DISSERTATION

DEVELOPMENT OF AN SCAAVIGF-I GENE THERAPEUTIC VECTOR FOR THE ENHANCEMENT OF CARTILAGE REPAIR

Submitted by

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ABSTRACT

DEVELOPMENT OF AN SCAAVIGF-I GENE THERAPEUTIC VECTOR FOR THE ENHANCEMENT OF CARTILAGE REPAIR

In this work, we hypothesize scAAVIGF-I can be efficiently engineered and used as a gene therapeutic vector to transduce cartilage and synovium and elicit biochemical changes consistent with cartilage repair mechanisms. Here we show joint tissues are permissive to serotype specific, efficient AAV transduction. This is in agreement with previous in vitro and in vivo studies demonstrating AAV as an efficient vector for gene therapy. Interestingly, we show a clinically relevant occurrence of pre-existing, serotype specific AAV neutralization. After creation of an optimized scAAVIGF-I vector, we show transduction of chondrocytes results in significantly increased IGF-I levels that translate to biochemical changes in glycosaminoglycan and proteoglycan content. NextGen RNA transcriptome sequencing and PCR analysis revealed significant differential regulation of genes in pathways including cartilage biology and cell death, but not significant in other pathways associated with IGF-I treatment such as Wnt and Akt regulation. The problem addressed by this research is ultimately answered in that scAAVIGF-I can both efficiently transduce joint tissue and increase IGF-I concentrations sufficiently to cause biochemical changes indicative of healthy cartilage, and the pathways associated with upregulation of IGF-I gene therapy are revealed.

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Chapter 1: Introduction

Dissertation Overview

Osteoarthritis is the leading cause of morbidity among the elderly. As such, it is a costly disease with over $185 billion being spent annually (Kotlarz et al. 2009). This dissertation project establishes in vitro evidence in support of a new gene therapeutic treatment. The first chapter begins with an introduction to the scope and reasoning of the presented research. Following is a comprehensive literature review supporting the various topic areas critical to the understanding and defense of this research. The second chapter is a report of a study analyzing the serotype specific adeno-associated virus (AAV) transduction efficiencies in explant and monolayer joint tissues and compares the differences in transduction of both and addresses the concept that transduction of explants of tissue is a more representative scenario of what occurs in vivo (within the equine joint). This report demonstrates the potential for differential transduction rates between explant and monolayer transductions, indicating results from in vitro monolayer experiments may not directly translate to the in vivo model. The third chapter is a research report analyzing the biochemical and gene expression response of chondrocyte transduction by the AAVIGF-I gene therapeutic. This is one of the first studies to examine the gene pathway changes through next generation (NextGen) sequencing subsequent to IGF-1 (insulin-like growth factor 1) gene therapy. This work results in novel information of how IGF gene therapy upregulates matrix and collagen production and provides valuable information into IGF-I function in chondrocytes. Finally, the fourth chapter is an analysis of this current research with regard to the broader scientific community, with insight on advancement and new directions.

In this work, we hypothesize AAVIGF-I can be efficiently engineered and used as a gene therapeutic vector to transduce cartilage and synovium and elicit biochemical changes consistent with cartilage repair mechanisms. Here we show joint tissues are permissive to serotype specific, efficient AAV transduction. This is in agreement with previous in vitro and in vivo studies demonstrating AAV as an efficient vector for gene therapy. Additionally, we show a clinically relevant occurrence of pre-

existing serotype specific AAV neutralization. In the third chapter of this dissertation it is shown that AAVIGF-I transduction of chondrocytes results in significantly increased IGF-I levels that translate to biochemical changes in glycosaminoglycan and proteoglycan content. NextGen RNA transcriptome sequencing and PCR analysis revealed differential regulation in gene ontologies for chondrocyte and cartilage development, collagen, and regulation of cell death and growth among numerous others. The problem addressed by this research is ultimately answered in that AAVIGF-I can both efficiently transduce joint tissue and increase IGF-I concentrations sufficiently to cause biochemical changes indicative of healthy cartilage and the pathways associated with upregulation of IGF-I gene therapy are revealed.

The work within this thesis both builds upon existing research and creates a foundation for future research endeavors as discussed below and in the final chapter of this report. Testing of several naturally occurring AAV serotypes has shown efficient, serotype specific transduction of joint tissues. This indicates a potential for AAV gene therapy vectors designed with further joint specificity and efficiency through directed evolution capsid design. The NextGen sequencing of AAVIGF-I transduced chondrocytes provides a snapshot of transcriptional activity in chondrocytes overexpressing IGF-I. The transient activity of overexpressed IGF-I leads to many research questions regarding both short-term and long-term activities. Additionally, in this research only in vitro chondrocytes were transduced. In clinical settings an AAVIGF-I gene therapeutic could be used as both an in vivo and ex vivo treatment of not only chondrocytes but stem cells as well. This will require additional research on these cell types and conditions. For this gene therapeutic to be evaluated as a true treatment for osteoarthritis, future research will need to be directed toward the transduction response in osteoarthritic cartilage or chondrocytes. Would the anabolic activity of IGF-I overexpression be sufficient to overcome the catabolism of osteoarthritis? Here we have shown that in normal chondrocytes this overexpression amplifies a healthy chondrocyte phenotype. However, ultimately we may need a combination of anabolic and anti-catabolic gene therapeutic vectors used in a comprehensive approach to effectively treat OA.

The Joint as an Organ

The primary functions of a joint are to facilitate efficient movement and transmit the loads produced by the musculoskeletal system (McIlwraith et al. 1996). Essential for these functions is the articular layer of hyaline cartilage found on the ends of long bones and the supporting role played by the synovium lining the joint capsule (Figure 1). This cartilage is a multi-zoned tissue consisting of 50% collagen, 35% proteoglycan,10% glycoproteins, and remainder minerals and fats by weight. The only

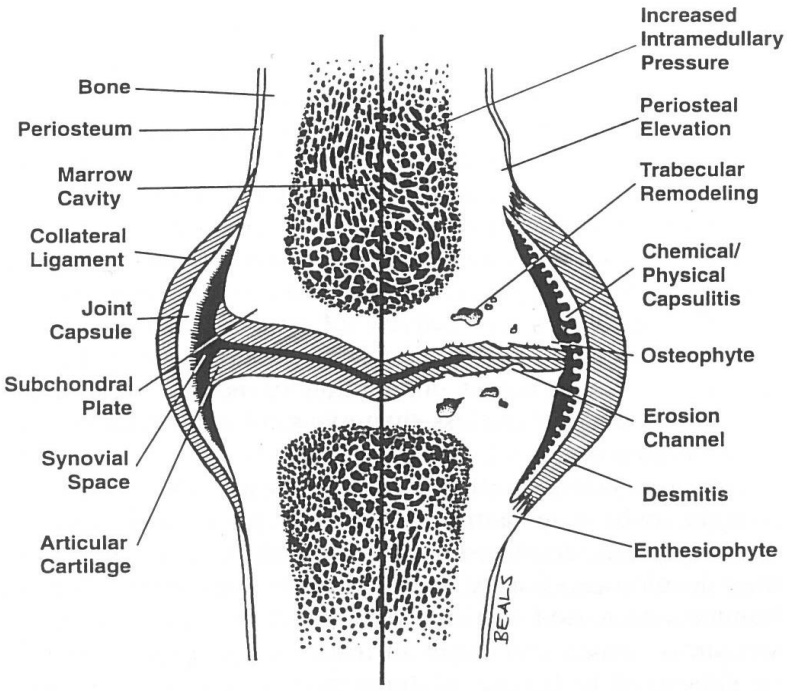

Figure 1: A normal synovial joint (left) and a joint showing OA pathology (right). Figure reproduced with permission (McIlwraith et al. 1996).

cellular components, chondrocytes, take up 1 to 12% of cartilage volume (McIlwraith et al. 1996). See Figure 2 for a comprehensive depiction of cartilage components. Each component of cartilage serves an important role in the functioning of a joint: collagen provides tensile strength with orientation in the superficial zones aligned with the axis of motion and orientation in deeper zones aligned perpendicular to the articular surface; the proteoglycan component provides compressive strength via hydrostatic pressure

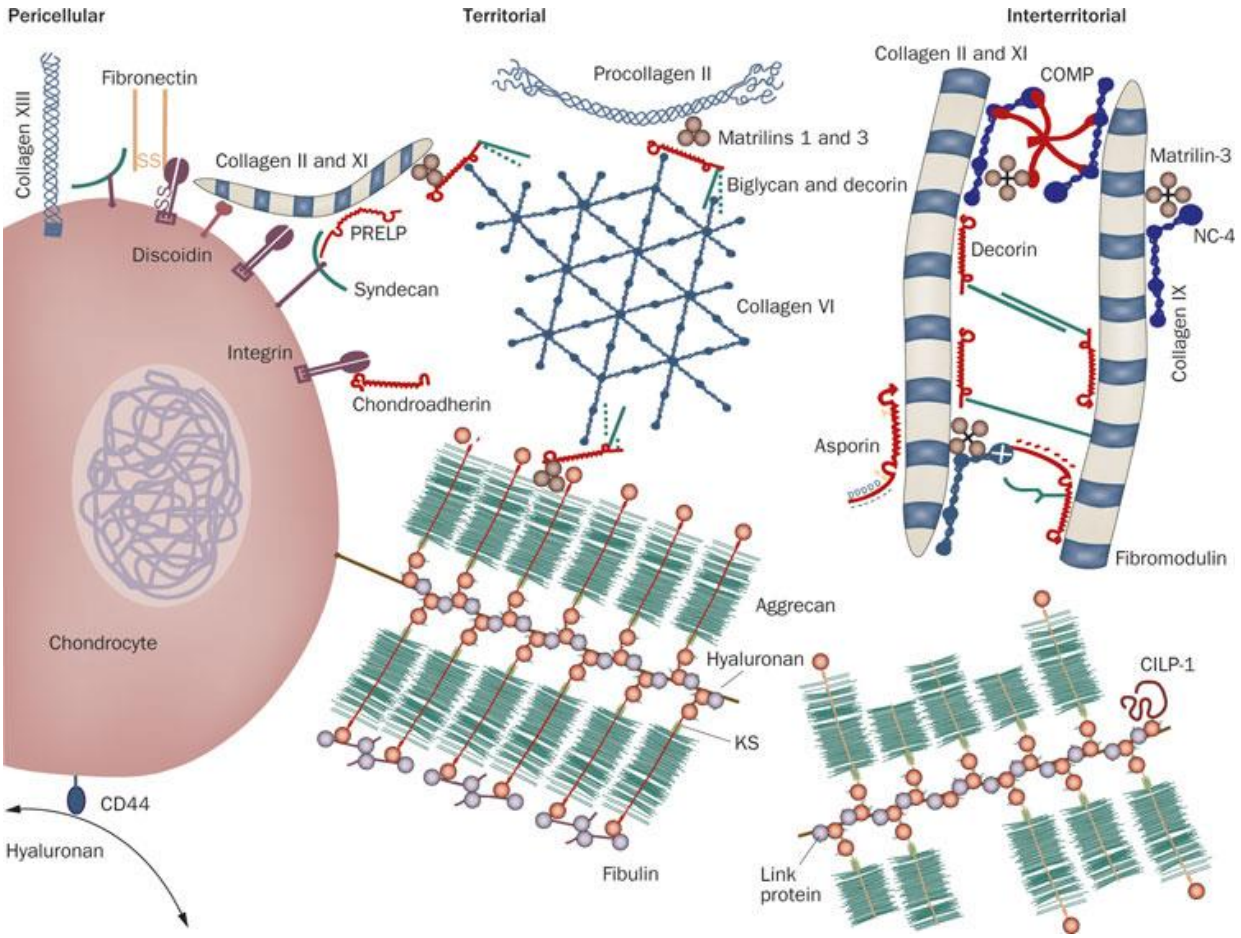

Figure 2: Cartilage matrix components. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Heinegård et al. 2011), copyright (2011).

maintained with negatively charged sulfated side chains; and chondrocytes and glycoproteins are the mechanism for active local maintenance of cartilage homeostasis (Broom et al. 1983). In support of the primary functions of the joint and cartilage, the synovium is a cellular tissue lining the joint capsule responsible for maintenance of the synovial fluid. The synovium contains macrophages and synoviocytes, the latter of which are fibroblastic cells that produce hyaluronan and other biological components active in joint physiology.

Osteoarthritis

Osteoarthritis (OA) is a disease that manifests as a degradation of articular cartilage and generalized joint inflammation. There is a strong correlation between age and the prevalence of OA (Valdes et al. 2008). This leads to the observations that OA is one of the most common morbidities in the

elderly, and age related changes may contribute to the disease etiology. Additionally, there have been heritable components of OA theorized (Valdes et al. 2008). Known contributors to a higher risk of OA include inflammation, general joint trauma, malaligned joint anatomy and traumatic focal defects in the cartilage surface. Inflammation causing deviation from the molecular homeostasis in the joint is becoming a prevalent theory in the genesis of OA (Berenbaum 2013). The joint is influenced by anabolic factors that contribute to cartilage matrix deposition and catabolic factors that contribute to matrix remodeling. Normally, these factors maintain homeostasis in a healthy joint. Inflammation in a diseased joint will lead to a net increase in catabolic factors resulting in the degradation of cartilage (Berenbaum 2013). Inappropriate, localized cartilage loading not normally experienced may lead to further breakdown of the cartilage, cartilage wear particles, and increased inflammation. Pain is the main symptom of clinical manifestation of OA, and usually by the time pain is experienced, the joint has suffered cartilage degradation and OA is in the later stages of pathophysiology (Evans et al. 2004; Jones 2013).

Cartilage undergoes a consistent low level of maintenance facilitated by a homeostasis between catabolic and anabolic factors (Mueller et al. 2011). The half life of proteoglycan is 3-24 years and that of collagen is more than 100 years in a normal joint (Goldring et al. 2009). Upon clinical manifestation, OA and the associated inflammation lead to a breakdown in the biological homeostasis of the joint characterized by an increase in catabolic inflammatory factors and the failure of growth-related anabolic factors to match the increase in catabolism (Mueller et al. 2011; Lee et al. 2013). Current non-surgical palliative treatments for OA include NSAID pain relievers, nutraceuticals such as glucosamine and chondroitin sulfate, cortisone injections, and joint stabilization through braces. Invasive treatments may lead to better cartilage healing, but still do not cure a person of the morbidity. These treatments include chondroplasty or smoothing of the cartilage surface, microfracture (puncture through a cartilage defect to allow bleeding and progenitor cells from the subchondral bone to fill the defect), osteochondral autograft or allograft transplants, and joint replacement, among others.

Pathways Important in Joint Physiology

There have been several biological pathways implicated in the OA process. Pathways that are regulated in response to catabolic factors in the joint include MAPK signaling through Erk1/2, JNK, p38, and NF-κB signaling (Fan et al. 2007; Elshaier et al. 2009), Wnt signaling, and TGF-β signaling (Stock et al. 2013; Jotanovic et al. 2014).

Wnt signaling can be divided into three distinct pathways of action. The "canonical signaling" or traditional pathway centers around Wnt activation of β-catenin. β-catenin localizes to the nucleus and leads to upregulation of transcription factors and eventually cell proliferation of Wnt targeted genes, c-jun and cyclins (Ogryzko et al. 1996; Hens et al. 2005; Robinson et al. 2006). The "non-canonical signaling" pathway consists of two pathways, one where PCP, then Rho and JNK (c-jun N-terminal kinase) are activated, and the second where PKC (protein kinase C) and CaMKII (calcium/calmodulin-dependent kinase II) are activated by Wnt/Ca++ (Park et al. 2002; Liu et al. 2008). A mouse model has been used to show that loss of the Wnt inhibitor FRZB leads to increased cartilage damage in OA models (Lories et al. 2007). The canonical signaling pathway is activated in response to Il-1β stimulation of chondrocytes leading to dedifferentiation and inhibition of apoptosis (Hwang et al. 2004). Inhibition of the canonical pathway in knockout mice leads to increased chondrocyte apoptosis and cartilage destruction (Zhu et al. 2008). Wnt function can be chondrogenic or inhibitory in a transient manner depending upon the current maturation state of the cartilage (Sassi et al. 2014).

The inflammatory factor IL-1 acts through four signaling pathways. Three of these pathways belong to the mitogen activated protein kinase (MAPK) system of pathways. The MAPKs are responsible for transcription of many factors. Some of the key enzymes are c-Jun amino-terminal kinases (JNK) 1/2, extracellular signal-regulated kinases (ERK) 1/2, and 38-kd protein kinases (p38) (Fan et al. 2007). The ERK proteins are produced in all cells and help regulate cell division through transcription regulation (Carter et al. 2000; Lai et al. 2001). Also involved in the pathway phosphorylation of ERKs is upstream control by the oncogene Ras, and components of the three-kinase module including c-Raf1, B-Raf, and A-Raf (Johnson et al. 2002). The JNKs are important in transcription regulation, and

downstream in the pathway they will bind the c-Jun component of the AP-1 transcription complex. AP-1 then regulates production of many cytokines. Upstream, JNKs are influenced by 13 MKK kinases (MKKKs) and multiple environmental stimuli in order to help control apoptosis (Tournier et al. 2000). The four members of the p38 kinases are both activated by and regulate the expression of inflammatory cytokines, leading to an important role in the immune response (Johnson et al. 2002). MAPK has been shown to be an upstream regulator of articular cartilage degradation through p38 mediated MMP expression and activity. In response to IL-1, a strong MMP-13 expression response was observed dependent upon p38, JNK and NF-κB (Mengshol et al. 2000). Runx-2 enhances this response through p38 MAPK pathway (Mengshol et al. 2001). P38 inhibition through inhibition of the Src family of proteins also shows that only MMPs are regulated by the p38 MAPK, not aggrecanases (Sondergaard et al. 2010). It was also shown that heat or mechanical stress of normal chondrocytes will increase phosphorylated p38 to higher levels as are found in osteoarthritic chondrocytes, and that this increase leads to increased apoptosis (Takebe et al. 2011). The MAPK-ERK1/2 pathway has been shown to be responsible for abnormal MMP and ADAMTS production in OA articular chondrocytes and subchondral bone osteoblast coculture (Prasadam et al. 2012). P38 and JNK, but not ERK1/2 or NF-κB have been shown to regulate MMPs in an all-trans retinoic acid treatment of OA chondrocytes model (Ho et al. 2005).

