bone, and mixture within the filled area), and mineralization activity (% calcein-labeled area within the filled area), by point-counting with the microscopic grids under 200X magnification. The five representative areas in the cortical bone filling in <5-mm depth from the top of drill hole were evaluated and averaged.

Similarly, the medullary filling zones were evaluated for the porosity, maturity, and mineralization activity, by point-counting with the microscopic grids under 200X magnification. The five representative areas of the medullary bone filling in <10-mm depth from the top of drill hole were evaluated and averaged.

The adjacent cortices were evaluated for the mineral apposition rate (MAR), osteon filling period, and osteon activation frequency. Within the 1-mm² cortical bone area under 200X magnification, the number of single-labeled osteon (sL.On) and double-labeled osteon (dL.On) were counted and, in the 2-3 representative dL.On within the 1-mm² area, the radii of Haversin canal (Hv.Rd), inner label (In.Rd), outer label (Out.Rd), and cement line (Cm.Rd) were measured using the microscopic grids, and these values were used for the following calculations.\(^{30,31}\)

\[
MAR (\mu m/day) = (Out.Rd - In.Rd) ÷ 7
\]

\[
Osteon filling period (days) = [\ln(Hv.Rd/Cm.Rd)] ÷ [\ln(In.Rd/Out.Rd) ÷ 7]
\]
Osteon activation frequency (#/mm²/year)

\[ = \frac{(sL.On)/2 + dL.On}{(Cm.Rd - Hv.Rd)/MAR} \times 365 \]

The five representative areas of the adjacent cortex in <5-mm distance from the edge of drill hole were evaluated and averaged.

The adjacent medullae were evaluated for the MAR, percent active bone surface, and surface activation frequency. Within the 1-mm² medullar bone area under 200X magnification, the trabecula width (Tr.Wd) and distance between two calcein labels (Lb.Dt) were measured in the 2-3 double-labeled surface and averaged, the intercept-counting of total bone surface (BS) and single/double-labeled mineralizing surface (MS) were performed with the microscopic grids, and these values were used for the following calculations.

\[ MAR (\mu m/day) = \frac{(Lb.Dt)}{7} \]

\[ Percent \ mineralizing \ surface = \frac{(MS/BS)}{100} \]

\[ Surface \ activation \ frequency (/year) = \frac{[MAR \times (MS/BS)]}{Tr.Wd \times 365} \]

The five representative areas of the adjacent medulla in <5-mm distance from the edge of drill hole were evaluated and averaged.

Ilium drill hole defect model

The ilium drill hole defects were used for in vivo cell tracking. Skin punch biopsy was performed at Day 26 to culture the efficient DFb. At day 40, the drill hole defects were created bilaterally in the tuber coxae regions of ilium as standing surgery. The horses were restrained in stocks, sedated with xylazine (IV, 1.1 mg/kg.), and the skin
overlying the tuber coxae was aseptic prepared and injected with local anesthesia (lidocaine; SQ, 100 mg). The 2-cm stab incisions were made on the lateral aspect of the tuber coxae and a low-speed cordless electric drill was used to create 8-mm diameter 10-mm deep defects on the tuber coxae. Two drill holes were made in each tuber coxae at 10-cm apart (i.e., four ilium defects per horse). The two drill hole defects in each side were created by the two separate stab incisions to prevent the periosteal communication between the defects, and were thoroughly flushed with sterile saline solution to remove all bone shards. Antibiotics and anti-inflammatory drug were administered at same dose and length as the rib drilling. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

The DFb were prepared by the same protocol as for the rib injection. The DFb were seeded in eighteen flasks at Day 47 (1 week after ilium drilling), treated with Ad-BMP2, Ad-GFP, or untreated at Day 52, and harvested and resuspended into a 500 µl total volume with GBSS containing 5×10⁷ cells for each of DFb, DFb-GFP, and DFb-BMP2 at Day 54 (48 hour after gene transduction). Horses were restrained in stocks, sedated, aseptically prepared, and the assigned ilium defects were treated by percutaneous ultrasound-guided needle injection of 5×10⁷ cells of DFb-BMP2, DFb-GFP, DFb, or GBSS in a 500 µl volume directly into the drill hole defects. The drill hole size and the cell dosage of DFb-BMP2, DFb-GFP, and DFb was exactly same as the rib drill hole defects making these two model comparable.

Immediately after the euthanasia at Day 56 (2 days after the cell injection), the tissue within the ilium drill hole defects were aseptically harvested. The RNA was
extracted from the half of the tissue by Trizol technique and the other half were digested by collagenase for 6 hours. Transgene expression of human BMP2 and GFP were quantified by SYBR real-time RT-PCR with the ABI PRISM 7000™ Sequence Detection System (Applied Biosystems, Foster City, CA). Mean fold change was calculated using custom designed primers for the *hbmp2* (Forward: 5’-AAAACGTCAAGCCAAACACAAA-3’, Reverse: 5’-GTCACTGAAGTCCACGTACAAAGG-3’) and *gfp* gene (Forward: 5’-CATGATATAGACGTTGTGGCTGTG-3’, Reverse: 5’-AAGCTGACCCCTGAAGTTCATCTGC-3’) in the DFb, DFb-GFP, and DFb-BMP2-treated drill hole tissue relative to expression of GBSS-treated drill hole tissue and relative to expression of the endogenous equine β-actin gene (Forward: 5’-GGGCATCCTGACCCTCAAG-3’, Reverse: 5’-TCCATGTCGTCCCAGTTGGT-3’) using the 2^−ΔΔCT method.34,35

