GENE-INDUCED CHONDROGENESIS OF MESENCHYMAL STEM CELLS THROUGH VIRAL GENE DELIVERY

By

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To my parents, Diana Bush and Jim Bush

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AAV	adeno-associated virus
ACT	autologous chondrocyte transplantation
BM	bone marrow
BMP	bone morphogenetic protein
bp	base pairs
^{o}C	degrees Centigrade
$CaPO_4$	calcium phosphate
CAR	Coxsackie-adenovirus receptor
CDMP	cartilage derived morphogenic protein
cDNA	complementary DNA
CMV	cytomegalovirus
CsCl	cesium chloride
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GDF	growth/differentiation factor
GFP	green fluorescent protein
hr	hour
IGF	insulin-like growth factor
Ihh	Indian hedgehog

kg	kilogram
MSC	mesenchymal stem cell
µg	microgram
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MMPs	matrix metalloproteases
ng	nanogram
nm	nanometer
OA	osteoarthritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PG	proteoglycan
RA	rheumatoid arthritis
rAAV	recombinant adeno-associated virus
RNA	ribonucleic acid
sc	self-complementary
scAAV	self-complementary adeno-associated virus
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ssAAV	single-stranded adeno-associated virus
TGF-β	transforming growth factor beta
trs	terminal resolution site
U	units

UV	ultraviolet
vg	vector genome
vp	virus particle
VSV-G	vesicular stomatitis virus G protein

Abstract of Dissertation Presented to the Graduate School
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GENE-INDUCED CHONDROGENESIS OF MESENCHYMAL STEM CELLS THROUGH VIRAL GENE DELIVERY

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Articular cartilage is a highly specialized tissue that allows for near frictionless motion of diarthrodial joints. When cartilage is damaged as a result of injury or disease, the body's innate capacity for repair is often insufficient due to the low cellular density, avascular and aneural nature of cartilage. Typically the repair tissue is a fibrotic scar which lacks the structural properties of native cartilage. Adult mesenchymal stem cells (MSCs) are well-suited for cell-based therapies for cartilage repair since they are readily available from many tissue sources and capable of differentiating along multiple mesenchymal lineages. Gene transfer to MSCs is a viable method for achieving sustained local expression of specific protein factors, which has been shown to induce chondrogenesis *in vitro* and may enhance chondrogenic differentiation *in vivo*.

Previous experiments have shown that delivery of the cDNAs for TGF-β and BMP-2 induces chondrogenesis of bovine MSCs in high density aggregate culture. In these studies, we expanded the analyses to include additional cDNAs whose protein products are associated with chondrogenic differentiation during development, including bone morphogenetic proteins BMP-4, BMP-7, developmental morphogen Indian hedgehog

(Ihh), transcription factor Sox9, and connective tissue growth factor (CTGF). We transduced early passage bovine MSCs with adenoviral vectors carrying the complete cDNAs for the candidate transgenes at doses ranging from 10 virus particles/cell to 100,000 vp/cell. Chondrogenesis was evaluated by gross examination of aggregate morphology, toluidine blue staining for proteoglycan expression and collagen types I, II, and X immunohistochemistry. The greatest biological responses for each transgene were observed in the dose range of 100-1000 vp/cell; higher viral doses appeared to inhibit chondrogenesis, while lower viral doses were insufficient to yield a pronounced effect. We found that gene transfer of the cDNAs for BMP-4, BMP-7, Ihh, and Sox9 induced chondrogenesis of bovine MSCs while CTGF was not chondrogenic. BMP-4 supports chondrogenesis as effectively as other members of the TGF-β superfamily such as BMP-2.

In further experiments to elucidate the most suitable gene delivery system for animal models and clinical applications, we evaluated vector systems currently available: adeno-associated virus (AAV), lentivirus, and non-viral transfection relative to adenovirus. Simple transfection alone was unable to generate the levels of expression necessary to promote chondrogenesis in our system. Both adenovirus and AAV transduction of MSCs resulted in robust BMP-4 expression over the course of 21 days, complete with chondrogenic differentiation and the expression of cartilage matrix proteins. Lentivirus, which offers the potential for long-term gene expression, was ill-suited for our application because it required long-term culture selection of cells, which altered cellular morphology and negatively impacted cell survival. These data indicate that scAAV serotype 2 delivery of BMP-4 promotes chondrogenesis of MSCs.

CHAPTER 1
INTRODUCTION

Cartilage Biology

Cartilage is a specialized connective tissue found in various locations throughout the human body and is subdivided into three main types: 1) hyaline (articular), 2) elastic, and 3) fibrocartilage[1]. Hyaline cartilage, named for its glassy appearance, covers articulating surfaces, such as those at the ends of long bones; therefore, it is also known as articular cartilage. Its unique architecture offers firm support with some pliability and consists of 80-90% water, with the remainder of the tissue composed primarily of collagen type II fibers and proteoglycans. Cells known as chondrocytes reside in the cartilage at low density and work to slowly remodel the matrix to keep it structurally and functionally sound[2]. In childhood, hyaline cartilage is a key component of the growth plates, the actively growing regions that add length to the ends of long bones[1] (Fig.1-1A).

Architecture of the Knee

Diarthrodial joints, such as the knee, have a specialized architecture that is capable of withstanding repeated extreme forces over the lifetime of an individual, often without problems for 80 or more years. Mesenchymal tissues in the knee joint, including cartilage, tendons, ligaments, synovium, and meniscus, have specific features that enable smooth locomotion and resistance to compressive and tensile forces (Fig. 1-2). Ligaments present within and without the joint capsule guide movement of the femur and tibia to control extension and flexion, secure the articulating bones when standing, and prevent hyperextension or overflexion of the knee joint. The knee can absorb vertical forces equal to nearly seven times body weight; however, the knee joint is

susceptible to damage from horizontal forces or twisting movements such as those that occur in football and other contact sports.

Articular Cartilage Structure

Articular cartilage covers and protects the joints at sites of articulation, such as the knee, ankle, elbow, and knuckle. To withstand the stress of movement of the human body, articular cartilage must be durable yet provide an effective cushion against load-bearing and impact, and it must be smooth to provide nearly frictionless motion. The nature and structure of articular cartilage impart these necessary properties.

The extracellular matrix (ECM) has unique properties that enable articular cartilage to rebound after impact. Moreover, it is remarkably durable and is capable of withstanding repeated exposure to extreme forces and pressure, which effectively cushions the ends of the bones. Extracellular matrix is synthesized and maintained by the resident population of chondrocytes through anabolic and catabolic mechanisms and is composed mainly of collagen fibrils and proteoglycans[3] (Fig. 1-3).

As depicted in Figure 1-1B, articular cartilage is highly organized and consists of four zones, or layers: 1) superficial, 2) transitional, 3) radial, and 4) calcified cartilage[3,4]. The superficial zone contains chondrocytes that are flattened and lie parallel to the surface. Collagen type II fibrils also have a parallel orientation in this layer, which allows the cartilage to withstand the shear forces generated during normal joint loading. The transitional zone, as its name suggests, exhibits a cell morphology and ECM composition intermediate to that of the superficial zone and the deeper radial zone. Transitional layer chondrocytes are rounded and produce more proteoglycan and less collagen than in the superficial layer. Deep to this zone lies the radial zone, where the cells are aligned in columns perpendicular to the joint surface[4]. The radial zone is the

largest layer, with the thickest collagen fibrils, the most concentrated proteoglycans, and the lowest water content. The radial zone is separated from the deepest zone, calcified cartilage, by the tidemark, an area that appears to help tether the cartilage by increasing the contact area between the layers[5]. The zone of calcified cartilage forms the transition between soft articular cartilage and the hard underlying bone[4,5]. Cells in this zone are isolated and almost completely surrounded by calcified cartilage, suggesting that they have a low metabolic rate, but it is known they are not completely inactive[5].

Throughout the body, articular cartilage is thicker, with greater proteoglycan content, in load-bearing areas and thinner in areas where loading is minimal[4]. Mechanical loading affects the morphology and the metabolic activity of chondrocytes[4], and controlled loading is thought to contribute to enhanced cartilage healing after injury.

Proteoglycans

The extracellular matrix consists of a network of proteoglcyans (PGs) and collagen fibers arranged to control the flow of water molecules to cushion the joint from forces associated with normal motion. Proteoglycans give the tissue its ability to resist compression and remain durable for up to 80 years, or more, in some humans.

Proteoglycans are proteins bound to long-chain polysaccharides known as glycosaminoglycans, or GAGs. Large aggrecating proteoglycans known as aggrecans are the most abundant proteoglycans in cartilage, consisting of a linear protein core with numerous GAG chains of chondroitin sulfate or keratan sulfate attached[6] (Fig. 1-4). In addition to large aggregating proteoglycans like aggrecan, small non-aggregating proteoglycans, including decorin, biglycan, and fibromodulin, bind to other matrix molecules and help to stabilize the matrix[7].

Hyaluronic acid (HA), or hyaluronate, molecules are the central organizing units of the cartilage matrix. They are long chains with up to 100 glycoproteins attached to and extending from them. Link protein connects each proteoglycan subunit to the hyaluronate backbone. The large concentration of negative charges from the GAG side chains attract and hold polar water molecules in the matrix by osmotic pressure[8]. This charge repulsion keeps the chains separated in a characteristic bottle-brush formation (Fig. 1-4). When hydrated, proteoglycans account for most of the physiological mass of cartilage. They provide a structure to hold and control the flow of water, the integral factor in cartilage's ability to provide its protective cushioning properties throughout lifetime of the individual.

The architecture of articular cartilage regulates the flow of fluid in the cartilage and cushions the joint from mechanical stresses such as compressive loads[9]. When cartilage is compressed during normal joint motion, water flows out to the joint cavity, mixing with the existing synovial fluid that lubricates the joint. The negative charges of the GAGs repel one another when forced into close proximity, enabling them to resist further compression. Additionally, collagen type II fibrils provide the strength needed to retain the shape of cartilage under compression and hinder the expansion of proteoglycans[7,10]. When the pressure is released, the cartilage springs back to its original form and water molecules are drawn back into the proteoglycans. This flow of liquid into and out of cartilage also carries nutrients to and wastes from chondrocytes, providing nourishment, which helps explain why periods of inactivity can result in weaker cartilage that injures easily[1].

If proteoglycans are damaged by trauma, infection or enzymes, such as those released during inflammatory disease, their structure degenerates and their water-holding capacity changes[8]. The collagen meshwork also begins to break down, which reduces constraint on the proteoglycans and allows them to imbibe additional water, thus reducing cartilage stiffness. Over time damaged cartilage loses its resilience to mechanical forces in the joint, and the subchondral bone can become exposed, causing severe pain and disability due to the abundant nerve supply and bleeding from the bone marrow and surrounding vasculature.

Due to the density of the extracellular matrix secreted and maintained by chondrocytes and the physical requirements of the tissue, there is no room for blood vessels or nerve fibers to permeate cartilage. Indeed, the permeation of the matrix with vascular or neural tissues would significantly compromise its protective properties. Instead, nutrients diffuse through the perichondrium, a well-vascularized dense irregular connective tissue that surrounds the surface of most cartilage structures[1].

The matrix, while continually undergoing remodeling by chondrocytes, also serves to protect chondrocytes from injury resulting from normal use of the joint. Because cartilage lacks innervation, injuries that occur within the cartilage layer often go unnoticed until there is penetration of the subchondral bone. Additionally, the structure of the matrix determines the types of molecules and which concentrations reach the cells encased within.

Chondrocytes

Chondrocytes are mature cartilage cells found in deep pits called lacunae[1] where they function to maintain cartilage homeostasis through the perpetual degradation and synthesis of matrix components[11]. Chondrocytes diffusely populate articular cartilage,

exhibit a low metabolic rate compared to tissues such as muscle, and are restricted from moving and dividing by the dense matrix fibers. The cells do not interact with one another, but instead interact with the ECM (Fig. 1-1B). In the deepest layers of cartilage, chondrocytes exist in a hypoxic environment, so metabolism is mainly anaerobic, with the conversion of glucose to lactic acid[4]. In normal articular cartilage, there is a low turnover of the extracellular matrix, and chondrocytes are thought to rarely divide[4,12]. When articular cartilage sustains damage, however, the chondrocytes form clusters and cellular activity increases[4]. Aging chondrocytes, on the other hand, gradually lose their ability to divide, which supports the observation that the cartilage in older individuals heals more slowly than that of young people[13,14]. Sports injuries in adolescence or young adulthood may set the stage for pain later in life as those early events damage cartilage, causing it to unnaturally erode. Damaged cartilage may calcify, escalating the extent of damage as this transition to bone results a loss of nutrient supply and subsequent chondrocyte death.

The cartilage microenvironment may have a profound influence upon chondrocyte lifespan. The chondrocyte has a finite lifespan in the epiphyseal growth plate, whereas it has a very long, stable phenotype evident in articular cartilage[15]. Chondrocytes of articular cartilage are long-lived cells of mesenchymal origin, but their lifecycle is unconfirmed. It is possible that they are post-mitotic and replaced by new cells infiltrating from the subchondral bone or that a small population of progenitor cells exists within the cartilage.

Chondrocyte dedifferentiation in culture. The stability of the phenotype of articular chondrocytes is critically dependent on physical environment and cell density.

In monolayer culture, these cells progressively lose their chondrogenic phenotype, transitioning from cubiodal cells with high ECM synthesis to spindle-shaped cells that primarily produce collagen type I. Dedifferentiation can be avoided or delayed by changing the geometry of the cell culture through the use of 3D matrices such as agarose or alginate, high density micromass cultures, or pellet cultures[15].

Collagens

The dry mass of articular cartilage is composed primarily of type II collagen and aggrecan, a proteoglycan. Collagen type II makes up 90% of the collagen present, with minor collagens types VI, IX, and XI making up the other 10% (Table 1-1). Collagen fibrils provide tensile strength, serving as a natural scaffold to anchor and organize cells and hydrated proteoglycans and possibly guiding cell signaling responses during development and repair[16].

Collagens differ in their ability to form fibers and to organize the fibers into networks (Table 1-1). The typical structure of fibrillar collagens is a right-handed triple helix, as shown in Figure 1-5, which arises from an abundance of three principal amino acids: glycine, proline, and hydroxyproline[2]. These form a repeating pattern of Gly-Pro-X where X is any amino acid. The side chain of glycine, a single H atom, is the only side chain capable of fitting into the crowded center of the triple strand helix.

Type I collagen fibrils have enormous tensile strength; that is, this type of collagen can be stretched without being broken. These fibrils, roughly 50 nm in diameter and several micrometers long, are packed side-by-side into parallel bundles, termed collagen fibers. In tendons, collagen type I fibers connect muscles with bones and must withstand enormous forces. Gram for gram, type I collagen is stronger than steel[2].

Collagen type II, the primary collagen of articular cartilage, has fibrils that are smaller in diameter than type I and are oriented randomly in the viscous proteoglycan matrix (Fig. 1-3). Such rigid macromolecules impart a strength and compressibility to the matrix and allow it to resist large deformations in shape. This property allows joints to absorb shock. Type II fibrils are cross-linked to proteoglycans in the matrix by collagen type IX, a collagen consisting of two long triple helices connected by a flexible kink (Fig. 1-5B). The globular N-terminal domain extends from the composite fibrils, as does a heparan sulfate molecule, a type of large, highly charged polysaccharide that is linked to the type IX collagen chain at the flexible kink. These protruding nonhelical domains are thought to anchor the fibril to proteoglycans and other components of the matrix. The interrupted triple-helical structure of type IX collagen prevents it from assembling into fibrils; instead, collagen type IX associates with fibrils formed from other collagen types and thus is called fibril-associated collagen (Table 1-1).

Post-translational modification of procollagen is crucial for the formation of mature collagen molecules and their assembly into fibrils; defects in this process have serious consequences[2]. For example, the activity of prolyl hydroxylases requires an essential cofactor, ascorbic acid (vitamin C). In cells deprived of ascorbate, as in the disease scurvy, the procollagen chains are not hydroxylated sufficiently to form stable triple helices at normal body temperature, nor can they form normal fibrils. Consequently, nonhydroxylated procollagen chains are degraded within the cell.

Type II collagen fibers are firmly embedded in subchondral bone and rise from there to the cartilage surface, where they bend to form arches[8]. The overlapping fibers

form a mesh framework that gives stability to cartilage (Fig. 1-3). Collagen fibers provide the framework that allows proteoglycans to hold water molecules within the matrix.

Adjacent cells and tissues support articular cartilage

The adjacent cells and tissues in the joint—synoviocytes, bone marrow, MSCs, meniscus, ligaments and tendons—provide support to articular cartilage. Most proximal to the bones and cartilage of the joint is the synovium, a thin layer of vascularized connective tissue that lacks a basement membrane. Two cell types are present: type A, macrophage-like cells, and type B, fibroblast-like cells. Macrophage-like or phagocytic cells remove microbes and the debris that results from normal wear and tear in the joint. Type B synoviocytes produce synovial fluid, which consists of hyaluronan, or hyaluronic acid, lubricin, proteinases, and collagenases, and serves to surround, lubricate, and nourish the joint space. This fluid forms a thin layer (roughly 50 μm) at the surface of cartilage and also seeps into microcavities and irregularities in the articular cartilage surface, filling all empty space. Normal synovial fluid increases the viscosity and elasticity of articular cartilage, and there is also some evidence that it helps regulate synovial cell growth.

The liquid present in articular cartilage effectively serves as a synovial fluid reserve. During movement, the synovial fluid held in the cartilage is squeezed out mechanically to maintain a layer of fluid on the cartilage surface (so-called weeping lubrication). Synovial fluid is chiefly responsible for reducing friction between apposing surfaces of cartilage and absorbing shock. In addition, movement of synovial fluid supplies oxygen and nutrients to and removes carbon dioxide and metabolic wastes from chondrocytes within articular cartilage.

Cartilage Development

Osteogenic and Chondrogenic Differentiation and Regulation in Development

Cartilage morphogenesis and osteogenesis are influenced by developmental signals. Current research is exploring which signals direct cartilage to remain at the ends of long bones when the rest of the bone ossifies. Cartilage serves as the blueprint for subsequent bone and joint morphogenesis as well as tendon and ligament insertions[15].

Pre-cartilage condensation observed in early limb buds is a transient phase of skeletogenesis, in which a cartilaginous framework serves as a scaffold for later ossification of skeletal elements[17]. To form cellular condensations, cells actively move toward a center, resulting in an increase in cells per unit area rather than an increase in cellular proliferation[17,18]. Mesenchymal cell condensation is crucial for chondrogenesis and is associated with an increase in cell-cell and cell-matrix interactions through cell-cell adhesion molecules and gap junctions, which facilitate intercellular communication[17,19]. Cell-cell interactions presumably trigger one or more signal transduction pathways that initiate chondrogenic differentiation. N-cadherin and N-CAM are two cell adhesion molecules expressed in condensing mesenchyme, and they play a role in mediating mesenchymal condensation. N-cadherin is responsible for cell-cell adhesion and its expression likely modulates the progression of chondrogenesis. The actions of N-cadherin can be modulated by Wnts, a family of secreted glycoproteins that influence cellular condensation and chondrogenic differentiation in early development[20].

During embryonic development, MSCs give rise to cartilage of two types: permanent and transient. Permanent hyaline cartilage arises from MSCs at the distal ends of developing bones. Following the initial cellular condensation event, MSCs

differentiate toward stable chondrocytes that synthesize the extracellular matrix of articular cartilage. Transient cartilage forms a skeletal framework which is later replaced by mineralized bone during the process of endochondral ossification.

When considering the candidate biological factors for enhancing the repair response of cartilage, members of the transforming growth factor-β (TGF-β) superfamily are of particular interest because of their abilities to promote chondrogenic activity *in vitro* and *in vivo*. The TGF-β superfamily comprises structurally related regulatory molecules that include the five TGF-β isoforms, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), activins, inhibins, nodal, and glial-derived neurotrophic factor (GDNF)[21].

TGF-β. TGF-β has been shown to induce mesenchymal chondrogenesis in cultures of cell lines and primary adult MSCs[22,23,24,25] and has been implicated in embryonic cartilage formation[26]. TGF-β also regulates the growth and synthetic processes of chondrocytes, stimulating extracellular matrix synthesis and chondrocyte proliferation in cell and organ culture[27,28,29]. Similar increases in matrix synthesis have been reported in chondrocyte cultures genetically modified with TGF-β1 cDNA[30]. Administration of recombinant TGF-β1 *in vivo* has been reported to increase proteoglycan synthesis and restore proteoglycan levels in the knees of arthritic mice[31].

TGF-β1 promotes cellular proliferation and initiates and maintains chondrogenesis of mesenchymal progenitor cells[32]. Other cell types, including both embryonic and adult fibroblasts, are able to undergo differentiation into a chondrogenic phenotype in the presence of chondrogenic inducers[33], demonstrating that TGF-β can influence differentiation of more cell types than just chondroprogenitors. TGF-betas have been

shown to activate intracellular signaling cascades, particularly those cascades containing MAP kinases, p38, ERK-1 and JNK to promote cartilage-specific gene expression[32].

Bone morphogenetic proteins. Bone morphogenetic proteins (BMPs) make up nearly one-third of the TGF-β superfamily and are closely linked to the formation of bone, cartilage and connective tissues *in vivo*[34,35]. BMPs regulate a diverse range of developmental processes during embryogenesis and postnatal development, and they control the differentiation of musculoskeletal tissues including bone, cartilage, tendon, and ligaments[36] (Table 1-2). The homeostasis of articular cartilage in the joint is maintained as a balance between anabolic morphogens such as bone morphogenetic proteins (BMPs) and cartilage-derived morphogenetic proteins (CDMPs) and catabolic cytokines such as IL-1, IL-17, and TNF-α[15].

Studies both *in vitro* and *in vivo* have shown that BMP signaling is required for the formation of cartilaginous condensations and for the differentiation of precursor cells into chondrocytes[37]. Like TGF-β, BMPs -2, -4, and -7 can stimulate chondrogenesis of mesenchymal progenitor cells *in vitro*[38,39,40]. BMP-2 is shown to accelerate the healing of osteochondral defects *in vivo* when delivered via a collagen sponge[41,42]. BMP-7 is used clinically for spinal fusion and fracture repair in the long bones[43]. Implantation of periosteal derived progenitor cells genetically modified to express BMP-7 improved repair of rabbit osteochondral defects, indicating the efficacy of a combined gene therapy and tissue engineering approach[44]. Further clinical applications of BMP-7/OP-1 include incorporation of the recombinant human protein into collagen scaffolds for repair of full-thickness canine osteochondral defects. Repair tissue in treated canine knee

defects had a hyaline cartilage-like appearance and integrated continuously with the intact cartilage adjacent to the defect[45]. Both BMP-2 and BMP-7 improve cartilage repair in studies of artificial cartilage plugs containing either collagen or hydroxyapatite blended with biodegradable polymers[46].