The fourth pathway of action for IL-1 that doesn't involve the MAPK system is the NF-κB signaling pathway. NF-κB is released via phosphorylation of its inhibitory protein IκB. Following this, NF-κB functions as a transcription factor after translocation to the nucleus (Fan et al. 2007). Some of the factors that NF-κB activates results in positive feedback to activate further NF-κB in a signal amplification loop. Otherwise NF-κB activation is triggered by many proinflammatory factors and helps with expression of many genes related to innate and adaptive immunity (Bacher et al. 2004).

The phosphoinositide-3 kinase (PI3K) is a pathway responsible for events leading to cell growth, migration and survival after phosphorylation in response to cell stimulation by growth factors. The phosphorylation activates PI3K to binding with guanisine triphosphate (GTP)-binding proteins (G

proteins) regulatory factors, serine-threonine kinases, and protein tyrosine kinases. These other proteins then accumulate near the cell membrane and initiate protein kinase cascades and assembly of signaling complexes (Cantley 2002). The protein serine-threonine kinases Akt are also a downstream target of PI3K. Once phosphorylated, Akt catalyzes phosphorylation of further proteins that target cell growth and survival. One example is the Akt phosphorylation of Bad, which creates a binding site for 14-3-3 proteins and takes away the binding opportunity of Bcl-2 family proteins. These Bcl-2 proteins then accumulate in the cytoplasm where they induce a cell survival response (Brunet et al. 2001; Lawlor et al. 2001; Cantley 2002).

TGF-β is a family of growth factors that play a role in cell proliferation and development. The signaling pathway is initiated with membrane bound serine/threonine kinase receptors of two types. See Figure 3 for a representation of the pathway. The receptors phosphorylate the intracellular Smad family of proteins. There are seven type I receptors, denoted activin receptor-like kinases (ALKs). Of these seven, ALKs 1, 2, 3, and 6 communicate via the Smad1, 5 or 8 proteins. ALKs 4, 5 and 7 communicate via the Smad2 or 3 proteins (ten Dijke et al. 1994; Goumans et al. 2003; Daly et al. 2008). Signaling is responsible for all phases of chondrogenesis. In early stages of differentiation TGF-β plays a stimulatory role, while in later stages it is inhibitory. The activity of TGF-β in general maintains the prehypertrophic phenotype of chondrocytes (Ballock et al. 1993). The transcription regulator Runx2 is a downstream target of various Smads, with some causing activation and some inhibition (Hjelmeland et al. 2005; Javed et al. 2009).

Proteins Involved in Joint Physiology

There are numerous proteins involved in joint physiology. The growth factors TGF-β1, bone morphogenetic protein 2 (BMP-2), BMP-7, insulin-like growth factor 1 (IGF-I), fibroblast growth factor 2 (FGF-2), FGF-18 and platelet-derived growth factor (PDGF) contribute to joint anabolism (Fortier et al. 2011). TGF-β1 research has shown contradicting roles in the joint. See Figure 3 for an overview of the TGF β superfamily pathway. It has been shown to stimulate proteoglycan and glycosaminoglycan production, but also has led to cartilage degradation in a contrasting study

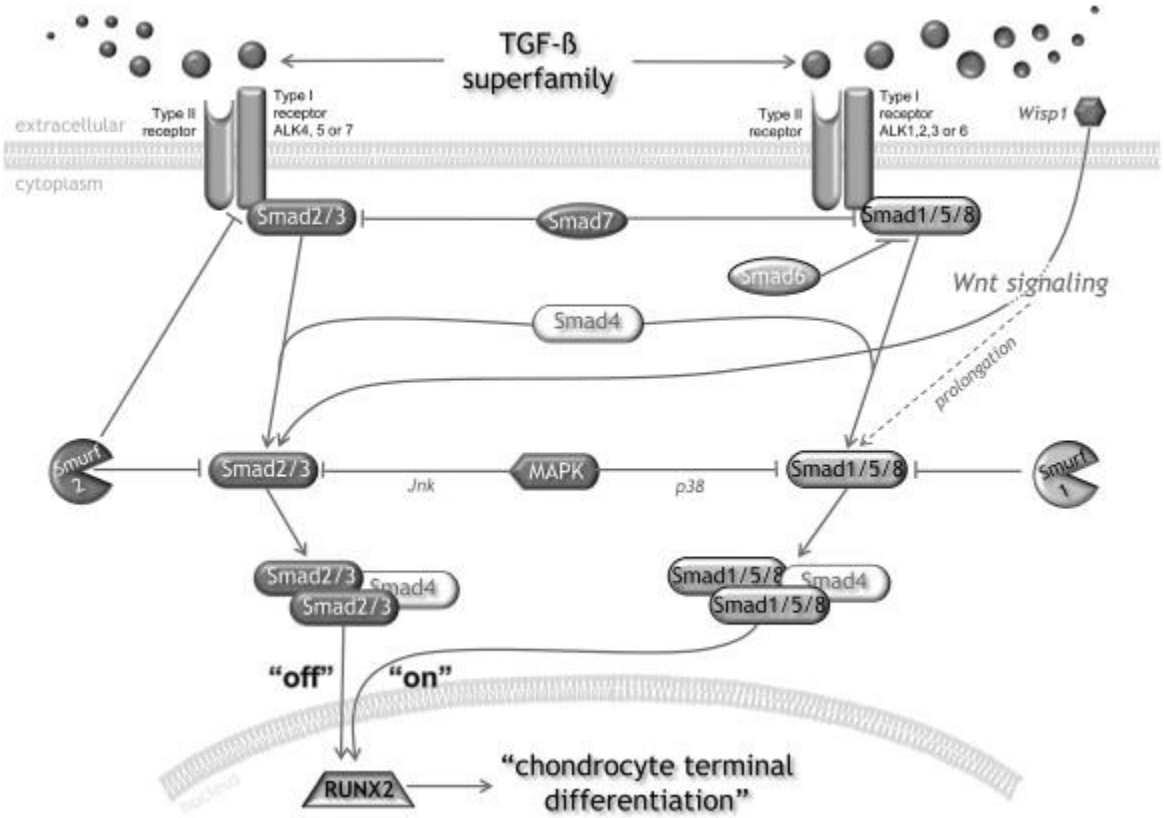

Figure 3: Pathway map of the TGF-β superfamily. Open access and copyright under Elsevier user license (van der Kraan et al. 2009).

(Glansbeek et al. 1998; Mi et al. 2003). Unfortunately with this increase in proteoglycan and GAG production, "impressive osteophyte formation" was also found (Van Beuningen et al. 1994; Bakker et al. 2001). Alternatively it has also been found TGF-β1 can greatly reduce inflammatory cell infiltration in a joint model of inflammatory polyarthritis from streptococcal cell wall fragments (Brandes et al. 1991). TGF-β1 also has varying effects on differentiation of in vitro chondrocytes. One study noted TGF-β1 induced the switch to hypertrophy in chondrocytes, with a loss in aggrecan and type II collagen (Narcisi et al. 2012), while it was also found that TGF-β1 in the presence of serum contributed to a slight dedifferentiation of chondrocytes in a fibrin matrix model (Fortier et al. 1997). A recent in vitro and in situ gene therapy study of TGF-β1 showed reduced expression of differentiation markers (type-X collagen, MMP-13, β-catenin) and also increased TGF-β1 receptor levels (Venkatesan et al. 2013). The increased receptor levels are crucial to address the age- and OA- related difference in TGF-β pathway signaling, specifically with regard to the ALK1/ALK5 ratio (van der Kraan et al. 2012).

Of the BMPs, BMP-2 and BMP-7, both members of the TGF-β super family, have shown potential as therapeutic growth factors (Fortier et al. 2011). BMP -7 has shown the greater promise for cartilage repair and is anticatabolic when treating cells that have been exposed to IL-1B (Elshaier et al. 2009). In a skeletogenesis model of the chick limb bud, both BMP-2 and -7 were shown to modulate joint development. It was shown that BMP-2 led to alteration in joint shape while BMP-7 led to inhibition of joint formation (Macias et al. 1997). As a potential treatment for cartilage defects, BMP-2 has been shown to be most effective when compared to BMP-4 and -6 at inducing chondrogenesis and in proteoglycan synthesis of MSCs (Sekiya et al. 2005). When compared to TGF-β1, BMP-2 also resulted in increased proteoglycan synthesis, however intra-articular injections only led to increased synthesis for 3-4 days compared to the 21+ days achieved by TGF-β1. In this same study chondrophyte formation was also noted at the growth pate - joint space interface (van Beuningen et al. 1998). In an osteochondral defect model in dogs, BMP-7 was shown to produce discontinuous, hyaline cartilage when used in conjunction with a type I collagen scaffold (Cook et al. 2003). In a gene therapy treatment of an equine focal chondral defect model, it was shown that allogeneic chondrocytes in a fibrin clot overexpressing BMP-7 led to superior healing at four weeks. However, at eight months the repair tissues were similar to the control defect, showing a transient response in this treatment (Hidaka et al. 2003). BMP-7 has also been shown to have anticatabolic activity (Chubinskaya et al. 2007). This action has specifically been shown to decrease expression of MMP-1,-3,-13 and the aggrecanase ADAMTS-4 (Im et al. 2003; Chubinskaya et al. 2007; Im et al. 2007).

IGF-I is an anabolic factor that contributes to cell proliferation, tissue differentiation, and prevention of apoptosis (Laviola et al. 2007). See Figure 4 for several signaling pathways triggered by IGF-I. Many of its functions mediate embryonic and postnatal development. These processes specifically include linear growth, metabolism, organ maintenance, immune development, and neurologic development (Powell-Braxton et al. 1993). IGF-I has also been investigated in tumor proliferation in several cancers (Rinaldi et al. 2010; Attias-Geva et al. 2011; Rohrmann et al. 2011; Casa et al. 2012). The IGF-I family also includes a superfamily of six IGF binding proteins (IGFBP-1,-2,-3,-4,-5, and -6)

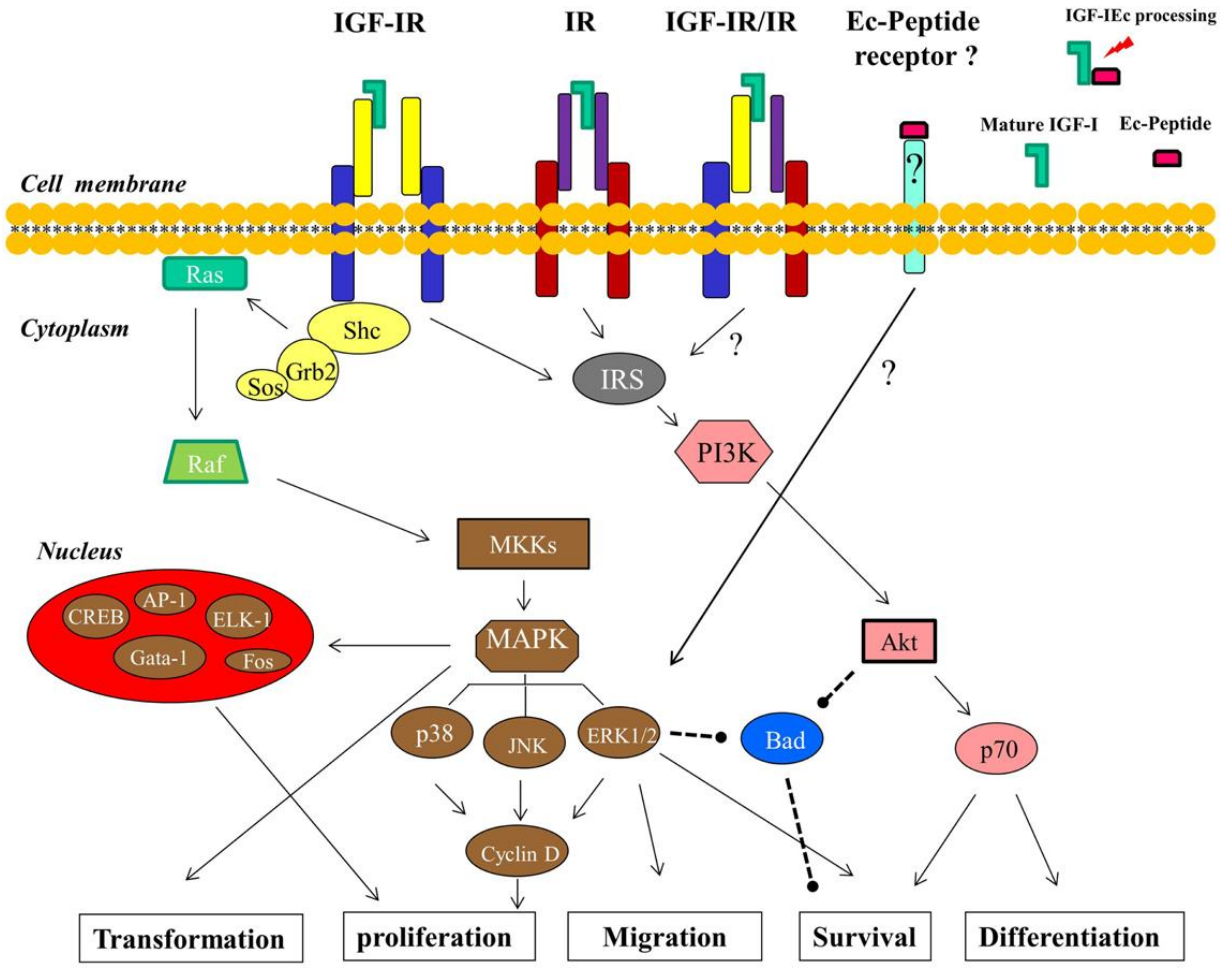

Figure 4: IGF-I signaling. Figure reproduced according to CC BY 3.0 (Philippou et al. 2013).

and four semi-structurally similar proteins that also bind IGF, IGFBP related proteins (IGFBP-rP1,2,3,4,5,6 also named IGFBP-7,8,9,10,NA,NA, respectively) (Hwa et al. 1999; Rosenfeld et al. 2000). These IGFBPs bind IGFs, the majority of which is by IGFBP-3 (Juul et al. 1995). The binding proteins contribute to modulation of IGF levels, bioavailability and even IGF-independent functions, prolonging IGF half-life, IGF transport, and enhancement of IGF actions (Collett-Solberg et al. 2000). More specifically in cartilage, IGF-I has been shown to increase proteoglycan, GAG, and cell proliferation (Sah et al. 1994; Madry et al. 2005). The intensity of these actions, however, decreases with age and particularly OA chondrocytes have been shown to be hyporesponsive to IGF-I due to (in part) an increase in IGFBPs- most specifically a disregulation in IGFBP-3 and -4 (Martel-Pelletier et al. 1998). Due to this hyporesponsiveness, one study showed IGF-I was unable to decrease catabolism but still able to exert

anabolic properties at sufficiently high levels (Morales 2008). MSC chondrogenesis is regulated by IGF-I, and complemented by TGF-β1 this is further enhanced (Worster et al. 2001). By combining IGF-I with BMP-7 in a treatment, cell survival and matrix synthesis was enhanced beyond that of either alone (Loeser et al. 2003; Chubinskaya et al. 2007). A decrease in IGF-I has been shown to lead to cartilage lesions in a rat model (Ekenstedt et al. 2006), and an increase in IGF-I levels has shown enhanced cartilage repair in equine cartilage defect models (Fortier et al. 2002; Goodrich et al. 2007). These responses will be discussed further, later.

Members of the fibroblast growth factor family have also shown anabolic activity in the joint. Specifically, FGF-2 and FGF-18 have both shown anabolic activity, while studies on FGF-2 have shown contradictory results (Ellman et al. 2008). FGF-2 has been shown to be an important cell cycle regulator in growth plate chondrocytes (Kilkenny et al. 1996), and led to increased viability through the reduction of apoptotic cells and a dedifferentiated phenotype including reduced collagen type II and aggrecan production (Schmal et al. 2007). In vivo gene therapy mediated delivery of FGF-2 in a rabbit osteochondral defect model led to improved repair through increased filling, cell morphology, and architecture. This study did not show significant changes in matrix synthesis (Cucchiarini et al. 2005). FGF-18 is a more well-known anabolic factor that leads to increased matrix formation and cell differentiation (Ellman et al. 2008). Specifically, in a meniscal injury model in rats, FGF-18 led to generation of new cartilage in chondral defects and increased chondrophyte size and subchondral remodeling (Moore et al. 2005). PDGF is another anabolic factor also important in cartilage development. Chondrocytes from the resting zone of cartilage, when cultured with PDGF, increased proliferation and proteoglycan production while inhibiting further hypertrophy (Schmidt et al. 2006).