The digested tissues were centrifuged, washed with GBSS, and seeded in 25-cm² culture flasks (Day 57). Four days later (Day 61), the culture medium were collected for the detection of BMP2 protein by ELISA. The lowest BMP2 standard (62.5 pg/mL) was considered as detection limit, because the mean ± 2SD optical density in triplicate wells did not overlap that of zero standard (0 pg/mL).

**Statistical analysis**

Repeated-measure analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC) was used to evaluate the effects of DFb-mediated BMP2 therapy with the post-test
multiple comparisons between the treatment groups using *Proc Mixed* statistical models for continuous outcomes (i.e., von Kossa bony nodule count, QCT, histologic evaluation, RT-PCR gene expression, and ELISA BMP2 concentration data) and *Genmod* statistical models for categorical outcomes (i.e., ALP stain uptake score). Repeated variables were considered to be nested within horse, and the distribution of data was assessed by use of a subset of normality tests (e.g., the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests). Fisher’s exact tests were used to compare the frequency outcomes (i.e., occurrence of fractures in rib drill hole defects) between the treatment groups. Significant level was set at $P<0.05$ for all analyses. The power calculations were made using the means, standard deviations, sample sizes, and 5% alpha-error level for all outcomes differed significantly in given treatment groups.
Chapter 6:
Inflammation and immune reactions of intra-articularly administered recombinant and self-complementary adeno-associate-viral or adenoviral vectors encoding bone morphogenetic protein-2

6.1 Summary

Adenoviral and recombinant adeno-associated-viral (rAAV) vectors, including self-complementary (sc) AAV vectors, have been used to deliver genes by intra-articular injection to treat osteoarthritis and rheumatoid arthritis. Our study quantified, in vitro, the relative gene transduction among Ad, rAAV, or scAAV vectors in articular cells, and, in vivo, compared the inflammatory and immune responses following their intra-articular administration. In equine chondrocytes and synovial cells, scAAV showed greater transduction efficiency and more sustained gene and protein expression. Intra-articular administration of scAAV and rAAV produced less joint inflammation than Ad vectors. Neutralizing antibodies were detected against Ad and AAV in synovial fluid from injected joints at higher titer than serum and contralateral joint fluid. Bone morphogenetic protein 2 (BMP2) was detected in synovial fluid of Ad-BMP2-injected joints but not in rAAV or scAAV-injected joints, even at high vector titer (1 x 10^{13} DRP/joint).
Adenoviral delivery of genes produced a robust gene expression and transient inflammation. Further work is necessary to optimize intra-articular administration of AAV vectors for reliable gene transduction for large species in vivo.

6.2 Introduction

Joint disorders including osteoarthritis and rhematoid arthritis remain major causes of the mobility losses in elderly people,\textsuperscript{1} and gene therapy has a great potential for the treatment of those conditions by utilizing the gene transfers of interleukin-1 receptor antagonist (IL-1Ra),\textsuperscript{2,3} transforming growth factor-\(\beta\)1,\textsuperscript{4} or tumour necrosis factor alpha antagonist protein.\textsuperscript{5} Although there are several different approaches for the arthritis gene therapy, direct intra-articular administration of gene delivery vectors has been considered as an attractive strategy to transduce the cartilage and synovial tissues with therapeutic genes, because joints are discrete and accessible cavities that can be easily injected.\textsuperscript{6}

Various types of viral vectors have been used for an introduction of therapeutic genes for the intra- and peri-articular tissues including adenovirus (Ad)\textsuperscript{7,8} and adeno-associate virus (AAV).\textsuperscript{9-11} The Ad vectors have shown to induce rapid and successful transgene expression, but its intra-articular usage has regarded as unfavorable due to the robust inflammatory and immune response and transient transgene expression.\textsuperscript{6,7,8} In contrast, recombinant AAV (rAAV) has gained much attention as a safe and injectable vector for the gene delivery to joints, because it is generated from non-pathogenic virus, immunologically inert, and can induce sustained transgene expression.\textsuperscript{6,9-11} In addition, rAAV-mediated joint therapy may be advantageous by its greater penetrating capability
into cartilage extracellular matrix on account of the smaller particle size. Moreover, AAV vector has been modified by adding double stranded DNA and bypassing the rate-limiting step of second-strand synthesis in order to improve the transduction efficiency and to broaden their tropisms. Such self-complementary AAV (scAAV) vectors have shown to effectively transfect chondrocytes and synovial cells, cartilage implants, and induce superior transgene expression in cartilage and synovium tissues. Therefore, an intra-articular administration of scAAV vectors has a great potential for future clinical application; however, the inflammatory and immune response of the scAAV have not been directly compared with rAAV or Ad vectors in vivo. Also, relative tropism of chondrocytes and synovial cells has not been evaluated among the Ad, rAAV, and scAAV vectors.