BMP-2 and BMP-4 are equally effective in promoting chondrogenesis in primary hMSCs in aggregate culture, but BMP-4 aggregates show a lower tendency to progress toward hypertrophy, a crucial characteristic to consider for cartilage repair[47]. Local delivery of BMP-4 by retrovirally transduced MDSCs (muscle-derived stem cells) shows enhanced chondrogenesis and significantly improved articular cartilage repair in rats[48].

IGF-1. Insulin-like growth factors are another class of molecules with potential for improving endogenous repair. IGF-1 is a major regulator of matrix synthesis in articular cartilage as it stimulates chondrocyte metabolism and promotes healing of cartilage lesions *in vivo*,[49,50] but its role in chondrogenesis is less clear. Ad.IGF-1 effectively transduces chondrocytes, MSCs, and synovial cells resulting in IGF-1 production sufficient to stimulate matrix gene expression and proteoglycan production[49]. When chondrocytes are cultured *in vitro* and exposed to varying concentrations of Ad.IGF-1, the cells are readily transduced; they produce significant amounts of IGF-1 to promote increased cartilage matrix gene expression and resist de-differentiation for 28 days[51]. IGF-1, when combined with TGF-β, can have synergistic effects in promoting chondrogenesis[52].

Sox9. In addition to growth factors, research has identified increasing numbers of biological molecules that are involved in regulation of chondrogenic differentiation, expression of cartilage matrix genes, and accelerated repair of cartilage defects in

animal models. Of these, the transcription factor SRY-related HMG box gene 9 (Sox9) is most closely associated with the expression of cartilage ECM genes and cartilage formation[53,54,55,56]. Mutations in Sox9 result in campomelic dysplasia, a semilethal skeletal malformation syndrome and XY sex reversal[57].

Sox9, dubbed the chondrogenic master gene, binds to regulatory sequences in the promoter region of several cartilage genes, thus enhancing their expression in chondrocytes[56]. Sox9 regulates the expression of chondrogenic genes such as aggrecan and collagen types II, IX and XI during chondrocyte differentiation[52]. In the collagen type II α1 gene (Col2a1), Sox9 binds within a 48 bp enhancer region located in the first intron and acts in concert with two cofactors: long form of SRY-related HMG-box gene 5 (L-Sox5) and SRY-related HMG-box gene 6 (Sox6)[58]. The two cofactors are normally expressed in MSCs; therefore, Sox9 gene delivery is sufficient to enhance the level of chondrocytic genes[58]. Because it functions intracellularly and cannot be delivered in soluble form, gene transfer of Sox9 cDNA to mesenchymal progenitor cells offers a means to investigate the reparative potential of this molecule.

Ihh and PTHrP. Chondrocyte proliferation and maturation are key points of regulation that may influence a repair process based on differentiation of progenitor cells. Indian hedgehog (Ihh), a member of the hedgehog family of cell surface-associated ligands, is expressed in prehypertrophic chondrocytes of the growth plate, and functions to inhibit chondrocyte hypertrophy by maintaining expression of parathyroid hormone related peptide (PTHrP) through a negative feedback loop[59]. Altering the expression of these proteins during chondrogenesis may serve to delay the onset of hypertrophy, chondrocyte apoptosis, and formation of bone, while increasing

the pool of proliferating chondrocytes. Indeed, addition of PTHrP has been shown to inhibit chondrocyte hypertrophy during *in vitro* chondrogenesis of primary adult MSCs[60,61].

BMP Receptors and Signaling

Two major types of membrane-bound serine/threonine kinase receptors are required for BMP signal transduction: BMPR-I and BMPR-II. Additionally, there are two options for intracellular signaling: the Smad and MAP kinase pathways. When BMPs bind to pre-formed heteromeric BMPR-I:BMPR-II receptor complexes, the Smad pathway is activated[36], whereas BMP ligand binding that induces the formation of heteromeric receptor complexes induces the MAPK (mitogen-activated protein kinase) pathway[35]. The BMP-specific Smad proteins include receptor-regulated Smads 1, 5, and 8 (R-Smads), a common-partner Smad 4 (Co-Smad), and inhibitory Smads 6 and 7 (I-Smads).

During joint morphogenesis, BMP binding proteins play a role in defining the boundaries between muscle, cartilage, perichondrium, and tendon/ligament[36]. BMP signaling is constrained on many levels by antagonists such as Noggin, Chordin, follistatin, ventropin, twisted gastrulation, Gremlin, Cerberus, and DAN[62]. Noggin binds to BMP-2 and -4 with high affinity and blocks their interaction with BMP receptors. Chordin also binds to BMP-2 and -4 to govern pattern formation (as originally studied in *Drosophila*). Antagonists, including Chordin, are proteolytically activated by an MMP, BMP-1, which was misidentified upon its discovery. BMP-1 is not a BMP *per se*, rather, it is a BMP inhibitor. DAN family members are newly identified BMP antagonists based on screens of cDNA libraries; their role in articular cartilage development and homeostasis as well as arthritis is not yet clear[63].

Cartilage Repair

Arthritic conditions affect over 70 million adults in the United States and cause an economic burden in excess of $60 billion annually. These numbers are projected to climb substantially as the population increases in age and the incidence of obesity continues to skyrocket.

Pathological conditions, such as osteoarthritis and rheumatoid arthritis, and traumatic conditions, such as intra-articular fracture or cartilage tearing from ligament injury, all yield damage to articular cartilage[64]. Articular cartilage defects fall into two categories: partial and full-thickness. Partial thickness defects are limited to the cartilage layer only, and do not penetrate through to the subchondral bone. In the absence of blood, a reservoir of stem cells and growth factors, there is little potential for the defect to repair spontaneously. These defects deteriorate with time and can lead to additional problems as the synovial lining becomes irritated by loose cartilage flaps, and the knee locks due to cartilage detachment[65]. Full-thickness defects, on the other hand, penetrate through the cartilage to the subchondral bone causing rupture of the local vasculature and access to the marrow. This provides an avenue for progenitor cells from the bone marrow to enter the defect and promote spontaneous healing through the formation of *fibrocartilaginous* repair tissue.

The Body's Natural Approaches to Cartilage Defect Repair

Unlike bone, which has great regenerative potential, cartilage has no vascularity or innervation; therefore, it has a low innate capability for self repair and regeneration. Injury to cartilage usually heals through formation of a fibrocartilage scar. Fibrocartilage, consisting predominantly of type I collagen fibrils with unordered proteoglycans and a random cell arrangement, has inferior mechanical and biological properties compared to

the highly ordered network of type II collagen fibrils, proteoglycans and chondrocytes present in hyaline cartilage. Over time, fibrocartilage repair tissue degenerates, resulting in permanent loss of structure and function and leading to severe pain[66].

Although they do not lead to cartilage healing, palliative therapies are the primary approach to treat symptoms of knee lesions. A combination of physiotherapy to maintain range of motion and strengthen the affected limb, weight loss to decrease forces exerted upon the knee, and NSAIDs for pain relief are among the conservative treatment procedures commonly used. Intra-articular injections of analgesics along with steroids will relieve pain, and injections with hyaluronate promote increased lubrication and decreased friction on joint surfaces, showing a modest improvement over placebo in clinical studies[11,65].

Surgical Approaches to Cartilage Repair

Nonreparative restorative techniques

Nonreparative restorative techniques for damaged cartilage include debridement, chondral shaving, and joint lavage[11]. These techniques can be performed arthroscopically, and are thought to relieve pain and improve mobility, but they do not on their own restore the structure and function of diseased cartilage[67,68,69]. One major drawback of nonreparative cartilage restoration techniques is that long-term benefits are reduced due to a loss of chondrocytes at the border between healthy and damaged cartilage after the injured cartilage is removed[70].

Debridement. The debridement procedure, developed by Magnuson in 1941, involves removal of inflammatory cells and other fragments such as chondral flaps, osteophytes, torn ligaments, degenerated menisci and other debris resulting from arthritis-mediated joint damage[71,72,73]. Debridement is intended to eliminate the

biochemical and mechanical factors that cause arthritis symptoms, resulting in pain relief and improvement of joint functions. The effects are short-lived and somewhat unpredictable, with 1/3 to 2/3 of patients showing improvement of symptoms at follow-up evaluation[73,74].

Chondral shaving. Chondral shaving, developed in 1908 by Budinger, excises damaged cartilage to relieve pain, using a motorized shaver. Shaving the cartilage is thought to convert the fibrillated damaged surface to a smooth surface; however, further examination has shown that shaving yields a rough surface with grooves[11]. Adverse effects on chondrocytes and cartilage matrix can result from chondral shaving, most notably chondrocyte death from the heat generated by frictional resistance to the tools used. Additionally, there is a risk of chondral tears that do not heal, leading to progressive degeneration of the remaining cartilage[4,75].

Knee joint lavage. Joint lavage is the technique of rinsing the joint with a physiological fluid to remove debris[76]. It is frequently coupled with a debridement procedure[77], and is usually performed when more conservative treatments, such as debridement and chondral shaving, prove inadequate. Suction of the fluid removes degradation products, inflammatory cells and degradative enzymes; however, the irrigation fluid can potentially harm cartilage and does nothing to halt disease progression[11,69].

Reparative procedures

Reparative strategies, also termed marrow stimulation techniques, aim to initiate bleeding from the subchondral bone, which releases progenitor cells, among other cells with chondrogenic potential, from the vascular system to the site of cartilage injury[78,79].

This causes a blood clot to form, plugging the injury site and paving the way for chondroprogenitor cells to generate a fibrocartilage scar[11,80]. Although fibrocartilage has inferior mechanical capabilities, it does serve a purpose in filling the chondral defect and covering the underlying bone, thus reducing pain and swelling[65]. While fibrocartilage fills the site of cartilage injury and temporarily alleviates pain, it is functionally inadequate as a replacement for articular cartilage in the long term[58]. Reparative procedures include arthroscopic abrasion arthroplasty, subchondral drilling, microfracture and spongialization, a modified technique that combines debridement and suchondral drilling.

Arthroscopic abrasion arthroplasty. Arthroscopic abrasion arthroplasty is a minimally invasive procedure that involves burring the exposed bone to access the vasculature of the subchondral plate, which promotes formation of a blood clot and subsequent formation of fibrocartilaginous repair tissue. In short term follow-up, nearly half of the patients showed improvement; however, some studies have shown that breakdown of repair tissue can occur as early as one year after the procedure[11]. Other studies indicate that when the joint is properly protected, fibrocartilaginous repair tissue maintains integrity for up to six years[76].

Microfracture. The microfracture technique was developed by Steadman to enhance chondral resurfacing by allowing the influx of marrow elements—mesenchymal stem cells, growth factors, and other proteins—to create a microenvironment that would promote new tissue formation and take advantage of the body's natural healing process[79]. This arthroscopic procedure involves a preparative debridement step to remove the damaged cartilage and expose the subchondral bone followed by a V-

shaped piercing using a specialized awl which makes multiple perforations approximately 3 mm apart[76,79]. Subchondral piercing causes bleeding from the bone marrow and leads to formation of a blood clot populated with platelets, growth factors, and progenitor cells, which adheres to the exposed bone surface, fills the defect, and progresses toward a cartilage-like repair tissue[11,79]. In younger, active patients (<35 years of age), microfracture remains the procedure of choice for cartilage lesions smaller than 2.5 cm[81]. Long-term follow-ups from 2 to 12 years report pain relief and restored knee function for 75% of patients with deep subchondral defects[4,60,76]. Conversely, another study shows a decline in positive clinical outcomes 2 years postoperatively, especially in older patients[82]. Drawbacks to microfracture include the poor biomechanical nature of the resulting fibrocartilage repair tissue, incomplete filling of the defect, and the potential for abnormal bone growth into the cartilage lesion[76]. Physical rehabilitation, typically with continuous passive motion and protected weight bearing, is an important step in improving cartilage repair after microfracture, possibly due to increased movement of synovial fluid throughout the joint space, which carries nutrients to chondrocytes and enhances synthesis of matrix proteins[83,84].

Subchondral drilling. Drilling into the subchondral bone is a technique established by Pridie in 1959 to develop bleeding channels through the subchondral bone to promote the formation of cartilage to resurface the exposed bone. It is reported that 85% of patients whose knees were treated with this procedure demonstrated satisfactory long-term outcomes[76]; however, in a rabbit model, the newly formed cartilage repair tissue lost its hyaline-like morphology within 1 year of treatment and instead resembled dense collagenous tissue[85]. Adverse effects of drilling include

damage to the subchondral bone from heat generated during the procedure and the potential for formation of a subchondral hematoma.

Spongialization. Spongialization is a modification of debridement and drilling, where the entire injured cartilage and the subchondral bone beneath it are removed, which exposes the spongy, or cancellous, bone[86,87]. In contrast to subchondral drilling, spongialization removes the highly innervated subchondral plate, thus removing a source of pain, and it may promote improved healing. Follow-ups by Ficat, the developer of this procedure, showed improved joint function and pain relief in 70-80% of patients, but this procedure has not gained popularity, perhaps because it is invasive and may cause thermal necrosis of the surrounding cells that are the target for stimulation[86,88].

Restorative strategies

Restorative strategies for the joint include high tibial osteotomy (HTO), knee replacement (either total or unicompartmental), and transplantation of bone, cartilage, or tissue. Knee replacement procedures involve removal of bone from femoral and tibial surfaces and resurfacing with prosthetic implants.

High tibial osteotomy. High tibial osteotomy (HTO) is a technique developed by Jackson in 1958, and is used in patients who experience cartilage degradation in the medial compartment of the knee. This procedure aims to transfer weight bearing from damaged regions to those with healthy cartilage to relieve pain and prevent further osteochondral damage[11]. Especially useful in younger, active patients who are not ready for knee replacement, HTO shows satisfactory clinical outcomes in 80% of patients 5 years post-op, and 60% at 10 years after treatment[11]. Since HTO is only

useful in a select group of patients, knee replacement is often the technique of choice for middle-aged and elderly patients since it has reliable long-term outcomes.

Partial knee replacement. With partial knee replacement, only the damaged surfaces of the knee are replaced with prostheses while intact surfaces are not altered. Typically, younger patients take advantage of partial knee replacement because it is less invasive, has a lower cost and has a shorter recovery time than total knee replacement surgery. Even though partial knee arthroplasty has a 98% survival rate at 10 year follow-up and delays the need for invasive total knee arthroplasty by 10 years or more, the cartilage in surrounding areas progressively deteriorates due to osteoarthritis as shown by radiographs of patients' knees.

Total knee replacement. When conservative treatments fail, total knee replacement, or total knee arthroplasty (TKA), is recommended as an effective and durable procedure to restore mobility and relieve pain in people suffering from end-stage knee lesions[11]. TKA is the most invasive of all the procedures and can be performed as revision surgery for each of the aforementioned procedures. Prostheses consist of a femoral and tibial component and some also involve patellar resurfacing[11]. The anterior cruciate ligament (ACL) is always removed and in some instances the posterior cruciate ligament (PCL) is substituted or removed. For elderly patients, total knee arthroplasty is currently the recommended technique to restore function of the entire knee after articular cartilage damage[11]. In patients greater than 70 years old, the 10 year follow-up success rate remains high, but in patients less than 50 years old, the failure rate is higher, likely due to the longevity and vigor of joint use. The average

duration of implant survival remains 10-15 years, with some reports of 20 years. When the implant degrades, revision surgery is necessary to restore joint mobility.

Reconstructive methods to treat cartilage defects

Reconstructive methods aim to fill the cartilage defect with autologous cells or tissues, and surgeons attempt to carry them out in the least invasive method possible[80]. These methods use either pieces of cartilage tissue (mosaicplasty) or autologous chondrocytes harvested from non-load-bearing areas (autologous chondrocyte transplantation)[58]. The main drawbacks are that there is a very limited supply of non-weight-bearing cartilage tissue available for harvest, and collecting tissue introduces new sites of damage to the articular cartilage of the joint[58].

Osteochondral transplantation or mosaicplasty. Osteochondral transplantation (osteochondral grafting) includes autologous and allograft transplants. Autologous osteochondral transplantation (OATS), also termed mosaicplasty, consists of removal of cartilage from the defect site down to the subchondral bone, followed by creation of small holes 15 mm deep and perpendicular to the cartilage surface[11,80,89]. Next, osteochondral grafts 10-15 mm long are harvested from non-weight-bearing surfaces of the patient's articular cartilage and inserted into the donor sites. As the site heals, fibrocartilage is the most prevalent repair tissue observed[11].

While mosaicplasty promotes pain relief and joint function improvement, there is risk of donor site morbidity, the original contour of the femoral condyle may be difficult to recreate, and chondrocyte death can result in degeneration of the graft. Additionally, there is a chance of bone and cartilage collapse. Removal of cartilage from non-load-bearing areas of the joint may lead to defects at those donor sites, resulting in more pain and additional cartilage damage from the lack of chondrocytes available for repair.

Allograft osteochondral transplantation is indicated for large osteochondral defects that exceed those which can be repaired via mosaicplasty and other less invasive techniques. Allografts replace the injured cartilage and its underlying subchondral bone with a histocompatible fresh or fresh frozen cartilage segment taken from an organ donor. Chondrocyte viability and biomechanical integrity deteriorate over time in storage; therefore, fresh osteochondral allograft (less than 2 weeks old) is recommended to optimize the success of the procedure[11]. Long-term follow-ups report 85% and 74% graft survival at 10 and 15 years post-operatively[11].

Autologous chondrocyte transplantation. Autologous chondrocyte transplantation (ACT), also dubbed autologous chondrocyte implantation (ACI), is a cell-based technique first described by Brittberg in 1994, to relieve pain and restore function in knees affected by either chondral or osteochondral defects[11,90]. Chondrocytes are harvested arthroscopically from a non-weight-bearing portion of the joint and cultured for approximately 6 weeks. After cell expansion *in vitro*, the cultured cells are surgically introduced to the cartilage lesion of the patient[11,80].

To prepare the defect, the site is debrided and covered by a periosteal flap harvested from the patient's tibia or femur. The periosteal flap is sealed or glued to the edge of the defect, and the cultured cells are delivered and held in place underneath the flap. The cells in suspension eventually attach and secrete extracellular matrix. ACT is useful for medium to large sized defects with positive results for up to 10 years[90]. It is reported that in the majority of patients (80-90%), hyaline-like repair tissue forms and pain relief and joint mobility improve[89,91,92]. Furthermore, integration of the newly formed repair tissue with the surrounding cartilage is reported in nearly 90% of patients at five

year follow-up[92]. Additional studies show, however, a high proportion of fibrocartilage present in the repair site, further demonstrating the difficulty of regenerating articular cartilage[93].

Disadvantages of ACT include the extreme expense, leakage of chondrocytes from beneath the periosteal flap at the recipient site, the need for two separate surgeries for harvest and implantation, the risk of chondrocyte dedifferentiation in monolayer cell culture, uneven distribution of cells introduced to the defect, and long recovery time following the operation[94]. Further complications following ACT include fibrous overgrowth of the periosteal flap and separation and detachment of the periosteum from the repairing cartilage[95].

An improved variation of ACT applied clinically is known as characterized chondrocyte implantation (CCI), where patients' chondrocytes are sorted and selected based on cell surface antigens that indicate greater potential to produce hyaline cartilage. This method is currently marketed at ChondroCelect by Tigenix of Belgium[11].

Gene- and Cell-Based Approaches to Cartilage Repair

What we observe is that no matter the restorative or reconstructive procedure used, the fibrocartilage repair tissue or the prosthesis eventually degrades. Nothing can match the native articular cartilage originally produced by the patient. The goal of tissue engineering, gene-, and cell-based therapies is to replicate the natural structure and durability of articular cartilage to enable long-lasting repair of damaged cartilage. Ideal strategies would restore the structural and functional elements of articular cartilage through transplant of a viable cartilage substitute, revised methods to enhance cartilage repair, or methods to prevent or halt cartilage damage.

Clearly there is a need for a cartilage repair strategy that introduces autologous, multipotent cells to the joint in a way that enables them to integrate and survive within the existing cartilage structure. MSCs appear as viable candidates as they are found abundantly in tissues throughout the body[22,23].

MSCs: the logical cell type for chondrogenesis

Cells derived from mesenchymal tissues with the ability to proliferate extensively, self-renew, and undergo multipotent differentiation are broadly defined as mesenchymal stem cells, or MSCs. Experiments performed by Friedenstein and others described the presence of mesenchymal stem cells (MSCs) in the bone marrow that could be isolated through their intrinsic property to adhere to tissue culture plastic[96,97]. These early observations led to the standard accepted assay used to identify MSCs, the colony-forming unit-fibroblast (CFU-F) assay, which identifies adherent, spindle-shaped cells that proliferate to form colonies.

There are no definitive surface markers for MSCs; however, they are frequently positive for STRO-1, CD73, CD90, CD105, CD106, CD146, CD166 and negative for CD45, CD11b, CD34, CD31, CD117[98,99]. Rather, they are characterized by their ease of isolation and their rapid growth *in vitro* while maintaining their differentiation potential, allowing for extensive culture expansion to obtain large quantities suitable for therapeutic use. These properties make MSCs ideal building blocks for tissue engineering efforts to regenerate tissues and repair structures damaged by arthritic conditions and cartilage injuries[100]. MSCs offer advantages for cell therapy because they are easier to culture and manipulate ex vivo than chondrocytes, which de-differentiate when removed from their native ECM and grown in monolayer.

MSCs can be readily harvested from multiple tissue sources including bone marrow, adipose tissue, synovial fat pad and periosteum[101]. Cells with stem-like characteristics can also be harvested from skin, liver, skeletal muscle, dental pulp and cartilage[102,103,104]. MSCs have been shown to differentiate into chondroprogenitors, osteoblasts, adipocytes, myoblasts and hepatocytes *in vitro*[105]. Mesenchymal stem cells contribute to the regeneration of mesenchymal tissues throughout the body such as bone, cartilage, muscle, ligament, tendon, adipose and marrow stroma[106].

MSCs play an important role in human development, growth, repair, regeneration, and homeostasis. Their multilineage potential makes them a useful model to investigate mechanisms of cartilage tissue development and regulation, especially following treatment with proteins from the TGF-β superfamily[32]. Few reports exist of human MSC implantation for cartilage repair, but studies are taking place to evaluate the use of MSCs rather than chondrocytes for repair of cartilage defects in the knee.

It is thought that with increasing age there is a significant decline in the abundance of MSCs, their lifespan, and their differentiation potential. This is a logical assumption since it has been observed that as chondrocytes age they generate smaller, less functional ECM proteins and are less responsive to growth factors and mechanical stimuli, although an equally poor repair response of cartilage to damage is observed throughout embryonic, immature, and mature cartilage[107,108]. However, MSCs extracted from the synovial fat pad have been shown to maintain their osteogenic differentiation potential throughout life and could perhaps offer more chondrogenic potential than bone marrow-derived MSCs[109].