There are numerous catabolic factors that can be found in the joint, including several from the following families: IL family of proteins, MMP family of proteins, ADAMTS family of proteins, and the protein TNF- α and prostaglandin PGE2 among other factors. Of the catabolic factors present in the normal joint and more prevalent in the OA joint, MMPs have been intensively studied. The MMP family consists of "classical" MMPs, the ADAMs (a disintegrin and metalloproteinase, adamlysins), the

membrane-bound MMPs (MT-MMPs), and the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motif). In all, there are more than 20 members in these families (Malemud 2006). These members also consist of collagenases, aggrecanases, gelatinases, stromelysins, and elastases (Woessner Jr 2001). MMPs are generally found in higher levels in young animals than in older animals. This is thought to be due to the decrease in growth and remodeling as animals age (Brama et al. 1998). Although it is generally thought that MMPs are detrimental to the joint, newer studies have suggested that ADAMTSs are responsible for cartilage destruction and MMPs are actually useful for modifying the pericellular space composition (Fosang et al. 1996; Sandy 2006; Struglics et al. 2006). In support of the previous argument are also studies showing a mouse model null for ADAMTS5 is protected from arthritic joint destruction (Glasson et al. 2005) and a mouse model null for MMP-3 is less protected from arthritic joint destruction (Clements et al. 2003). Of the two major aggrecanases in the ADAMTS family applicable to OA research, ADAMTS-4 is expressed in response to proinflammatory cytokines and ADAMTS-5 is constitutively expressed in chondrocytes and synoviocytes (Fosang et al. 2008). However, there is still debate on whether one is more important to cartilage destruction than the others. Studies that have used RNA silencing and antibodies to ADAMTS-4 and -5 show ADAMTS-4 plays a significant role (Tortorella et al. 2001; Song et al. 2007) but studies of null expression in mice reveal ADAMTS-5 plays the more significant role (Glasson et al. 2005; Stanton et al. 2005). The MMPs 1, 3, 8, 13 and 14 are produced by OA chondrocytes. MMP-13 and has been closely studied as it specifically cleaves type II collagen (Knäuper et al. 1996). These fragments in addition to fibronectin fragments may further increase MMP-13 expression through positive feedback with cell-surface integrins (Goldring et al. 2011). Additionally, a mouse model of MMP-13 overexpression shows OA-like joint changes (Neuhold et al. 2001). In a TMJ model of arthritis in rats, MMP-3 and -9 were upregulated in response to IL-1 α (Ijima et al. 2001).

IL-1 actually refers to two different cytokines, IL-1α and IL-1β. Both of these cytokines are inflammatory; IL-1α is mainly cell associated whereas IL-1β can be released from its primary cell sources, monocytes and macrophages (Jacques et al. 2006). IL-1 has been shown to act as an

inflammatory protein in the joint, specifically through activation of T cells, prostaglandin E2 and I2, IL-6, MMPs, ADAMTSs, and others (Kirkham 1991). This protein is expressed in low levels in normal articular chondrocytes, and at increased levels in osteoarthritic chondrocytes and the associated, inflamed synovium (Fan et al. 2007). IL-1 has been specifically shown to be produced in response to synovium monocyte exposure to collagen breakdown particles (Goto et al. 1988). In concert with oncostatin M, IL-1 has also been shown to increase expression of MMP-1,-3,-8,-13,-14, and ADAMTS-4 but not -5 (Koshy et al. 2002). An IL-1β knockout mouse study indicated no development of joint destruction following streptococcal wall fragment intra-articular injection (Lubberts et al. 2001). Conveniently, IL-1 receptor antagonist was discovered and has been shown to reduce the severity of many IL-1 mediated diseases such as arthritis (Dinarello et al. 1991). Other pro-inflammatory factors include IL-6, IL-17, tumor necrosis factor alpha (TNF- α), prostaglandin E2 (PGE2). These factors also contribute to pain in the joint (Lee et al. 2013). TNF destructive activity in the joint has been shown to be mediated solely by IL-1 (Zwerina et al. 2007).

Gene Therapy

As of 2012, there have been over 1800 gene therapy clinical trials completed or approved (Ginn et al. 2013). In brief, gene therapy is the therapeutic transfer of genes to individuals. It was originally intended to treat heritable genetic diseases, but may also be used to treat acquired diseases. Only somatic cells are targeted in current research. Although gene therapy offers the ability to treat germ-line cells, this challenges ethical concerns including eugenics or the artificial and heritable improvement of individuals (Evans et al. 1995). Most of the clinical trials for gene therapy have targeted cancers, with the most commonly transferred gene being the tumor suppressor p53 (Ginn et al. 2013). The first successful treatment of a genetic disease was in a 2000 trial in which X-linked severe combined immunodeficiency (SCID-X1) was targeted with an ex vivo transduction of CD34+ cells with a Maloney retrovirus vector encoding the gene for the gammac cytokine receptor subunit (Cavazzana-Calvo et al. 2000). The majority of the 20 patients were treated successfully. However, 5 of the patients went on to develop a T-cell leukemia from vector integration that had occurred near a proto-oncogene promoter (Hacein-Bey-

Abina et al. 2003; Hacein-Bey-Abina et al. 2003). This setback has not stopped research into a better cure as evidenced by a new gene therapy clinical trial for this disease with both an improved vector and promoter begun in 2010 (Herzog et al. 2010). An additional gene therapeutic success was from a trial targeting X-linked adrenoleukodystrophy, a disease that leads to demyelination of the central nervous system. Of the two patients currently referenced, demyelination had stabilized and the corrected X-ALD gene had been shown to be stably inserted into hematopoetic stem cells (Cartier et al. 2009; Cartier et al. 2012). A gene therapy trial showing a successful length of transgene expression was reported by Buchlis et al. This study showed continued transgene expression of Factor IX ten years after intramuscular transduction in sever hemophilia B patients (Buchlis et al. 2012). Another successful use of a gene therapeutic treating cancer was shown in a metastatic melanoma targeting treatment. Autologous lymphocytes were transduced ex vivo with a retrovirus encoding a T-cell receptor. Two of the seventeen patients treated showed dramatic improvement with circulating engineered cells still present one year after infusion. Although the engineered cells were still found in the other fifteen patients, it is theorized the specific lymphocytes were unable to infiltrate the tumors effectively (Morgan et al. 2006). One final gene therapeutic example is AAV mediated treatment of Leber congenital amaurosis. Treatment of patients in an advanced stage of the disease led to improvement in vision and similar canine based models of the disease have shown persistence of over eight years (Bainbridge et al. 2008; Hauswirth et al. 2008; Maguire et al. 2008).

In contrast to the successes presented above, there have been specific inadvertent fatal outcomes from gene therapy trials. One fatal gene therapy outcome with significant lessons learned was that of a trial including 18 year old Jesse Gelsinger (Raper et al. 2003). This trial was for treatment of ornithine transcarbamylase deficiency, a disease that has a high mortality and morbidity but can be acceptably treated in some individuals. The adenovirus vector induced a fulminant innate immune response that subsequently proved fatal. This trial indicated that high doses of adenovirus vector, and even minor differences at high doses could have drastically varying outcomes (Raper et al. 2003). This also led to more emphasis being directed to safer vector design. An additional fatal gene therapy incident of interest

was the clinical trial of a TNF-α antagonist gene carried in an AAV2 vector for the treatment of rheumatoid arthritis. One of the study participants who was on additional anti-TNF-α antibody therapy died 22 days after a second administration of vector. This death was due to the opportunistic infection of *Histoplasma capsulatum*, and the inability of the patient's immune system (compromised by significant levels of TNF inhibitors for the treatment of the rheumatoid arthritis) to successfully target and clear the pathogen (Furst et al. 2007; Frank et al. 2009). Together, these negative incidents among the many successes offer both lessons on how to further develop improved gene therapy treatments and reminders of the comprehensiveness required in the study of gene therapy in order to prevent fatal outcomes.

The majority of gene therapy protocols employ the use of viral based vectors. Most historically studied vectors have been those based upon adenovirus. In addition to adenovirus, other less studied virus based vectors include those based upon vaccinia/poxvirus or herpes virus. These three examples are all highly immunogenic, as evidenced above in the Jesse Gelsinger clinical trial death (Heilbronn et al. 2010). This leaves AAV and lentiviral based vectors as the most promising candidates for future studies. Lentiviral vectors, however, have shown insertional mutagenesis whereby the transgene insertion modifies the enhancer regulation of nearby genes, with cancerous effects (Rothe et al. 2013).

Clinical Gene therapy vectors are traditionally virus based; however gene therapy can be conducted with non-viral vectors. Other demonstrated vectors include electroporation, gene gun, ultrasound, hydrodynamic pressure, chemical approaches and novel carrier molecules (Niidome et al. 2002). Most of these vectors are not as efficient as virus vector based transductions, however novel non-viral vectors approaching viral efficiency have been developed (Li et al. 2000). In vivo examples of non-viral transductions include electroporation which has been shown for the purpose of skin DNA vaccinations, and DNA covered gold particles have been used with the gene gun delivery tool (Lin et al. 2000; Drabick et al. 2001). Liposomes and biodegradable polymers also show potential, especially when modified with certain peptides (Lee et al. 2001; Turk et al. 2004). Peptides for the nucleus localizing signal have also been used to modify DNA carriers to facilitate transport to the nucleus and transduction efficiency in non-dividing cells (Bremner et al. 2001; Tachibana et al. 2001).

Adeno-Associated Virus

AAV is a small, single-stranded DNA virus. Traditional recombinant AAV vectors only have space for a transgene capacity of 4.5 kb, and this space is halved for self-complimentary vectors. There are numerous wild-type serotypes (strains) of AAV that infect man and nonhuman primates with no noticeable pathogenicity. The viral capsid is composed of VP1, VP2, and VP3, three structural proteins that make up a 20nm icosahedral shell in a 1:1:10 ratios, respectively. The wild-type virus contains just two genes, rep and cap, and short 145 bp sequences on either end of the genome called inverted terminal repeats (ITRs). The ITRs are responsible for the packaging signals, mediate integration into the host chromosome, and contain the origins of DNA replication. There are three promoters in the genome, with p5 and p19 responsible for four overlapping rep gene variants and p40 responsible for three cap gene variants due to differential splicing and translation initiation (Muzyczka et al. 2001). Wild-type infection requires a helper virus for replication; these viruses include herpes simplex, cytomegalovirus, or adenovirus, through which identification in a co-infection gave AAV its name. Without a helper virus co-infection, AAV integrates its genome favorably into a site called AAVS1 on human chromosome 19, where it lies latent until rescue with a helper virus co-infection (Samulski et al. 1991). AAVs bind different cell surface glycans depending on the serotype of the AAV. AAVs 2, 3, and 6 bind heparan sulfate, 4 binds O-linked sialic acids, and 1, 5 and 6 bind N-linked sialic acids (Summerford et al. 1998; Wu et al. 2006; Wu et al. 2006). Additional co-receptors include human fibroblast growth factor receptor 1, hepatocyte growth factor receptor, and the integrins αv-β5 and α5-β1 (Summerford et al. 1999; Kashiwakura et al. 2005). AAV production is accomplished by transfection into the human 293 cell line of the various AAV genes and helper genes required for lytic replication. The 293 cell line is constitutive for the helper genes E1A and E1B from adenovirus, and a helper plasmid containing the adenovirus helper genes VA-RNA, E2A, and E4, and the AAV rep and cap genes is used in the co-transfection with the vector plasmid containing the ITRs on either ends of the transgene of interest (Grimm et al. 1998; Xiao et al. 1998). Following this, vector purification can occur via density gradient or affinity column.

AAV has both benefits and challenges to being used as a gene therapy vector. Two of these are illustrated in a follow up study to a clinical trial using AAV2 to transduce muscle cells with human factor IX for the treatment of hemophilia B (Buchlis et al. 2012). The follow up by Buchlis et al. detected transgene expression 10 years after the initial treatment. This shows the persistence of AAV transgene expression, specifically in muscle, but could be extrapolated to other tissues where cellular turnover is low, as in cartilage. The challenge to AAV use as illustrated in the study however was that factor IX levels were sub-therapeutic. One of the challenges facing AAV is historically lower transduction efficiency when compared to other vectors such as adenovirus (Smith-Arica et al. 2003). Advances in AAV vector technology such as self-complimentary vectors greatly increase transduction efficiency, with one study in muscle showing 10-15 fold increase in scAAV compared to ssAAV. While the ssAAV may transduce the same number of cells, it does not equate to the same level of transgene expression (Wang et al. 2003; Blankinship et al. 2006; McCarty 2008). Nucleotide optimization of the transgene of interest is another method to increase transduction efficiency (Nathwani et al. 2011; Ward et al. 2011).

Models for Research of the Joint

There are several models for the study of joint disease. In vitro models utilizing chondrocyte and synoviocyte cell culture monolayers are used to roughly approximate their respective tissues. In vivo models routinely use the horse due to its similarity to the human joint (Frisbie et al. 2006; McIlwraith et al. 2011). In vitro models are routinely less expensive than in vivo models, however they may not actually provide accurate results pertaining to what actually occurs in the body- the ultimate question that needs answering when conducting medical research. In one study using an anti-inflammatory for cancer treatment, there were no cytotoxic effects in an in vitro model, but the drug proved attenuating in the in vivo model (Williams et al. 2000). In one study comparing 39 treatments in a cancer xenograft model, in vivo activity did not closely match human cancer histology. If a treatment was found active in at least one third of the models, there was a close correlation to activity in Phase II clinical trials (Johnson et al. 2001). In a review by An et al. of in vitro and in vivo models for intervertebral disc degeneration, a key takeaway is the fact that each model has its advantages and disadvantages. Primarily, each model

answers specific questions, but the applicability to the actual pathology must be considered (An et al. 2006). A further study discussed species differences in the same model (Hunter et al. 2004). Specifically in the joint, Chu et al. discussed different animal models. Several important points included: small animal (mice) models allowed knockout, cheap and efficient proof of concept models, but translational value was limited due to small joint size, thin cartilage and quicker healing than humans. An equine model provides a reliable model for the study of cartilage defects in humans, having a similar thickness of cartilage unlike many other model species (Frisbie et al. 2006); however, larger animal models can be more expensive and carry more ethical and logistical concerns (Chu et al. 2010). Finally, Chu et al. make the argument that the scientific goal should be the determining factor in what model is ultimately utilized.

Articular Cartilage Repair

There are currently many articular cartilage repair treatment options presently under study. One option under investigation is perichondrial or periosteal grafts. This tissue has shown chondrogenic and repair promoting properties, but neoformation of cartilage like tissue is only slight and tissue filling of voids is minimal (Cohen et al. 1955; Rubak 1982; Carranza-Bencano et al. 1999). Similar to these grafts are autologous osteochondral transplantation. There is little prospective research on this method, but retrospective research reveals good to very good amelioration of pain and increase in joint functionality (Brent 1992; Hangody et al. 2001). In addition to autologous, there is also allogeneic osteochondral transplantation, however concerns exist and surround host rejection and death of transplant material (Stevenson 1987; Stevenson et al. 1991). Tissue engineering is used to mediate the formation of repair tissue. The goal is to reconstruct tissue both structurally and functionally with the primary variables of design being the matrix scaffold, signaling molecules, and finally cellular components. Classes of matrix materials include protein-based (i.e. fibrin, collagen), carbohydrate-based (i.e. alginate, hyaluronan), and artificial polymers (i.e. Dacron, Teflon) (Hunziker 2002). Recent studies by Watson et al. and Goodrich et al. show promise with an AAV IL-1Ra intra-articular gene therapy treatment (Goodrich et al. 2013; Watson et al. 2013). These studies have resulted in both therapeutic levels and extended duration of protein production. Intra-articular injection of MSCs post arthroscopy is another method that has been

shown to be safe, and may lead to a clinically beneficial outcome as measured by improvement in ability to work (Ferris et al. 2014). Autologous bone marrow mesenchymal stem cells have further been explored for use in several matrices, several of which are fibrin composites, platelet-rich fibrin glue and a hyaluronic acid hydrogel (Haleem et al. 2010; Erickson et al. 2012; Ahmed et al. 2013). Platelet-rich plasma (PRP) intra-articular injection has been shown to decrease pain and improve knee function and quality of life. These results are retained for a duration of 12 to 24 months (Filardo et al. 2011). Autologous cultured serum increases the anti-catabolic factors in a patient's own blood, through enhanced levels of interleukin receptor antagonist and other beneficial cytokines (Hraha et al. 2011). This then can be used for partial relief of joint pain and destruction.

IGF-I

The IGF-I signal transduction pathway begins with the activation of IGF-I receptor (IGF-IR). Pathways that then carry this signal include MAPK/ERK-1/2 and PI3K/Akt. Final signal transduction proteins include Elk-1 and SOX9 which both are transcription factors that impact cell proliferation and survival. These factors also are responsible for modulating extracellular matrix components including proteoglycans and type II collagen (Bell et al. 1997; Loeser et al. 2008; Beier et al. 2010). In a mouse knockout model, a loss of IGF-IR led to decreased type II collagen and Indian Hedgehog (Ihh) expression, and in increased parathyroid hormone-related protein PTHrP (Wang et al. 2011). Another pathway stimulated by IGF-I is the Bcl-2 cell survival pathway (Chand et al. 2012). Overexpression of Bcl-2 leads to reduced apoptosis in cells(Hockenbery et al. 1993; Adams et al. 1998). In previous studies, expression of Bcl-2 in normal cartilage was found to be significantly higher than in osteoarthritic cartilage, while Bcl-2 expression in lesional OA areas was found to be lower than in non-lesional OA areas(Kim et al. 2000).