Consequence of the intra-articular placement of gene delivery vectors also needs to be concerned when certain viral vectors are used to accelerate articular fracture healing. Both Ad and AAV vectors have been applied for the direct gene transfer of osteogenic growth factor such as bone morphogenetic protein-2 (BMP2) to accelerate and enhance the healing of various fracture models. When osteogenic gene transduction was performed in the bones adjacent to joints, the viral vectors may be hematogenously migrated or iatrogenically misplaced into the articular spaces, in which an ectopic articular tissue mineralization or ossification may subsequently result. Authors’ laboratory has demonstrated that an administration of Ad-BMP2 into articular fracture gap can lead to periarticular ossification and joint ankylosis in immunoincompetent animals. Although direct BMP2 gene transfer is a promising therapeutic strategy to
accelerate bone healing, the potential adverse effects of intra-articular BMP2 gene transduction must be investigated prior to a clinical application of fracture gene therapy.

The objectives of this study are to assess the relative gene transduction among Ad, rAAV, scAAV vectors in chondrocytes and synovial cells, and compare the inflammatory and immune responses by the intra-articular administration of Ad, rAAV, and scAAV vectors, and examine potential adverse mineralization of intra-articular BMP2 gene delivery by these vectors in equine joints. We hypothesized that the scAAV vectors would show greater transduction efficiency in vitro compared to rAAV, induce less inflammatory and immune responses in vivo compared to Ad vectors, and intra-articular BMP2 gene transfer induce minimu to none adverse mineralization in an immunocompetent animal. Use of large animal models would allow the collection and evaluation of joint fluid samples for multiple time points without physiologically affecting the joint environments, and could demonstrate a feasibility of applying arthritis gene therapy in large size of human joints.

6.3 Methods

Viral Vector Production

Recombinant E-1 defective serotype-5 adenovirus preparations encoding green fluorescent protein (GFP) or BMP2 genes under the control of the cytomegalovirus (CMV) promoter were generated as previously described.20 Viral titers of Ad vectors were determined by optical density at 260 nm and diluted to a concentration of $1 \times 10^{12}$ particle/mL in phosphatebuffered saline.21 Recombinant serotype-2 AAV and scAAV
preparations encoding GFP or BMP2 genes were produced by the triple-transfection method under the control of the CMV promoter as previously described. The viral titers of rAAV and scAAV vectors were determined by DNase-resistant particle (DRP) values using real-time PCR assay as described previously. Presence of transgene expression by Ad, rAAV, and scAAV vectors was verified.

**In Vitro Experimental Design and Vector Administration**

Articular cartilage and synovium were harvested from the tarsocruaral joint from healthy adult 6 horses (3 horses were used to obtain each cell type), and chondrocytes and synovial cells were isolated by collagenase tissue digestion and cultured in DMEM supplemented with L-glutamine (300 μg/mL), penicillin (30 μg/mL), streptomycin (30 μg/mL), and 10% fetal bovine serum at 37°C in a 5% CO2 atmosphere. Following the sufficient cell expansion at 3-5 passage, the equine chondrocytes and synovial cells were placed in 48-well plates at a density of 10,000 cells/well (day -1). Twenty-four hours after the final seeding (day 0), the DMEM was aspirated and replaced with the serum-free 200 μL Gey’s Balanced Salt Solution (GBSS) containing Ad-GFP or Ad-BMP2 at 1×10^2, 1×10^3, or 1×10^4 particles/cell (1×10^6, 1×10^7, or 1×10^8 particles/well, respectively), or rAAV-GFP, rAAV-BMP2, scAAV-GFP or scAAV-BMP2 at 1×10^4, 1×10^5, or 1×10^6 DRP/cell (1×10^8, 1×10^9, or 1×10^10 DRP/well, respectively). At each dosage of each vector, equine chondrocytes and synovial cells that were seeded in duplicate wells for each of the 5 horses were transfected. After 2 hours incubation at 37°C in a 5% CO2 atmosphere, the GBSS was aspirated and replaced with 1 mL DMEM. The chondrocytes
and synovial cells were cultured for 6 weeks (42 days), while the DMEM were changed at days 7, 14, 17, 21, 24, 28, 30, 32, 35, 37, 39, and 42.

*In Vitro Gene Transduction and Protein Production*

To evaluate the %transduction, the Ad-GFP, rAAV-GFP, and scAAV-GFP-treated cells were assessed by a fluorescent microscope by calculating the number of GFP-expressing cells divided by the total number of cells counted within the 5 fields of 25×25-μm area under 200X magnification that were expressed as a percentage.21 The %transduction was assessed at days, 2, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 42. The %transduction values from the duplicate culture wells were averaged.