MSCs provide an autologous cell source, eliminating much of the risk of disease transmission and rejection of donor cells. There is also controversial evidence that MSCs may have immunosuppressive effects[110]. MSCs can be prompted toward chondrogenic differentiation to provide cells for direct delivery to articular cartilage defects or to provide cells to seed scaffolds which are then implanted into defects. When used in a gene therapy approach, MSCs can be genetically enhanced to express specific growth and differentiation factors which could not only influence their differentiation in a paracrine manner, but also stimulate neighboring cells present in the cartilage implant site.

Stem cells can serve as vehicles for gene delivery to damaged articular cartilage by transfecting cells with recombinant cDNAs encoding chondrogenic proteins and growth factors[52]. Furthermore, viral transduction of MSCs with chondrogenic cDNAs can result in longer expression of the gene product and greater potential to influence cartilage defect repair.

Mechanical stimulation to promote cartilage

Mechanical stimulation is a natural component of the chondrocyte environment and is known to affect gene expression and re-differentiation of chondrocytes[58]. Chondrocytes, and other cell types such as MSCs, respond to dynamic compression by changing their gene expression profile[58]. In therapeutic applications, it is important to remember that controlled movement post-implantation could be essential for adequate healing and development of articular cartilage rather than fibrocartilage.

Regeneration and Repair Using MSCs: Gene Therapy and Orthopaedics

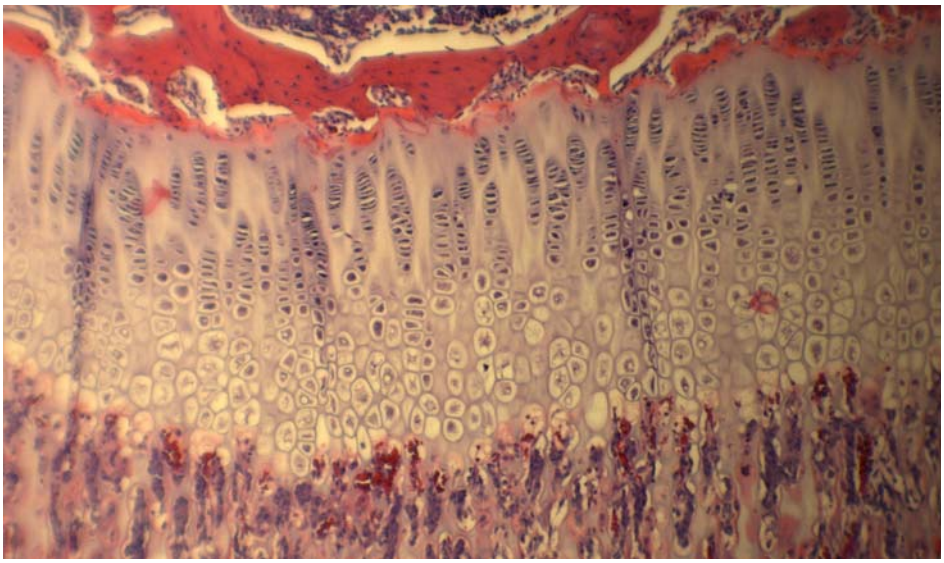
MSCs are ideally suited for cartilage gene therapy applications as they can be prompted to differentiate along a chondrogenic lineage by growth and differentiation

factors. Members of the TGF-β superfamily play an integral role in cartilage development; thus, they are logical choices for cartilage development in vitro. Gene delivery using viral vectors enables rapid, robust expression of chondrogenic proteins in MSCs, prompting their differentiation along a chondrogenic lineage. Such modified MSCs can then be implanted into cartilage defects, where they will continue to express the transgene products, thus impacting neighboring chondrocytes embedded in the ECM. Differentiated MSCs can take the place of the limited number of chondrocytes that are currently harvested for transplant procedures and will serve to fill cartilage defects with chondroprogenitor cells which secrete cartilaginous matrix and repair the site of damage with a natural tissue.

As the field of gene therapy expands, numerous approaches are being explored to improve treatments for cancer, AIDS, hemophilia, cystic fibrosis and an array of other diseases. Studies of gene therapy applications for orthopaedic conditions arose from research directed toward the treatment of rheumatoid arthritis[111]. Rather than a gene therapy method to correct a genetic abnormality, this work was based upon the concept of using gene transfer as a protein delivery system to treat chronic joint disease. By delivering cDNAs encoding naturally-occurring anti-inflammatory or anti-arthritic proteins to cells in the synovial lining, these cells would then serve as factories for the local overproduction and secretion of the therapeutic proteins into the joint space and surrounding tissues. Studies in several laboratories have shown that exogenous cDNAs can be efficiently delivered and expressed at levels within the joint sufficient to have beneficial effects in a variety of animal models[112].

The success of gene therapy studies in rheumatoid arthritis has led to the exploration of the application of gene delivery for other orthopaedic applications such as bone,[113,114,115] ligament, tendon,[116,117] and cartilage[44] healing. Most of these investigations use gene delivery in the same manner, as a system for localized, sustained production of bioactive molecules to promote healthy regeneration. Gene transfer can be used as a means to achieve sustained synthesis of specific proteins within a cartilaginous lesion, and this can be used to augment the differentiation of mesenchymal stem cells toward chondrogenesis *in vivo*. Gene transfer can also be used to stimulate existing cells and tissues, such as muscle, to repair large segmental defects in bone[118]. A similar technique can be applied to repair cartilage defects with chondrocytes (ACT) or MSCs that are stimulated by virally-delivered chondrogenic transgenes.

A).



B).

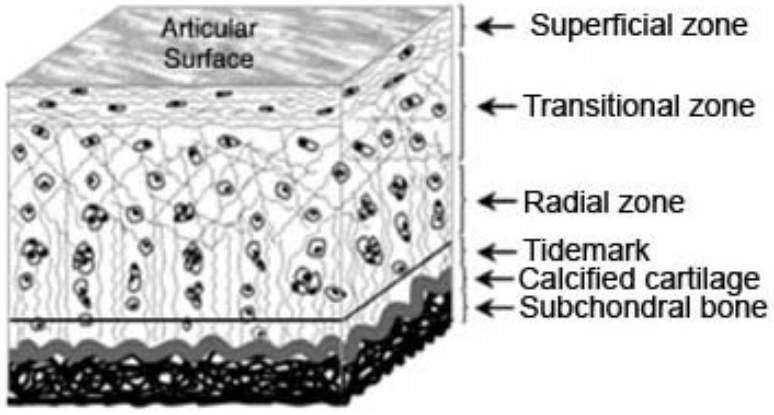


Figure 1-1. The four zones of articular cartilage stretch from the superficial surface to the deep zone, where hypertrophic chondrocytes are replaced by calcified matrix. A) Growth plate of proximal tibia. Columns of prehypertrophic chondrocytes are present in the radial zone, progressing to enlarged, rounded chondrocytes that undergo hypertrophy, followed by apoptosis and replacement by calcified cartilage. B) Depiction of the zones of articular cartilage with resident chondrocyte population. The collagen fibrils, depicted as pale gray lines, differ in their orientation throughout the zones to allow movement, provide structural support, limit the expansion of proteoglycans, and absorb stresses. (Part B adapted from Ahmed and Hincke, *Tiss Eng Part B*, 2010).

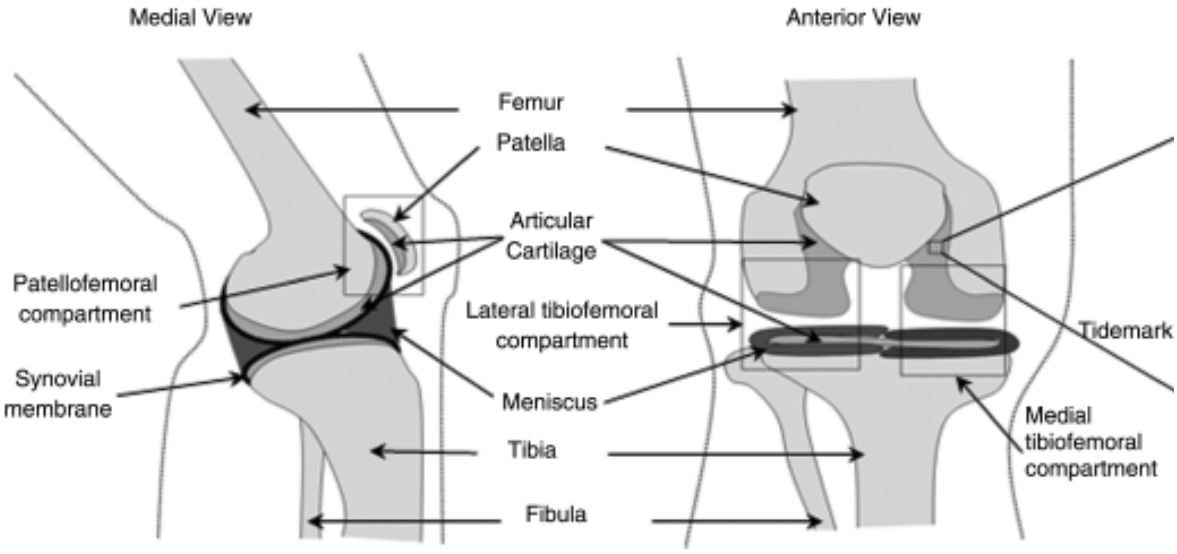


Figure 1-2. The human knee contains specialized structures to withstand the forces of movement. Depicted here, articular cartilage lines the ends of the femur and tibia and the inner patella to enable near frictionless motion of the knee joint. The specialized architecture of articular cartilage can withstand repeated extreme forces, often for more than 80 years, without damage. Image adapted from Ahmed & Hincke, *Tiss Eng Part B*, 2010.

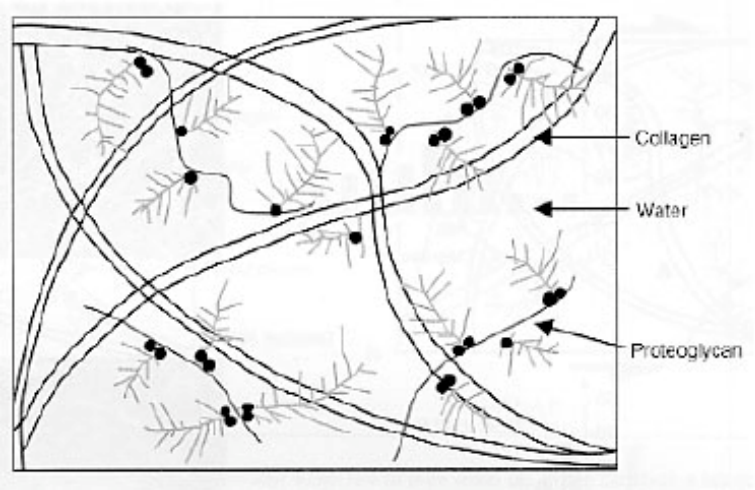


Figure 1-3. The arrangement of structures in the articular cartilage matrix is designed to absorb forces, especially compression. Collagen fibers form a supportive framework for cartilage, and they constrict the expansion of hydrated proteoglycans. Chondrocytes (not shown), the resident cells of cartilage, populate the matrix at a low density and serve to remodel the extracellular matrix. Image adapted from Mow and Ratcliffe, *Biomechanics of Diarthrodial Joints*, “Structure and Function of Articular Cartilage and Meniscus,” 1990.

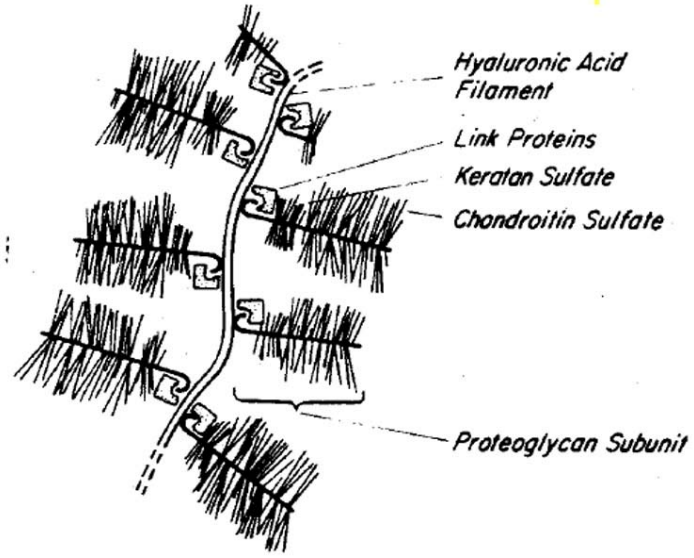


Figure 1-4. A proteoglycan aggregate is made up of many proteoglycan subunits attached to a hyaluronic acid backbone via link protein. Proteoglycans consist of a protein core with glycosaminoglycan side chains of keratan sulfate or chondroitin sulfate attached. The negative charges of the side chains repel one another and form their characteristic bottlebrush-like structure. Image adapted from Mow and Ratcliffe, *Biomechanics of Diarthrodial Joints*, "Structure and Function of Articular Cartilage and Meniscus," 1990.

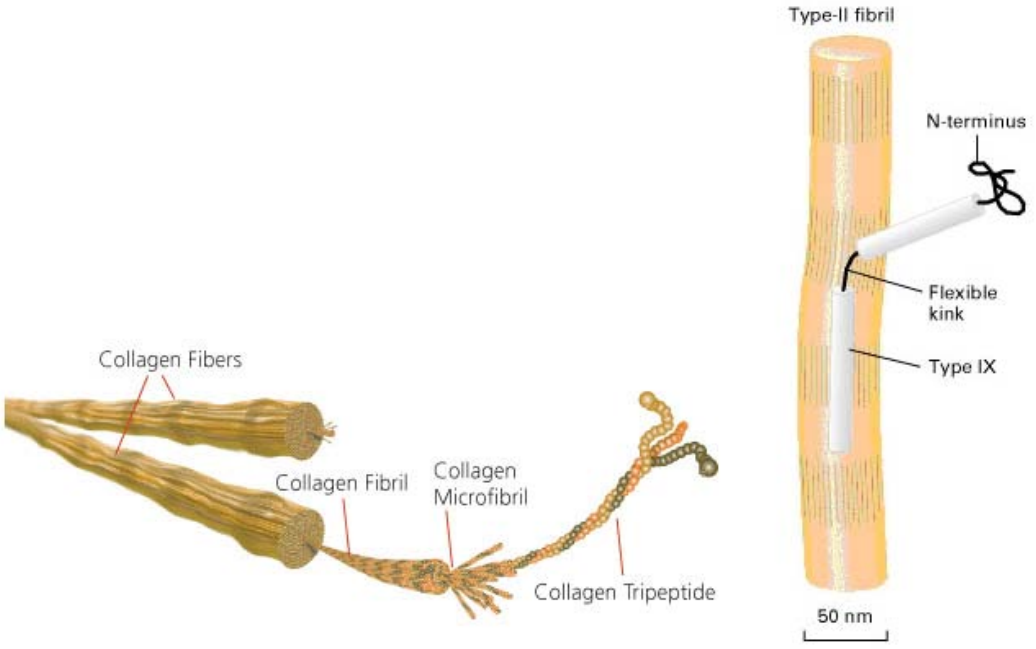


Figure 1-5. Fibrillar collagens, including collagen type II, form a characteristic triple helix. Multiple tripeptides are bundled together to form fibrils, which in turn, are packed into parallel bundles to yield fibers with incredible strength. Collagen type IX, with its flexible kink, crosslinks collagen type II fibrils to proteoglycans in the cartilage extracellular matrix. Figures are adapted from Sigma-Aldrich Life Sciences, 2009, and Lodish, et al. *Molecular Cell Biology,* 1999.

Table 1-1. Collagen types.

Collagen type	Representative Tissues	References
Fibrillar collagens		
I	Abundant throughout human body; skin, bone, scar tissue, fibrocartilage, tendon, ligaments, dentin, artery walls, interstitial tissues	2,119
II	Articular cartilage and vitreous humor of the eye	2,119,120
III	Produced by young fibroblasts prior to type I collagen, reticular fibers, skin, muscle, blood vessels, intestines, uterus	2
V	Interstitial tissues, similar to type I, also associated with placenta (fetal tissues), synovial membranes and cell culture	2,119
XI	Hyaline cartilage	119
Nonfibrillar collagens		
IV	Basal lamina, lens of the eye, part of filtration system of capillaries and glomeruli of the kidney nephrons	
VI	Most interstitial tissues, similar to type I, blood vessels, skin, intervertebral disc	119
VII	Forms anchoring fibrils in dermal epidermal junctions	
VIII	mainly endothelial cells	121
IX	Hyaline cartilage, associated with type II and XI fibrils in cartilage, vitreous humor, FACIT collagen	121
X	Hypertrophic and mineralizing cartilage, growth plate	119
XII	Embryonic tendon and skin, periodontal ligament, FACIT collagen	119
XIII	Endothelial cells	119
XIV	Fetal skin and tendon, FACIT collagen	119

Collagens are an essential part of the integumentary system. Of the 29 types of collagen identified and described in literature, the fourteen most prevalent are listed above. Collagen type I is the most abundant collagen in the human body. Collagen type II is the main component of articular cartilage, and is supported by types IX and XI. A subgroup of nonfibrillar collagens, including types IV and VIII, form sheets that create structures such as basement membranes that surround tissues. FACIT, Fibril Associated Collagens with Interrupted Triple Helices, refers to a type of collagen which is also a proteoglycan. FACIT collagens include types IX, XII, XIV, XIX, and XXI.

Table 1-2. Morphogens and Growth Factors in Cartilage Development

Morphogen	Name	Alternate Descriptors	Function(s)
Bone Morphogenetic Protein (BMP)			
	BMP-2	BMP-2A	Cartilage and bone morphogenesis, used clinically for bone repair[36]
	BMP-4	BMP-2B	Cartilage and bone morphogenesis
	BMP-3		Bone formation, inhibits activity of BMP-2[46]
	BMP-3B	GDF-10	Membranous bone formation
	BMP-5		Bone morphogenesis
	BMP-6		Cartilage hypertrophy, bone formation via alternate mechanism to BMP-2 or BMP-4
	BMP-7	OP-1 (osteogenic protein 1)	Osteogenic differentiation, used clinically to augment bone repair[36]
	BMP-8	OP-2	Bone formation, esp. active in early phase of fracture healing[46]
	BMP-9		Anabolic factor in juvenile cartilage
	BMP-10		Not chondrogenic; regulates cardiac growth and heart chamber maturation
Cartilage derived morphogenic protein (CDMP)		GDF (Growth/ differentiation factor)	
	CDMP-1	GDF-5, BMP-14	Mesenchymal condensation, chondrogenesis
	CDMP-2	GDF-6, BMP-13	Cartilage development and hypertrophy, cartilage formation *in vitro* similar to BMP-2
	CDMP-3	GDF-7, BMP-12	Ligament and tendon development, cartilage formation *in vitro* similar to BMP-2

Table 1-2. Continued.

Morphogen	Name	Alternate Descriptors	Function(s)
Transcription Factor			
	Sox9	SRY (sex-determining region Y) HMG box 9	Binds regulatory sequences in Col2a1 promoter region; necessary for mesenchymal condensation
Other Morphogens			
	Indian hedgehog (Ihh)		Cartilage morphogenesis; esp. in prehypertrophic chondrocytes

Many growth factors, morphogens, and transcription factors play integral roles in cartilage morphogenesis and are activated during the body's natural cartilage repair responses. Many of these same factors, especially BMPs, are involved in bone morphogenesis and are delivered in clinical applications to enhance non-union fracture healing.

CHAPTER 2
MATERIALS AND METHODS

In Vitro Cell Culture

HEK293 and 293FT Cell Culture

Immortalized cell lines were cultured in 75 cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM), with 10% FBS (Gibco), 1% glutamine (Gibco), and 1% penicillin-streptomycin (Gibco), hereafter referred to as complete DMEM, at 37°C in a 5% CO_2 environment. For adenovirus propagation, HEK293 cells were grown to approximately 70-80% density in 175 cm^2 flasks prior to viral infection. For lentivirus preparation, 293FT cells were cultured in either 75 cm^2 or 175 cm^2 flasks and treated as indicated below.

Harvesting and Culturing Bovine MSCs

We harvested bone marrow from the long bones of 3-day old Holstein-Fresian bull calves from the University of Florida Department of Large Animal Sciences. The bones were cut open with a table saw, marrow was removed with a spatula and diluted, to prevent clotting, in MSC medium, which contains DMEM (Gibco) with 10% MSC-qualified FBS (Gibco), 1% glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Addition of antimycotic agents to the medium were not beneficial as these substances interfered with adherence, growth, and expansion of MSCs. The cells were cultured at 37°C in a 5% CO_2 environment for 48-72 hours, at which point blood cells were removed by changing the media. The plastic-adherent cells that remained were grown and expanded for up to five passages for use in experiments.

Aggregate (Pellet) Culture

The pellet culture system allows cell-cell interactions analogous to those that occur in precartilage condensation events during embryonic development[122]. We used aggregate culture as a means to evaluate the chondrogenic potential of multiple transgenes *in vitro*. We adapted our "high-throughput" aggregate culture system based upon results that showed the efficiency of 96-well plates over the use of individual 15 mL conical vials when screening numerous treatment groups[123].

As depicted in Figure 2-1, we transduced early passage bovine MSCs with adenoviral vectors carrying the complete cDNAs for the candidate transgenes, denoted Ad.BMP-2, Ad.BMP-4, Ad.BMP-7, Ad.Ihh, Ad.Sox9, Ad.CTGF, and so on, at doses ranging from 10 virus particles/cell to 100,000 vp/cell. Similar dose ranges were used for scAAV-BMP-4 transduction. MSCs were grown in monolayer in 75 cm^2 flasks and transduced with virus. Twenty-four hours later, cells were trypsinized, counted, and 2.0 x 10^5 cells in a 300 to 350 µl volume were pelleted by centrifugation within individual wells of 96-well V-bottom polypropylene plates (Corning). The resulting cellular aggregates were grown for 21 days in serum-free chondrogenic medium. Chondrogenesis was evaluated by gross examination of aggregate morphology, histological staining for proteoglycan expression, and immunohistochemical analysis for collagen types I, II, and X.

Chondrogenic Media Formulation

Cell aggregates in 96-well plates were maintained in 350 µl chondrogenic medium, which consists of serum-free DMEM (Gibco), 1% ITS (insulin, transferrin, selenium) (Sigma), 1% penicillin-streptomycin (Gibco), dexamethasone (10^{-7} M), ascorbate-2-phosphate (50 µg/mL) (Sigma), proline (40 µg/mL) (Sigma), and 1 mM sodium pyruvate

(Gibco). Media were changed every 48 to 72 hours, except for days 3, 7, 14, and 21, when media were changed to allow 24-hour accumulation of proteins for detection via ELISA.