IGF-I gene therapy was predominantly studied using adenovirus as the vector of choice before using AAV. Transduction of chondrocytes and synoviocytes was accomplished with adenovirus-IGF-I, and produced high levels of IGF-I through at least 28 days of monolayer culture (Nixon et al. 2000). An equine in vivo full depth chondral defect model revealed that IL-1ra/IGF-I combination adenovirus gene

therapy increased defect proteoglycan and collagen production (Morisset et al. 2007). Goodrich et al. demonstrated improved early healing and long term healing by genetically modifying chondrocytes with adenovirus-IGF-I prior to transplantation in an equine cartilage defect model (Goodrich et al. 2007). A gene therapy treatment utilizing an episome delivery vehicle for IGF-I antisense DNA was used to remove tumorigenicity from glioma cells in a rat model (Trojan et al. 1993). Ex vivo transduction of rabbit chondrocytes with IGF-I and FGF-2, encapsulation in alginate, and finally transplantation in osteochondral defects showed beneficial repair tissue when compared to controls (Orth et al. 2011).

AAV gene therapy with the IGF-I transgene has been used in many studies to date. Several studies revealed AAVIGF-I treatment for a mouse model of ALS (Kaspar et al. 2003; Kaspar et al. 2005; Dodge et al. 2008). One study by Zhu et al. revealed cerebral AAVIGF-I delivery to improve motor function, increase neovascularization and neurogenesis following infarction (Zhu et al. 2008). A rat model of myocardial infarction demonstrated a strong increase in capillary density after AAVIGF-I delivery to the infarct area (Dobrucki et al. 2010). In a study on aging and muscle mass, an intramuscular injection of AAVIGF-I increased muscle mass and strength in young adult mice, and resulted in an even greater percentage increase in muscle strength in older mice (Barton-Davis et al. 1998). A diabetic neuropathy mouse model showed AAVIGF-I treatment reversed hypoalgesia, improved motor function and corrected sensory nerve function (Chu et al. 2008). In a rat model of joint damage, AAVIGF-I intra-articular injection was shown to express IGF-I for at least 8 weeks, but did not show a difference in joint health between treated and untreated joints (Izal et al. 2008)l. Another AAVIGF-I study on in vitro chondrocytes and in situ cartilage explants showed long term reconstruction, and a decrease in IGFBP-3 and -4 and increase in IGFBP-5 and the IGF-I receptor (Weimer et al. 2012). Together, these studies demonstrate various diseases in which AAVIGF-I has been applied, some successfully and some unsuccessfully. This niche also proves a market for which current AAVIGF-I vectors can be refined for additional treatment success.

Methods to Analyze Cartilage

There are several platforms with which to analyze cartilage including macroscopically, histochemically, histologically, and biochemically (Morisset et al. 2007). For cursory analysis of chondrocyte functioning, biochemical analysis provides reliable and repeatable, quantitative measures. Glycosaminoglycan (GAG) quantification is a measure of the structurally supportive molecule. This assay can be performed on tissue digestions to gauge functional content, or on media samples to gauge release of GAG and in theory catabolism (Dechant et al. 2005). There is natural turnover of the matrix, however, and this must be taken into account. Collagen type II is the molecule that provides tensile strength, and can be measured via ELISA from tissue digestion as well. Polymerase chain reaction (PCR) measures mRNA transcripts of genes. This allows for a targeted analysis to gather a general sense of the cell programming with regard to metabolic signaling. This is only cursory, however, as splice variants, post-transcriptional silencing, post-translational modification and sequestration can occur to modify the mRNA's effect on cellular metabolic activity (Olsson et al. 2007).

NextGen Sequencing

A significant number of osteoarthritis studies have been performed using microarrays. One study examined the transcriptional response to mechanical injury in ex vivo cartilage transplants (Dell'Accio et al. 2008). Another looked at the differences in gene expression between OA and healthy cartilage (Karlsson et al. 2010). However, microarray based research has begun to reach the limits of its applicability when compared to other technologies such as next-generation high-throughput sequencing (NextGen) (Bloom et al. 2009). Whereas microarrays have a set dynamic range with which cDNAs are quantified, NextGen uses a census based scoring method that allows advantages in dynamic range and quantification (Jiang et al. 2009). Additionally, microarrays can only quantify cDNAs for which they are designed. cDNAs not found in the library (such as small- and microRNAs) will not be quantified, and those cDNAs with mutations will not be accurately quantified, even if they are included in the library. Mismatches can significantly affect the hybridization efficiency (Van Vliet 2010). These mismatches would be sequenced by a NextGen analysis, however, leading to additional avenues of potential research.

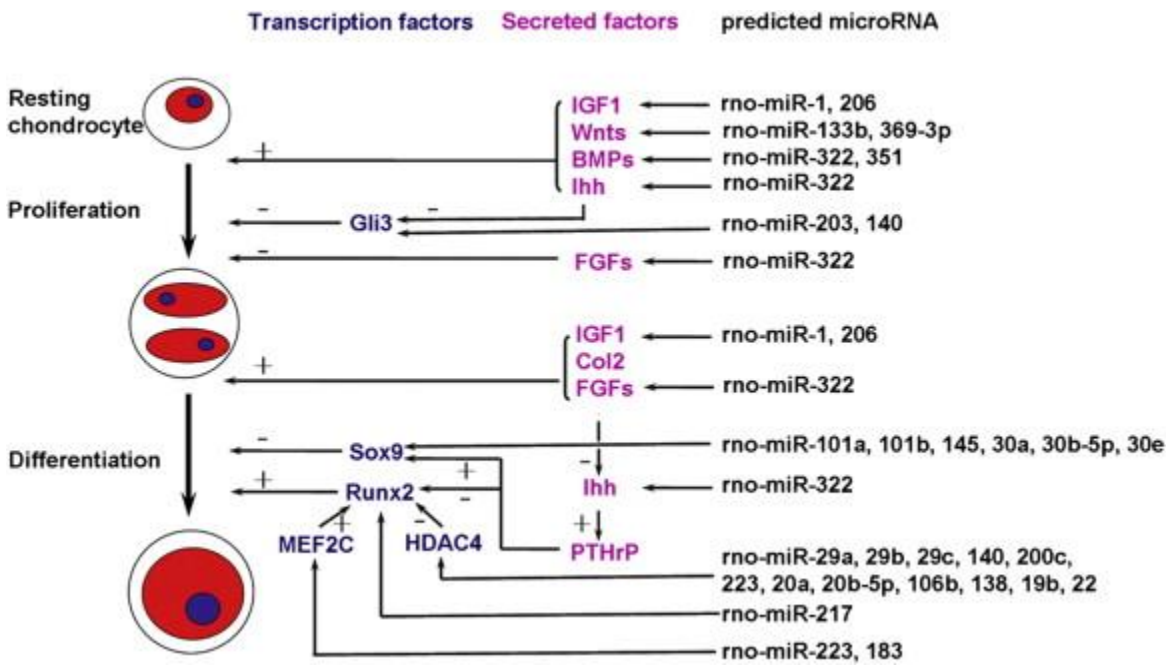

Figure 5: Predicted miRNA roles in chondrocyte regulation. Open access and copyright under Elsevier user license (Sun et al. 2011).

Recently there have been several NextGen analyses in OA cartilage. In one study, OA cartilage was compared to normal and found to show hierarchical clustering and differential expression previously shown in microarrays. In addition, novel associations with several canonical pathways were found, and the authors proposed additional research from the data to investigate alternative splicing events, novel RNAs and different post-transcriptional events (Xu et al. 2012). In another study, sequencing of rat cartilage samples to investigate different developmental stages discovered 86 novel miRNA candidates (Sun et al. 2011). See Figure 5 for a representation of how the predicted microRNAs may contribute to regulation of chondrocyte proliferation. These two studies demonstrate how NextGen sequencing can allow previously unfeasible research into OA.

Overview: State of the Art in 2008 and Where We Are Now

The research progress from 2008 to the present (2014) has been steady and incremental in nature. For instance, in 2008 a study by Izal et al. showed no effect of AAVIGF-I treatment on joint damage in a rat model, while a later study in 2012 by Weimer et al. reported reconstruction of monolayer chondrocytes and in situ cartilage explants after AAVIGF-I treatment(Izal et al. 2008; Weimer et al.

2012). With regard to TGF-β1, one study in 2008 looked at chondrogenesis of mesenchymal progenitor cells in the infrapatellar fat pad treated with TGF-β1 (Lee et al. 2008), and in a study in 2009 the authors reported chondrogenesis of similar cells in a fibrin-based matrix loaded with TGF-β1 (Jung et al. 2009). The idea of recurring but ultimately repetitive research is corroborated by Evans in his 2008 article on the state of arthritis gene therapy: "… we have made the case that, because proof of principle has been overwhelmingly demonstrated for local arthritis gene therapy in joints, priority should be given to implementing clinical studies" (Evans 2008). However, this review also discusses the rapidly accelerating costs when taking research from in vitro, to in vivo, to clinical trials. In this light, it would be beneficial to create models that more cheaply simulate the in vivo environment and to pursue research that incorporates new elements with the greater potential to contribute to clinical research.

A search of clinicaltrials.gov for "AAV" with trials reported after January 1, 2008, reveals 46 trials. In contrast, a search of "osteoarthritis gene therapy" responds with 11 results, only four of which are actually gene therapy treatments for osteoarthritis (and all four are variations using the same product, TissueGene-C – an allogeneic, ex vivo, retrovirally transduced and then gamma irradiated, TGF-β1 chondrocyte treatment). Modifying the search to use "arthritis gene therapy" returns 47 trials; no further osteoarthritis gene therapy trials, but several of note include: a comparison of proliferation and differentiation of MSCs from OA vs. healthy donors, a gene survey on Rheumatoid arthritis patients, a biomarker study to define anti-TNF-α unresponsive RA patients, and a study on the use of umbilical cord-derived mesenchymal stem cells for RA treatment. A search for "IGF" comes up with 456 trials, none of which are for the treatment of osteoarthritis. An analysis of the clincaltrials.gov searches indicates a very weak progress on clinical OA gene therapy advancements over the last six years. There has been relatively more clinical research on AAV, and vastly more research on clinical applications of IGF.

Chapter 2: Adeno-Associated Viral Vectors Show Serotype Specific Transduction of Equine Joint Tissue Explants and Cultured Monolayers

Introduction

Gene therapy is currently being considered as a promising treatment for musculoskeletal diseases with considerable emphasis placed on arthritis (Ghivizzani et al. 1998; Evans et al. 2004; Goodrich et al. 2006; Goodrich et al. 2007). Intraarticular gene therapy would target tissue using a vector that can infect articular cartilage and the synovial lining of the joint which contain the cell types chondrocytes and synoviocytes, respectively (Watanabe et al. 2000; Madry et al. 2003). Osteoarthritis is characterized in the joint by tissue wear particles originating from the cartilage surface and increased inflammatory factors in the joint fluid (Goldring et al. 2004). Gene therapy has focused on supplementation of anabolic factors including insulin-like growth factor-I, transforming growth factor beta, bone morphogenetic protein 2, and inhibition of pro-inflammatory molecules using interleukin 4, soluble tumor necrosis factor receptor, and interleukin-1 receptor antagonist protein which inhibits the pro-inflammatory interleukin-1 (Glansbeek et al. 1997; Glansbeek et al. 1998; Lubberts et al. 2000; Frisbie et al. 2002; Kim et al. 2002; Goodrich et al. 2006). Treatment with these biological molecules requires long term and steady state dosing and both of these requirements are difficult to impossible using recombinant protein treatments but well suited for the gene therapy approach (Watanabe et al. 2000; Evans et al. 2011). Various viral vectors have been studied for gene therapy use in the joint including herpes simplex virus, HIV based lentiviral vectors, adenovirus and adeno-associated virus (AAV) (Oligino et al. 1999; Gouze et al. 2002; Madry et al. 2003; Goodrich et al. 2006). Adeno-associated virus as a therapeutic vector currently shows the most promise as it can genetically modify a variety of cells including non-dividing cells with minimal pathologic effects. As a result the gene of interest is expressed long term, and tissue tropism is only limited by capsid serotype receptor accessibility (Evans et al. 2004; Ulrich-Vinther 2007). The wide tissue tropism partly comes from the existence of different serotypes, at least 12 of which have been

described with over 100 variants in different animal species (Choi et al. 2005; Gao et al. 2005; Wu et al. 2006; Schmidt et al. 2008).

One challenge facing AAV as a gene therapy vector is the prevalence of neutralizing antibodies found in the patient population (>50% for AAV 2) (Cottard et al. 2004; Halbert et al. 2006; Boissier et al. 2007; Boutin et al. 2010). The IgG antibodies from nonpathogenic AAV infections have been proven to induce a neutralizing effect on AAV *in vitro* (Cottard et al. 2004). These antibodies drastically decrease the transduction efficiency of the vector resulting in a treatment with limited to no success (Scallan et al. 2006). Due to the similarity between AAV capsid serotypes and the cross reactivity antibodies can have to the capsid, the most effective assay that determines transduction inhibition is a neutralization assay which directly tests whether the virus is not only bound by antibodies, but actually neutralized. These experiments have been performed in humans, but not in the horse to our knowledge (Gao et al. 2005; Halbert et al. 2006).

Cells *in vitro* are different in several ways from those in explants. A cell monolayer typically results in certain cell types based on adherence and growth conditions. Further cell passaging can induce dedifferentiation and phenotypic changes as shown in chondrocytes (Lin et al. 2008). Exposure to cell surface and cell doubling is significantly different for monolayer versus explants, all critical variables that may influence vector transduction (Alexander et al. 1996). Many prior studies have been done with monolayer cell cultures due to the simplicity and ability to better control for variation in experiments. Because of the potential differences that may exist between transduction in monolayer and transduction of cells *in situ*, our goal was to test whether cell monolayers are a good approximation for transduction of tissue explant cultures.

The objective of this study was to determine whether transduction efficiencies in the monolayer culture model are an accurate representation of transduction efficiencies in tissue explants, a model more closely related to *in vivo* transduction. We hypothesized that there may be differences in transduction efficiencies due to the increased amount of extracellular matrix in explant tissues. Further, to maximize transduction efficiency *in vivo* we sought to investigate whether neutralizing antibodies existed in the

joint fluid or the serum of the horse. We hypothesized that neutralizing antibodies would most likely exist to some of the AAV serotypes that have efficient transduction in equine synoviocytes and chondrocytes.

Materials and Methods

Transduction Efficiencies

Tissues were harvested post mortem from four horses, aged two to five years old,and euthanized for other reasons. Joints displayed no OA pathology. Synovium was aseptically excised from the inside of the fetlock joint capsule and cartilage from the patella. Similarly sized explants approximating 5mm squares were cut from the larger pieces and kept in wells of 48 well plates with 500μL Ham's F12 media (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1X antibiotic antimycotic (HyClone, Logan, UT, USA), and 1N HEPES buffer (Invitrogen) changed every other day. The remaining synovium and cartilage pieces were diced and digested as previously described (Nixon et al. 1992). Cells were plated in appropriately sized tissue culture flasks at a density of 100,000/cm^2 for synoviocytes and 200,000/cm^2 for chondrocytes. Cells were grown for four days, then trypsinized and replated in 48 well plates at the aforementioned plating density. Two days following plating, cells and explants were transduced with scAAV-eGFP serotypes 1, 2, 2.5, 3, 4, 5 and 6 from the Gene Therapy Center Vector Core Facility (Chapel Hill, NC, USA). The vector scAAV-eGFP (pHpa-trs-SK) construct and cross packaging has been described previously (McCarty et al. 2001; Rabinowitz et al. 2002). Transduction was preceded by rinsing cells in PBS and then 250μL Ham's F12 media was put into the wells. scAAV-eGFP was added to the wells at a concentration of 8000 virus particles per cell (VPC). The virus incubated with the cells for four hours at 37ºC and 5% CO_2 and was then aspirated and replaced by complete F12 media. The day of transduction was considered day zero. On days 4, 8, 12, 16, and 20, fluorescent microscopy pictures were taken of the cells and explants. The microscope used was an Olympus IX70 (Center Valley, PA, USA) with a filter cube with excitation at 495nm and emission at 521nm and at 100x magnification. Software used to capture images was QCapture by QImaging (Surrey, BC, Canada) and no post processing occurred. On day thirty, explants

were individually digested and plated immediately into wells according to the prior digestion protocol. The explant cells adhered to the well for two days and then all wells were trypsinized. PBS with 0.2% FBS was added and the suspension analyzed by flow cytometry. Suspensions were run through a CyAn ADP Analyzer (Beckman Coulter, Brea, California, USA) and data analyzed with Summit 4.3 software (Dako, Carpinteria, CA, USA). The percent of cells transduced was the final data set analyzed. A mixed model ANOVA was used with horses being considered random variables. Statistics were considered significant with a p-value < 0.05.

Enzymatic Desialylation and AAV Transduction

Explants of cartilage and synovium and monolayers of chondrocytes and synoviocytes were isolated from a horse as described prior. Cultures were treated with heparinase I and III (Sigma-Aldrich #H2519 and #H8891, respectively) and/or neuraminidase III (Sigma-Aldrich #N7885) as described (Shen et al. 2011). Briefly, cultures were treated with 3U/ml heparinase I, 1.5U/ml heparinase III, and/or 50mU/ml neuraminidase in incomplete Ham's F12 media for two hours at 37ºC and 5% CO_2. Cultures were rinsed and scAAV-eGFP of 1000 VPC was added in incomplete Ham's F12 media and incubated for four hours at 37ºC and 5% CO_2. The lower level of 1000 VPC was shown to produce a more noticeable difference between enzyme treated and control samples as similarly found previously (Boyle et al. 2006). Finally, the virus suspension was removed and complete Ham's F12 media was added. At seven days post transduction the explants were digested and plated as previously described. On day ten the cells were analyzed with flow cytometry as described above. A fixed effects ANOVA was used for statistical analysis and significance was established at a p-value < 0.05.

