OPTIMIZING ADENO-ASSOCIATED VIRUS AS A GENE THERAPY VECTOR FOR TREATING ARTHRITIS

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To my parents, Thomas and Linda Kay

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ºC	degrees centigrade
$CaPO_4$	calcium phosphate
CAR	coxsackie-adenovirus receptor
CDC	Centers for Disease Control
cDNA	complementary DNA
CMV	cytomegalovirus
CO_2	carbon dioxide
DMARD	disease modifying anti-rheumatic drug
DMEM	Delbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DRP	DNAse resistant particle
EDTA	ethylenediaminetetraacetic acid
EGFR-PTK	epidermal growth factor receptor protein tyrosine kinase
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
FGFR-1	fibroblast growth factor receptor – 1
FKBP52	FK506 binding protein
FPLC	fast protein liquid chromatography
GBSS	Grey's balanced salt solution
GFP	green fluorescent protein
HGFR	human growth factor receptor
HIV	human immunodeficiency virus
HSPG	heparin sulfate proteoglycan

hr	hour
HSV	herpes simplex virus
IL-1β	interleukin-1 beta
IL-1Ra	Interleukin-1 receptor antagonist
ITRs	inverted terminal repeats
LLnL	N-acetyl-L-leucyl-L-leucyl-L-norleucine
kg	kilogram
mg	milligram
mL	milliliter
mM	millimolar
MMPs	matrix metalloproteases
NAB	neutralizing antibody
ng	nanogram
nm	nanometer
NO	nitric oxide
NSAID	non steroidal anti-inflammatory drug
OA	osteoarthritis
pg	picogram
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PDGFRα	platelet derived growth factor receptor alpha
RA	rheumatoid arthritis
rAAV	recombinant adeno-associated virus
RBE	rep binding element
RNA	ribonucleic acid

sc	self-complementary
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
scAAV	self-complementary adeno-associated virus
ssAAV	single-stranded adeno-associated virus
TNF-α	tumor necrosis factor alpha
trs	terminal resolution site
U	units
UV	ultraviolet
vg	vector genome
Vp	virus particle
VSV-G	vesicular stomatitis virus G protein
zLLL	carbobenzoxy-L-leucyl-L-leucyl-L-leucinal

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Gene-based therapies offer enormous potential for the treatment of chronic joint conditions, presenting the capacity to directly change the biology of diseased or damaged tissues. Adeno-associated virus (AAV), a parvovirus with a single stranded DNA genome, has emerged as the most favorable viral vector for use in human clinical applications, due primarily to its safety profile. However, many challenges still face AAV-based gene therapy technology, as AAV is completely dependent on cellular mechanisms for entry, nuclear trafficking, and second strand synthesis of its genome. The recent development of self-complementary (sc) vectors, which bypass the need for second strand synthesis, and the capacity to cross-package the AAV2 vector into different capsid serotypes have expanded both the efficacy and versatility of this system.

Determining the effectiveness of scAAV was the first step of our study. To accurately compare conventional single-stranded AAV (ssAAV) and scAAV vectors, identical expression cassettes were inserted into both viral genome types, and were packaged in serotype 2 capsids for use in vitro and in vivo. These two viral genome

types were used for infection of cells in culture and for intra-articular injections of rabbits. Measurements of transgene expression in vitro verified an earlier onset and higher levels of gene expression when using scAAV. The gene for the anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) was used as the transgene for animal experiments, and therapeutic results were achieved with the self-complementary vector.

Another step for optimization of AAV based gene transfer is selecting the most effective capsid serotype for the target tissue. Various joint tissues from rat, horse, and human were used to screen a battery of AAV serotypes. The outcomes of these screens showed that types 2 and 5 were most effective overall, but also that equine tissues were highly receptive to any AAV transduction with any serotype. When scAAV.IL-1Ra was injected into equine joints, the result was transgene expression levels matching the therapeutic levels seen in the rabbit study. Further enhancement of AAV transduction can be achieved through mutations of the capsid that inhibit intracellular degradation.

These data continue to support the viability of AAV as a safe and effective gene therapy vector for treatment of joint diseases.

CHAPTER 1
INTRODUCTION

Arthritis

Joint inflammation is the general definition of arthritis, but this simple explanation masks the enormous complexity that exists in diseases of the joint. There are two major forms of arthritis: rheumatoid arthritis (RA) and osteoarthritis (OA). Although they have different causes, mechanisms of actions, and many different symptoms, both forms of arthritis ultimately degrade the cartilage that is necessary for proper joint function.