To evaluate the BMP2 production, the 150 µL of DMEM were collected from the culture wells of Ad-BMP2, rAAV-BMP2, and scAAV-BMP2-treated cells, and the BMP2 concentration were quantified using enzyme-linked immunosorbent assays (ELISAs) for recombinant human (rh) BMP-2 (QuantiKine1, R&D Systems, Minneapolis, MN) that were expressed as picograms/milliliter/day.20 The BMP2 production was assessed at days, 2, 7, 14, 21, 28, 35, and 42. The BMP2 concentrations from the duplicate culture wells (measured by the duplicate ELISA wells) were averaged.

*In Vivo Experimental Design and Vector Administration*

The in vivo experimental design was summarized in Figure 6.1. By using 8 healthy adult horses, the metacarpo/tarso-pharangeal joints were bilaterally treated by the intra-articular administrations of GBSS (n=2 joints), Ad-GFP (5×10¹¹ particles/joint:
n=2), Ad-BMP2 (5×10^{11} particles/joint: n=2), rAAV-GFP (5×10^{11} DRP/joint: n=2), rAAV-BMP2 (5×10^{11} DRP/joint: n=2), scAAV-GFP at low (5×10^{11} DRP/joint: n=2) or high dosages (1×10^{13} DRP/joint: n=2), or scAAV-BMP2 at low (5×10^{11} DRP/joint: n=2) or high dosages (1×10^{13} DRP/joint: n=2). In 4 horses, one randomly assigned joint were injected twice, GBSS at day 0 and Ad-GFP or Ad-BMP2 at day 14, and the contralateral joints were injected once, rAAV-GFP or rAAV-BMP2 at day 0. In other 4 horses, both joints were injected once, scAAV-GFP or scAAV-BMP2 (randomly assigned) at low or high dosages. The treatments were assigned as a block design so that the left and right joints always received different genes (GFP or BMP2) and, for scAAV-GFP and scAAV-BMP2 vectors, the left and right joints always received same dosage (5×10^{11} or 1×10^{13} DRP/joint).

Intra-articular administrations of viral vectors were performed while horses were standing and sedated with xylazine hydrochloride (i.v., 1 mg/kg). After the aseptic preparation of skin over the lateral aspect of the metacarpo/tarsal-pheangeal joints, the limb was held off the ground at approximately 120 degree flexion, and a 20G 1.5 inch needle was inserted into the joint space between the proximal sesamoid bone and lateral distal condyle of the third metacarpal/tarsal bone by passing through the lateral collateral sesamoidean ligament. The intra-articular administrations of assigned treatments were performed only after the 2 mL joint fluid was collected to ensure the needle placement. All treatments (GBSS, Ad, rAAV, scAAV) were in 500 µL volume, except the high dosage (1×10^{13} DRP/joint) of scAAV-GFP/BMP2 which were in 1 mL volume.
In Vivo Protein Production and Inflammatory and Immune Response

Physical examinations were performed weekly after the joint injection by evaluating circumference of the injected joints and pain-free range of joint motion.

Figure 6.1 – In vitro experimental design and assignments in 8 horses used.
Circumference of the injected joint was recorded as the mean of 3 measurements obtained over the injection site by use of a cloth measuring tape.\textsuperscript{23} Range of pain-free motion was recorded as the mean of 3 goniometer measurements of the flexed joint immediately before elicitation of an aversion response such as lifting of the head or movement of the limb forward/backward.\textsuperscript{23}

Joint fluid samples were collected weekly by using the same technique described above, and analyzed for cell count and total protein concentrations. Also, the BMP2, interleukin 1 beta (IL-1β), and IL-1Ra protein concentrations in the joint fluid were quantified by ELISA (Quantikine\textsuperscript{1}, R&D Systems, Minneapolis, MN). Serum samples were also collected weekly, and neutralizing antibody (NAb) titer for Ad/AAV-vectors were measured for serum and joint fluid samples. Titers were determined by analyzing the ability of serum antibody to inhibit Ad-GFP (1×10\textsuperscript{4} particles/cell) or rAAV/scAAV-GFP infection (1×10\textsuperscript{6} DRP/cell) on Human carcinoma cells (HeLa cells: 5×10\textsuperscript{3} cells/well in 96-well plates) at 48 hours. By applying twofold dilution series of sample serum, the NAb titer was calculated as the highest serum dilution inhibiting Ad/rAAV/scAAV-GFP transduction by >50%.

\textit{Evaluation of Ectopic Mineralization and Cytotoxic Effect}

Radiographs of the bilateral metacarpo/taso-pharangeal joints were obtained weekly to monitor if any ectopic mineralization of intra- and peri-articular tissue was developed. Eight weeks following the injections (day 56), horses were sedated with
xylazine hydrochloride (i.v., 1 mg/kg) and euthanized with by lethal intravenous overdose of pentobarbital (Beuthanasia, i.v., 2.2 mg/kg). Immediately after the euthanasia, both metacarpo/taso-pharangeal joints were dissected at the levels of proximal phalanx and third metacarpal/tarsal bone, and quantitative computed tomography (QCT) (Picker PQS Helical CT Scanner, Philips Medical Systems, N.A., Bothell, WA) was performed in transverse sections at 1-mm intervals. The entire metacarpal/tarsal-pharangeal joint along with 1 cm margins proximal and distal extra-articular regions were imaged. The area of ectopic soft tissue mineralization/ossification was scanned by the visual observation of each slices and the identification of any areas with threshold level of 600-3000 hounsfield units using an image analysis software (Mimics software tutorial manual, Materialise, Ann Arbor, MI).