AAV Neutralizing Antibodies

Synovial fluid and serum were collected post mortem from four horses euthanized for other reasons, and independent to the horses prior. 293 cells were plated and grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented as described above in 48-well plates to act as an indicator of AAV neutralization. Two days after plating, synovial fluid and serum dilutions of 1:12, 1:25, 1:50, 1:100 and 1:200 were created from the four animals with incomplete DMEM media. To the

dilutions were added 8000 VPC of scAAV-eGFP serotypes 2, 2.5, 3, 5 and 6. The virus and serum or synovial fluid dilutions were incubated at 37ºC and 5% CO_2 for one hour. Following this incubation the mixtures were used in a four hour transduction of 293 cells. Post transduction the mixtures were replaced with complete DMEM. Six days post transduction the cells were analyzed with flow cytometry as described above. Neutralization was defined as a concentration of synovial fluid or serum that inhibited a transduction by at least 50% compared to the positive control (AAV treatment with no synovial fluid or serum). Only the neutralizing dilution with the weakest concentration of synovial fluid or serum was indicated for each combination of treatment type and serotype. Statistical analysis was performed using a fixed effects ANOVA, and significance was established at a p-value < 0.05.

Results

Flow Cytometry of Vector Transductions

Flow cytometry was performed on monolayer and tissue explant cultures to analyze the transduction efficiencies of AAV serotypes. The percentage of cells transduced and expressing GFP provided an indication of the proportion of the cells in the population that were permissive to an AAV transduction. In brief, cartilage and synovium explants and chondrocyte and synoviocyte monolayers were cultured and transduced with AAVs 1, 2, 2.5, 3, 4, 5 and 6 at a dose of 8,000 virus particles per cell (VPC). All AAVs were self-complimentary and encoded the gene for green fluorescent protein (GFP). This allowed for the flow cytometric determination of which AAV produced the highest percentage of cells expressing GFP, and consequently which AAV was the most efficient at transducing the cell type/culture of interest. The results divided AAV serotypes into three classes based on preference for transducing explants or monolayers more efficiently: class I or those that transduced explants more efficiently than monolayer, class II or those that showed no difference between explant and monolayer transduction, and class III or those that transduced monolayer more efficiently than explants. In cartilage derived cultures class I consisted of AAV 2 and 2.5; class II consisted of AAV 3 and 4; class III consisted of AAV 1, 5 and 6. In cultures derived from synovium, class I consisted of AAV 1, 2, 2.5 and 6. Class II consisted of AAV 3, 4 and 5, and there were no serotypes that could be categorized as class III (Figure 6).

Flow cytometry revealed AAV2 and 2.5 to be the most efficient serotypes at transducing all tissue and culture types (Figure 7). Between the two, AAV 2 was consistently more efficient than 2.5, but this was not significant. AAV 3 was similar to 2 and 2.5 in all cultures except for synovial explants and AAV 5 in all but synovial and cartilage explants. AAV 1, 4 and 6 had varying amounts of statistical similarity to the other serotypes but consistently transduced cells the least efficiently of all serotypes. The difference in transduction efficiencies between cartilage explants and chondrocyte monolayers is not significant while averaging across all serotypes. This difference is significant however, when comparing synovial explants and synoviocyte monolayers.

In a qualitative analysis of all the cultures (Figure 8 being a specific example) AAV 2 consistently appeared to have the best overall transduction efficiency. AAV 2.5 did not appear as efficient as the quantitative analysis showed. And finally AAV 1 and 4 often appeared to not transduce cells at all, while AAV 3, 5 and 6 had mixed results but usually presented transductions that were somewhat less efficient than AAV 2.

Role of Cell Surface Glycans

We next wished to compare the role of cell surface receptors with serotype specific transduction in explants versus monolayers. It was hypothesized that the differences in transduction efficiencies between explants and monolayers seen in Figure 6 could be attributed to varying concentrations of cell surface receptors for AAV. A previous report (Boyle et al. 2006) indicated that lack of heparan sulfate led to only a moderate decrease in AAV 2 transduction of cells. While the previous report examined the role of heparan sulfate, we also sought to determine the relationship of sialic acid (the other primary receptor specific to AAV 1, 4, 5 and 6) (Kaludov et al. 2001; Wu et al. 2006). An experiment was performed where the primary cell surface receptors for AAV were enzymatically cleaved and transduction efficiencies compared to those of untreated controls. These experiments were carried out in cell monolayer and joint tissue explants at 1000 VPC in the presence of heparinase or neuraminidase. Enzyme treatment to remove cell surface receptors for AAV 2 included heparinase I and III, AAV 2.5 included both heparinase I and III and neuraminidase III, and AAV 5 included neuraminidase III.

After enzyme treatment, there was no longer a statistical difference in transduction efficiencies between synovial explant and synoviocyte monolayer for AAV 2 and 2.5. The enzyme treatment also removed the statistical difference in transduction efficiencies between cartilage explant and chondrocyte monolayer for AAV 5 transduction. Of note, enzyme treatment did not remove the statistical difference in transduction efficiencies between cartilage explant and chondrocyte monolayer for AAV 2 and 2.5. It was interesting to note that AAV 2.5 is a rational design chimeric capsid containing 5 amino acids from AAV 1 spliced into the backbone of AAV 2 (Bowles et al. 2012). While this capsid still utilizes heparan sulfate as the primary receptor, the inclusion of the additional five amino acids plays a significant but still unknown role in enhancing vector transduction in muscle. AAV 2.5 was the first example of a chimeric AAV vector used in a clinical trial (Bowles et al. 2012). The ability to mimic or partially mimic successful *in vivo* transduction with joint tissue explants offers the unique opportunity to exploit capsid library evolution that may confer a syntropic capsid for the joint superior to any of the several analyzed in this study.

Impact of Neutralizing Antibodies from Equine Serum and Synovial Fluid on Serotype Transduction

We next sought to understand the role of neutralizing antibodies. An experiment was performed to determine the concentrations of serum and synovial fluid that would prove to neutralize AAV transduction. In brief, serial dilutions of serum or synovial fluid were created from 1:12 to 1:200 with incomplete F12 media as the diluent. The 1:12 dilution would have the greatest concentration of serum or synovial fluid and consequently neutralizing antibodies (if present). These concentrations were then pre-incubated with AAVGFP particles for one hour prior to synoviocyte cell transduction. If neutralizing antibodies were found in any treatment, this would have prevented the AAVGFP particles from efficiently transducing the cells, resulting in fewer cells expressing the GFP transgene as quantified by flow cytometry measurement compared to a control transduction. Specifically, any neutralizing serum or synovial fluid treatment was recorded only if it inhibited at least half of the transduction compared to the untreated, positive control. Additionally, as an increase in concentration implies a commensurate increase in antibody concentration, only the lowest concentration dilution of serum or synovial fluid was recorded

as this would identify the minimum absolute amount of neutralizing antibodies required to effect a 50% change in transduction. This would additionally differentiate between the strength of neutralization against specific serotypes. For example, if a neutralizing concentration of 1:25 was demonstrated, the stronger concentration (1:12) would also cause neutralization. And for example, if neutralization at 1:200 was found against AAV1 and 1:25 against AAV2, this would imply a higher concentration and/or greater specificity of antibodies to AAV1 than to AAV2. It was found that no synovial fluid samples had neutralizing effects on AAV transduction (Table 1). Serum samples were neutralizing to AAV 5 at the two highest concentrations tested in all four horses and only to AAV 2 and 2.5 in serum samples from one horse. Horses A and D were neutralizing to AAV 5 at a 1:12 dilution while horses B and C were neutralizing at a 1:25 dilution. Horse A was neutralizing to AAV 2 and 2.5 at only a 1:12 dilution.

Discussion

Using GFP transduction and neutralization assays we demonstrated differences between explant and monolayer transduction efficiencies in the joint tissues of the horse using a wide range of AAV serotypes. We observed three classes of AAV capsid transduction. The varying preference with which AAV serotypes transduce culture types was in agreement with findings by Mason et al. (Mason et al. 2012). The serotype most effective at transduction overall was AAV 2. AAV 2.5, 3, 5 and 6 also were effective in varying culture conditions. This is in agreement with work done in monolayer cultures by Goodrich et al. that showed AAV 2, 3, 5 and 6 (AAV 2.5 was not included in the study) to be the most efficient serotypes tested (Goodrich et al. 2009). In this study we extended the above analyses to the well-documented explant tissue model, previously shown to better represent *in vivo* joint tissue and surface glycans (Collier et al. 1991; Collier et al. 1995; Flannery et al. 1999). AAV 6 was found to be efficient in chondrocyte monolayers as in the previous study but less efficient in cartilage explants. Of note, AAV 2 and 2.5 were more effective in explants while AAV 5 and 6 were more effective in chondrocyte monolayers than in cartilage explants. This would predict that AAV 2 and 2.5 would perform better *in vivo*, all other variables being held constant. In fact, a recent study analyzing AAV 2 and 2.5 demonstrated persistent (80+ days) of increased transgene expression (400 ng/ml) supporting our

explant studies (Goodrich et al. 2013). The ability to mimic or partially mimic *in vivo* success with joint tissue explants offers the unique opportunity to exploit capsid library evolution that may offer syntropic capsids that are superior to the collection analyzed in this study.

To address the differences between explants and monolayers, an experiment was performed to enzymatically remove the cell surface receptors for AAV and determine if this equalized the AAV transduction efficiencies between the two. AAV 2 enzyme treatment included heparinase I and III, AAV 2.5 included both heparinase I and III and neuraminidase III, and AAV 5 included neuraminidase III. AAV 2 uses the proteoglycan heparan sulfate as the major binding receptor, and human fibroblast growth factor receptor 1 (FGFR1), hepatocyte growth factor receptor (HGFR), and the integrin αVβ5 as co-receptors (Summerford et al. 1998; Qing et al. 1999; Summerford et al. 1999; Kashiwakura et al. 2005). It has been previously shown that AAV 2 transduction is not mediated by chondroitin sulfate moieties (Summerford et al. 1998). Perlecan, however, has been theorized to be a binding site for AAV based upon immunohistochemistry and a BLASTN search of AAV Rep recognition sequences within the human genome (Wonderling et al. 1997; Konishi et al. 2008). AAV 5 uses the sialylated glycan α-2,3-N-linked sialic acid for the major binding receptor (Walters et al. 2001). AAV 2.5 is a chimera composed of AAV 2 with 5 amino acid substitutions from AAV 1 (which uses α-2,3- and α-2,6-N-linked sialic acid as major receptors). The primary receptor is still heparan sulfate and the role of the five amino acid substitutions is unknown (Wu et al. 2006; Wu et al. 2006). This experiment revealed that a difference in AAV 5 transduction efficiency between cartilage explant and chondrocyte monolayer could be attributed to varying amounts of sialylated glycan. The difference in AAV 2 and 2.5 transduction efficiencies between cartilage explant and chondrocyte monolayer cannot be attributed to differences in heparin sulfate, and heparan sulfate or sialylated glycan, respectively since removal of these receptors did not reduce transduction. Finally, the difference between synovial explant and synoviocyte monolayer transduction efficiencies for AAV 2 and 2.5 can be explained by differences in heparan sulfate, and heparan sulfate or sialylated glycan, respectively. For experiments where changes in transduction efficiency between explants and monolayers are not explained by differences in primary cell surface receptors, there are

several AAV secondary cell surface receptors (Wu et al. 2006; Wu et al. 2006) that could be contributing to the variable efficiencies. The concentrations of enzymes used to remove cell surface glycans followed the procedures by Shen et al. and were nearly 1000 times those demonstrated to significantly reduce AAV transduction by Summerford et al. (Summerford et al. 1998; Shen et al. 2011). In several cases, the results of the AAV transductions did not match the efficiencies from Figure 9. This could be due to either using a lower titer of virus (which was necessary to observe the effects of the enzyme) of 1000 VPC (as opposed to 8000 VPC) or the fact that the cells were analyzed at ten days (as opposed to thirty). Although we expected a greater decrease in transduction in the positive control, this degree of drop in transduction has been reported in the past with this dose of viral particles per cell (Boyle et al. 2006). In contrast, Summerford and Samulski found an 80% decrease in rAAV transduction after heparinase treatment; however, this finding used a viral dose of 2 VPC. The significantly higher dose of AAV used in this study is hypothesized to have led to a smaller enzyme treatment mediated decrease in transduction due to increased binding by AAV to co-receptors HGFR, αVβ5, and FGFR1 (Qing et al. 1999; Summerford et al. 1999; Kashiwakura et al. 2005). Lower viral doses result in greater drops, however below 1000 viral particles per cell in joint tissues results in very low transduction efficiencies (Goodrich et al. 2013). Regardless, it is of interest to determine if library select capsids with joint tropism would utilize sialic acid or heparan sulfate or a combination.

There were several horses that demonstrated various levels of AAV neutralization. Tests of horse samples showed no synovial fluid neutralization to any serotype of AAV but serum neutralization of varying degrees to AAV 5. Of note, bovine AAV shares capsid homology with AAV 5 (Johnson et al. 2004). The dilutions of serum that proved neutralizing in our study (1:12 and 1:25) could both be considered non-neutralizing according to an arbitrary cutoff of 1:50 that was used in the study by Boissier et al., and the 1:12 dilution could be considered non-neutralizing by the cutoff of 1:20 used in the study by Halbert et al. (Halbert et al. 2006; Boissier et al. 2007). The cutoff established by Halbert et al indicated dilutions of less than 1:20 can exhibit non-specific neutralization of AAV transduction, meaning AAV is neutralized by factors other than antibodies specific for the virus serotype. Horse A also revealed

serum neutralization to AAV 2 and 2.5 but only at the highest concentration and neither of these neutralizations would be considered neutralizing by the previous cutoffs. There is known immune cross reactivity between serotypes so this test cannot prove whether a specific neutralized AAV serotype was responsible for a primary infection, if the neutralizing capability of the serum is indeed due to pre-existing antibodies. However, the experiment reveals that, in a clinical setting, it is favorable to use AAV 2, 2.5, 3 or 6 for gene therapy transduction in the horse. Others could theoretically be used if neutralization is tested before the gene therapy treatment. As neutralization was only seen in the serum, it would be most accurate for testing to use serum rather than synovial fluid for neutralization assays. This trend is in contrast to the work of Cottard et al. which demonstrated a strong correlation between serum and synovial fluid inhibition in humans (Cottard et al. 2004).

In conclusion, this study reveals that AAV transduction efficiency can differ between explants and monolayers. One of the contributing factors could be the increased amount of extracellular matrix found in the explant. This suggests that monolayer cultures could provide an adequate, relative model for testing transduction efficiency for AAV serotypes *in vivo*, but explants may offer a more accurate model. Additionally, we have shown there is a possibility of serum neutralization to AAV 5 in some horses. This should encourage clinicians to perform AAV 5 neutralization tests before administration of AAV gene therapy vectors to horses.

Table-1: Values for the minimum dilution of synovial fluid (SF) and serum ("SF / serum") at which neutralization of AAV was found. For example, if a neutralizing concentration of 1:25 was found, the stronger concentrations (1:12) would also cause neutralization. "-" indicates no statistically significant neutralization at any dilution. Neutralization is defined as the weakest concentration of synovial fluid or serum treatment giving a transduction statistically, significantly less than 50% of the positive control (AAV treatment with no synovial fluid or serum).

	Virus Serotypes:				
	AAV2	**AAV2.5**	**AAV3**	**AAV5**	**AAV6**
Horse A:	- / 12	- / 12	- / -	- / 12	- / -
Horse B:	- / -	- / -	- / -	- / 25	- / -
Horse C:	- / -	- / -	- / -	- / 25	- / -
Horse D:	- / -	- / -	- / -	- / 12	- / -

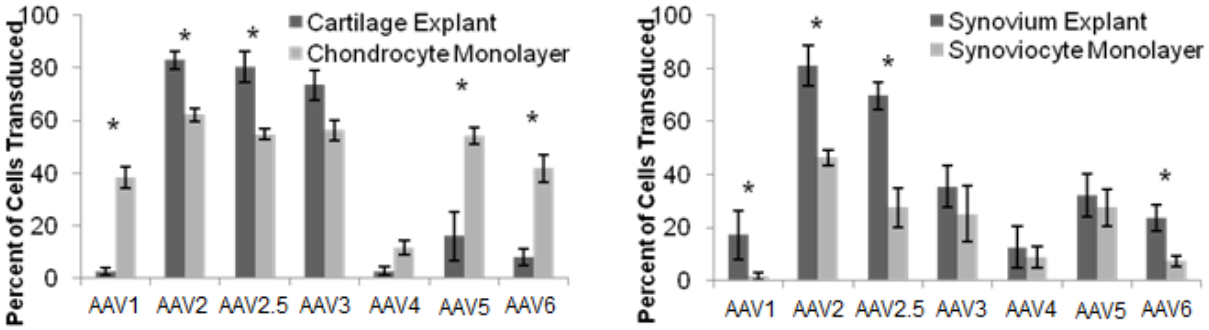

Figure 6: Comparison of transduction efficiencies within the same tissue of origination but different culture types. The graphs show flow cytometry analysis of the percent of cells transduced. Columns indicate mean percent and bars indicate standard error of the mean. Asterisks denote a significant difference between the explant and monolayer data in the specific AAV serotype tested. P-values < 0.05 are considered significant.