Virus Preparation and Transgene Expression

Construction and Generation of Recombinant Adenoviruses Containing Chondrogenic Transgenes

Serotype 5, E1- E3-deleted recombinant adenoviruses containing BMP-2, BMP-4, BMP-7, TGF-β1, Indian hedgehog, Sox9, GFP, and others were generated through the *Cre-lox* recombination system developed by Hardy[124]. Each transgene was inserted directionally into the adenoviral shuttle plasmid, pAdlox, containing the 3' inverted terminal repeat of the virus, a ψ packaging signal, a cDNA expression cassette driven by the cytomegalovirus (CMV) promoter/enhancer, and a *loxP* Cre recombinase recognition sequence. Cotransfection of Cre8 293 cells, which constitutively express high levels of Cre recombinase, with linearized Adlox plasmid and ψ5 adenoviral genomic DNA flanked by *loxP* sites generates recombinant adenovirus[124]. Specifically, Cre-mediated recombination occurs between the *loxP* site in pAdlox vector and the 3' *loxP* site in the adenoviral backbone. Any nonrecombined ψ5 adenovirus that is present can be separated from the recombinant adenoviral particles via subsequent propagation on Cre8 293 cells, whose Cre recombinase will delete the packaging signal of ψ5 virus.

Adenovirus Propagation and Amplification

To generate the quantities of replication-deficient adenovirus needed for large-scale infections, 293 cells were grown in complete DMEM in 175 cm^2 flasks. At 70-80% confluence, the media were removed and the cells were rinsed with PBS. A small aliquot (2-5 µl) of the desired adenovirus was added to a minimal volume of serum-free

media (10 mL) and applied to the cells for 4-6 hours, at which point 12-15 mL complete DMEM was added. Cells were incubated at 37°C with 5% CO_2 until cells rounded up and developed a granular appearance (signs of the cytopathic effects of a lytic virus) usually occurring after 2-3 days. Based on the method developed by Palmer[125], cells were collected using a cell scraper just before the virus caused them to lyse, then transferred, along with the media, to a 50 mL centrifuge tube. Following tabletop centrifugation at 2000*g* for 10 minutes at 4°C, the media were discarded in bleach, and the pellet was resuspended in 4 mL Tris, pH 7.0, and stored at -80°C. Cells were lysed by three rounds of freeze/thaw, digested with benzonase (Sigma) and the final supernatant was collected and stored on ice until cesium chloride (CsCl) gradient purification. CsCl gradients were prepared by layering approximately 3 mL of 1.4 g/mL CsCl solution on the bottom, 3 mL of 1.2 g/mL CsCl solution in the middle, and 4 mL of viral cell supernatant on the top layer in chilled ultracentrifuge tubes (Beckman). After centrifugation at 40,000 *g* for 1 hour at 4°C, viral bands localized at the interface of the two CsCl layers. The viral band was harvested by puncturing the centrifuge tube with a needle and syringe. If two bands were visible, the lowest band containing the infectious particles was harvested. The harvested band was diluted 2- to 4-fold in 10mM Tris-HCl, pH 8.0, for recentrifugation. Three consecutive gradients were performed on each viral prep. After the third CsCl gradient purification, the harvested adenovirus fraction was transferred to dialysis tubing and placed in dialysis buffer at 4°C for 6 to 8 hours. Following three rounds of dialysis, the virus was stored at -80°C in 50 µl aliquots. Viral titers were estimated by optical density[125].

Generation of Lentivirus

We produced lentiviral vectors by implementing a four plasmid transfection procedure adapted from Invitrogen's ViraPower expression system. Transducing vectors expressing the desired transgenes (GFP and BMP-4) were generated via the insertion of the specific cDNAs into the pLenti4/V5-DEST vector via homologous recombination. The resulting expression plasmid was mixed with the three necessary packaging plasmids, denoted pLP1, pLP2, and pLP-VSVG. Plasmid DNA was complexed with lipofectamine and delivered to monolayer cultures of 293FT cells. Twenty-four hours following transfection, the cell culture medium was replaced. Conditioned medium was harvested at 48 and 72 hours to allow collection of virus. Medium was concentrated through ultracentrifugation at 20,000*g* at 4^{o}C for 2 hours. The resulting pellet was resuspended in Opti-MEM (Gibco) and used immediately or aliquotted and stored at -80^{o}C until use.

Construction and Generation of scAAV Vectors

For generation of scAAV vector plasmids, the cDNA encoding GFP was directionally inserted into the conventional AAV packaging vector pTRUF2 as a NotI-SalI fragment. For generation of scAAV vector plasmids, the cDNAs for GFP and BMP-4 were directionally inserted into the SacII and NotI sites of the pHpa-trs-SK plasmid. PCR was used to modify BMP-4 cDNA to introduce SacII and NotI sites at the 3' and 5' ends. The insert plasmid contains the CMV promoter/enhancer and the cDNA of interest surrounded by ITRs from AAV2. The pDG-2 plasmid contains the *rep* and *cap* genes from AAV2 and complementing adenoviral functions required for amplification and packaging of the AAV genome. Similarly, to generate serotype 5 scAAV, the pxyz-2 plasmid was used.

AAV vectors were propagated using an adenovirus-free, two plasmid transfection system. Using 10-layer cell factories (Nunc), the respective AAV vector plasmids were co-transfected into 293 cells by $CaPO_4$ precipitation with the pDG-2 or pxyz-5 packaging/helper plasmid. Sixty hours post-transfection, cells were harvested with PBS containing 10mM EDTA, pelleted, resuspended in buffer containing 150 mM NaCl and 50 mM Tris, and lysed by three successive rounds of freeze-thaw. Cellular nucleic acids were digested by incubation with Benzonase (Sigma). Purification of AAV from the crude lysate was performed over iodixanol gradients followed by FPLC affinity chromatography over mono-Q columns. The eluate was desalted and concentrated with a Millipore Biomax 100K filter, aliquotted and stored at -80ºC. Viral titers were determined by competitive quantitative PCR assay relative to well-characterized AAV viral reference standards. Each viral preparation was examined for purity by resolution of the viral proteins by SDS PAGE and silver stain.

Gene Transfer to MSCs to Induce and Enhance Chondrogenesis

Plasmid DNA transfection

Monolayer cultures of bovine MSCs were transfected with DNA-lipofectamine complexes. Liposomes containing each BMP-4 expression vector were generated by incubation of DNA with lipofectamine (Invitrogen) in Opti-MEM (Gibco). Following a 20 minute incubation, the complexes were added to cells in a minimal volume of serum-free medium. One to three hours later, complete medium was added to cells. Medium was changed 24 hours post-transfection. At 48 hours post-transfection, conditioned media were collected and BMP-4 expression was characterized by ELISA. GFP expression was observed visually in the transfection controls.

Transgene delivery using adenovirus

MSCs are receptive to adenoviral transduction since they possess the CAR receptor. Adenovirus readily infects dividing and non-dividing cells so this was not a limiting factor in carrying out viral transduction prior to forming cell aggregates.

Methods to Detect Transgene Products

Western blot

Since there is no commercially available ELISA to quantify expression levels of Ihh or Sox9, Western blots of conditioned media or cell lysates, respectively, were used to verify expression. MSCs were grown in monolayer culture, then transduced with Ad.Ihh or Ad.Sox9 in a minimal volume of Opti-MEM (Gibco). Twenty-four hours later, media were removed and replaced with serumless DMEM. After an additional 24 hours, conditioned media was removed from Ad.Ihh-treated wells for detection of protein expression. Since Sox9 is expressed intracellularly, Ad.Sox9 infected cells were harvested with a cell scraper and lysed in chilled homogenization buffer. The resulting cell extract was used for protein detection. As these were human transgenes expressed in bovine MSCs, we were able to distinguish between endogenous and exogenous protein production.

A BCA assay was completed to gauge total protein content and ensure consistency in loading samples. A total of 10 µg total protein per lane was loaded into 10% or 15% Tris-HCl pre-cast gels (Bio-Rad) for Sox9 and Ihh detection, respectively. Proteins were transferred to Immun-Blot PVDF membranes (Bio-Rad) in buffer containing 25mM Tris, 192 mM glycine with 20% methanol and 0.1% SDS. Following transfer, the membranes were blocked with 5% milk for 1 hour prior to application of primary antibodies: 1:2000 rabbit anti-Sox9 (Santa Cruz Biotechnlogy, Inc.) and

1:10,000 rat anti-Ihh (R&D Systems). Membranes were soaked in primary antibody solution overnight on a low speed orbital shaker at 4°C. Following incubation, the membranes were rinsed with TBS-T and secondary antibodies conjugated to horseradish peroxidase were applied for 45 minutes: 1:15,000 anti-rabbit-HRP (Bio-Rad) and 1:12,000 anti-rat-IgG-HRP (Sigma-Aldrich), respectively. Membranes were rinsed and proteins were detected with the Immun-Star HRP Chemiluminscent Kit (Bio-Rad). For detection of β-actin as a loading control, antibodies were stripped from the membrane using 0.5M NaOH for 20 minutes at room temperature. As before, membranes were rinsed with TBS-T, and blocked with 5% milk for 45 to 60 minutes. Mouse anti-β-actin-HRP antibody (Sigma-Aldrich) was applied to the membranes at a concentration of 1:50,000 overnight at 4°C. β-actin was visualized via chemiluminescence.

ELISA to detect secreted transgene products

Concentrations of secreted protein products present in conditioned media were quantified using commercially available Duo-Set ELISA kits (R&D Systems) for BMP-2, BMP-4, BMP-7, and IGF-1 as directed by the manufacturer. The conditioned chondrogenic media from three or more replicate aggregates were used for all data points. Unless otherwise noted, media were placed onto cells 24 hours prior to collection, then used immediately or stored at -20°C until use.

Histology and Immunohistochemistry

Aggregates were removed from culture after 21 days and fixed in 4% paraformaldehyde solution overnight. The aggregates were paraffin embedded, cut into 5 μm sections, and mounted on plus-charged slides (Fisher Scientific). Slides were deparaffinized and rehydrated through a series of xylenes and graded alcohols, after

which they were stored in water for 2 minutes. Appropriate sections were stained with toluidine blue, while alternate sections were examined for collagen type I and type II content. Initially, heat mediated antigen retrieval was performed in Dako Target Retrieval Solution (DakoCytomation) for 20 minutes at 95°C. While this procedure works well for many tissue samples, the heat proved too intense for aggregates, and they often became detached at this step. We omitted the heat retrieval and found that aggregate sections remained attached for the entire procedure, and its omission did not impact detection of collagen.

Samples were treated with chondroitinase ABC (Sigma-Aldrich) at 0.2 U/mL for 15 to 30 minutes at 37°C to cleave chondroitin sulfate polysaccharide chains. Nonspecific binding was blocked in 15% normal serum matched to the secondary antibody species. Slides were incubated overnight at 4°C or 1 hour at room temperature with commercially available antibodies: rabbit anti-collagen I (Chemicon) at 1:1000 and rabbit anti-collagen II (Chemicon) at 1:500. Although the primary species reactivity was mouse, the antibodies were shown to cross-react with bovine tissues. The fluorescent secondary antibody, Alexa Fluor 488 donkey anti-rabbit, was used at a 1:200 dilution and allowed to incubate for 1 hour in the dark. Samples were washed with buffer (TBS-T), counterstained with Dapi, and coverslipped using Vectashield mountant.

RNA Extraction, RT, and rtPCR

For each treatment, ten additional pellets were harvested at days 3, 7, and 21 for total RNA isolation. Total RNA was isolated from treated and control cell pellets using the RNeasy mini kit (Qiagen) as directed. Aggregates were stored in RNALater (Qiagen) until RNA extraction was performed, at which time each group of treated pellets were frozen in liquid nitrogen and pulverized using a mortar and pestle. The

pulverized tissue was added to lysis Buffer RLT, homogenized using a 20-gauge needle, and RNA was harvested using RNeasy spin columns following the manufacturer's protocol (Qiagen).

We completed semi-quantitative real time RT-PCR analyses to verify transgene expression in cases where there was no ELISA for detection (Ihh) or chondrogenic gene products of interest were intracellular (Sox9). Real time PCR was carried out using the Eppendorf Realplex machine and software. To synthesize cDNA, 1 µg of total RNA from each group was reverse transcribed using random hexamer primers and MMLV reverse transcriptase (Invitrogen). Specific primer sets were used to amplify type II collagen α1 chain, type I collagen α2, type X collagen α1, aggrecan, osteopontin, and fibronectin.

In Vivo Experiments

Intra-Articular Injections

To examine the effects of chondrogenic transgene expression on collateral tissues in the joint, we performed intra-articular injections in both knee joints of Male Wistar rats weighing 100-150 g. The adenoviral vectors of interest were suspended in phosphate buffered saline (PBS) to a 50 µl volume and injected into the joint space of the knee through the infrapatellar ligament. The knee diameter was measured with calipers daily, and rats were weighed daily for 7 days. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida.

Harvesting Tissue, Decalcification, and Histology

Seven days after intra-articular injection, joint tissues were harvested and stored in 5% formic acid overnight at low speed on an orbital shaker to speed decalcification. Following a PBS rinse, the knees were stored in 0.5 M EDTA solution on a low speed orbital shaker at 4°C until bones were sufficiently decalcified (approximately one week).

The decalcified knees were sliced medially and cut into 5 μm sections for hematoxylin & eosin staining.

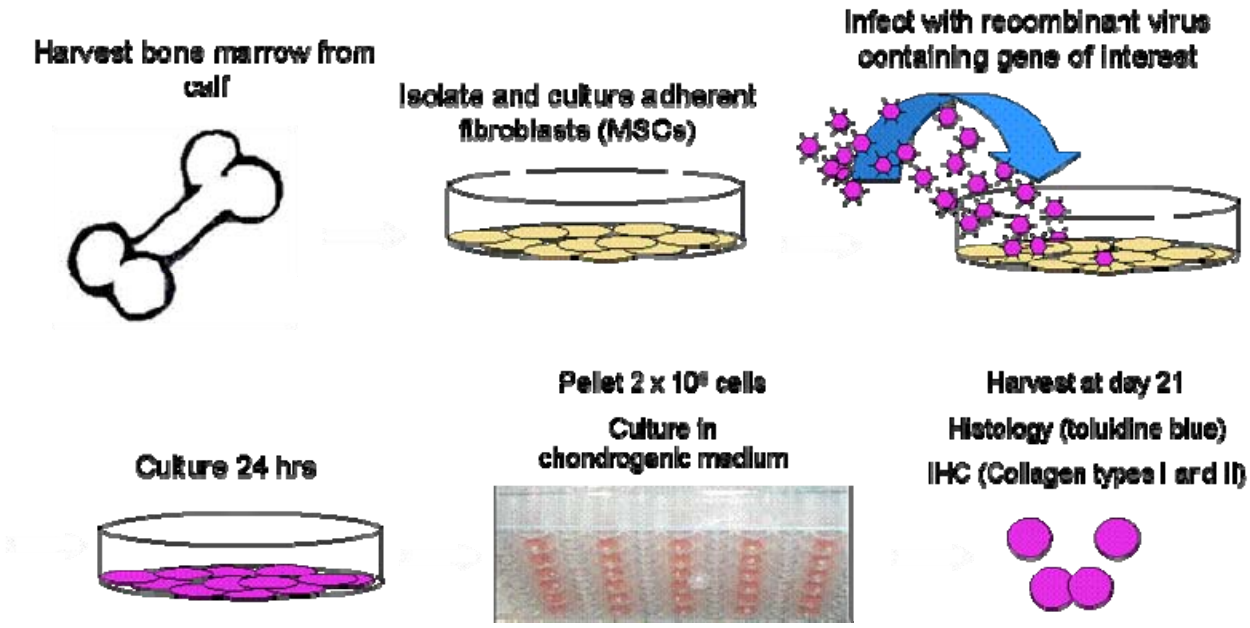


Figure 2-1. Assay for *in vitro* chondrogenesis. We harvested bone marrow from 1 to 3-day old bull caves, cultured the plastic adherent cells and expanded them *in vitro*. To generate cell aggregates, we virally transduced cells in monolayer and 24 hours later, pelleted them by centrifugation. Cells were grown in chondrogenic medium for 21 days, then evaluated for hypertrophic differentiation, cellular proliferation, and matrix protein production.

CHAPTER 3
GENE DELIVERY STIMULATES CHONDROGENESIS OF MSCS

Introduction

Articular cartilage is a highly specialized tissue that allows for near frictionless motion of diarthrodial joints. When cartilage is damaged as a result of injury or disease, natural repair processes are often insufficient to regenerate the tissue due to the lack of vascularity, dense extracellular matrix (ECM) and low cellular density of cartilage. Typically the repair response, if any, generates a fibrocartilage scar which lacks the unique architecture and structural properties of native articular cartilage. In most cases, though, damage or lesions of a significant size remain permanently. These injuries often initiate a degenerate cycle that over time leads to generalized cartilage loss and osteoarthritis.

Biology offers a number of potential approaches to enhancing the natural repair response of bone marrow progenitor cells *in vivo*. As shown in Table 1-2, several growth factors, morphogens and more recently, transcription factors, have been shown to promote differentiation along chondrogenic lineages. While these substances have shown promise in animal models of cartilage repair and regeneration, their clinical application is hindered by delivery problems. The half-lives of many proteins are limited *in vivo*, so they are difficult to administer to sites of cartilage damage at therapeutic concentrations for sustained periods of time. Localized delivery of these agents without involvement of non-target organs has also proven to be problematic. These limitations may be overcome by developing techniques to transfer genes encoding chondrogenic gene products to cells at the appropriate sites and to express those genes locally for the

necessary period of time. In this manner, the proteins of interest are synthesized locally by cells and are presented to the microenvironment in a natural fashion.

Numerous experimental approaches are currently being explored to enhance cartilage regeneration and repair. These include tissue engineering and gene and cell-based therapies. Adult mesenchymal stem cells (MSCs) are a well-suited cellular platform on which to base therapies for cartilage repair and tissue regeneration since they have the capacity to self renew and can differentiate into multiple mesenchymal tissues, including cartilage and bone[105,106]. They are also readily available from a variety of tissue sources, including bone marrow, synovium, fat, skin, and muscle. MSCs maintain their multilineage capacity over several passages in culture, making them amenable to various applications, including ex vivo therapies[126].

Within articular cartilage, the extracellular matrix constantly undergoes remodeling by chondrocytes, which exclusively populate the matrix at low density. Adult MSCs *in vivo* serve as replacements for differentiated cells of mesenchymal tissues that naturally expire or succumb to injury or disease[127]. This process of native stem cell-generated cell replacement peaks between ages 20 through 29 and decreases with age[14,122], but it could be re-charged by the introduction of modified MSCs to sites of cartilage damage. To date, no repair strategy has been shown to generate a durable repair tissue that can withstand the functional demands required of articular cartilage *in vivo*[128].

Delivery of MSCs alone is not sufficient to generate appropriate repair tissue in cartilaginous lesions, as the microenvironment is not adequate to drive and maintain chondrogenic differentiation. Gene transfer to MSCs, however, can be adapted to achieve sustained local expression or synthesis of specific protein factors, which may

be used to induce chondrogenesis *in vitro* and may enhance chondrogenic differentiation *in vivo*. If appropriate stimulatory factors are delivered to sites of cartilage damage, it may be possible to trigger signaling pathways in resident and introduced cells that drive the cells to synthesize repair tissue identical to the original in structure and form. Growth factors which are delivered as gene products offer advantages over recombinant protein delivery in that the proteins are presented in a natural context, they can be synthesized locally at the site of need for extended periods of time, and may be less costly.

We used an aggregate culture system to evaluate the ability of candidate transgenes to stimulate bovine MSCs toward chondrogenesis *in vitro*. High-throughput aggregate culture of MSCs offers a useful means to evaluate chondrogenic potential of multiple transgenes *in vitro*. Using a similar method, Johnstone et al. demonstrated chondrogenesis in MSC aggregates in defined medium containing dexamethasone and TGF-β1 resulting in aggregates that synthesized extracellular matrix characteristic of articular cartilage, containing proteoglycan and type II collagen[22].

Growth factors such as TGF-β, BMP-2, BMP-4, BMP-7, IGF-1 and FGF-2 have stimulatory effects on cartilage function. Animal studies document the benefits of exogenous growth factors in stimulating MSCs and grafted chondrocytes toward chondrogenesis[129]. Chondrogenic genes, such as Sox9, Ihh, and BMPs 2, 4, and 7 play key roles in the development of the cartilage anlagen within the embryo and subsequent formation of permanent cartilage, as seen in the joints, or transient cartilage that undergoes replacement by skeletal elements elsewhere in the body. The method of

introducing modified MSCs to cartilage defects has considerable potential to improve the cartilage repair process.

This study was performed to evaluate BMP-4, BMP-7, Sox9, Ihh and other genes for their chondrogenic potential when expressed by MSCs as transgenes. While there are numerous other factors that could be studied, our evaluation includes gene products considered to be among the principle contributors to the process of chondrogenesis and those reported to be effective in cartilage repair in animal models. These experiments were used to identify the most effective gene and gene combinations for stimulating chondrogenic differentiation of MSCs *in vitro*. Thus, the selected cDNAs should offer a reasonable assessment of the potential utility of gene delivery in this application.

Rationale

Multiple growth factors, morphogens, and transcription factors, including TGF-β1, BMP-2, BMP-4, IGF-1, Ihh and Sox9, are known to play a role in chondrocyte differentiation and proliferation. Chondrogenic effects of TGF-β1, BMP-2, and IGF-1, have been well-characterized in an aggregate culture system when delivered to MSCs via adenoviral vectors[130]. Although TGF-β1 and BMP-2 showed strong chondrogenic activity, they tended to drive the cells toward hypertrophy, a preliminary step in bone formation. With the goal of identifying transgenes whose products may be more suitable for cartilage repair, we expressed BMP-4, BMP-7, Sox9, Ihh, IGF-1, and CTGF as transgenes in MSCs and characterized their relative abilities to induce chondrogenesis within the pellet culture system singly and in combination. The results of these studies provide new insight into the biological activity of each cDNA when administered to MSCs as a gene product, and they form the basis for selection of candidate transgenes to evaluate *in vivo*.

Results

Our group has shown previously that MSCs in culture are highly amenable to infection and subsequent transduction with recombinant type 5 adenovirus vectors. For the majority of transgenes tested, adenoviral-mediated delivery to MSCs yielded robust expression of protein products that typically persisted for two to three weeks. The efficiency of adenovirus gene transfer coupled with the relative ease of generating novel recombinants and the ability to readily propagate the vector to high titers, led us to use this technology to evaluate the chondrogenic activity of our candidate transgenes.

Cre-lox Recombination and Adenovirus Propagation

We obtained the cDNAs for BMP-4, BMP-7, Ihh, Sox9 and CTGF from the American Type Culture Collection (ATCC) as IMAGE clones. Following amplification of each associated vector, we isolated and directionally inserted each cDNA into the pAdlox shuttle vector. This plasmid contains the 3' inverted terminal repeat of the adenovirus, a native ψ packaging signal, and a cDNA expression cassette driven by the cytomegalovirus promoter/enhancer followed by a *loxP* Cre-recombinase recognition sequence. Following verification of each pAdlox construct by diagnostic restriction digestion, we transfected each into cultures of 293 cells to assay for synthesis of the respective transgene products. Forty-eight hours post-transfection the conditioned media or cell lysates, as appropriate, were analyzed qualitatively for expression of the respective transgene products: BMPs -4 and -7 by ELISA; Ihh, Sox9, and CTGF by Western blot (data not shown).