Histological evaluations were performed for the articular cartilage and synovium tissue from all the injected joints. The synovium tissues in the distal palmar/plantar pouch of the joint capsules were harvested from each joint, fixed overnight with 10% neutral-buffered formalin, paraffin-embedded, sectioned at 5 µm (minimum four sections per specimen), and stained with H&E. The synovium sections were viewed by microscopy to determine the formation of ectopic mineralizing nodules or disturbance of normal synovial villi structures. Similarly, the articular cartilage tissues on the lateral condyle of the third metacarpal/tarsal bone were harvested from each joint, fixed overnight with 10% neutral-buffered formalin, decalcified for 24 hours in formic/hydrochloric acid, paraffin-embedded, sectioned at 5 µm (minimum four sections per specimen), and stained
with Toluidine Blue. The cartilage sections were viewed by microscopy to determine the formation of ectopic mineralizing nodules, or disturbance of normal extracellular matrix.

**Evaluation of In Vivo Gene Expression and Vector Biodistribution**

The GFP or BMP2 gene expression analysis was performed using RNA from the cartilage samples in 3 locations such as (i) lateral condyle of distal third metacarpal/tarsal bone, (ii) proximal lateral articular eminence of proximal phalanx, and (iii) dorsal articular surface of lateral sesamoid bone, and the synovium samples in 3 locations such as (i) dorsal pouch, (ii) proximal palmar/plantar pouch, and (iii) distal palmar/plantar pouch of each joint. The RNA was also collected from the cartilage and synovium samples in the uninjected metacarpal/tarsal-phalangeal joints. Total RNA was extracted by TRIzol (Invitrogen Life Technologies, Carlsbad, CA) from the homogenized bone tissues using established protocol. Transgene expression of \( hbmp2 \) and \( gfp \) were quantified by SYBR real-time RT-PCR with the ABI PRISM 7000TM Sequence Detection System (Applied Biosystems, Foster City, CA). Mean fold change was calculated using custom designed primers for the \( hbmp2 \) (Forward: \( 5’-\text{AAAACGTCAGCCAAAACACAAA-3’} \), Reverse: \( 5’-\text{GTCACTGAAGTCCACGCAAAGG-3’} \)) and \( gfp \) gene (Forward: \( 5’-\text{CATGATATAGACGTTTGCTGTGGTTG-3’} \), Reverse: \( 5’-\text{AAGCTGACCCTGAAGTTCATCTGC-3’} \)) relative to expression of the uninjected joint tissue and relative to expression of the endogenous equine \( \beta \)-actin gene (Forward: \( 5’-\text{-} \)
GGGCATCCTGACCCTCAAG-3’, Reverse: 5’-TCCATGTCGTCACCCAGTTGGT-3’) using the 2^{-\Delta\Delta C_t} method.24

Genomic DNA was extracted from the cartilage and synovium samples in the same location as RNA described above, as well as abdominal organs such as liver, kidney, spleen, lung, and ovary/testicle, using QIAamp Mini Kit (Qiagen1, Valencia, CA). Quantitative polymerase chain reaction (qPCR) was performed using primers that amplify CMV sequences, which was detected by the internal fluorogenic probe labeled with FAM reporter dye, and ABI Prism 7000 sequence detection instrument (PE Applied Biosystems, Foster City, CA).16 Each of triplicate reactions contained the 100 ng of test DNA. A standard curve was generated using plasmid containing 5×10^1 through 5×10^7 copies of CMV. DNA was also isolated from HeLa cells (3×10^6 cells in a 75-cm^2 flask) with and without Ad-GFP transduction (1×10^3 particles/cell) and included in each qPCR plate as positive and negative controls. The copy numbers of CMV were averaged among the 3 locations of articular cartilage and snovium per joint.

**Statistical Analysis**

Repeated-measure analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC) was used to evaluate the effects of Ad/rAAV/scAAV-mediated GFP/BMP2 gene deliveries with the post-test multiple comparisons between the treatment groups at each time point using Proc Mixed statistical models for continuous outcomes and Genmod statistical models for categorical outcomes. Repeated variables were considered to be nested within horse, and the distribution of data was assessed by use of a subset of
normality tests (e.g., the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests). Significance level was set at p<0.05 for all analyses.

**Figure 6.2** – In vitro transduction efficiency and transgene protein production in equine chondrocytes and synovial cells tranfected by adenoviral (Ad), recombinant adeno-associated viral (rAAV), or self-complementary AAV (scAAV) vectors encoding green fluorescent protein (GFP) or bone morphogenetic protein-2 (BMP2) genes at three different dosages. The scAAV-GFP/BMP2 vectors showed greater and more sustained GFP %transduction (a, b) and BMP2 protein production in culture medium (c) compared to the Ad and rAAV vectors.
6.4 Results

In Vitro Gene Transduction and Protein Production

Self-complementary AAV vectors showed greater and more rapid GFP gene transduction (Fig 6.2a and 6.2b) and BMP2 protein production (Fig 6.2c) compared to rAAV vectors in equine chondrocytes and synovial cells. Also, scAAV vectors showed more sustained GFP gene transduction (Fig 6.2a and 6.2b) and BMP2 protein production (Fig 6.2c) compared to Ad vectors in equine chondrocytes and synovial cells, where the onset of GFP gene transduction (Fig 6.1a and 6.2b) and BMP2 protein production (Fig 6.2c) were equally rapid to Ad vectors.