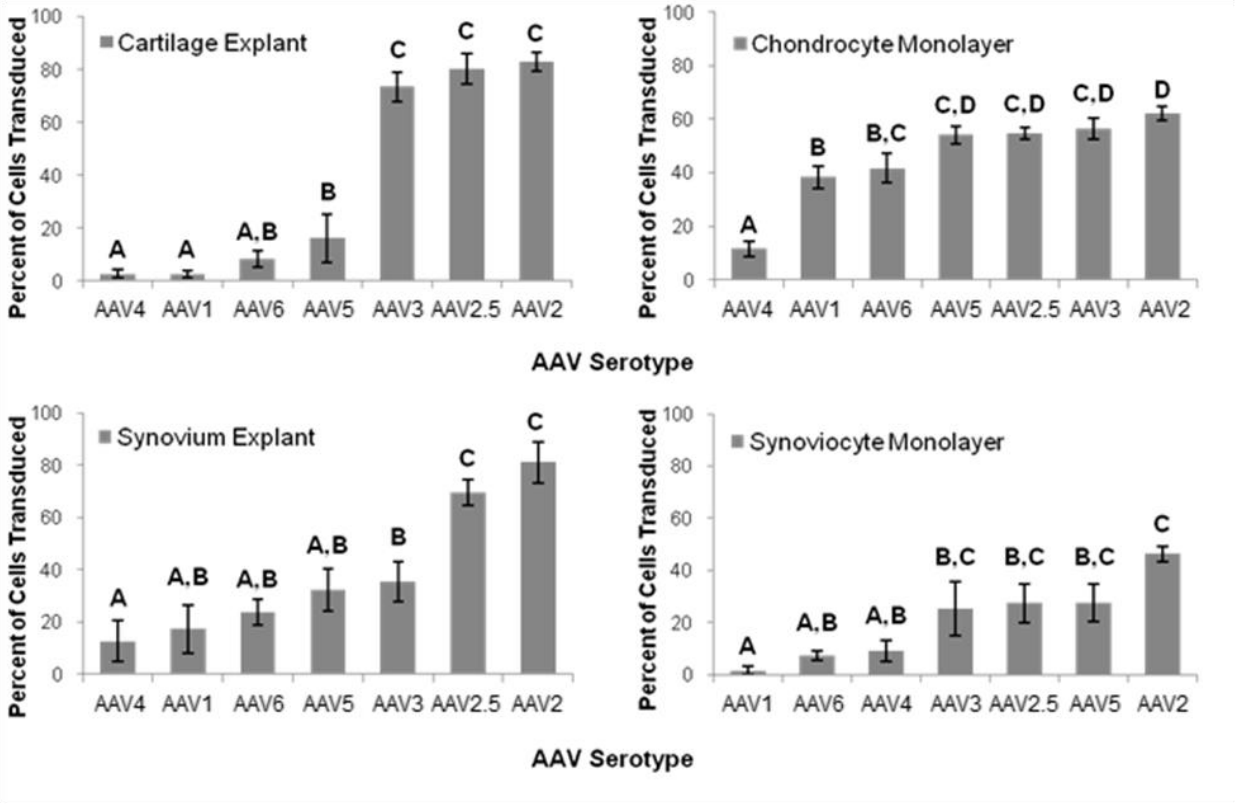

Figure 7: Ranking of AAV transduction efficiency as the percent of cells transduced in the cell and culture types tested. Columns indicate mean percent and bars indicate standard error of the mean. Significance between serotypes is shown with letters. P-values < 0.05 are considered significant.

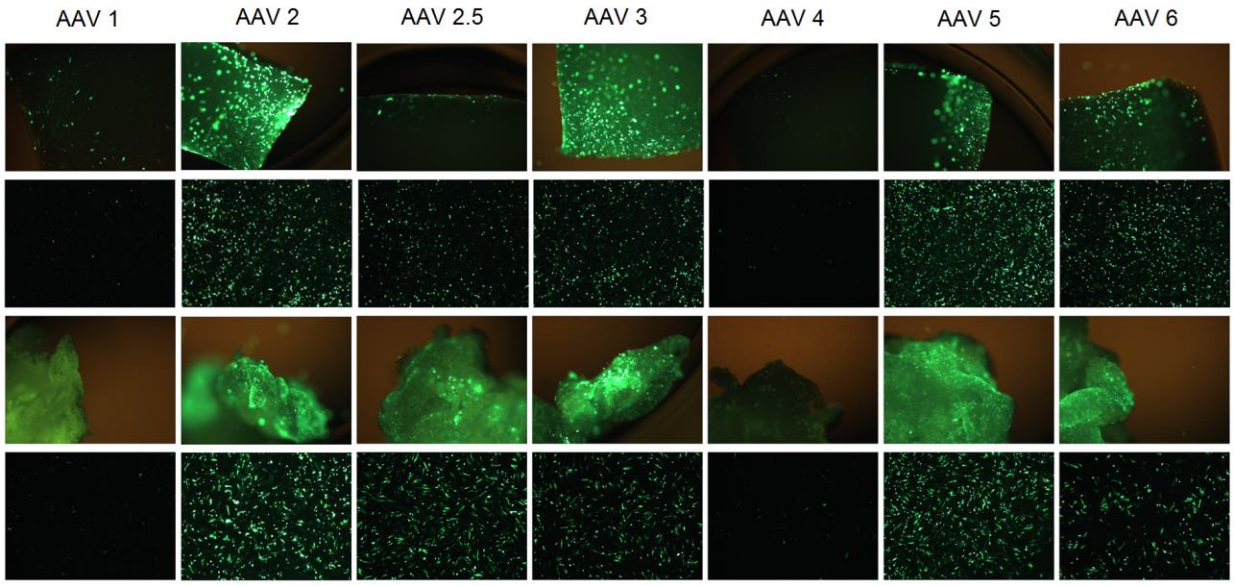

Figure 8: Fluorescence micrographs of the cell and culture types tested showing the presence or not of AAV transduction with the vectors tested. Pictures are from a single animal twelve days after transduction. From top row to bottom row: cartilage explants, chondrocyte monolayers, synovial explants, and synoviocyte monolayers.

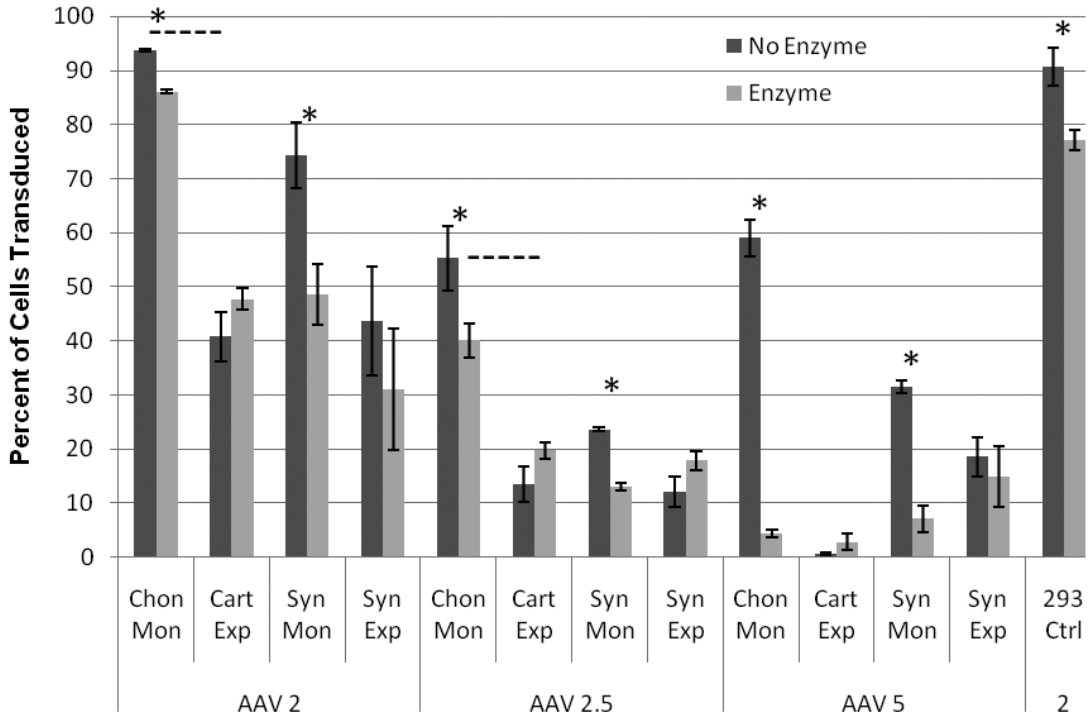

Figure 9: Comparison of AAV transduction rates as percent of cells transduced in cultures treated with heparinases I and III (for AAV 2 and 2.5) and neuraminidase (for AAV 2.5 and 5). Positive controls are denoted with dark fill and enzyme groups with light fill. Asterisks denote a significant difference between the enzyme treated and control group of the specific cell, culture, and virus type

tested. “-----" denotes a significant difference between enzymatically treated explant and monolayer cells of the same derived tissue within the specific serotype.

Chapter 3: Creation and NextGen RNA-Seq Evaluation of a scAAVIGF-I Gene Therapy Vector

Introduction

Osteoarthritis (OA) is a disabling disease being investigated for treatment with gene therapy. Currently there are no treatments to cure OA but only palliative remedies to allay pain and inflammation (Evans et al. 2004). Potential gene therapy treatments have been used with various viral and non-viral vectors and several transgenes encoding biological molecules of interest. Adenovirus, retrovirus, and adeno-associated virus (AAV) have been utilized in gene therapy clinical trials, and transgenes of interest in past trials have included IL-1RA and TGF-β_1 (Evans et al. 2011). In addition, candidates for transgenes include IGF-I, BMP-2, -7 and FGF as anabolic factors, IL-4, sIL-1R and sTNFR as catabolic antagonists, and SOD, Sox-5,-6,-9, SMAD and BCL-2 as various other factors that regulate free radicals, transcription, or apoptosis (Evans et al. 2004). This study specifically examines IGF-I as the transgene of interest in an AAV vector and the potential repair mechanisms and molecular pathways that may be specifically associated with enhancement of matrix production and collagen upregulation.

IGF-I is one of a handful of anabolic factors associated with cartilage repair (Nixon et al. 2000; Goldring et al. 2004; Goodrich et al. 2009). It has a brief biological half-life which leads to transiently waning effects of therapeutic treatment with recombinant IGF-I (Guler et al. 1989; Fortier et al. 2001; Fortier et al. 2002). The therapeutic treatment, however, does lead to increased production of proteoglycan and collagen type II, the normal constituents of healthy articular cartilage (Trippel 1995; Nixon et al. 2000). Mesenchymal stem cells (MSCs) can also benefit from IGF-I in designing treatments for cartilage defects (Worster et al. 2001; Goepfert et al. 2010). Supplementation of MSCs with IGF-I increases the chondrogenic potential of the cells (Worster et al. 2001; Longobardi et al. 2006).

AAV is an ideal vector for gene therapy due to its lack of pathogenesis, ability to infect numerous and non-dividing cell types, and the ability to promote long-term transgene expression. The wild type virus is a single stranded DNA virus. To circumvent the rate limiting second strand synthesis, self-complimentary AAV is used (Madry et al. 2003). As osteoarthritis is a long term, chronic disease, a long-

term constitutive expression of IGF-I is preferred. Chondrocytes rarely divide in the cartilage and thus have little capacity for DNA replication in the cell. This fact also affects the speed of the second strand synthesis, allowing self complimentary AAV (scAAV) an increased advantage over single stranded (Levy et al. 1975; Johnson et al. 2004). scAAV requires a transgene of only half the size available to single stranded AAV, but IGF-I has a size of only 888 base pairs which meets this criterion, far shorter than the 2300 base pair limit for scAAV.

Activation of the IGF-I receptor (IGF-IR) leads to autophosphorylation of this receptor and phosphorylation of a cascade of protein kinases. Eventually mitogen activated protein kinase (MAPK) can be phosphorylated through this cascade, and activate nuclear proteins and transcription factors that lead to the apoptotic and proliferative effects of IGF-I (LEROITH et al. 1995; LeRoith et al. 2003). In myoblast cells, IGF-I regulates molecular markers of hypertrophy and atrophy through activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway (Latres et al. 2005). IGF-I has also been shown to work through MAPK, but not phosphatidylinositol 3-kinase pathways in a fibroblast cell line (Mulligan et al. 2002). Also in this fibroblast cell line, Wnt-1 causes the upregulation of IGF-I expression which mediates apoptosis and shows an increase in PKB/Akt phosphorylation (Longo et al. 2002). In controlling apoptosis, IGF-I has been shown to work in muscle by upregulating transcription of TWIST through the MAPK pathway (Dupont et al. 2001).

This study was designed to create and evaluate a gene therapy vector delivering the IGF-I gene in vitro. Simple biochemical analyses were used to establish whether there was a differential production of factors conducive to a healthy cartilage environment, and RNA-seq was used for expression profiling. We hypothesized treatment of chondrocytes with AAVIGF-I would increase factors associated with healthy cartilage and increase gene expression of anabolic pathways, compared to both AAVGFP positive control and a negative cell culture control. The biochemical analyses indicated increased production of anabolic factors, and transcriptome sequencing indicated differential expression of known anabolic genes and pathways that may play an important role in cartilage repair.

Materials and Methods

Creation of scAAV2IGF-I

The nucleotide sequence for the primary peptide form of equine IGF-I as deduced by (Nixon et al. 1999) was optimized by GeneOptimizer (Life Technologies, Grand Island, NY) and purchased from GeneArt (Invitrogen, Carlsbad, CA). The optimization process ensures the most efficient use of codons, GC content, and avoids stable secondary RNA structures. This sequence was excised from its carrier plasmid using NotI and AgeI restriction endonucleases (NEB, Ipswich, MA) and ligated into a vector plasmid. The pTRs-ks vector plasmid (UNC Vector Core, Chapel Hill, NC), a mammalian expression vector, contained a CMV promoter and poly-A tail following the optimized IGF-I sequence. This plasmid is used for the creation of self-complementary AAV (scAAV). The completed IGF-I vector plasmid was sequenced to ensure a correct construction. The pTRs-ks IGF-I was produced in an scAAV2 capsid (UNC Vector Core, Chapel Hill, NC) as described in Grieger et al. (manuscript in preparation). Serotype 2 was considered the prime candidate for vector production based upon previous studies (Goodrich et al. 2009; Goodrich et al. 2013) and Hemphill et al. (manuscript in preparation).

Evaluation of scAAV2IGF-I

Cartilage was aseptically taken post-mortem from the trochlear ridge of three cadaver horses. All horses were between the age of two and five years. The pieces of cartilage were diced and digested as previously described (Nixon et al. 1992). Digested tissue was plated at 140,000 cells/cm^2 in complete Ham's F12 media (Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich, St. Loius, MO), 1N HEPES buffer (Invitrogen) and 1X antibiotic/antimycotic (HyClone, Logan, UT). The cells were detached using AccuMax (EMD Millipore, Billerica, MA) and passaged two remaining times in this same media. Upon 90% confluency in the final passage chondrocytes were frozen in a freeze media solution of 5% DMSO (Sigma-Aldrich) and 95% FBS in liquid nitrogen until required for further use. When chondrocytes were required for further use, they were thawed and immediately mixed with complete F-12 media and centrifuged at 500G for five minutes. Media was removed and chondrocytes were resuspended in complete F-12 and plated at 140,000 cells/cm^2 in 48 well plates with 500µL of complete F-12 media.

After three days in culture, chondrocytes were transduced with scAAV2IGF-I at a dose of 40,000 virus particles per cell (VPC) or scAAV2GFP at a dose of 8,000 VPC in 250μL of Ham's F-12 media, or received a negative control of 250μL of Ham's F-12 media for a four-hour transduction. Transduction media was removed and replaced with complete F-12 media and subsequently changed two days and five days post transduction. On the seventh day, media was frozen for further analysis and chondrocytes taken for analysis.

Biochemical Analysis

Media at day seven was tested for IGF-I protein content using a human IGF-I ELISA (R&D Systems, Minneapolis, MN). The media samples were pre-treated according to the manufacturer's directions to release IGF-I from binding proteins. Media samples were also tested for glycosaminoglycan content with dimethylmethylene blue (DMMB) spectral analysis after undergoing an initial papain digestion as described(Farndale et al. 1986). Chondrocytes were analyzed for cellular collagen type II content with the Type II Collagen Detection Kit, Multi-Species (Chondrex, Inc., Redmond, WA) as directed. Quantitative real-time Polymerase chain reaction (qRT-PCR) was also performed on the chondrocytes to determine differential expression of genes of interest. mRNA was isolated from cells as directed by QIAshredder and RNeasy kits (Qiagen, Germantown, MD). cDNA was generated with oligo(dT) primers and a SuperScript III first strand synthesis kit (Invitrogen). mRNA expression from the genes aggrecan, collagen type I, collagen type II, and IGF-I were compared to 18S mRNA as a control. Also used to run the PCR analysis were TaqMan Universal Master Mix (Applied Biosystems, Life Technologies) and primers and probes from Eurofins MWG Operon (Huntsville, AL) in a ABI Prism 7000 thermocycler (Applied Biosystems, Life Technologies). Primers and probes had the following sequences: 18S forward, 5'-CGGCTTTGGTGACTCTAGATAACC, reverse, 5'-CCATGGTAGGCACAGCGACTA, probe, 5'-FAM-CCCATTCGAACGTCTGAAATATCAACTTTCG-TAMRA-3'; aggrecan forward, 5'-ACCACTTTACTCTTGGCGTTTG, reverse, 5'-GCGAGTTGTCAGGGTCTGAA, probe, 5'-FAM-CTCTGAGGGTCATCACAGCGGCCATCTC-TAMRA-3'; collagen type 1 forward, 5'-

ATTTCCGTGCCTGGCCCCATG, reverse, 5'-GCCTTGGAAACCTTGGGGAC, probe, 5'-FAM-TCCTTCTGGTCCTCGTGGTCTCCCTGG-TAMRA-3'; collagen type 2 alpha 1 forward, 5'-AAACCATCAACGGTGGCTTCCA, reverse, 5'-GCAATGCTGTTCTTGCAGTGGT, probe, 5'-FAM-AGATGACAACCTGGCTCCCAACACTGCCAA-TAMRA-3'; IGF-I forward, 5'-TGTACTGCGCACCCCTCAA, reverse, 5'-TGCGTTCTTCAAATGTACTTCCTTC, probe, 5'-FAM-CCAGCGCCACACCGATATGCC-TAMRA-3'. PCR data was analyzed using the comparative Ct method with 18S as the housekeeping gene. For all assays statistical analysis was performed with SAS (SAS Institute Inc., Cary, NC).