Once it was confirmed that the vector constructs were indeed functional, they were used to generate recombinant adenovirus. For this, the respective plasmids were linearized by restriction digest and co-transfected with purified, ψ5 adenoviral DNA into

cultures of 293 cells engineered to constitutively express the Cre recombinase (Cre8 cells). (The ψ5 adenoviral genome packaging sequence (ψ) is flanked by loxP sites: novel recombinant adenovirus is generated by Cre-mediated recombination between the loxP site in the Adlox shuttle vector and the 3' loxP site in the ψ5 viral backbone.) If the far left hand portion of the viral genome contained in the Adlox plasmid was successfully linked to the adenoviral backbone, plaques in the monolayer of cells were usually detectable within 5-7 days. After this point the culture medium was no longer changed, allowing the virus released from the lytic plaques to accumulate and infect neighboring cells, generating widespread cytopathic effects in each culture. The cells and medium were then collected, and following successive rounds of freeze-thaw, the lysates were used to infect new cultures of Cre8 cells. Since the packaging signal of the ψ5 adenoviral backbone is removed by the Cre recombinase, the Cre8 cell line is non-permissive for its replication. Therefore, each new vector construct was passaged at least three times in Cre8 cells to eliminate any contaminating ψ5 virus that may have been propagated during growth and amplification of the new vector.

To determine the purity of each new adenovirus preparation, the genomic DNA was isolated, digested with appropriate restriction enzymes and analyzed following electrophoresis in 0.7% agarose gels. Once the vectors were found to be free of detectable ψ5, subsequent amplifications were performed in 293 cells. In an effort to eliminate as much contaminating cellular DNA and debris as reasonably possible, adenoviral preparations were banded over three successive CsCl gradients and were then dialyzed against multiple changes of dialysis buffer prior to aliquotting and storage.

Isolation of MSCs

We elected to use the bone marrow of bovine calves as a source of MSCs, as it would provide several advantages for our studies. Foremost is that the long bones of these animals, at birth, are at least 5 to 10 times greater in size and volume than the corresponding bones in the adult rabbit, which is among the largest of the common experimental animals. This large size enabled the isolation of an abundance of primary MSCs (usually > 5 x 10^7) from a single bone (generally the head of the femur), requiring minimal expansion of the cells in culture prior to their use in experiments. Further, since male calves are routinely sold from the dairy farm soon after birth, we could make use of these animals and in the process obtain cells from a newborn animal. In this regard, the cells would be expected to have maximal proliferative and differentiation potential, and thereby provide a robust readout of chondrogenic stimulation in our *in vitro* assays. The use of these animals was facilitated by the Large Animal Sciences Department whose facilities on the University of Florida campus are in close proximity to the Health Sciences Center.

Following procurement immediately after the death of the animal, the femurs were transected with a band saw. The red marrow was removed from the epiphyseal ends of the femurs and tibiae, and both red and yellow marrow was scooped from the medullary cavity with a spatula. The semi-solid marrow was placed in MSC medium (as described in Chapter 2) and passed through a syringe several times to disaggregate the loose stroma. Following digestion of the mixture with collagenases types I and II and neutral protease (Worthington Biochemical Corporation) for 30 minutes at 37°C, the cells were passed through a 40 µm nylon cell strainer (BD Falcon) and plated. After the first preparation, we found that the enzymatic digestion step was not necessary; the cells

would adhere to the culture flasks as long as the stroma was sufficiently homogenized by hand. Although somewhat variable between preparations, typically enough of the disaggregated marrow was obtained to seed 5 to 6 175 cm^2 flasks, which would be ~30-40% confluent with adherent fibroblastic cells at 24 to 48 hrs. Cells were allowed to adhere for 72 hours prior to the first change in media, which removed all of the non-adherent blood cells. Following expansion to confluence, the cells were either used immediately for experimentation or were aliquotted for storage in liquid nitrogen.

Gene-Mediated Chondrogenesis of MSCs

Our studies of gene-induced chondrogenesis were performed using high density aggregate culture systems. In early experiments we followed the methods of Palmer et al. whereby approximately 2.0 x 10^5 MSCs suspended in culture medium were aliquotted into 15 mL conical vials (Corning). During centrifugation at low speed, the cells were forced into aggregates, forming pellets in the bottom of each tube. Each cell pellet was cultured in 750 µl of chondrogenic medium supplemented with dexamethasone in its individual 15 mL tube for the following 21 days. Media were changed every 48 hours, with additional changes to allow for 24-hour conditioned media collection at days 3, 7, 14, and 21. While this method proved useful for a small number of samples, it proved unmanageable for the simultaneous culture of multiple treatment groups, each with multiple replicates. The frequent handling of large numbers of tubes and their individual screw caps was unwieldy, leaving the cultures prone to fungal contamination. Supplementation of the culture medium with antimycotic agents was of no benefit as these appeared to interfere with cell-cell adhesion and inhibited chondrogenic differentiation.

To provide a method amenable to the analysis of large numbers of samples, we worked to develop a “high-throughput” *in vitro* chondrogenesis system whereby numerous MSC aggregates could be formed and cultured simultaneously in multi-well plates. In this technique, the MSCs were genetically modified in monolayer, detached using trypsin 24 hrs later, suspended in a minimal volume of culture medium and then counted. Approximately 2.0 x 10^5 cells were then delivered in a 300 µl volume of chondrogenic medium (described in Chapter 2) to individual wells of a V-bottom 96-well plate. Once the plate was loaded with the samples of interest, using appropriate plate adaptors, it was spun at 4°C in a table top centrifuge at 500*g* for 5 minutes to form cell aggregates in the bottom of each well. The wells were topped off with an additional 100 µl of chondrogenic media and grown at 37°C. Over the course of 24 hours, the cells formed rounded aggregates that did not adhere to the polypropylene plate. After 24 hours, aggregates were disturbed by pipetting the media vigorously. This provided a means to observe whether all cells were incorporated into each aggregate rather than disassociating upon movement. The medium for each sample was changed routinely, as before, and the cells were cultured for 21 days.

Although the maintenance of the tiny pellets remained a somewhat challenging and tedious procedure, we found this approach to be far more manageable and provided more consistent results than the use of 15 mL tubes. We found, however, that certain parameters were essential to its application. For example, useful aggregates were generated only with the use of Corning brand, polypropylene, V-bottom, 96 well plates. We tested similar plates from other manufacturers, such as Nunc, but were

unable to consistently generate usable pellets following centrifugation since the “V” of their well was a slightly different angle.

Adenoviral-Mediated Delivery of BMP-4, BMP-7, Ihh, and Sox9 Drives Chondrogenic Differentiation of MSCs

Having developed suitable techniques for the isolation of low passage MSCs and their use in high-throughput *in vitro* chondrogenesis assays, we used these methods to evaluate the relative chondrogenic activity of BMP-4, BMP-7, Ihh, Sox9 and CTGF following adenoviral-mediated gene delivery.

Initially we focused our efforts on Ad.BMP-4 and Ad.BMP-7, since the chondrogenic potential of the recombinant proteins has been demonstrated in several reports. First or second passage bovine MSCs were seeded in monolayer and expanded to ~90% confluence. To ensure that the full potential of each cDNA was represented in our assays, we infected separate monolayer cultures with each adenoviral vector over a range of doses spanning 10 to 10^5 vp/cell. The genetically modified cells were harvested 24 hours later, and ~2.0 x 10^5 cells were seeded per well for each vector and dose. The cells were pelleted by centrifugation and cultured in defined medium containing dexamethasone for 21 days. The levels of the secreted transgene products were measured in conditioned medium over the course of the experiment. At the end of the incubation period, the cell pellets were harvested and processed for histology and immunohistochemistry or were pooled and used for isolation of RNA for subsequent analysis by qRT-PCR. Chondrogenesis was evaluated by examination of aggregate morphology, histological staining for proteoglycan content and immunohistochemical analysis for production of collagen types I, II, and X.

For both Ad.BMP-4 and Ad.BMP-7, the aggregates formed from MSCs infected at the 10^5 vp/cell dose were not viable and disintegrated within 3 to 7 days of culture (data not shown). As shown in Figure 3-1, ELISA measurements of the respective transgene products produced by the remaining cell pellets between the two vectors reflected considerably different levels of protein production and response ranges. In general, peak production occurred near days 3 and 7, and gradually tapered over the 21 day incubation period. Transgenic expression was largely dose dependent for both Ad.BMP-4 and Ad.BMP-7; however, the cell pellets transduced with Ad.BMP-7 showed a somewhat higher level of protein production relative to viral dose (Fig. 3-1B).

In response to Ad.BMP-4, the cells receiving the 10, 100 and 1000 vp/cell doses produced peak expression levels of ~0.8, 2.0 and 7.0 ng/mL, respectively (Fig. 3-1A). While the 1000 vp dose showed the greatest initial levels of expression, it also showed the most precipitous reduction over time to less than 2 ng/mL. Interestingly, the pellets infected at the 10,000 vp/cell dose produced somewhat less BMP-4 than the 1000 vp dose throughout, and expression was more variable. BMP-4 was not detected in the conditioned medium of the control pellets at any time.

Transgene expression from the Ad.BMP-7-infected pellets at day 3 ranged from less than 1 ng/mL for the 10 vp/cell dose to over 50 ng/mL for the 10,000 vp/cell dose (Fig. 3-1B). With the exception of the day 7 time point where the 1000 vp/cell dose showed a jump in expression from 32 ng/mL to nearly 70 ng/mL, expression levels showed a modest but gradual reduction in expression.

Histologic examination indicated distinct evidence of transgene-induced chondrogenesis of the MSCs stimulated with BMP-4 and BMP-7. However, we found

the level of chondrogenesis to be reproducibly greater and more consistent when BMP-4 was supplied as a transgene. As seen in Figure 3-3, relative to controls, MSC pellets infected with either 10, 100 or 1000 vp/cell of Ad.BMP-4, expressing between 0.8 and 8.0 ng/mL of the transgene product, were highly cellular and showed positive staining for toluidine blue and corresponding positive immunostaining for type II collagen, characteristic of articular cartilage matrix. Pellets infected at 100 and 1000 vp/cell doses, expressing 2-8 ng/mL BMP-4 over the 21 days produced a more dense, uniform matrix populated with rounded chondrocytic cells in lacunae, morphologically similar to the transitional zone of articular cartilage (Fig. 1-1B). Pellets infected at the 10,000 vp/cell dose were much smaller and appeared as loose cell aggregates with no evidence of extracellular matrix production or cellular differentiation. This is consistent with previous findings that excess viral load can have toxic effects, which would account for the reduced expression of the BMP-4 transgene and lack of a biological response in these pellets.

For the pellets transduced with Ad.BMP-7, pronounced chondrogenesis was observed only in the pellets infected at the 1000 vp/cell dose, which produced between 30 and 70 ng/mL of BMP-7. As shown in Figure 3-4, these pellets synthesized a dense, uniform extracellular matrix enriched for proteoglycans and collagen type II. Rounded chondrocytic cells were evident; however, many appeared to have begun to advance to hypertrophy, indicated by their increased cytoplasmic volume. MSC pellets infected at the lower doses, producing less than 10 ng/mL of BMP-7, showed minimal metachromatic staining with toluidine blue and little evidence of cellular differentiation or cartilage ECM synthesis. The pellets infected at the 10,000 vp/cell dose were largely

similar to those infected at the same dose with Ad.BMP-4, and despite continued production of BMP-7 of between 30-50 ng/mL over the 21 days, showed no evidence of chondrogenesis.

Following these experiments, we wanted to adopt a similar strategy to evaluate the chondrogenic effects of adenoviral mediated gene transfer of Ihh and Sox9 to MSCs. Unfortunately, since Sox9 is an intracellular transcription factor and commercial ELISAs were not available for Ihh, it would not be possible quantify transgene products synthesized by the pellets over time. Therefore, to verify effective gene delivery to MSCs and gain insight into the relationship between viral dose and the level of transgenic expression, we performed Western blot analyses of MSC cultures infected with Ad.Sox9 or Ad.Ihh at 3-fold dose increments between 10 and 10,000 vp/cell. Cultures of uninfected MSCs were processed in parallel and used as negative controls. Based on the negative results obtained above with extremely high vector doses, we set 10,000 vp/cell as the upper limit for infection.

As seen in Figure 3-2, detectable human Sox9 protein was found in lysates of cells infected with as few as 30 vp/cell of Ad.Sox9, with peak expression associated with doses of 100 and 300 vp/cell. Analogous to the expression data for Ad.BMP-4, a marked reduction in Sox9 production was seen at 1000 vp/cell and higher doses, such that it was below the limit of detection in cells infected with 3000 and 10,000 vp/cell.

Western blots of media conditioned by MSCs infected with Ad.Ihh also showed dose dependent expression, but with a slightly different profile. Protein bands were faintly visible in the 300 vp/cell dose lane increasing in intensity with viral dose to a maximum at 3000 vp/cell (Fig. 3-2). At the 10,000 vp/cell dose, however, there was no

visible protein band. The dramatic reductions in transgene expression seen with high vector doses were again consistent with toxicity from excessive viral load.

Having established a working relationship between viral dose and transgene expression for Ad.Sox9 and Ad.Ihh, we infected additional cultures of MSCs with each vector over a similar dose range. As described above, we then seeded the genetically modified cells into aggregate culture and assayed for chondrogenic induction 21 days later.

Histologic analysis of the recovered cell pellets showed that both Ihh and Sox9 were capable of driving chondrogenesis when expressed as transgenes; however significant differences were noted in their stimulatory capacities. For MSCs infected with Ad.Ihh, pellets formed from cells at the 300 and 1000 vp/cell doses demonstrated the most robust chondrogenesis (Fig. 3-5). The majority of the cells in each had a rounded, mature, chondrocytic phenotype and produced large quantities of ECM enriched for proteoglycans and collagen type II, with low collagen type I content. Although chondrogenesis was readily discernable in pellets at the 3000 vp/cell dose, they were noticeably smaller, with less abundant matrix and reduced cellularity. Aggregates formed from MSCs at the 10,000 vp/cell dose never increased in size, became fragmented within 1 to 2 weeks and disassociated by day 21. Aggregates formed from MSCs infected at doses below 100 vp/cell gradually diminished in size and at the end the incubation period were too small to paraffin embed.

Aggregates formed from MSCs infected with Ad.Sox9 showed the greatest response to the transgene at the 100 vp/cell to 1000 vp/cell range (Fig. 3-6). The volume and cellularity of the aggregates was considerably greater than those infected at

lower doses and the uninfected controls. The pellets showed robust extracellular matrix production with increased proteoglycans (indicated by metachromatic toluidine blue staining) and increased collagen type II content, consistent with that of articular cartilage. Distinct from the aggregates expressing the other growth factors, the morphology of the cells appeared considerably less hypertrophic. The cells (and their surrounding lacunae) were noticeably smaller in diameter, and contained less cytoplasmic volume. While aggregates from the 10,000 vp/cell dose also showed pronounced synthesis of cartilaginous matrix, these pellets were smaller in diameter.

During endochondral bone growth, the synthesis of collagen type X by growth plate chondrocytes, in conjunction with increased cellular volume, is indicative of their maturation to a terminal hypertrophic state. From this, the cells undergo apoptosis, and the residual cartilage matrix is replaced by bone. The morphology of the chondrogenic cells in the pellets expressing BMP-4, BMP-7 and Ihh suggests that prolonged stimulation with these factors has driven the differentiation of the MSCs toward an early hypertrophic phenotype, more so than overexpression of Sox9. To further examine this, we used immunohistochemistry to stain for the presence of collagen type X in chondrogenic sections from Ad.BMP-4 and Ad.Sox9 infected pellets. As shown in Figure 3-7, strong collagen type X staining is seen throughout the outer regions of the Ad.BMP-4 pellet, while the Ad.Sox9 pellets show little positive staining over background.

Adenoviral Delivery of CTGF Stimulates Proliferation of MSC Aggregates

In an effort to identify additional cDNAs with chondrogenic potential that might be of use in cartilage repair, we also tested the effect of adenoviral mediated expression of connective tissue growth factor (CTGF) in MSCs in aggregate culture. Similar to TGF-

β1, CTGF stimulates fibroblast proliferation, differentiation and extracellular matrix synthesis, and is important for chondrocyte differentiation and maturation[131].

As above, early passage of MSC cultures were infected with a range of doses of Ad.CTGF and placed into aggregate culture for 3 weeks. As with the other transgenes, the aggregates infected with certain vector doses visibly increased in size relative to controls, during incubation. Histologic analysis showed that the increase in pellet volume could be attributed to intense cellular proliferation, which was particularly evident at the 1000 vp/cell dose (Fig. 3-8). However, despite a clear biological response to the transgene product, there was no apparent chondrogenic differentiation or cartilage matrix protein production. At higher levels of Ad.CTGF infection, such as those at 10,000 vp/cell and higher, the cells did not proliferate; the aggregates remained small, and they fragmented after 21 days of culture.

Combinations of Sox9 with BMPs Induced Chondrogenesis

The process of chondrogenesis *in vivo* is complex, requiring the orchestrated expression and interplay of numerous growth and transcription factors. Using gene delivery we have shown that overexpression of certain of these proteins individually is sufficient to initiate chondrogenic differentiation of mesenchymal progenitors *in vitro*. The resulting phenotype of the cells and their synthesized matrix demonstrate key hallmarks of articular cartilage, but fall short of the architecture of the native tissue. Indeed it is likely that successful regeneration of articular cartilage will require the delivery of multiple factors and temporal regulation of their expression. Toward this direction we initiated preliminary studies of the effects of gene combinations on *in vitro* chondrogenesis. For these experiments, we co-expressed the most potent of the chondroinductive genes we have tested thus far, BMP-4 and BMP-2 (characterized in

earlier studies in our group), with the chondrogenic transcription factor Sox9. We hypothesized that the enhanced expression of Sox9 would help to stabilize the chondrocyte phenotype and block progression to hypertrophy, significantly improving the quality of the resulting cartilaginous tissue.

In exploring gene combinations, we infected MSCs in monolayer with pairs of vectors at doses that individually produced a robust chondrogenic response: i.e. Ad.BMP-4 at 100 and 1000 vp/cell; and Ad.BMP-2 at 1000 vp/cell were mixed with Ad.Sox9 at 100 or 1000 vp/cell. We then seeded the genetically modified cells into aggregate culture and analyzed the effects of co-expression of the respective transgenes on chondrogenesis in our *in vitro* assay.

During the incubation period, the majority of the cell aggregates showed a steady increase in diameter, and none of the gene combinations caused the pellets to reproducibly disintegrate. Histological analysis showed enhanced production of cartilage matrix components in pellets from each of the various treatment groups, relative to controls, indicating that the transgene products were functionally expressed. However, none of the gene combinations appeared to enhance chondrogenesis *in vitro* relative to single gene delivery.

While the results of these experiments were somewhat negative, they serve to emphasize the complexity of cell signaling pathways and cellular differentiation. The adenoviral vector, with its CMV-promoter driven expression cassette, is designed to provide extraordinarily high levels of transgenic expression, and likely serves to continually saturate ligand-specific surface receptors and downstream signaling pathways. Effectively modulating the effects of such a powerful stimulus may prove to

be challenging, requiring far more sophisticated gene delivery approaches than the crude systems used here.

Wistar Rat Responses to Adenoviral Transgene Delivery

When designing a gene-based strategy for cartilage repair, the selection of an appropriate transgene is fundamental to the overall success of the procedure—its efficacy and its safety. For most applications, it is probable that gene delivery vectors, their secreted transgene products, or genetically modified cells will emigrate from the repair site to adjacent tissues. Because many of the agents considered to have the greatest chondrogenic activity are pleiotropic, with broad stimulatory activities in various tissues and cell types, it is critical to thoroughly understand the possible impact of their constitutive, local overexpression on the joint tissues and the health of the prospective patient.

Using our *in vitro* assay we have identified several proteins (BMP-4, BMP-7, Ihh, Sox9) with the capacity to drive mesenchymal chondrogenesis when expressed in MSCs as transgenes. In earlier related studies we found that the cDNA for BMP-2 was also highly effective. Having established the relative chondrogenic activity of these genes *in vitro*, we wanted to determine their potential to stimulate pathologic side effects when over-expressed intra-articularly. For these *in vivo* studies, we selected the knee of the rat as our animal model since the rat is the smallest common experimental animal with a defined joint space that can be reliably targeted by intra-articular injection.

We delivered the adenoviral vectors containing BMP-2, BMP-4, BMP-7, Sox9 and Ihh bilaterally at doses of 5×10^8 and 2×10^9 vp/µl to the knees of Wistar rats (2 rats per vector and dose). Parallel groups of rats were similarly injected with Ad.GFP to control for the effects of adenoviral delivery. At 7 days post injection the animals were killed,

and the knees were harvested, decalcified and processed for histologic analysis. (The vector doses used in these experiments were based on our previous experience with adenoviral gene transfer to joints. They were intended to provide sufficient transgene expression to enable a robust depiction of the stimulatory properties of each transgene product intra-articularly without endangering the overall health of the animals.)

H&E stained sections of the knees receiving Ad.GFP showed a mild leukocytic infiltration and a slight thickening of the synovial intima, typical of an adenoviral injection into the joint. Otherwise, the morphology of the tissues was normal. In stark contrast, adenoviral delivery of the various chondrogenic cDNAs elicited a wide variety of biological responses. The most dramatic effects occurred in the joints injected with Ad.BMP-2. The representative field shown in Figure 3-10 shows massive hypertrophy of the synovium and capsular tissues that completely displaced the adipose layer that normally supports the synovial lining. The expanded tissue was fibrotic in many areas and populated with spindled fibroblasts. About half of the tissue mass, (particularly those areas proximal to articular cartilage), was strikingly chondrogenic and was heavily populated with chondrocytes and chondroblastic cells. The joints receiving Ad.BMP-4 and Ad.BMP-7 showed a dramatic hypertrophy of the synovial lining, caused by extensive chondrometaplasia throughout the subsynovium. There was little if any fibrotic component to the expanded tissues; the increased volume was comprised almost exclusively of chondroblastic cells.

Distinct from overexpression of the BMPs, adenoviral delivery of Ihh induced a mild to moderate fibrosis broadly across the synovial lining, marked by an increase in both collagen fibers and fibroblastic cells. Ad.Sox9 was associated with a moderate

synovitis throughout the lining, with increased numbers of synovial fibroblasts and infiltrating leukocyes relative to the Ad.GFP control.