In Vivo Protein Production and Inflammatory and Immune Response

The detectable concentration of BMP2 protein was induced by an intra-articular injection of Ad-BMP2 at day 2 and 7 (Fig 6.3a). The Ad vectors induced greater articular inflammation compared to rAAV and scAAV vectors with significantly greater joint fluid IL-1β concentration (P<0.001: Fig 6.3b), joint fluid cell counts (P<0.03: Fig 6.3d), joint circumference (P<0.04: Fig 6.3e), and range of joint motion (P<0.02: Fig 6.3f), although, all parameters were recovered in normal values within 4-5 weeks. For the rAAV/scAAV-injected joints, all inflammatory parameters were not significantly different than GBSS-injected joints, except the significantly greater joint fluid protein concentration compared to the GBSS-injected joint at day 2 (Fig 6.3c). None of inflammatory parameters were significantly different between rAAV, scAAV at low dose, and scAAV at high dose groups.
Figure 6.3 – In vivo inflammatory response of equine metacarpal/tarsal-pharangeal joints treated by the intra-articular administration of adenoviral (Ad), recombinent adeno-associated viral (rAAV), or self-complementary AAV (scAAV) vectors encoding green fluorescent protein (GFP) or bone morphogenetic protein-2 (BMP2) genes. *Significant greater than all other groups (P<0.04). There were no significant differences in joint inflammation between transgene products (i.e., GFP v.s. BMP2) in each type of vectors. The BMP2 protein was detected only in the Ad-BMP2-injected joints. The Ad-injected joints showed significantly greater inflammatory responses compared to the rAAV/scAAV-injected joints, although all parameters were returned within normal range in 4-5 weeks after the joint injection.
The NAb titers against Ad/AAV were significantly greater in the Ad/AAV-injected joint fluid compared to the serum or uninjected contralateral joint fluid ($P<0.03$: Fig 6.4a and 6.4b), in which the titers were remained high (>100) by the end of study period. The
NAb titers against Ad/AAV had corresponding movement between the serum and uninjected contralateral joint fluid (Fig 6.4a and 6.4b) that were peaked at 2 weeks after intra-articular administration and decreased afterward.

**Ectopic Mineralization and Cytotoxic Effect**

Radiography and QCT analysis did not show any signs of ectopic mineralization in the intra- and peri-articular tissue of all joints. Also, histological evaluation of the articular cartilage and synovium did not show any signs of mineralizing nodule formation, extracellular matrix disturbance, or increases of the empty/pyknotic lacunae (Fig 6.5).

**In Vivo Gene Expression and Vector Biodistribution**

The *gfp* and *bmp2* gene expression were not detected in any articular cartilage and synovium tissues from any joints. The CMV promoters were detected in the cartilage tissues from the Ad-GFP and Ad-BMP2-injected joints (253 ± 83 [Mean ± SE] and 506 ± 110 copies per 100ng DNA, respectively), and the synovium tissues from the Ad-GFP (1783 ± 284 copies) and Ad-BMP2-injected joints (1,783 ± 284 and 1,434 ± 330 copies per 100ng DNA, respectively). The CMV promoters were not detected in any cartilage or synovium tissues from any rAAV/scAAV-injected joints and any abdominal organs (eg, liver, kidney, spleen, lung, ovary, and testicle).
6.5 Discussion

An intra-articular administration was shown to be effective in Ad vectors by inducing robust BMP2 production in the joint fluid, although detectable BMP2 concentration did not sustain beyond the 7 days after the gene transduction. This is in agreement with previous reports where the intra-articular Ad vectors induced transient production of growth factors. The inflammatory responses shown in the Ad-injected joints were expected results, but appeared to be only transient effect. The signs of swelling, pain, and increased joint fluid protein and cell counts were significantly greater in Ad-GFP/BMP2 compared to rAAV/scAAV vectors; however, all parameters were
returned to normal values within 4-5 weeks (Fig 6.3b to 6.3f). In addition, structural and cellular damage of articular cartilage and synovium were not evident on the histology of the Ad-GFP/BMP2-injected joints (Fig 6.5). Therefore, this result may suggest that the adverse effects associated with an intra-articular Ad vectors is temporary and might be clinically acceptable. However, because of the short-term induction of the transgene products, repeated administrations of Ad vector may be necessary to effectively treat the joint disorders which are predominantly chronic conditions.