NextGen Sequencing

Chondrocytes were isolated and transduced as described earlier. Seven days post transduction mRNA was isolated as described above. Total mRNA from chondrocytes was rRNA depleted then sequenced by the Infectious Disease Research Center Next Generation Sequencing Core at Colorado State University using an Ion Proton (Life Technologies) and the "Ion Total RNA-Seq Kit v2" protocol, including the use of RNA spike-in control. Further analysis was performed with Avadis NGS software (Strand Scientific Intelligence, San Francisco, CA). Within Avadis, alignment of the fastq sequencing files was done against "Transcriptome and genome together (with novel splices)" using the "Ensembl Genes and Transcripts (2012.12.15)" transcript model. During this analysis, data from the three horses was analyzed as "pooled" and averaged across replicates (for each treatment) for the Avadis Z-test workflow, and genes with p-values less than 0.05 and fold changes greater than 1.5 were considered significant for further analysis.

Specific Settings Used in Avadis Analysis

Avadis NGS version 1.5.1 Build 196070 was used for analysis. Alignment parameters used were: minimum percent identity=90; maximum percent gaps=5; maximum number of splices=1; minimum match length=25; maximum number of matches to be reported per read=1. Trimming parameters used were: number of bases to trim from 3' end=0; number of bases to trim from 5' end=0; trim 3' end with average quality less than=10. Quantification parameters used were: "Step 2 of 5"- defaults; "Step 3 of 5"-

defaults; “Step 4 of 5” – “normalization algorithm DESeq”, “threshold normalized counts t0”=1; “Step 5 of 5” – “Baseline to” = median of all samples. “Create Interpretation” using AAVGFP and AAVIGF samples, and “Average over replicates in conditions”=”Averaged”.

Results

Chondrocytes were isolated from trochlear ridge cartilage of cadaver horses. These chondrocytes were passaged twice and upon the third passage used for analysis of the AAVIGF-I vector. Seven days post transduction with the AAVIGF-I vector, chondrocytes were assessed for IGF-I levels with ELISA first in a dosing study (Figure 10) and next extracellular matrix activity with glycosaminoglycan and collagen type II assays (Figure 11). The ELISA results from the dose response experiment indicated peak IGF-I production at the highest transduction VPC of 40,000. Transduction also showed significantly increased production of IGF-I with AAVIGF-I treatment compared to AAVGFP positive control and non-treated negative control. IGF-I levels on average were 608 ng/ml in the treated group, with individual horses reporting from 428 ng/ml to 914ng/ml. Collagen type II was also significantly increased in chondrocytes treated with AAVIGF-I, but media GAG was not found to be statistically increased despite being produced in higher levels on average than in the controls (p-value=0.11). rt-PCR was utilized to examine differential regulation of mRNAs in scAAVIGF-I treated chondrocytes as compared to scAAVGFP treated positive controls (Figure 12). Aggrecan and collagen type II were found to be statistically upregulated 3.9 fold and 10.1 fold, respectively. Collagen type 1 was found to be upregulated 1.5 fold and endogenous IGF-I downregulated 1.3 fold, both statistically insignificant. We hypothesized that due to the mRNA sequence differences in the optimized IGF-I transgene, the PCR primers and probes for IGF-I did not recognize the transgene transcripts but only quantified the transcripts from endogenously transcribed, natural IGF-I mRNA.

Also at seven days post transduction, RNA was isolated from the chondrocytes, depleted of rRNA, and sequenced. Once again, all comparisons were AAVIGF-I treated chondrocytes as compared to the AAVGFP positive control treated chondrocytes. A Z-test for differential expression analysis revealed 885 genes upregulated greater than 1.5 fold and 1320 genes downregulated greater than 1.5 fold

between the two treatments. In addition, there were 4939 genes that were transcriptionally active, but not differentially regulated greater than 1.5 fold.

Gene ontology (GO) analysis indicated 113 GO terms significantly differentially regulated with a corrected p-value<0.10. The GO analysis was performed on the subset of genes that was differentially regulated greater than 1.5 fold. The 20 most significant differentially regulated GO terms were listed in order of significance (Table 3) and another 25 GO terms applicable to IGF-1 treatment and joint physiology were also listed (Table 4). A more in depth look at seven of the GO terms (Figure 13) indicates the proportion of genes within the GO term that were found to be differentially regulated, and specifically how the number of upregulated genes compared to the number of downregulated genes. Ranking of the fold change indicated IGF-I and several types of collagen were the greatest upregulated genes found, and that IGF2, BACH2, MMP17, BCL2 and ADAMTS1 were among the most downregulated genes (Table 2).

Discussion

Biochemical analyses indicated increased activity of constituents associated with healthy cartilage. It was found previously that IGF-I increases collagen type II content in addition to graft incorporation (Fortier et al. 2002; Madry et al. 2005) in agreement with our collagen type II results. Despite our findings of no significant increase in GAG media content, previous studies have shown increased GAG production due to IGF-I supplementation of chondrocytes (Elisseeff et al. 2001). It is hypothesized this lack of GAG increase is due to the relatively short seven day growth that was used to establish an early transcriptome analysis. If the cells had been cultured for a longer period, there would most likely have been a significantly increased production of GAG (Sah et al. 1994). An increase in aggrecan production has also been noted with IGF-I supplementation of chondrocytes (Yaeger et al. 1997) in agreement with PCR results.

IGF-I gene therapy was predominantly studied using adenovirus as the vector of choice before using AAV. Transduction of chondrocytes and synoviocytes was accomplished with adenovirus-IGF-I, and produced high levels of IGF-I through at least 28 days of monolayer culture (Nixon et al. 2000).

Goodrich et al. demonstrated improved early healing and long term healing by genetically modifying chondrocytes with AdIGF-I prior to transplantation in an equine cartilage defect model (Goodrich et al. 2007). Other studies have shown in vivo transduction of joint tissues with similar AAVIGF-I vectors (Izal et al. 2008; Weimer et al. 2012), but we have demonstrated far superior levels of IGF-I; these levels even surpass the previous highs of AdIGF-I induced protein production by over 5-fold (Nixon et al. 2005).

The findings of differentially regulated gene ontology terms with AAVIGF-I treatment creates a fingerprint of various GO terms that could be used to compare and contrast these results to transductions with other transgenes of interest. Two of the GO terms differentially regulated, regulation of MAPKKK cascade and regulation of ERK1 and ERK2 cascade have been found to be signaling pathways through which IGF-I activates proteoglycan synthesis, proliferation, and hypertrophy of cells (Starkman et al. 2005; Pratsinis et al. 2007). In addition to FGF-18,-21 and BMP-4, IGF-I and NOX4 are involved in the regulation of ERK1 and ERK2 cascade GO term. These are also differentially expressed in the regulation of MAPKKK cascade GO term. There were several collagen types showing upregulation. Differentially regulated COL1A1, COL2A1, COL9A1 and COL11A1,2 are all components in the cartilage development GO term. Collagen fibrils in cartilage are composed of heterotypically assembled type II, IX, and XI collagens (Mendler et al. 1989). Thinner collagen fibrils have collagen type IX as a component, while larger fibrils do not (Hagg et al. 1998). Collagen type XI has also been found to regulate fibril diameter (Blaschke et al. 2000). Collagen fibrils containing type II will not form without type XI present. In addition to the collagens, aggrecan, BMP-4, FGF-18, and TGF-β_1 are also found differentially regulated in the cartilage development term. These factors in addition to several of the collagens are also found differentially regulated in the skeletal system development and chondrocyte differentiation GO terms. TGF-β_1 increases ROS via NOX4, where the increase in NOX4 is via a SMAD2 dependent mechanism. NOX4 and BMP-4,-7 are also found in the regulation of MAP kinase activity GO term. A decrease in NOX4 activity by heme oxygenase 1 in IL-1β stimulated chondrocytes leads to cell death in addition to other macroscopic osteoarthritic related changes (Rousset et al. 2013). Nox4 is responsible for the IL-1β

mediated increase in reactive oxygen species (ROS) (Grange et al. 2006) and upregulation of NOX4 causes decreased aggrecan expression (Lugo et al. 2013).

GO terms related to cell death and apoptosis are also expected, as IGF-I has been shown to prevent apoptosis (Lo et al. 2004) including through a Bcl-2 mediated pathway (Kooijman 2006) and also MAPK and ERK pathways (Shakibaei et al. 2001). Bcl-2 in addition to COL2A1, BMP-4,-7, and IGF-I are found differentially expressed in the regulation of apoptosis GO term. The addition of FGF-1,-18,-21, NOX4 and TGF-β_1 lead to a subset of the genes differentially expressed in the regulation of cell proliferation GO term. Overexpression of Bcl-2 leads to reduced apoptosis in cells (Hockenbery et al. 1993; Adams et al. 1998). In previous studies, expression of Bcl-2 in normal cartilage was found to be significantly higher than in osteoarthritic cartilage, while Bcl-2 expression in lesional OA areas was found to be lower than in non-lesional OA areas (Kim et al. 2000). One possibility for the decrease we found in Bcl-2 after AAVIGF-I treatment involves the role it plays in cell cycle regulation. Increases in Bcl-2 lead to cell cycle exit, possibly preventing hypertrophy of chondrocytes (Brochhausen et al. 2009). The primary mode of action of Bcl-2 is also through caspases, and all caspases sequenced showed down regulation in AAVIGF-I treated cells (Adams et al. 1998).

We have shown that AAVIGF-I genetic modification of chondrocytes mirrors the collagen type II and gene expression changes observed in vitro with IGF-I supplementation. By transitioning this vector to an in vivo model, we hypothesize it would show upregulation of IGF-I to therapeutic levels and enhance long term cartilage healing. NextGen sequencing also resulted in a global analysis of genes differentially regulated as a result of this treatment, and subsequently differential expression within GO terms. This analysis creates a differential expression fingerprint that can be used in future studies to help identify new potentially anabolic cartilage factors. This fingerprint can also help with further analysis of any one of the individual genes found differentially expressed, as with this data additional hypotheses may be tested.

Table 2: A listing of the 50 most differentially expressed genes between the AAVIGF-I treatment and the AAVGFP control group.

Fold Change	Gene Symbol	Description	Ensembl ID
244.82	IGF1	Insulin-like growth factor I [Source:UniProtKB/Swiss-Prot;Acc:P51458]	ENSECAG00000010109
23.08	COL11A2	collagen, type XI, alpha 2 [Source:HGNC Symbol;Acc:2187]	ENSECAG00000017342
19.13	COL9A2	collagen, type IX, alpha 2 [Source:HGNC Symbol;Acc:2218]	ENSECAG00000010971
10.49	COL5A3	collagen, type V, alpha 3 [Source:HGNC Symbol;Acc:14864]	ENSECAG00000007647
10.16	COL2A1	collagen alpha-1(II) chain [Source:RefSeq peptide;Acc:NP_001075233]	ENSECAG00000022313
9.38	MIA	melanoma-derived growth regulatory protein [Source:RefSeq peptide;Acc:NP_001116128]	ENSECAG00000023165
9.02	DCLK1	doublecortin-like kinase 1 [Source:HGNC Symbol;Acc:2700]	ENSECAG00000007300
8.33	FAT2	FAT tumor suppressor homolog 2 (Drosophila) [Source:HGNC Symbol;Acc:3596]	ENSECAG00000023329
7.93	KIF26A	kinesin family member 26A [Source:HGNC Symbol;Acc:20226]	ENSECAG00000018772
7.13	MMP15	matrix metallopeptidase 15 (membrane-inserted) [Source:HGNC Symbol;Acc:7161]	ENSECAG00000000196
6.56	ENSECAG00000008725		ENSECAG00000008725
6.47	SCUBE1	signal peptide, CUB domain, EGF-like 1 [Source:HGNC Symbol;Acc:13441]	ENSECAG00000000951
6.44	NOXRED1	NADP-dependent oxidoreductase domain containing 1 [Source:HGNC Symbol;Acc:20487]	ENSECAG00000017196
6.3	PRX	periaxin [Source:HGNC Symbol;Acc:13797]	ENSECAG00000023423
6.18	HAPLN1	Hyaluronan and proteoglycan link protein 1 [Source:UniProtKB/Swiss-Prot;Acc:Q28381]	ENSECAG00000016477
6.18	ENSECAG00000016787		ENSECAG00000016787
5.88	SSC5D	scavenger receptor cysteine rich domain containing (5 domains) [Source:HGNC Symbol;Acc:26641]	ENSECAG00000019043
5.7	BEND4	BEN domain containing 4 [Source:HGNC Symbol;Acc:23815]	ENSECAG00000021008
5.42	DUSP2	dual specificity phosphatase 2 [Source:HGNC Symbol;Acc:3068]	ENSECAG00000007242
5.21	GPC2	glypican 2 [Source:HGNC Symbol;Acc:4450]	ENSECAG00000008170
5.13	APOA1	apolipoprotein A-I [Source:HGNC Symbol;Acc:600]	ENSECAG00000009752
4.98	FAM122C	family with sequence similarity 122C [Source:HGNC Symbol;Acc:25202]	ENSECAG00000021939
4.93	AFAP1L2	actin filament associated protein 1-like 2 [Source:HGNC Symbol;Acc:25901]	ENSECAG00000014979

4.82	PVALB	parvalbumin alpha [Source:RefSeq peptide;Acc:NP_001152807]	ENSECAG00000021062
4.72	SNORA70	Small nucleolar RNA SNORA70 [Source:RFAM;Acc:RF00156]	ENSECAG00000026512
Fold Change	Gene Symbol	Description	Ensembl ID
-5.46	ANKRD1	ankyrin repeat domain 1 (cardiac muscle) [Source:HGNC Symbol;Acc:15819]	ENSECAG00000016983
-5.5	C1orf216	chromosome 1 open reading frame 216 [Source:HGNC Symbol;Acc:26800]	ENSECAG00000005102
-5.52	TICRR	TOPBP1-interacting checkpoint and replication regulator [Source:HGNC Symbol;Acc:28704]	ENSECAG00000021453
-5.53	ZBTB16	zinc finger and BTB domain containing 16 [Source:HGNC Symbol;Acc:12930]	ENSECAG00000024358
-5.84	NOX4	NADPH oxidase 4 [Source:HGNC Symbol;Acc:7891]	ENSECAG00000010054
-5.88	ENSECAG00000027634		ENSECAG00000027634
-5.91	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1 [Source:HGNC Symbol;Acc:217]	ENSECAG00000016339
-5.94	ENSECAG00000007830		ENSECAG00000007830
-6.12	SLC25A46	solute carrier family 25, member 46 [Source:HGNC Symbol;Acc:25198]	ENSECAG00000022177
-6.17	BCL2	B-cell CLL/lymphoma 2 [Source:HGNC Symbol;Acc:990]	ENSECAG00000020437
-6.24	RSPO2	R-spondin-2 [Source:RefSeq peptide;Acc:NP_001103151]	ENSECAG00000024853
-6.37	CDH2	cadherin 2, type 1, N-cadherin (neuronal) [Source:HGNC Symbol;Acc:1759]	ENSECAG00000009663
-6.43	FAM105A	family with sequence similarity 105, member A [Source:HGNC Symbol;Acc:25629]	ENSECAG00000017059
-6.47	THBS4	thrombospondin 4 [Source:HGNC Symbol;Acc:11788]	ENSECAG00000019665
-6.56	VIT	vitrin [Source:HGNC Symbol;Acc:12697]	ENSECAG00000003318
-6.72	RBPJL	recombination signal binding protein for immunoglobulin kappa J region-like [Source:HGNC Symbol;Acc:13761]	ENSECAG00000013463
-6.73	BHMT	betaine--homocysteine S-methyltransferase [Source:HGNC Symbol;Acc:1047]	ENSECAG00000015626
-6.95	SLC22A4	solute carrier family 22 (organic cation/ergothioneine transporter), member 4 [Source:HGNC Symbol;Acc:10968]	ENSECAG00000024253
-7	ADAMDEC1	ADAM-like, decysin 1 [Source:HGNC Symbol;Acc:16299]	ENSECAG00000019922
-7.39	MMP17	matrix metallopeptidase 17 (membrane-inserted) [Source:HGNC Symbol;Acc:7163]	ENSECAG00000013201
-8	eca-mir-671	eca-mir-671 [Source:miRBase;Acc:MI0012701]	ENSECAG00000026454

-8.45	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2 [Source:HGNC Symbol;Acc:14078]	ENSECAG00000010522
-8.51	MSMP	microseminoprotein, prostate associated [Source:HGNC Symbol;Acc:29663]	ENSECAG00000010570
-8.7	IGF2	insulin-like growth factor II precursor [Source:RefSeq peptide;Acc:NP_001108011]	ENSECAG00000024524
-9.75	GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2 [Source:HGNC Symbol;Acc:21531]	ENSECAG00000016679

Table 3: The top 20 significant pathways identified as differentially regulated between AAVIGF-I treatment and the AAVGFP control. P-values are corrected with the Benjamini Yekutieli method.