Discussion

Our experiments demonstrate that adenoviral-mediated delivery of chondrogenic growth factors to MSCs serves as an effective method to induce chondrogenic differentiation of these cells in aggregate culture. In comparing multiple growth factor cDNAs delivered across a range of adenoviral doses, we found that delivery of ~100 to 1000 vp/cell provided the most effective levels of transgene expression. Of the factors we evaluated, BMP-4 induced the most robust chondrogenesis, with aggregates exhibiting a dense extracellular matrix populated with chondrocytic cells resembling those found in deep articular cartilage. BMP-7 and Indian hedgehog exhibited less desirable chondrogenic responses; while aggregates showed extracellular matrix production and the presence of chondrocytic cells, these secreted factors were prone to drive cells toward terminal differentiation and hypertrophy. Sox9, while less prone to induce hypertrophic differentiation, induced production of cartilaginous matrix and maintained the cells at a prehypertrophic state, as shown with collagen type II expression and the absence of collagen type X. CTGF induced proliferation of the cells at the 1000 vp/cell dose but failed to promote chondrogenesis in our system.

Members of our lab have shown previously that gene delivery of BMP-2 and its expression in the 10-100 ng/mL range is required to achieve optimal chondrogenic differentiation of MSCs. Here, using a similar system, we have shown that BMP-4 production of <10 ng/mL is sufficient to promote robust chondrogenic differentiation of the cells. These data indicate that BMP-4 is highly potent, and it induces chondrogenic responses equal to those observed at higher ng/mL levels of BMP-2 production.

Overall, in pellets modified to express secreted growth factors, we observed more substantial chondrocytic differentiation than in those expressing the intracellular factor Sox9. With secreted factors, even though a proportion of MSCs within the pellet express the transgene, they have both autocrine and paracrine effects, which may enhance chondrogenesis throughout neighboring cells within the aggregate. Conversely, transgene products that function intracellularly may have advantages for use in studies *in vivo* because the risk of exposing cells in neighboring tissues such as the synovium, meniscus, tendon, and muscle to pleiotropic growth factors will be significantly reduced.

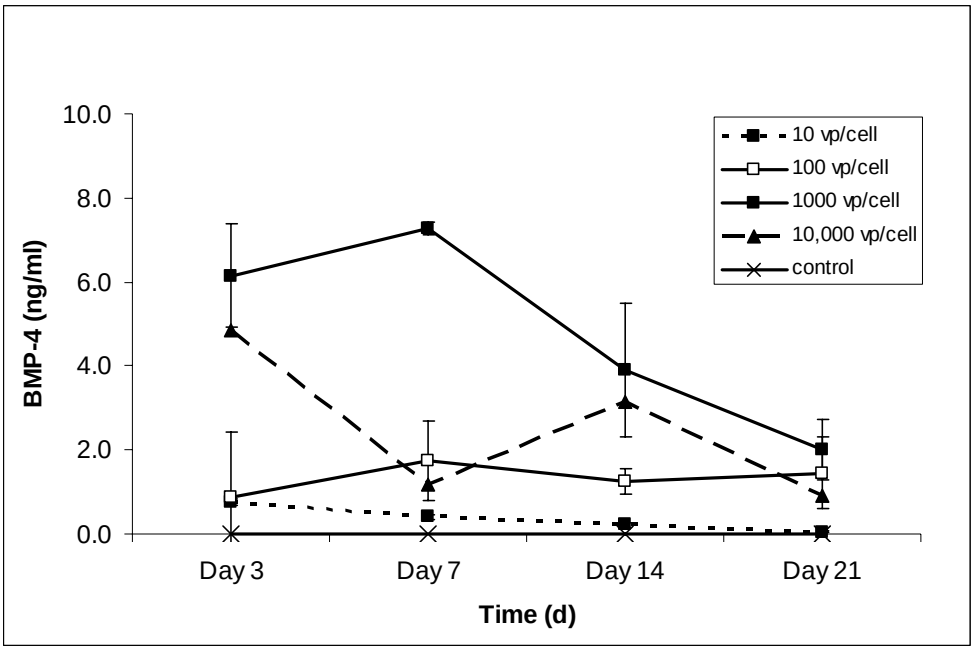
Chondrogenesis *in vivo* is a complex process involving the coordinate interplay of numerous factors. As such, it is probably unrealistic to expect complete articular cartilage regeneration via transfer and expression of a single gene. Based upon preliminary work demonstrating enhanced chondrogenesis when IGF-1 was delivered to MSCs in combination with TGF-β1 or BMPs, we opted to similarly test our battery of transgenes in combinations. As all potential combinations of chondrogenic cDNAs and doses are too numerous to test feasibly, we used the information gathered about each single transgene to deduce the most effective strategy. We hypothesized that delivery of Sox9, which is expressed intracellularly, combined with the secreted proteins, BMP-2 or BMP-4, would provide an effective combination to drive chondrogenic differentiation in MSCs while maintaining cells in a pre-hypertrophic state. Although the combinations we evaluated did not significantly alter chondrogenesis, they illustrate the complexity of modulating cell signaling.

Although each transgene tested induced measurable chondrogenic effects on bovine MSCs in aggregate culture, the microenvironment of the joint space *in vivo* is

quite different. Intra-articular delivery of Ad.BMP-2, -4, -7, Ad.Ihh, and Ad.Sox9 to the intact knees of Wistar rats enabled us to evaluate potential side effects of ectopic growth factor expression. BMP-2, -4 and -7 prompted cellular expansion and ectopic cartilage formation to varying degrees while Ihh caused a weak fibrosis across the synovial lining and Sox9 induced mild inflammation. Although these rat knees likely demonstrate the worst case scenario of potential side effects of gene enhanced cartilage repair, they provide a vivid representation of the potency of the various transgene products and their capacity to impact the biology of the articular connective tissues. Conversely, they likewise demonstrate the exquisite sensitivity of synovial fibroblasts to proliferate and differentiate following stimulation with certain BMPs. Methods designed to induce cellular differentiation in cartilage and bone repair *in vivo* should take into account the high capacity for toxic side effects in adjacent tissues.

Although adenoviral vectors have a reputation for causing adverse immune responses *in vivo*, this vector system may have a future in ex vivo approaches to tissue repair. With their ability to readily infect MSCs and induce a high level of gene expression, adenoviral vectors can serve as efficient protein delivery vehicles to modify cells that are introduced to sites of tissue damage. Such a method will bypass the potential for host immune responses while allowing for potent expression of chondrogenic cDNAs.

A).



B).

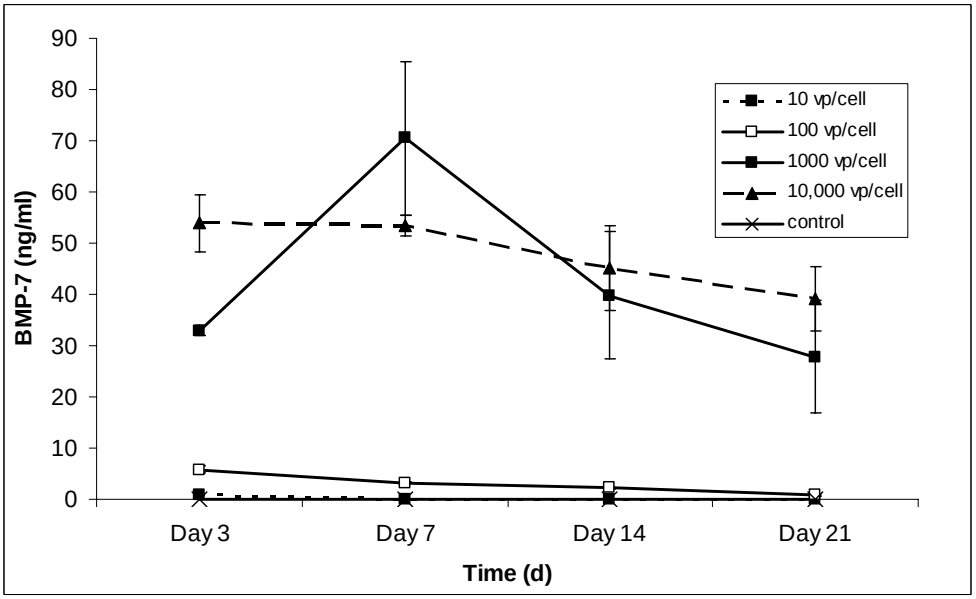


Figure 3-1. ELISAs of conditioned media from MSC aggregates transduced with BMP-4 and BMP-7 indicated protein production in response to viral doses administered. Triplicate samples of conditioned media were obtained at days 3, 7, 14, and 21 following a 24 hour incubation, and protein levels were quantified by ELISA. Data are shown as the mean ± SEM. A) BMP-4 ELISA indicates a dose-dependent response following Ad.BMP-4 transduction. The decrease in protein production following the 10,000 vp/cell dose indicates toxicity in response to excessive adenovirus. B) BMP-7 ELISA shows protein production in response to Ad.BMP-7 transduction at higher concentrations than those observed with BMP-4.

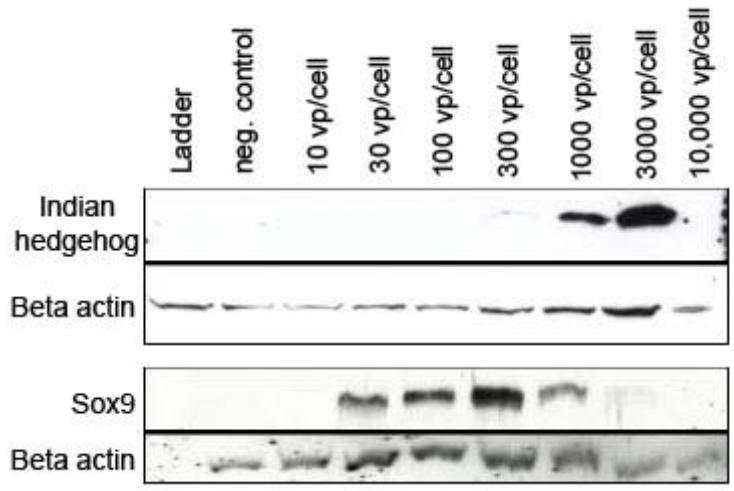


Figure 3-2. Western blotting confirmed transgene expression following adenoviral gene delivery to MSCs. As shown in the top panel, Ad.Ihh was delivered at a range of doses to bovine MSCs in monolayer, in a minimal volume of media. Media were replaced 24 hours later, and harvested at 48 hours post-infection for detection of Ihh protein. The presence of bands at 300, 1000, and 3000 vp/cell doses correlated with the matrix synthesis and chondrogenic differentiation observed with toluidine blue staining of Ihh aggregates at these doses (Fig. 3-5). Following detection of Ihh, antibodies were stripped from the membrane, and β-actin was detected as a loading control. In the bottom panel, Ad.Sox9 was delivered at a range of doses, and 48 hours post-transduction, cells were harvested with a cell scraper and lysed with chilled homogenization buffer. Because the transcription factor Sox9 remains intracellular, cell lysates were used to detect Sox9 production. As with Ihh, the range in which Sox9 bands were visible correlated to the dose range in which proteoglycan synthesis was most robust as shown through toluidine blue staining (Fig. 3-6). Again, β-actin was used as a loading control.

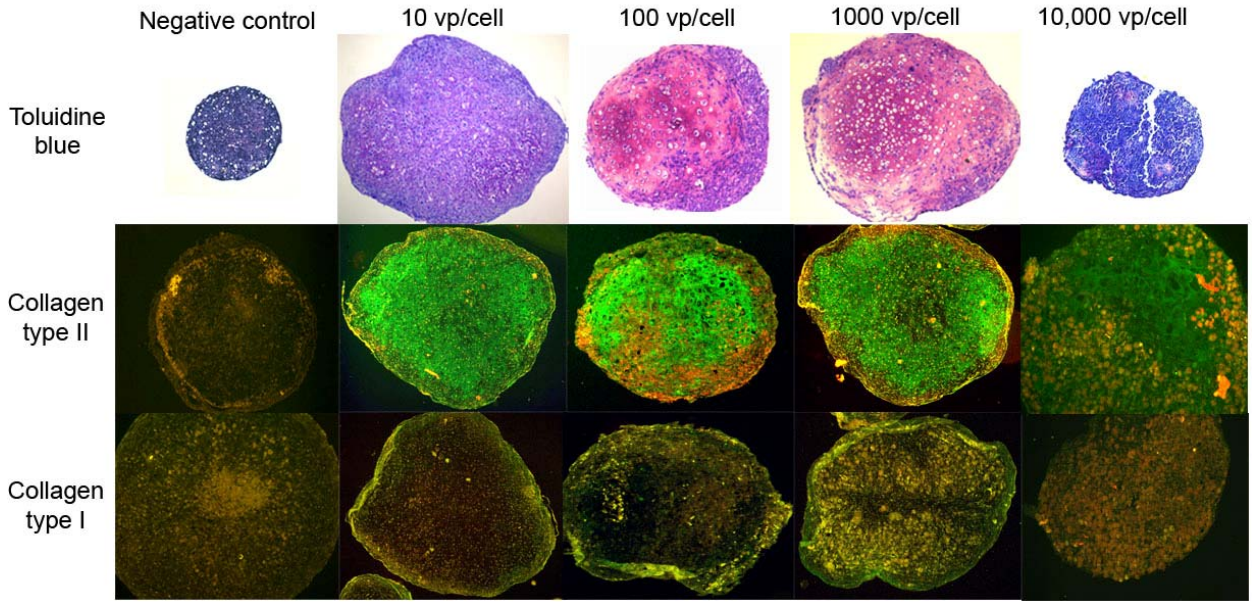


Figure 3-3. MSC aggregates expressing BMP-4 were evaluated for chondrogenesis through toluidine blue staining of proteoglycans and immunohistochemistry for collagen types I and II. Aggregates infected with 10, 100, or 1000 vp/cell were highly cellular, the proteoglycans of their synthesized matrix stained deep purple with toluidine blue, and they showed positive immunostaining for collagen type II, as shown by green fluorescence. The 10,000 vp/cell dose resulted in fragmented pellets with little to no cellular differentiation or matrix production. All aggregates were negative for collagen type I production.

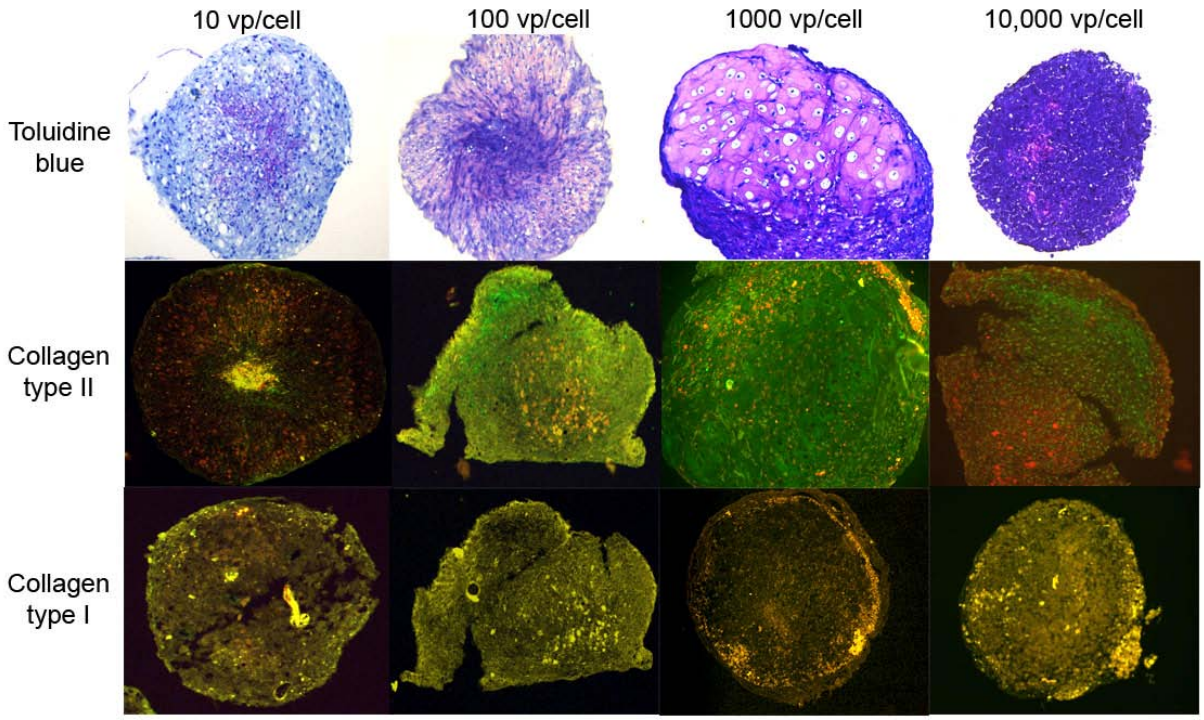


Figure 3-4. MSC aggregates infected with Ad.BMP-7 over a range of doses yielded pronounced chondrogenesis at the 1000 vp/cell dose only. Doses of 10 and 100 vp/cell showed minimal toluidine blue staining and poor collagen type II expression. Aggregates receiving 1000 vp/cell synthesized dense, uniform extracellular matrix enriched for proteoglycans and collagen type II. Rounded, chondrocytic cells were present in the matrix, and many appeared to advance toward hypertrophy, with increased cytoplasmic volume as shown above. Pellets receiving 10,000 vp/cell showed no evidence of matrix protein production or chondrogenic differentiation. The high dose of adenoviral vector proved toxic. All aggregates were negative for collagen type I production.

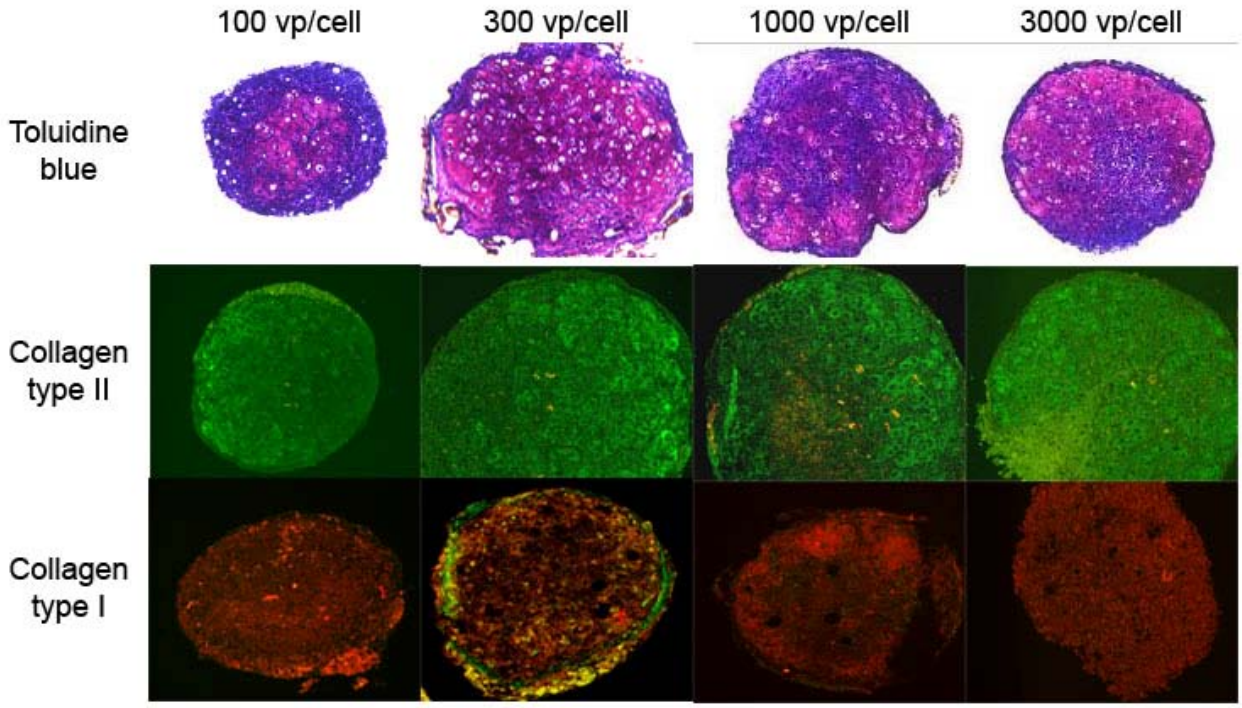


Figure 3-5. Indian hedgehog aggregates expressed proteoglycan and collagen type II following adenoviral gene delivery. Indian hedgehog (Ihh) expression in pellets after 21 days in culture revealed a dose-dependent response. Aggregates in the range of 300 to 1000 vp/cell demonstrated the most robust chondrogenesis by forming large, rounded aggregates that secreted proteoglycans, forming a cartilage matrix that contained chondrocytic cells. MSCs receiving doses of 100 vp/cell or less or 10,000 vp/cell or greater formed fragmented aggregates that did not proliferate, were smaller than the non-transduced controls (shown in Figure 3-3), and disassociated by the 21-day timepoint. All aggregates were negative for collagen type I.

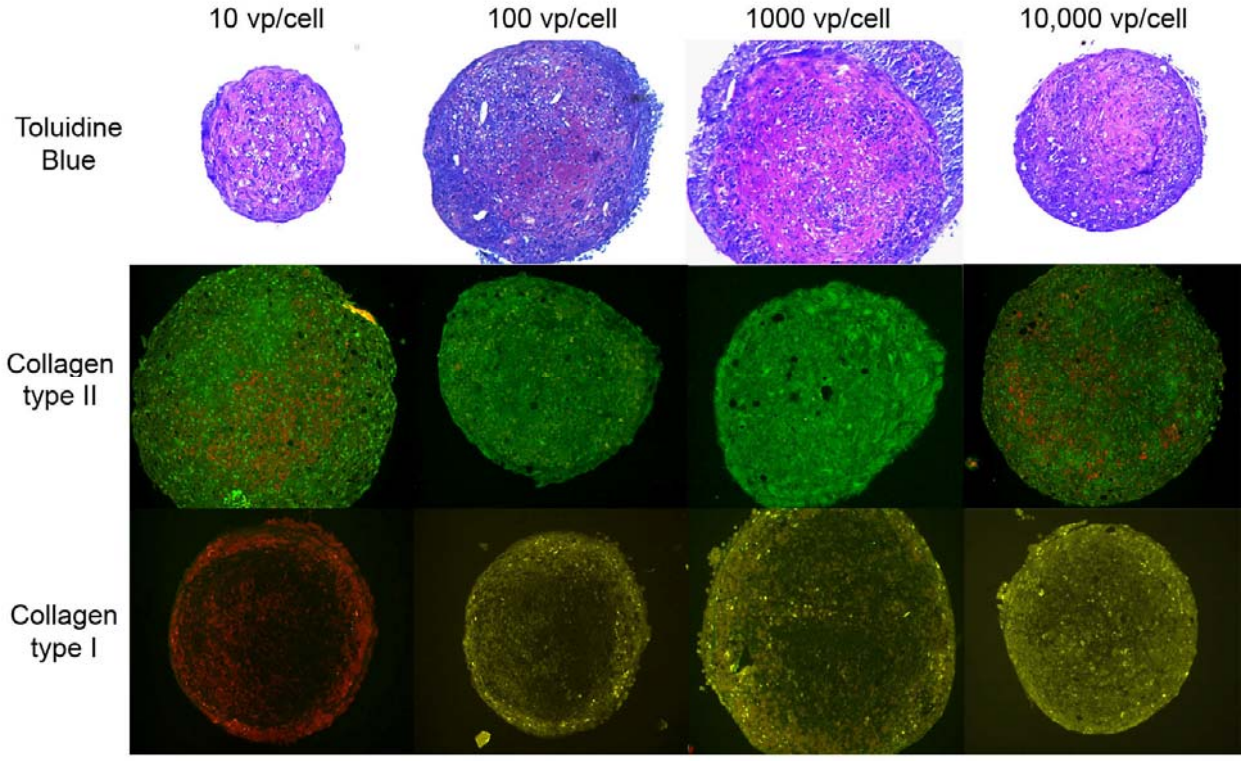


Figure 3-6. Sox9 induced cellular proliferation and matrix synthesis with less differentiation than other transgenes tested. Sox9 expression in MSCs resulted in aggregates of a uniform round shape consisting of proteoglycan matrix and prehypertrophic-like cells. Similar to the other transgenes tested, the most chondrogenic effects were observed after delivery of 100 to 1000 vp/cell. Doses above and below this range continued to promote collagen type II expression, yet they were insufficient to promote robust cellular proliferation. All aggregates were negative for collagen type I production.