Self-complementary AAV vectors showed the promising efficacy in vitro. Equine chondrocytes and synovial cells showed greater, more rapid, and sustained gene transduction by scAAV vectors compared to rAAV (Fig 1a to 1c). As the result, even 100 times lower dosage of scAAV-GFP vectors (1×10⁴ DRP/cell) was able to induce the approximately equal %transduction to rAAV-GFP vectors (1×10⁶ DRP/cell) in equine chondrocytes (Fig 6.1b). Bypassing rate-limiting second-strand DNA synthesis is expected to be largely beneficial to transfect the certain types of cells with low DNA replication; therefore, use of scAAV vector may be greatly advantageous for an intra-articular administration due to the low cell division rate of chondrocytes and synoviocytes.¹⁵ Our study also showed that, for the first time, there were no significant differences in the inflammatory and immune responses between the intra-articular rAAV and scAAV vectors (Fig 6.3b to 6.3f and Fig 6.4b). This is an expected result because, even though scAAV vectors contain double strand DNA and therefore carry twice larger amount of viral genomic materials, the rAAV and scAAV vectors used in this study were same serotype and the innate and adaptive immune responses are primarily initiated by
recognition of the capsid proteins. In concert, the dosage of scAAV vectors can be decreased to induce an equivalent gene transduction to rAAV which certainly will reduce the inflammatory and immune responses. This is an important finding since previous studies showed that, while the pre-existing immunity by host exposure of the wild-type AAV (80% of human population) is a significant limiting factor for rAAV gene transfer, the humoral immunity to the AAV capsid could be prevented by lowering the AAV particles administered.

To authors’ knowledge, this is the first study to report that the intra-articularly administered Ad and AAV vectors induced the antibody production in serum and joint fluid. The NAb production in the joint fluid may inhibit the second injection of the gene delivery vectors in the same joint. The previous study reported that, after intra-articular IL-1Ra gene transduction by scAAV vectors, the re-injection of the vectors could not generate detectable levels of IL-1Ra expression. In this regard, the modifications of gene delivery vectors might be necessary to prevent the immune responses against the vectors and successfully perform the repeated gene therapy application, including the capsid epitope alterations to decrease an immunogenicity of the vector, the cross-packaging genomes of different AAV serotype into the viral capsid to overcome pre-existing immunity, or the application of transient immunosuppression at the time of the second vector administration. Interestingly, the NAb titers in the Ad/AAV-injected joints were greater than serum titer and remained high (>100 NAb titer) until end of the study period, even after the serum Ad/AAV titers were faded away (Fig 6.4a and 6.4b). This may indicate the persistent viral infection of the Ad/AAV-injected joints. The detectable level
of CMV promoters in the articular cartilage and synovium tissue at 6 weeks after the injection may partly support this theory, at least for the Ad-injected joints. In addition, antigen-presenting cells that were activated by the viral vectors might be escaped from the Ad/AAV-injected joints and led to the systemic NAb production only in the initial inflammatory phase when the permeability of joint capsule and synovium was increased. In general, the dendritic cells exposed to antigens become the mature helper-2 T-lymphocytes (Th2), one of the key antigen-presenting cells in adaptive immune response, and undergo apoptosis within few weeks. Correspondingly, the serum Ad/AAV NAb titer was peaked at 2 weeks after the joint injection in our study. Since normal synovium poorly infiltrates immune cells, the Th2 cell may be sequestrated within the Ad/AAV-injected joints after the initial joint inflammation was resolved so that the sustained antibody production was secluded in the joint fluid of the Ad/AAV-injected joints.

Another important finding in our study is that the NAb titer was also increased in the synovial fluids from the contralateral joints where Ad/AAV-vectors were not administered (Fig 6.4a and 6.4b). The increment and peak of the NAb titer in the contralateral joints were corresponding to the serum NAb titer suggesting an infiltration of Ad antibodies from systemic circulation into the contralateral joint cavities. This may suggest that treating one joint with viral vectors may limit an efficacy of repeated application of intra-articular gene therapy in both the previously treated joints and the distact joints, at least for 6-8 weeks after the initial joint injection. Similar phenomenon has been noticed where an intra-ocular administration of AAV-vectors resulted in the
systemic antibody production and blocked transgene expression upon readministration in the contralateral eye.33

Unfortunately, the intra-articular administration of rAAV/scAAV-BMP2 vectors in this study was unable to produce the detectable levels of BMP2 protein in the joint fluid in vivo, at least in this equine metacarpo/tarso-pharangeal joint model and at the dosages used. Successful gene transductions of the articular cartilage and synovium were reported by the intra-articular administrations of $4.7 \times 10^{11}$ particles of rAAV in the mice knee joints, $5 \times 10^{11}$ particles of scAAV in the rabbit knee joints,3 and $1.5 \times 10^{12}$ particles of rAAV in the rabbit knee joints.11 The capacity of these rodent joints was estimated to be equal to human metacarpal-pharangeal joints that is a frequent site of rheumatoid arthritis.3 The dosage of rAAV/scAAV vectors used in our study ($1 \times 10^{13}$ DRP/joint) would be considered as high as technically and economically applicable in the size of equine metacarpo/tarso-pharangeal joints with the average normal synovial fluid volume of 4.4 mL.34 Therefore, our results may alert that, despite the promising results seen in the rodent models, the delivery methods of AAV vectors may need to be optimized for efficient and reliable gene transduction for sizeable joints such as human knee joints, where osteoarthritis and rheumatoid arthritis commonly affect and the average synovial fluid volumes are approximately 4.5 and 7.0 mL for healthy and arthritic joints, respectively.35 In our results, the synovial fluid in the rAAV/scAAV-injected joints had an increased total protein and cell count suggesting the intra-articular administrations of vectors were satisfactory. This may imply that the inadequate BMP2 production was due to the inhibitory effect by synovial fluid, rather than low transduction efficiency or
abortive post-translational modification of the transgene products. Reportedly, synovial fluid has shown to inhibit the in vitro AAV-mediated gene transfer in chondrocytes\textsuperscript{35} and intra-articularly administered rAAV vectors,\textsuperscript{36} therefore, the joint lavage prior to the vector injection may facilitate a successful gene transduction by intra-articular AAV vectors.