Joint Structure

Articular cartilage covers and protects the ends of the bones at sites of articulation. It must provide an effective cushion and be very durable to withstand the mechanical stress of motion, and also very smooth, to provide frictionless and effortless movement. The nature and structure of articular cartilage impart these properties. Cartilage is made mostly of collagen and proteoglycans with many other minor components with similar properties. Collagen II is the most common type of collagen in cartilage, making up 80-90% of the collagen content.[1] It forms an intricate, cross-linked network together with collagens types IX and XI, giving shape and tensile strength to the cartilage. Proteoglycans make up the majority of the remaining dry mass of cartilage. These molecules, made of highly sulfated aggrecan attached to hyaluronic acid, bind water, which accounts for most of the physiological mass of cartilage. When under compression, the collagens, due to their strength, retain the shape of the cartilage and hinder the expansion of the proteoglycans.[2,3] This architecture constricts the flow of

water out of the cartilage when it is under mechanical stress, thereby cushioning the joint.[4,5]

Chondrocytes are the cells specific to the cartilage that are responsible for its maintenance. They sparsely populate the cartilage tissue and continuously remodel the matrix by simultaneously degrading and synthesizing cartilage matrix components. Chondrocytes are long-lived cells of mesenchymal origin, but their lifecycle is unconfirmed. It is possible that they are post mitotic, and replaced by infiltration of new cells from the subchondral bone,[6] or that a small population of progenitor cells also exists within the cartilage.[7]

Cartilage is also unique in that it contains no blood vessels, nerve fibers, nor lymphatics.[8] Due to the lack of vascularity, the chondrocytes receive nutrients and have their waste removed by the flow of synovial fluid. The compression and subsequent decompression of the cartilage from normal movement and joint loading provides sufficient force to drive this flow.

While the ends of the bone are covered by cartilage, the whole structure is contained within a joint capsule of fibrous tissue. Several ligaments hold the bones together to maintain joint alignment, while tendons attach the bones to the muscles to allow movement. Together, the ligaments and tendons facilitate smooth joint function, while being critical to the biomechanical stability of the joint. Most proximal to the bones and cartilage is the synovium, a thin layer of cells of two types: type A, macrophage-like, and type B, fibroblast-like. Type B synoviocytes make up more than two-thirds of the synovium and are responsible for maintaining the synovial fluid, the liquid that surrounds, lubricates, and nourishes the joint space.[9] Both the volume and viscosity of

the fluid can be regulated by secretion of hyaluronan and other similar factors, with the mechanical stresses of expanded fluid volume acting as a negative feedback mechanism.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic inflammatory disease where an immune response is mounted against several of the body's organs including the joints. Approximately 1.5 million Americans have RA, although the prevalence is decreasing, according to the CDC. RA is characterized by the formation of a pannus, an aggressive hyperplasic tissue of synovium which invades and destroys the cartilage, due to increased production of metalloproteinases, serine proteases, and aggrecanses.[10] The pannus is unique to RA, almost tumor-like in its growth, and causes destruction of the joint. While anti-inflammatory drugs such as non steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids can provide pain relief, what is desired are disease modifying agents of rheumatic disease (DMARDs) which can both treat the symptoms and halt the joint destruction. Small molecules such as methotrexate have some effectiveness, but the state of the art has moved towards biologics such as soluble TNFα receptors and interleukin-1 receptor antagonist protein (IL-1Ra).[11] New biologics under clinical investigation include antibodies against CD20, which is present on B cells, and soluble receptors to IL-6 as well as CD28, which are present on T cells.[12]

Osteoarthritis

Osteoarthritis (OA) is a painful condition in joints that arises from degeneration of articular cartilage. OA affects nearly 40 million individuals in America, and these numbers are expected to rise to as many as 60 million as the population ages.[13] OA is generally associated with aging, but can occur in younger people following joint injury.

Once onset occurs, OA typically progresses until there is great pain and loss of joint mobility, although the rate of progression is highly variable among individuals. OA is not only incurable and its treatment inadequate, but it is also very debilitating, leading to physical impairment, reduction in quality of life and lost working days.[13]

Both age and injury contribute to the degradation of cartilage, but the continued synthesis of inflammatory cytokines by the synoviocytes and chondrocytes is thought to drive the progression of disease. As the body ages, chondrocyte numbers decrease, they are less able to respond to mitogenic signals from growth factors, and cellular metabolism and proliferation are reduced.[14-16] Cartilage wear leads to the generalized loss of cartilage across the articulating surfaces. When damage occurs to ligaments and tendons, joints can become misaligned, causing unnatural movement. This pathologic articulation is sensed by the chondrocytes whose biology becomes skewed, leading cells to degrade the matrix faster than it is synthesized, leading to gradual loss of cartilage and OA.[17,18]

Pain is the presenting symptom of OA, but as there are no nerves in cartilage, the early stages of the disease are not felt. This lack of pain in the early stages may result in the continuation of behaviors that caused the initial damage. Although the original injury may in itself be moderate, the loss of structural soundness can lead to a progressive, often irreversible cycle of joint deterioration.[19]