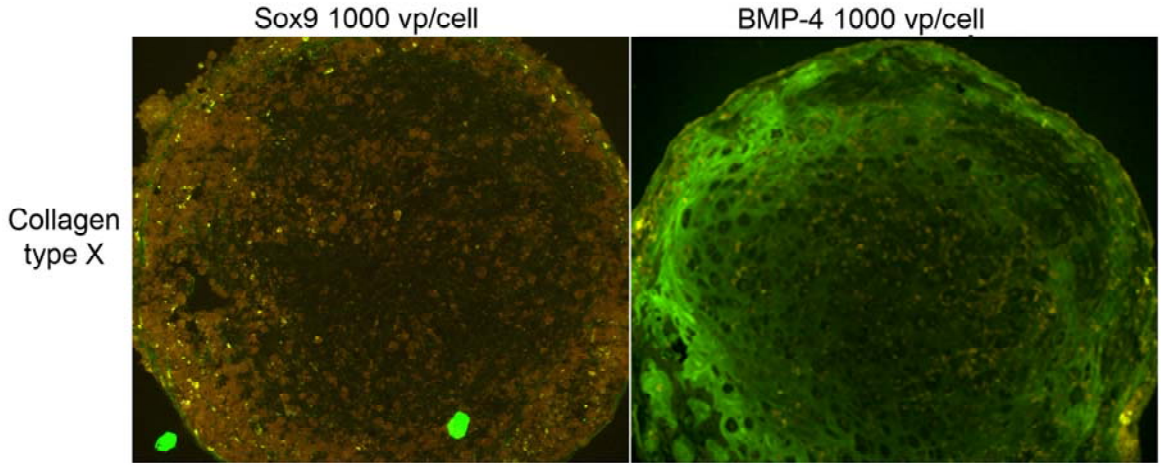


Figure 3-7. Collagen type X staining of Sox9 and BMP-4 aggregates. BMP-4 aggregates express collagen type X and undergo differentiation toward a terminal hypertrophic state, shown through green fluorescence. Sox9 pellets show no signs of collagen type X, with staining equivalent to background levels, indicating their maintenance at a prehypertrophic state of differentiation. 20x magnification.

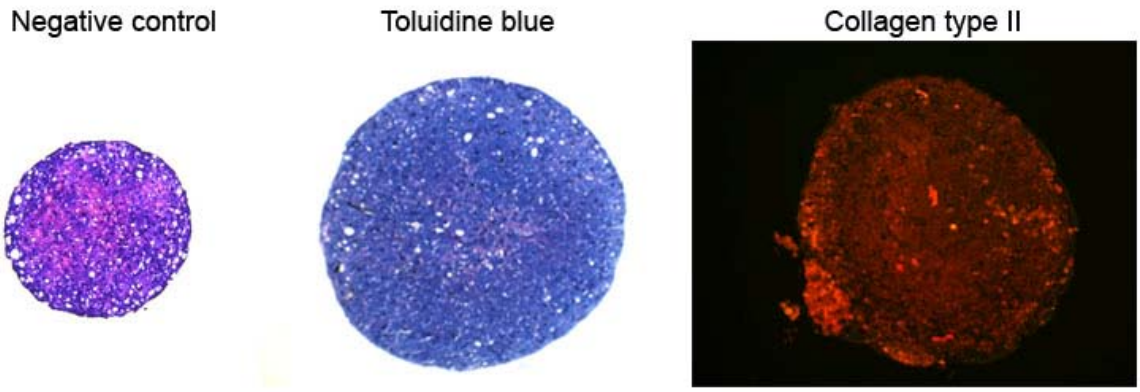


Figure 3-8. CTGF expression in MSC aggregates promotes cellular proliferation but no chondrogenic differentiation. Delivery of 1000 vp/cell of Ad.CTGF resulted in cellular proliferation and large aggregates; however, toluidine blue staining showed no evidence of proteoglycan matrix production or chondrogenic differentiation. Likewise, aggregates were negative for collagen type II production (lack of green fluorescence) when evaluated immunohistochemically. Cells receiving viral doses of 10 and 100 vp/cell formed aggregates that were too small to paraffin embed. Viral doses of 10,000 vp/cell or greater caused aggregates to fragment and disassociate in culture. 10x magnification.

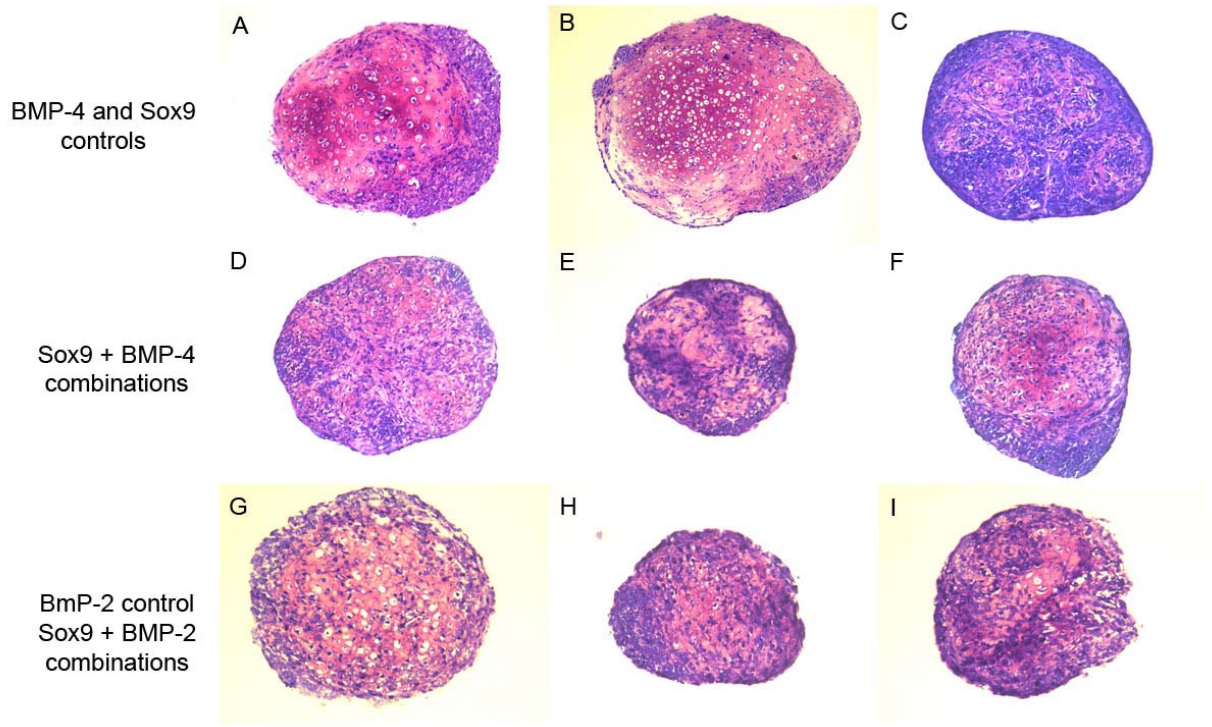


Figure 3-9. Sox9 in combination with BMP-4 and BMP-2 in MSC aggregate culture had no additive effects upon chondrogenesis. Panels A, B, C, and G are single doses, D-F, H-I are combinations. Toluidine blue staining shows matrix synthesis at all doses; however, combinations resulted in smaller aggregates that showed less matrix synthesis and less chondrogenic differentiation. A) Ad.BMP-4 100 vp/cell, B) Ad.BMP-4 1000 vp/cell, C) Ad.Sox9 1000 vp/cell, D) Ad.Sox9 1000 vp/cell + Ad.BMP-4 1000 vp/cell, E) Ad.Sox9 100 vp/cell + Ad.BMP-4 1000 vp/cell, F) Ad.Sox9 100 vp/cell + Ad.BMP-4 100 vp/cell, G) Ad.BMP-2 1000 vp/cell, H) Ad.Sox9 1000 vp/cell + Ad.BMP-2 1000 vp/cell, I) Ad.Sox9 100 vp/cell + Ad.BMP-2 1000 vp/cell. Images are displayed at 10x magnification.

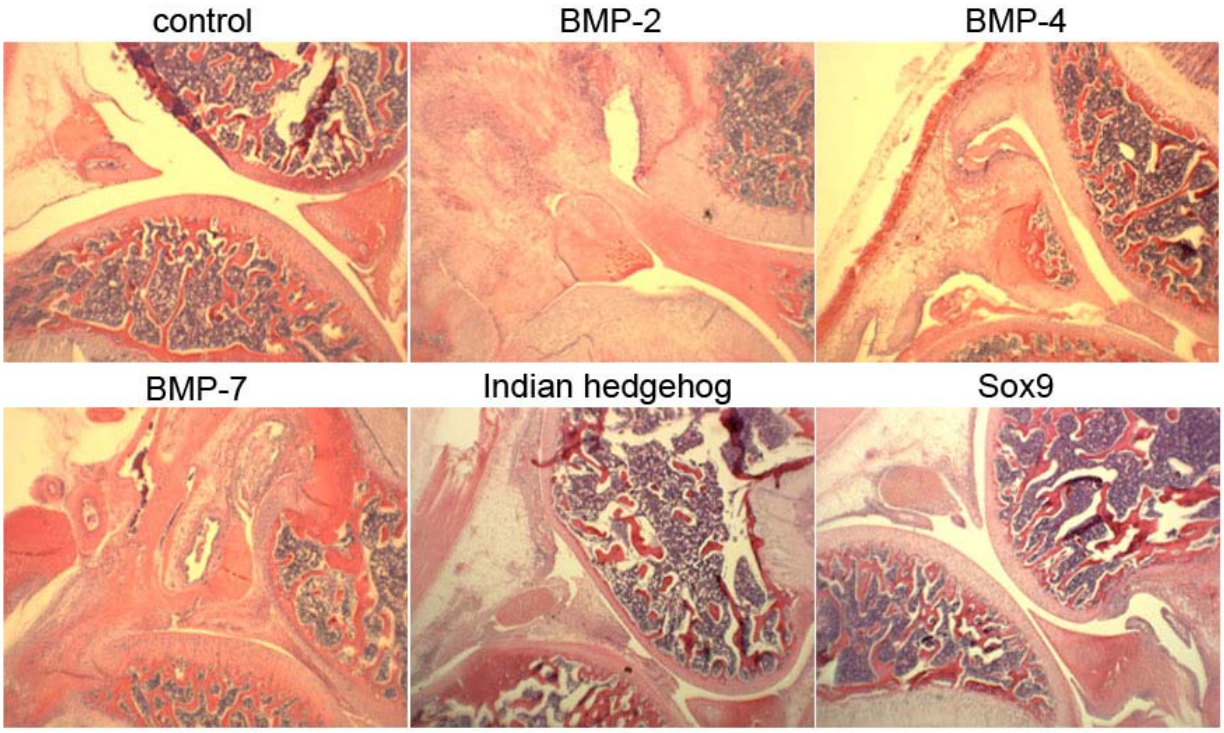


Figure 3-10. Wistar rat knees exhibited varying responses to adenoviral delivery of pleiotropic transgenes. Adenoviral vectors containing GFP and the chondrogenic cDNAs BMP-2, BMP-4, BMP-7, Ihh, and Sox9 were injected intra-articularly into both knee joints of healthy male Wistar rats at doses of 5 x 10^8 and 2 x10^9 vp/µl. Effects from BMPs ranged from the massive synovial hypertrophy, fibrosis, and ectopic chondrogenesis resulting from Ad.BMP-2 to moderate synovial hypertrophy and chondrometaplasia with little fibrosis from Ad.BMP-4 and Ad.BMP-7. Indian hedgehog (Ihh) induced synovial fibrosis and Sox9 was associated with a mild synovitis, comparable to that observed in the Ad.GFP control animals.

CHAPTER 4
COMPARISON OF THE EFFECTS OF ADENOVIRAL, LENTIVIRAL, AND AAV TRANSGENE DELIVERY TO MESENCHYMAL STEM CELLS

Introduction

Our studies have focused on the genetic modification of MSCs for use in the repair and regeneration of articular cartilage. We have found that adenoviral-based vectors efficiently transduce these cells, and readily enable the expression of transgene products at biologically relevant levels. Indeed, adenoviral-mediated delivery and expression of certain growth factor cDNAs in MSCs can be used to effectively direct the differentiation of these cells along chondrogenic pathways. While highly useful tools for studies *in vitro* and in experimental animals, the most widely used generations of this vector still contain the majority of the native adenoviral genes. Low-level expression of these genes in transduced cells is frequently associated with inflammatory reactions *in vivo* and elimination of transduced cells by cytoxic T cells in immunologically competent hosts.

The relative ease of use of the adenoviral vector system, coupled with its highly efficient gene transfer, provided us with a valuable tool to screen a battery of candidate cDNAs for their chondrogenic potential. From this screen we have identified several that merit evaluation *in vivo* in cartilage repair models. While the adenovirus has been the workhorse of our *in vitro* studies, its immunogenic profile excludes it from use in humans and may negatively impact the process of tissue repair. In an effort to identify a gene delivery system more suitable for use in animal models and possible clinical application, we evaluated the utility of the well-characterized vector systems currently available, (lentivirus, adeno-associated virus and non-viral transfection) for their capacity to effect gene-mediated chondrogenesis of MSCs.

For these studies, we first inserted the cDNA for BMP-4 into AAV, lentivirus and plasmid-based vectors. We then determined the capacity of each vector to deliver the BMP-4 transgene to bovine MSCs and stimulate chondrogenesis in the aggregate culture system. The BMP-4 cDNA was selected for these studies as it appeared to have the greatest chondrogenic activity in adenovirally-transduced aggregates. Thus, the ability to functionally deliver this cDNA would set the minimum standard for efficacy.

Results

Plasmid DNA Transfection of MSCs Results in Transient Expression

To generate the plasmids needed to produce recombinant viruses, we inserted the cDNA for human BMP-4 into specific CMV-promoter driven vector constructs: AAV (pHPA-trs-sk-BMP-4), adenovirus (pAdlox-BMP-4), lentivirus (pCDH-puro-BMP-4). We then used these plasmids in DNA transfections to gauge expression resulting from nonviral gene transfer. Plasmid DNA transfections of BMP-4 cDNA were carried out in 24-well plates of bovine MSCs grown to approximately 80% confluence. DNA was diluted in Opti-MEM (Gibco) and mixed with lipofectamine (Invitrogen) at a ratio of 1µg of DNA to 2.5 µl of lipofectamine. DNA:lipofectamine complexes were added drop-wise to cells and allowed to incubate for 24 hours. At 48 and 72 hours post-transfection, conditioned media were collected for quantification of BMP-4 by ELISA. As shown in Figure 4-1, transfections with each plasmid construct yielded BMP-4 in the 1.0 ng/mL range, which was significantly higher than background levels from control cells. However, transgene expression was transient. ELISAs of conditioned media at 72 hours following transfection showed concentrations of BMP-4 near background levels (data not shown). Such low levels of short-lived expression were not sufficient to drive chondrogenesis in our aggregate culture assay.

Lentiviral Transduction of MSCs Proves Challenging

Preliminary data showing robust GFP expression in 293 cells following lentiviral transduction established a basis for the use of lentivirus to deliver chondrogenic transgenes, such as BMP-4, to bovine MSCs in monolayer (Fig. 4-2). To produce recombinant lentivirus, we implemented a four plasmid transfection procedure adapted from the ViraPower expression system (Invitrogen). Transducing vectors expressing the desired transgenes (GFP and BMP-4) were generated via the insertion of the specific cDNAs into the pLenti4/V5-DEST vector via homologous recombination. The resulting expression plasmid was mixed with the three necessary packaging plasmids, denoted pLP1, pLP2, and pLP-VSVG. Plasmid DNA was complexed with lipofectamine and delivered to monolayer cultures of 293FT cells. Despite numerous attempts, we were unable to generate sufficiently high viral titers with the ViraPower system. In an effort to improve viral production, we then evaluated the plasmid, pCDH-puro, for its potential to generate appreciable titers of lentivirus.

Lentivirus generated using the pCDH-puro-BMP-4 construct induced BMP-4 expression in monolayer MSCs (Fig. 4-3), but again the viral titers generated were insufficient to induce chondrogenesis within our pellet culture system. In an effort to enhance this, we selected for cells that expressed BMP-4 using puromycin. We cultured the cells under selection in preparation for subsequent expansion and use in aggregate culture; however, the combination of BMP-4 expression and puromycin in the cell culture medium appeared to inhibit MSC growth and proliferation, rendering the cells unsuitable for aggregate culture. Despite repeated attempts with each system, we were unable to produce reasonable titers for use directly without the need for cell selection.

Adenoviral-Mediated Delivery of BMP-4 to MSCs Induces Chondrogenesis

As discussed extensively in Chapter 3, bovine MSCs were transduced in monolayer with an E1, E3-deleted serotype 5 adenoviral vector carrying human BMP-4 cDNA and grown in aggregate culture in our *in vitro* chondrogenesis assay. Histologic evaluation of aggregates after 21 days in culture showed that Ad.BMP-4 effectively induced chondrogenesis in MSC aggregates as indicated by staining for matrix proteoglycans and type II collagen. Aggregates infected with 100 to 1000 vp/cell were large, round, and produced a dense, uniform matrix populated with rounded chondrocytic cells in lacunae, morphologically similar to the transitional or deep zones of articular cartilage (Fig. 3-3).

Self-Complementary AAV-Mediated Delivery of BMP-4 to MSCs is Comparable to Adenovirus

Numerous AAV capsid serotypes are available for cross-packaging of vector genomes, and each one changes the tropism of the virus. In an effort to identify the most effective AAV serotype to target bovine MSCs, we packaged scAAV vector containing GFP into serotypes 1, 2, 5, and 8. Following administration of 10^4 vg/cell to MSCs in monolayer, transduction efficiency was determined visually by GFP expression. As shown in Figure 4-4, after 24 hours, GFP expression in transduced cells was evident for serotypes 2 and 5, and the intensity increased over the course of 7 days. By day 7, all 4 serotypes exhibited detectable GFP expression, but type 2 was by far the most robust, followed by type 5 (Fig. 4-4). The order of efficacy for AAV transduction of MSCs was AAV2>5>1>8.

Based upon these data, we packaged the scAAV vector plasmids containing BMP-4 (pHPA-trs-sk-BMP-4) to generate AAV serotypes 2 and 5 for use in our *in vitro*

chondrogenesis assay. We infected MSCs over a 3-fold range of doses from 10^2-10^4 vg/cell. After 21 days in culture, the pellets were evaluated histologically with toluidine blue staining for proteoglycans. As shown in Figure 4-5, consistent with data from GFP expression, infection with scAAV2-BMP-4 induced the most complete chondrogenic response observed to date. The 3000 vg/cell dose yielded aggregates that were large, round, highly cellular, and strikingly uniform in appearance. Aggregates showed robust proteoglycan synthesis with chondrocytic cells uniformly distributed throughout. Aggregates receiving the 1000 vg/cell dose exhibited similarly intense matrix production, yet the periphery of these pellets was differentiated. Conversely, scAAV5-BMP-4 delivered across the same range of doses produced aggregates with little apparent chondrogenic differentiation but showed evidence of enhanced proteoglycans synthesis.

Discussion

Using our pellet culture system, we compared the ability of recombinant lentivirus, AAV, adenovirus and plasmid transfection to deliver and functionally express the BMP-4 cDNA in bovine MSCs, as indicated by chondrogenic differentiation *in vitro*. The purpose of these studies was to identify from among the most developed gene transfer systems, the one most suitable for use *in vivo* in studies of cartilage repair. As shown in Table 4-1, each vector system offers advantages for gene therapy with certain limitations for human applications.

In our hands, plasmid transfection and lentiviral transduction were unsatisfactory for our *in vitro* chondrogenesis assay. While DNA transfection of MSCs provided BMP-4 expression near the minimal functional level (~1 ng/mL), it persisted for just over 48 hrs, which was insufficient to induce a meaningful response. With the lentivirus, technical

difficulties prevented us from reproducibly generating vectors at usable titer. While we had intended to evaluate these systems based on transgene expression profiles, in the case of lentivirus, vector production proved too cumbersome to be practical for our purposes.

Recombinant AAV is emerging as the vector of choice for human gene therapy applications because of its perceived safety, as the transduced cells do not express viral genes. In order to improve the efficiency of AAV transduction in our investigations, we utilized self-complementary AAV vectors. In a study completed by Kay et al., scAAV2 transduced synovial fibroblasts far more efficiently than conventional single-stranded AAV vectors, and expression levels of the hIL-1Ra transgene were similar to that noted previously for adenovirus and lentivirus vectors[132].

Self-complementary AAV mediated gene delivery of BMP-4 induced a strikingly robust and uniform chondrogenic response, generating cartilaginous pellets qualitatively superior to any other method. If these results hold, they indicate that AAV is clearly the optimal vector system for this type of application. Our goal was to identify a system that could provide functional transgene expression but was less immunogenic than adenovirus. In most gene transfer applications, relative to AAV, adenoviral vectors generate considerably higher levels of transgene expression. Based on this, we expected that AAV would provide only borderline functional levels of expression, forcing a trade-off between efficacy and low immunogenicity. That AAV may be capable of mediating more effective transgenic expression and a more favorable immune profile than adenovirus represents a significant finding.

At this point we do not know what may have contributed to the enhanced chondrogenic response provided by scAAV BMP-4. It is possible that it was fortuitous, caused by changes in culture conditions beyond our awareness. Alternatively, it is possible that the differences in morphology between pellets genetically modified with adenovirus and AAV can be attributed to the nature of their vector genomes. The adenoviral vector genome is about 35 kb in length and contains nearly all of the native viral genes. The scAAV vector is ~2.5 kb and is comprised only of an expression cassette flanked by small DNA hairpins. In our chondrogenic assays we infected MSCs in culture with a range of adenoviral vector particles. Pellets receiving the highest viral doses frequently showed toxic effects reflected by reduced transgene expression or loss of viability. At lower doses these effects may be less pronounced, but still negatively influence the biology of the infected cells, resulting in pellets whose morphology is asymmetric.