Corrected P-value	GO Term	GO ACCESSION	Genes in GO Term w/ FC>1.5	Total Genes in GO Term
0.0000	extracellular matrix	GO:0031012	91	300
0.0000	extracellular region part	GO:0044421	144	658
0.0000	proteinaceous extracellular matrix	GO:0005578	63	212
0.0000	extracellular region	GO:0005576	181	990
0.0000	cellular process	GO:0009987| GO:0008151| GO:0050875	1064	8595
0.0000	extracellular matrix part	GO:0044420	38	120
0.0000	Binding	GO:0005488	1425	12126
0.0000	protein binding	GO:0005515| GO:0045308	1080	8833
0.0005	regulation of phosphorylation	GO:0042325	109	605
0.0006	multicellular organismal development	GO:0007275	331	2340
0.0006	fibrillar collagen	GO:0005583	9	11
0.0010	developmental process	GO:0032502	357	2572
0.0011	system development	GO:0048731	301	2113
0.0012	extracellular space	GO:0005615	83	436
0.0012	positive regulation of biological process	GO:0048518| GO:0043119	341	2446
0.0012	regulation of metabolic process	GO:0019222	443	3306
0.0013	anatomical structure development	GO:0048856	328	2345
0.0015	cartilage development	GO:0051216	30	104
0.0019	negative regulation of biological process	GO:0048519| GO:0043118	291	2053
0.0030	collagen fibril organization	GO:0030199	14	30

Table 4: This table presents a further 25 gene ontologies of interest in IGF-I supplementation in cartilage biology. These were also identified as differentially regulated between AAVIGF-I treatment and the AAVGFP control. P-values are corrected with the Benjamini Yekutieli method.

Corrected P-value	GO Term	GO ACCESSION	Genes in GO Term w/ FC>1.5	Total Genes in GO Term
0.0045	collagen	GO:0005581	18	49
0.0045	extracellular matrix organization	GO:0030198	33	129
0.0045	negative regulation of apoptosis	GO:0043066	77	416
0.0061	regulation of ERK1 and ERK2 cascade	GO:0070372	24	81
0.0184	regulation of MAPKKK cascade	GO:0043408	47	229
0.0212	endochondral bone morphogenesis	GO:0060350	14	37
0.0213	regulation of cell proliferation	GO:0042127	120	761
0.0257	regulation of apoptosis	GO:0042981	123	788
0.0328	regulation of programmed cell death	GO:0043067| GO:0043070	123	794
0.0348	chondrocyte differentiation	GO:0002062	14	39
0.0404	cartilage development involved in endochondral bone morphogenesis	GO:0060351	9	18
0.0436	regulation of cell death	GO:0010941	124	809
0.0492	skeletal system development	GO:0001501	58	318
0.0515	chondrocyte development	GO:0002063	8	15
0.0526	positive regulation of cell proliferation	GO:0008284	73	428
0.0977	regulation of cell growth	GO:0001558	35	170
0.1064	skeletal system morphogenesis	GO:0048705	32	152
0.1079	negative regulation of ERK1 and ERK2 cascade	GO:0070373	8	17
0.1139	negative regulation of cell proliferation	GO:0008285	58	333
0.1156	bone development	GO:0060348	32	153
0.1197	bone morphogenesis	GO:0060349	16	56
0.1449	insulin-like growth factor binding	GO:0005520	9	22
0.1589	regulation of pathway-restricted SMAD protein phosphorylation	GO:0060393	10	27
0.1754	cell differentiation	GO:0030154	202	1485
0.1887	positive regulation of ERK1 and ERK2 cascade	GO:0070374	16	59

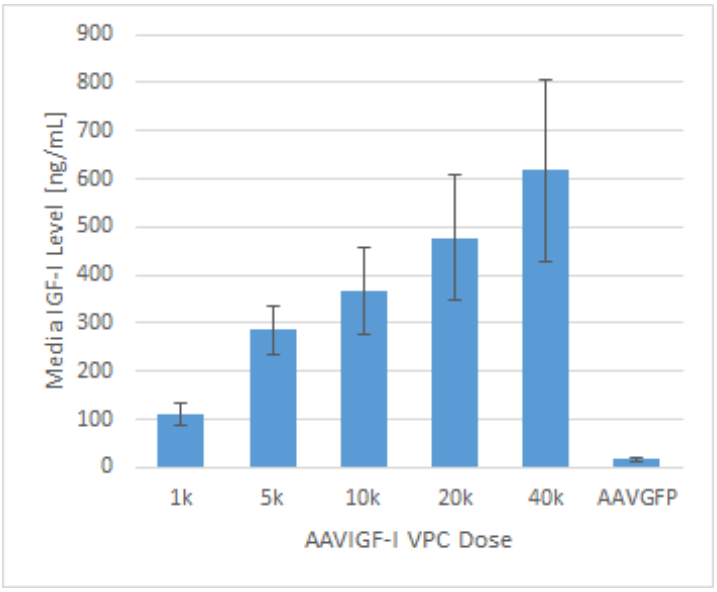

Figure 10: IGF-I response and dosing of AAVGF-I as number of virus particles per cell (VPC) in chondrocytes. Previous study of dosing with levels up to 100,000 VPC indicated plateau of response curve at ~20,000 VPC (data not shown).

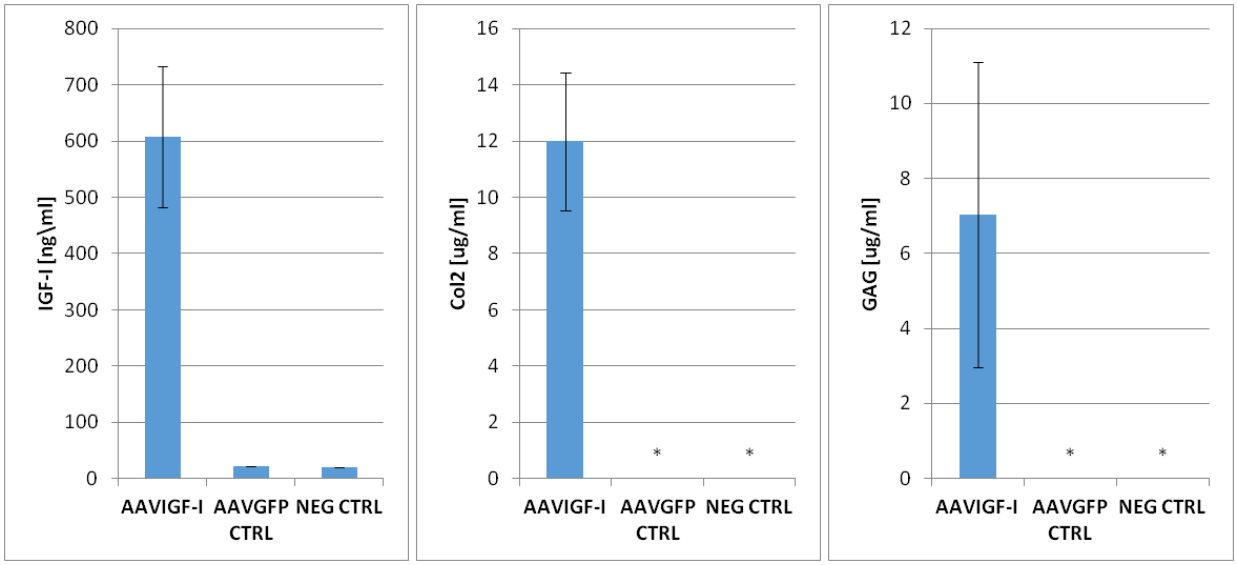

Figure 11: A) ELISA results of IGF-I levels in media seven days following transduction. AAVIGF-I treatments produces significantly higher concentrations of IGF-I with a p-value<0.01. B) Type II collagen cellular concentration. Asterisk indicates a level below the limit of detection. AAVIGF-I treatment produces statistically higher levels of Col2 with a p-value<0.01. C) Glycosaminoglycan concentration of media samples seven days post transduction. Asterisk indicates level below the limit of detection. The increased levels in AAVIGF-I treatment is not statistically significant with a p-value = 0.11. In all figures bars shown are standard error.

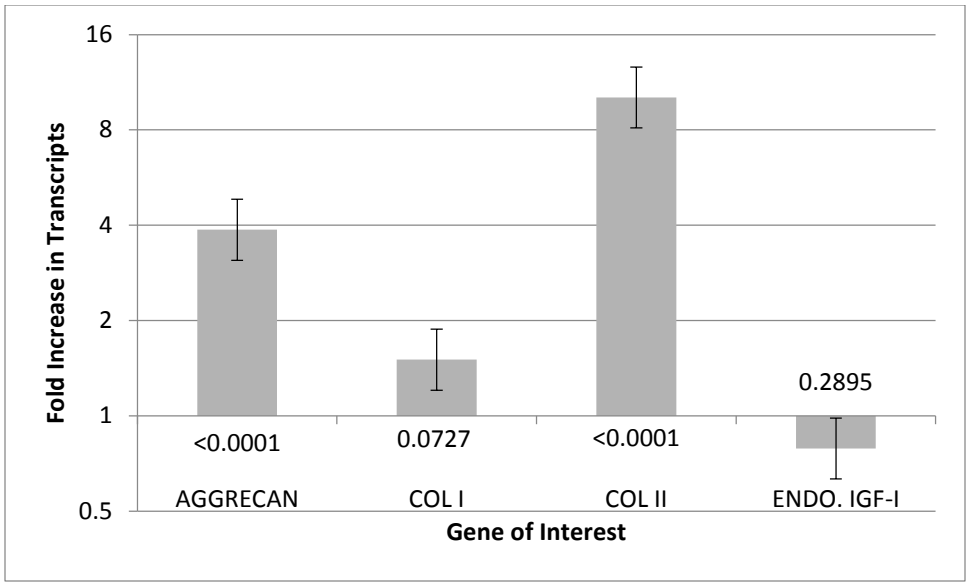

Figure 12: rtPCR data indicating the gene expression fold change in AAVIGF-I treatment compared to AAVGFP positive control. Statistically significant change in expression was noted in aggrecan and type II collagen mRNA expression with p-values as indicated. Type I collagen and endogenous IGF-I did not show significant differential expression. Bars shown are for standard error. We can conclude IGF led to the increased production of aggrecan and collagen type II in support of a healthy cartilage phenotype.

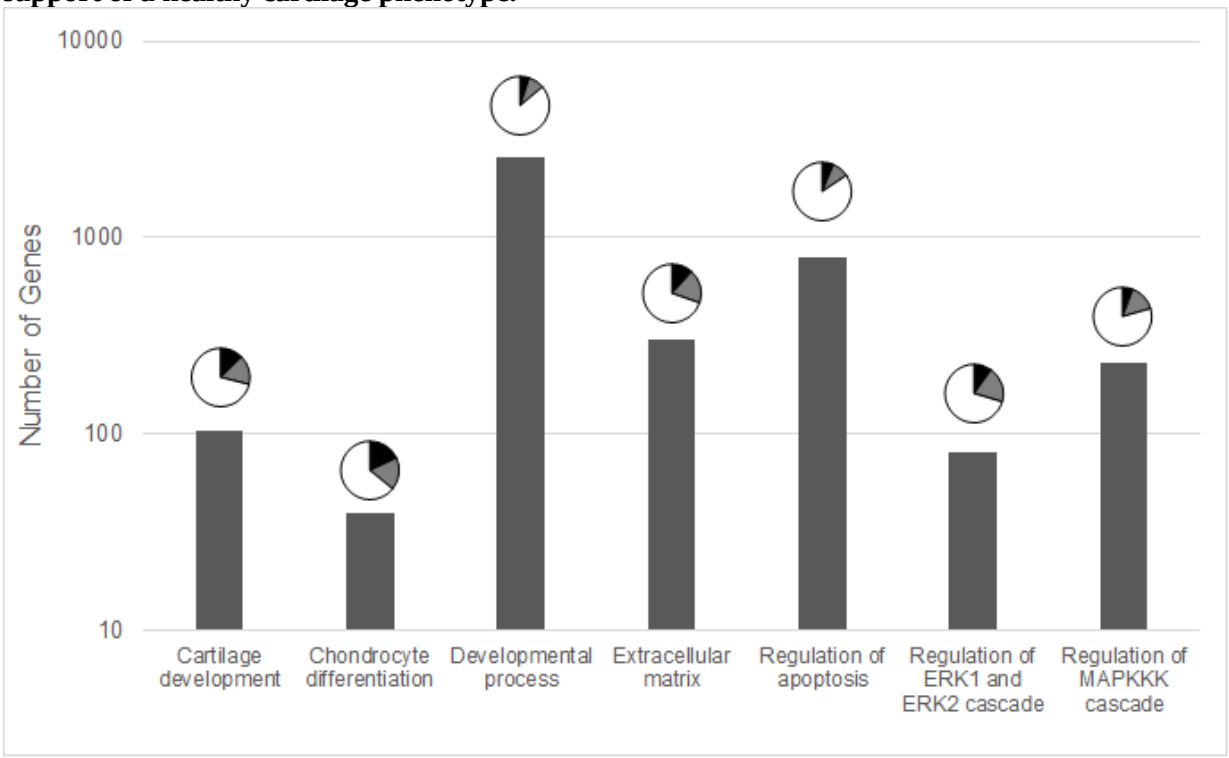

Figure 13: This figure compares the total number of genes found in gene ontology pathways of interest, and the ratio of upregulated to downregulated genes all differentially expressed between AAVIGF-I treatment and AAVGFP with p-values less than 0.10. A gene ontology represents the

behavior of a group of genes within a cellular context. Gene ontologies are curated by the Gene Ontology Consortium(Consortium 2000). Pie charts above columns indicate number of genes differentially expressed greater than 1.5 fold, specifically upregulated genes (black shading) and downregulated genes (gray shading). The white pie section indicates the proportion of genes in the gene ontology pathway that were not found differentially expressed greater than 1.5 fold in this analysis but which have been characterized to the pathway.

Discussion

The combined research presented here is evidence in support of further pursuit of AAVIGF-I as a gene therapeutic. Additionally, our work was the first research we are aware of that showed therapeutic levels of IGF-I from an optimized IGF-I transgene in a gene therapy vector. We are planning to apply in situ to further evaluate, and if positive results are revealed, an effective vector that will enhance cartilage repair may be proven.

We have shown efficient transduction of the various tissues in the joint in vitro but also in explants in situ. We have shown therapeutic levels of IGF-I production via the vector in vitro, and we have shown this IGF-I is biologically active and is beneficial to cartilage as has been observed in past studies. Other studies have shown in vivo transduction of joint tissues with similar AAVIGF-I vectors (Izal et al. 2008; Weimer et al. 2012), but we have demonstrated far superior levels of IGF-I; these levels even surpass the previous peaks of AdIGF-I induced protein production by over 5-fold (Nixon et al. 2005). While this study uses tissues from young, healthy animals for experiments, this does present a limitation in the research. Ultimately the vector would be used in older animals suffering from OA. Tissues harvested from OA joints represent much more difficult targets functionally due to general age related changes in cells, tissue degradation and altered disease state biological processes present. Sequencing of the transduced OA, and normal tissues would add a significant pool of data to analyze, with the potential for numerous reports. Extending the duration of culture would also enable additional clinically relevant analyses as OA is a chronic disease. With the current sequencing data, there are also opportunities to further inspect for miRNAs and other small coding and non-coding elements not yet recognized in the transcriptome. Regarding tissue types, MSCs offer a great direction for future research with this vector. Many new cartilage defect treatment strategies involve attaining chondrogenesis of autologous or allogeneic MSCs, which are more readily available than chondrocytes (Kisiday et al. 2013). One final limitation includes only analyzing the transcriptome of the cells when the proteome can also be

analyzed. RNA can undergo modifications prior to translation that limit the potential for using RNA analysis to predict relative phenotypic changes. A proteomic analysis would provide increased power to use measurements to predict relative phenotypic changes.

This report provides several additions to the scientific knowledge. First, we have provided an insight to serotype specificity in joint tissue transductions. We demonstrated that with the differences in extracellular matrix, there could be differences between in vitro and in situ models, and researchers should be aware of this as they transition from in vitro work to animal research models. Additionally, we found wild type, low level neutralization of AAV5 by serum of horses. This indicates the necessity of pre-treatment quantification, especially for AAV5, before gene therapy to ensure for the most efficient transduction possible and an abscence of inflammation due to an immune mediated event. This may also indicate a modality for any previous AAV studies that did not result in expected outcomes (Izal et al. 2008). Finally, we have shown the increased protein production capability of optimized gene transcripts from a gene therapeutic. The large increase in protein levels allows for new studies in which authors may consider even higher protein doses than have previously been offered via gene therapy vectors.

This research contributed new data to the field of osteoarthritis gene therapy and added evidence in support of previous studies (Nixon et al. 2005; Goodrich et al. 2009). The ultimate goal of this research is to develop a clinical therapeutic. As was stated by Evans et al. this also requires significant resources, as just creating good laboratory practice vector can incur costs of over $500,000 (Evans et al. 2009). Additionally, further research on the vector would be expected before clinical trials. The most pressing research project would be the establishment of efficacy of the vector in vivo in an animal model. While the equine model offers the most relevant model to human clinical use(McIlwraith et al. 2012), a mouse or small mammal model would be more efficient in producing the sample size- statistical power likely to be required before a human clinical trial is granted (Chu et al. 2010). Further research could be conducted into a more optimized AAV vector. Directed evolution capsid design is one method that could produce AAVs with even more efficient transduction of targeted tissues. Research interest has also been expressed in co-administration of IGF-I gene therapy with other vectors such as BMP-7 or IL-1Ra

(Morisset et al. 2007; Fortier et al. 2011). This would be important to explore concurrently with an AAVIGF-I clinical trial, as the use of co-administrations would be firstly contingent upon safety and efficacy of the AAVIGF-I trial. If a gene therapy vector such as AAVIGF-I became a valid treatment option that could regenerate cartilage, it would result in significant savings due to the morbidity and treatment costs associated with OA.

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