The serotype-5 AAV vectors have shown to have greater gene transduction capacity to synovium tissue than the serotype-2 AAV vectors.\textsuperscript{37,38} The enhanced gene transfer by rAAV5 over rAAV2 vectors were likely associated with presence of co-receptor improving for cell surface attachment and internalization\textsuperscript{39} or more efficient trafficking to nucleus.\textsuperscript{38} In contrast, the CMV promoters were undetectable in the synovium tissue of all rAAV/scAAV-injected joints in our study suggesting that the rAAV/scAAV vectors were inhibited by joint fluid prior to the vector-to-cell contact. Reportedly, serotypes 1 and 2 AAV was the most inhibited by joint fluid, whereas inhibition was weak for serotypes 5 and 8.\textsuperscript{36} We selected serotype-2 AAV vectors to investigate whether (1) the inflammatory and immune responses differ between rAAV and scAAV vectors, and (2) the enhanced gene delivery by self-complementation shown in vitro can overcome an insufficient in vivo transduction efficiency by intra-articularly administered AAV vectors in order to induce a clinically relevant amount of protein production. The insufficient transgene expression by scAAV, even at the high dosage, further advocates that the use of other serotype may be advantageous for intra-articular administration of AAV vectors. Moreover, the previous study showed that an inflammed synovium could be more permissive for gene transfer,\textsuperscript{10,38} but the increased joint fluid
volume in inflammed joints may dilute the viral vectors to reduce the transduction efficiency; therefore, the relative efficay of intra-articular rAAV and scAAV vectors may be further studied using arthritic joints in large animal models.

Our study demonstrated the safety of BMP2 gene therapy for articular fracture, because the direct intra-articular administrations of Ad-BMP2, rAAV-BMP2, or scAAV-BMP2 did not cause of mineralization or ossification of articular cartilage and synovium tissues. The dosage of intra-articular Ad-BMP2, $5 \times 10^{11}$ particles per joint, was sufficient to induce robust bone formation in equine models, but did not show any signs of ectopic bone formation on computed tomography and histology in this study. The amount of misplaced vectors into the synovial space would even be smaller, especially when a delayed injection was made in a mature and firm granulation tissue. This would advocate a safety of gene therapy application to accelerate the healing of fractures adjacent to the synovial structures such as joint, tendon sheath, or synovial bursa.

In summary, direct intra-articular administration appeared to be an efficient vector delivery method for Ad vectors due to the robust BMP2 production in joint fluid and the transitory inflammatory responses within the Ad-injected joints. Self-complementary AAV vectors have a great potential for arthritis gene therapy by inducing greater and more sustained in vitro gene transduction in equine chondrocytes and synovial cells compared to Ad or rAAV vectors. Because of an inadequate in vivo gene transduction by intra-articular administration, delivery methods of AAV vectors will need to be optimized. The effects of repetitive gene therapy application may be diminished by the
increased neutralizing antibody titers in both the Ad/AAV-injected joints and the distant joints.
Appendix A: Microarray data
Genes differentially regulated (>2-fold change) in second metatarsal bones (Mt2)

**Intact**: an intact Mt2 with no ostectomy

**Untreated**: Mt2 osteotomy with no treatment at 8-weeks after surgery

**Ad-BMP-2**: Mt2 osteotomy with Ad-BMP-2 treatment at 6-weeks after gene transduction (8-weeks after surgery)

**Ad-BMP-6**: Mt2 osteotomy with Ad-BMP-6 treatment at 6-weeks after gene transduction (8-weeks after surgery)

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<th>Ad-BMP6 vs Untreated</th>
<th>Ad-BMP2 vs Ad-BMP6</th>
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Chapter 1: Cell-Mediated and Direct Gene Therapy for Bone Regeneration


Chapter 2: Relative permissiveness and cytotoxicity of equine chondrocytes, synovial cells, and bone marrow derived mesenchymal stem cells to adenoviral gene delivery


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Chapter 4: Dermal fibroblast-mediated BMP2 therapy to accelerate bone healing in an equine osteotomy model


Chapter 5: Comparative efficacy of dermal fibroblast-mediated and direct adenoviral bone morphogenetic protein-2 gene therapy for bone regeneration in an equine rib model

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