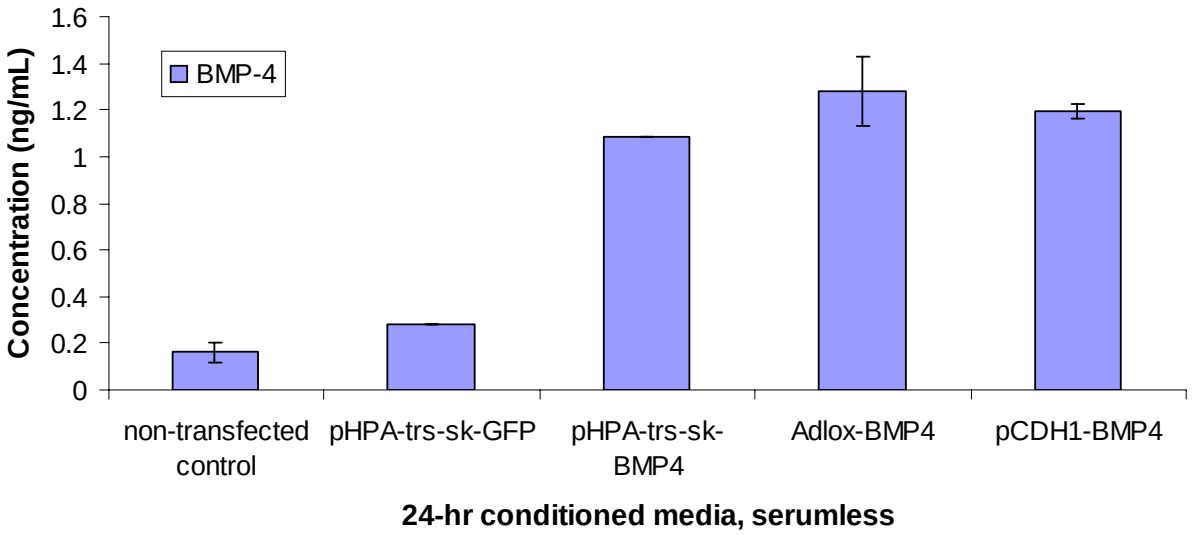


Figure 4-1. ELISA of conditioned media demonstrates expression of BMP-4 as measured 48 hours post-transfection. Bovine MSCs in monolayer were transfected with lipofectamine:cDNA complexes as directed by the manufacturer (Invitrogen). BMP-4 expression from MSCs transfected with AAV (pHPA-trs-sk), adenovirus (Adlox), and lentivirus (pCDH1) plasmid constructs carrying human BMP-4 cDNA was significantly higher than non-transfected control cells; however, this expression was transient. After 72 hours, BMP-4 expression matched that of negative controls (not shown).

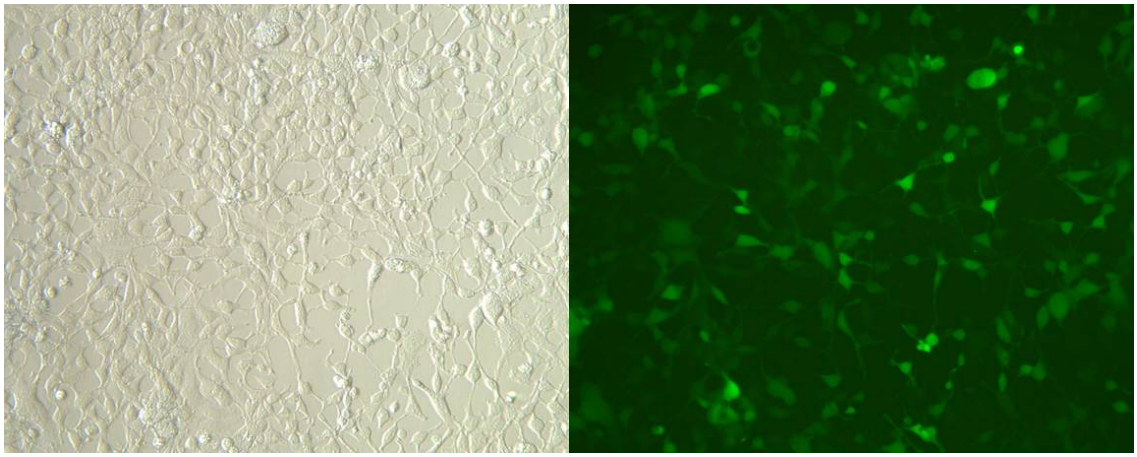


Figure 4-2. GFP expression in 293 cells 72 hours after infection with 15 µl of LV-GFP in a minimal volume of serumless media. A) brightfield B) fluorescence, 20x magnification. This demonstrates our ability to generate lentivirus expressing GFP. This virus was then applied to bovine MSCs to gauge expression of lentivirus in primary cells prior to generating lentivirus carrying BMP-4 cDNA.

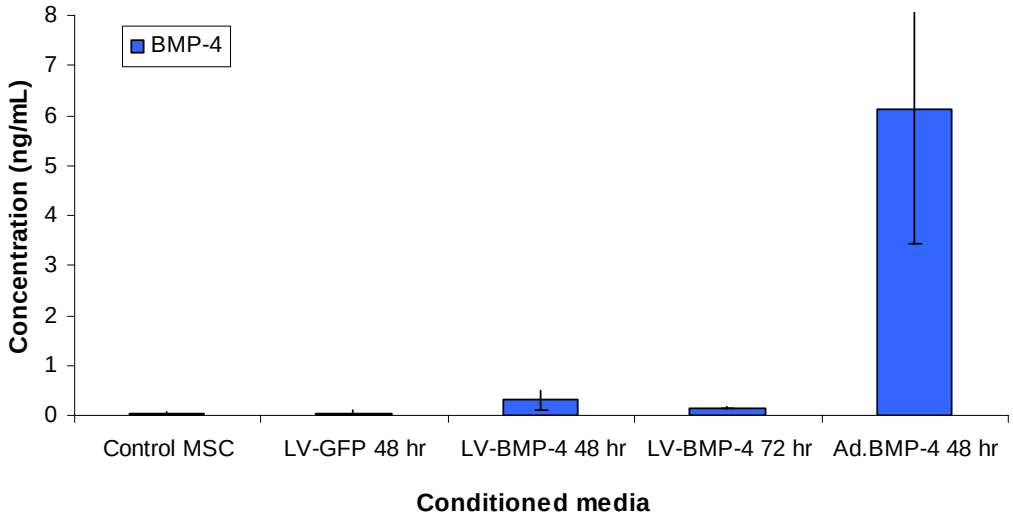


Figure 4-3. LV-BMP-4 expression was significantly less than Ad.BMP-4 expression in bovine MSCs. Bovine MSCs were grown to 70% confluence in monolayer prior to infection with virus in a minimal volume of media. 48 hours after infection, BMP-4 production was measured from 24-hour conditioned media. While Ad.BMP-4 equivalent to 1000 vp/cell yielded 6 ng/mL of BMP-4, lentivirus yielded less than 0.5 ng/mL. Data are shown as mean ± SEM.

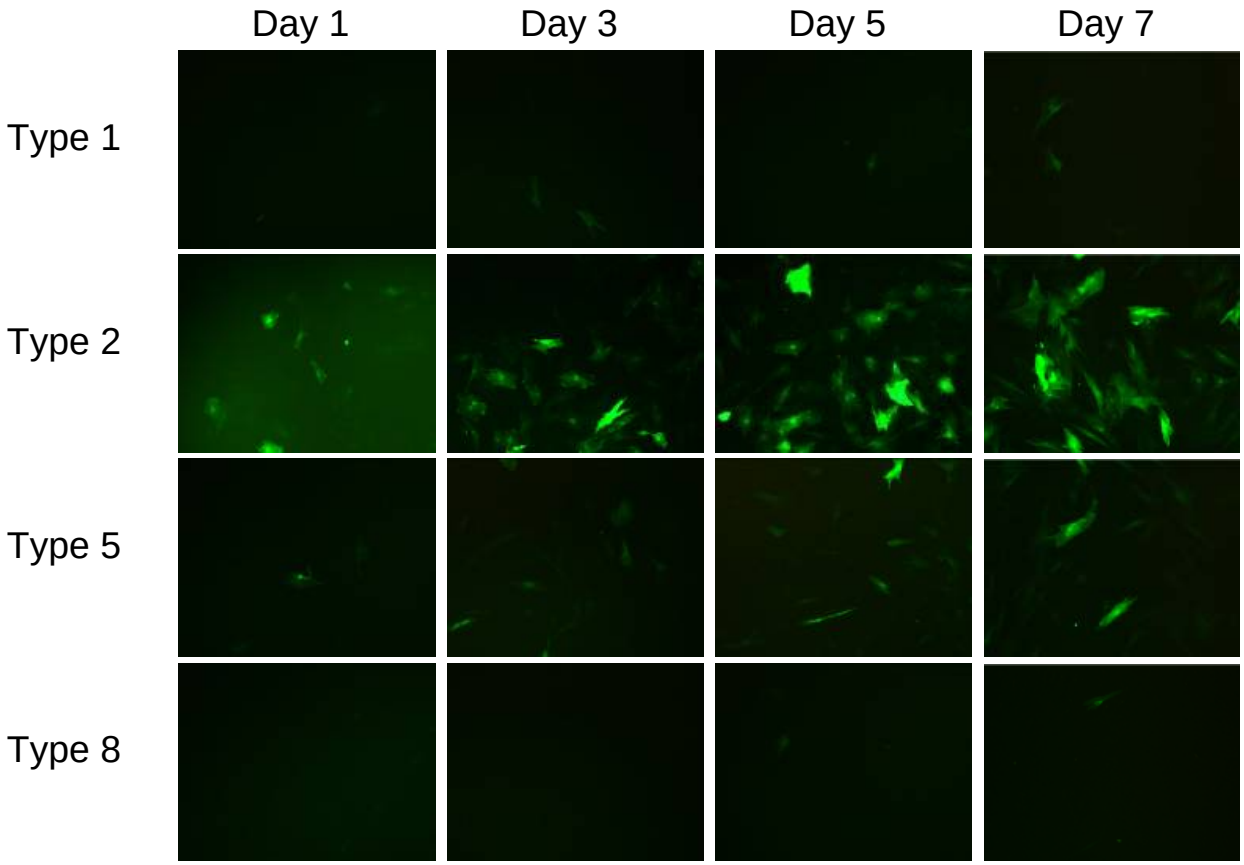


Figure 4-4. AAV serotypes 1, 2, 5, and 8 were screened for transduction efficiency on low passage bovine MSCs in monolayer. Self-complementary AAV-CMV-GFP was delivered at a dose of 10^4 vg/cell, and transduction efficiency was evaluated visually with GFP. Types 2 and 5 were most effective, as shown by robust GFP expression beginning at day 1 and increasing in intensity over the course of 7 days. Serotypes 1 and 8 were less effective at the same dose. We chose to use serotypes 2 and 5 for subsequent AAV constructs carrying BMP-4.

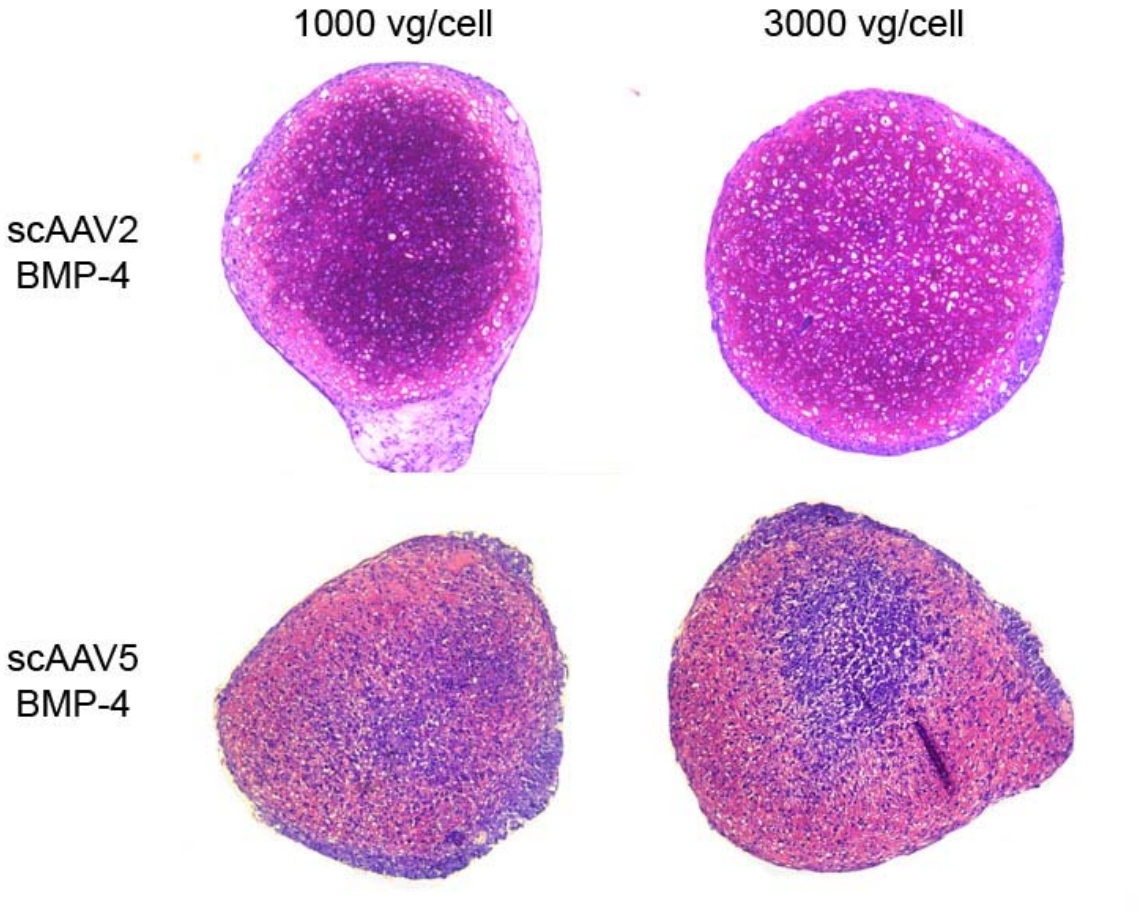


Figure 4-5. MSCs transduced with scAAV-BMP-4 serotypes 2 and 5 demonstrate matrix protein synthesis in aggregate culture. Toluidine blue staining shows that scAAV2-BMP-4 promotes prehypertrophic differentiation in MSC aggregates while scAAV5-BMP-4-aggregates show limited chondrogenic differentiation but substantial proteoglycan production.

Table 4-1. Comparison of gene delivery vectors reveals pros and cons

Vector	Advantages	Disadvantages
Adenovirus	Highly efficient gene transfer Readily infects MSCs Rapid, robust expression Long-term expression not required for chondrogenesis to occur Useful as a protein delivery system	Expression tapers off after 21d Hyperplasia in synovium when virus is injected freely—need to control delivery for ex vivo applications
Lentivirus	Infects MSCs Potential for sustained, long-term expression of growth factors Can select for cells that express protein products	Difficult to achieve high viral titers Selection is required Selection conditions are inhibitory to cell expansion and subsequent use in chondrogenesis assay
scAAV	Readily infects MSCs over a range of doses Effectively delivers chondrogenic transgenes Regarded as safe for clinical applications	Potential for less efficient gene delivery than other viral vectors Pre-existing humoral immunity to many serotypes
Plasmid DNA	Ease of production Stability Safety	Inefficient gene transfer Transient expression Cannot readily apply a dose range

Comparison of gene delivery vectors reveals advantages and disadvantages of each system, with adenovirus and AAV offering the most potential for safe, efficient delivery and expression of chondrogenic cDNAs.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

The unique architecture of articular cartilage limits its ability for self-repair, and since cartilage is frequently damaged by traumatic injury or disease, enhancing methods to repair damaged cartilage is of great clinical relevance. Tissue engineering techniques along with stem cell and gene-based therapies have the potential to improve cartilage repair and may eventually eliminate the need for invasive surgical procedures and total knee replacement.

Tissue engineering techniques and stem cell therapies hold great promise for improvements to articular cartilage repair, especially in the knee joint. Current cell-based surgical treatments employ the use of healthy chondrocytes harvested from non-weight-bearing areas of cartilage. Chondrocytes are available in very limited quantities and require additional time for expansion *in vitro* prior to introduction to the cartilage lesion. Autologous mesenchymal stem cells, on the other hand, can be harvested in large quantities from bone marrow, fat, muscle and other tissues, and they can be virally transduced to express chondrogenic transgenes. Modified MSCs introduced to sites of cartilage damage may prompt the generation of functional hyaline-like cartilage rather than a fibrocartilage scar.

In order to apply these ex vivo techniques, however, a number of challenges must be overcome. These include further work on identifying the optimal combination of stem cells, scaffolds and growth factors, and refining the conditions to enhance cell expansion and chondrogenesis *in vitro* and integration of the cells and scaffolds with existing cartilage *in vivo*. Future research efforts may focus on biodegradable scaffolds

laden with cells that will promote chondrogenesis and integrate at the wound margins to yield fully functional articular cartilage rather than fibrocartilage.

The mode of delivery of cells to repair articular cartilage depends upon the size of the defect. Small, localized defects may be repaired by direct application of modified MSCs to the site, similar to delivery of chondrocytes in current chondrocyte transplant procedures, whereas larger cartilage lesions would rely upon scaffolds to fill the defect and hold the cells in place. Modified MSCs that express growth factors and other chondrogenic proteins will provide the appropriate cues to initiate repair responses within cartilage. Caution must be applied, though, as induction of certain signaling pathways may trigger osteogenic differentiation.

Surgical attempts to repair damaged cartilage mentioned in Chapter 1 include autologous chondrocyte transplantation (ACT), which is limited to treating trauma, and is unable to repair large cartilage full-thickness defects. Gene-based therapies, on the other hand, deliver transgene products to injured tissue to catalyze a healing response without the need for surgery. A current phase 1 clinical trial uses a modified version of ACT to initiate cartilage repair by infecting primary autologous chondrocytes with a virus modified to express TGF-β1. The modified cells are cultured and expanded *in vitro*, then injected into the knee joint of patients with degenerative arthritis (TissueGene, Gaithersburg, MD). This type of approach bypasses the risk of inflammatory responses to adenovirus, and it ensures that only the desired cell type is exposed to the potent, chondroinductive transgene.

In Chapter 3, we set out to evaluate the ability of candidate chondrogenic transgenes to stimulate MSCs toward chondrogenic differentiation. Based upon findings

that TGF-β1 stimulates local proliferation and chondrogenic differentiation in mesenchymal progenitor cells, we designed our study to include members of the TGF-β superfamily as well as other developmental morphogens and transcription factors (Table 1-1). These factors included BMPs 2, 4, and 7, Indian hedgehog, and Sox9 as well as connective tissue growth factor (CTGF). Of all transgenes evaluated in our aggregate culture system, we found that BMP-4 expression resulting from doses of Ad.BMP-4 of 100 to 1000 vp/cell led to the most robust chondrogenic response. When Ad.BMP-4 was injected intra-articularly in rat knees, however, this secreted factor promoted dramatic cellular expansion and chondrometaplasia of the synovial layer. Because such potent chondrogenic inducers can impact the highly receptive progenitor cells of the synovial lining, it remains important to consider transgenes that are expressed intracellularly, such as Sox9. Although we selected BMP-4 for our studies in Chapter 4, Sox9 remains an important transgene for consideration in developing cartilage repair models, especially in preventing ectopic cartilage formation while still promoting defect healing.

Our evaluation of viral vectors in Chapter 4 enabled us to determine the optimal vector for delivery of chondrogenic transgenes—in this case BMP-4—to MSCs. We discovered that scAAV2-BMP-4 infects bovine MSCs optimally, and when administered to MSCs at 3000 vg/cell, it drives BMP-4 expression that promotes more robust chondrogenic differentiation and matrix synthesis than that shown in Ad.BMP-4 aggregates. Moreover, aggregates treated with scAAV2-BMP-4 were uniformly round without the irregular edges and asymmetry often observed in Ad.BMP-4 aggregates.

Delivery of modified stem cells could become a method of choice for clinical applications, in both human and veterinary medicine. Patients' own cells can be harvested, modified with scAAV-BMP-4 (or a virus carrying other chondrogenic factors), and reintroduced to the cartilage defect site within one surgical procedure to augment repair of cartilage damaged by sports injury (as in the case of football players and racehorses) or arthritic disease (such as in older adults and in dogs). A gene- or modified cell-delivery approach is not strictly limited to cartilage applications; this technique can be used to enhance bone fracture healing, heart tissue repair, and numerous other conditions that impact animals and people.

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BIOGRAPHICAL SKETCH

Marsha Lynn Bush was raised in the tourist town of Cave City, Kentucky, where she grew up on a farm, in a zoo, and next door to the longest cave in the world. After graduating valedictorian from Barren County High School in 1999, Marsha attended the University of Kentucky. She spent the summers of 2000, 2002, and 2003 serving as a guide at Mammoth Cave National Park. It was here that Marsha learned of the variety of research projects that were taking place spanning the fields of geology, biology, hydrology, microbiology, anthropology, genealogy, and paleontology. During the summer of 2001, Marsha began her own research pursuits as a student in the Kentucky Young Scientist Summer (KYSS) Research Program. Under the guidance of Dr. Charles (Chuck) Fox, Marsha took part in a population genetics experiment examining maternal effects on offspring lifespan in the seed beetle and stored grain pest, *Callosobruchus maculatus*. An offshoot of this project became her primary research project for completion of her Bachelor's degree in agricultural biotechnology. Prior to graduating in December 2003, Marsha spent the spring semester as the biotechnology lab intern at The Land at Epcot. As part of Epcot Science, Marsha learned about plant tissue culture as she propagated a variety of species used within The Land greenhouses and grew, packaged, and managed the sales of Mickey's Mini Gardens at four locations within Walt Disney World. This internship experience opened the door for Marsha's return to Florida in the spring of 2004 as a reproductive biology intern at Disney's Animal Kingdom Wildlife Tracking Center. This experience cemented her desire to take part in research efforts that would benefit both animals and people.

In August 2004, Marsha began studies in the Interdisciplinary Program (IDP) in Biomedical Sciences at the University of Florida College of Medicine. In May 2005,

Marsha joined the lab of Dr. Steve Ghivizzani in the Department of Orthopaedics and Rehabilitation where she has investigated the induction of chondrogenesis in mesenchymal stem cells following delivery of trangenes via viral vectors. Her work may contribute to the understanding of gene therapy treatments for repair of articular cartilage damage resulting from injuries or diseases such as arthritis. Following completion of her Ph.D. in 2010, Marsha will continue her education at the University of Wisconsin-Madison School of Veterinary Medicine, as she combines her interests in veterinary medicine and translational research.