Articular cartilage is also avascular, which limits its ability for self repair. In most other tissues, injuries lead to the rupture of blood vessels, releasing platelets and associated factors which form a clot. Progenitor cells are recruited, and differentiate into the local cellular phenotype or synthesize repair tissues.[20,21] In contrast, there is no

bleeding in a cartilage injury, and repair is limited to the local chondrocytes. They may respond by enhanced matrix synthesis, but they cannot fill a large void. Therefore, focal, chondral lesions usually remain for life.[22,23] When injuries extend deep enough into the chondral layer and reach the subchondral bone and underlying vasculature, local bleeding and clot formation lead to the generation of space filling repair tissue. This repair tissue only somewhat resembles the native cartilage: it contains a high amount of type 1 collagen. This fibrocartilaginous scar tissue does not have the same architecture and composition as original cartilage, nor the same structural properties, and can degenerate over time.[20,24,25]

There are many hypotheses about how mechanical stress or damage is transmitted to the chondrocytes, but what is clear is that they up-regulate their production of aggrecanase, which degrades proteoglycans and collagenase destroying collagens.[26] There is also new evidence that cathepsin K in particular is responsible for degrading collagen II.[27] It is believed that an increase in fibril fragments within the joint irritate the synovial membrane and activate an inflammatory response. Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) are the primary mediators of inflammation that accelerate matrix destruction pathways while inhibiting matrix synthesis.[28,29] Synthesized within the joint, IL-1 stimulates chondrocytes to produce matrix metalloproteases (MMPs) and aggrecanases, as well as other factors associated with OA such as nitric oxide (NO) and prostaglandin E2. IL-1 is also responsible for upregulation of TNFα expression, which in turn enhances IL-1 expression.[30]

Treatments for Arthritis

Most drugs that are taken for OA are analgesic, which do nothing to stop or reverse the progression of the disease. Until adverse side effects were reported, cyclooxygenase inhibitors were the primary class of drugs prescribed to those with OA, but now NSAIDS such as ibuprofen and naproxen are most prevalently taken. Other treatment options are intra-articular injection of corticosteroids.[31] Although commonly advertised, oral supplementations of glucosamine, hyaluronan, and other nutraceuticals have not been found to significantly affect outcomes of arthritis.[32] Surgical options, such as arthroscopic lavage, debridement, and osteotomy, can be performed to aid patients with late stages of OA. These procedures can provide symptomatic relief, though they are generally viewed as tactics that delay joint replacement, the final option for treatment. For this, the entire joint is surgically removed and replaced with a prosthesis. This removes the joint and the source of the inflammatory pain, but prosthetics have limits to their function and tend to wear out over several years and need replacement themselves.

Biologics

Although immune suppressants such as methotrexate act as DMARDs, more desirable outcomes can be achieved with biologics. Recombinant forms of IL-1Ra (anakinra) along with TNF inhibitors (etanercept, infliximab, adalimumab) are currently available for treatment of RA. Their method of action is more rational than analgesic drugs, and they are far less invasive than surgery, but they are not ideal solutions. They are costly to manufacture and therefore expensive for patients to buy: $13-15,000 annually. They must also be administered systemically with high frequency: anakinra must be injected daily to be effective, while most TNF inhibitors must be given weekly.

The short half-life of these proteins and their frequent systemic administration makes them less than ideal for treatment of OA because OA is restricted to one or a limited number of joints. However, frequent intra-articular injections are neither safe nor feasible.

IL-1Ra

Interleukin-1 is an inflammatory cytokine found in elevated levels in the synovial fluid of arthritic joints.[30] IL-1Ra is a naturally occurring competitive inhibitor of IL-1 signaling. It is closely related to IL-1, but does not activate the receptor upon binding.[33] While both IL-1 and IL-1Ra bind with about the same affinity to the receptor there is a strong spare receptor effect so a 100-fold molar excess of IL-1Ra is necessary before a 50% inhibition can be seen.[30]

A clinical formulation of IL-1Ra as a recombinant protein, anakinra (r-metHuIL-1ra), has proven safe and effective for treating RA, even in combination with methotrexate.[34-36] However, daily subcutaneous injections are needed daily to achieve therapeutic benefit.[37]

Experimentally, full length human IL-1Ra has been successfully delivered to joints with a variety of gene transfer modalities. In rodent models of induced inflammatory arthritis, IL-1Ra was successfully transferred to rodent joint tissues with herpesvirus,[38] retrovirus,[39] adenovirus[40,41] and AAV vectors,[42,43] with measurable therapeutic benefit. The use of ex vivo approaches has also been under investigation. Reintroduction of synoviocytes, transduced with retrovirus to overexpress IL-1Ra, to the joints of rats significantly reduced the severity of arthritis symptoms and attenuated cartilage erosion.[44] It was estimated that the local IL-1Ra production was 40-fold more effective than when given systemically. This approach was taken into two clinical trials for

patients with severe RA.[45,46] Autologous, retrovirally transduced synoviocytes were injected into certain metacarpophalangeal joints, as well as control cells. The studies lasted a few weeks, as at that point the joints underwent synovectomy, but no adverse effects were recorded, and reductions in pain and swelling were observed. Although no long term studies have been completed, sustained local production of IL-1Ra appears to be beneficial.

TNF-α Inhibitors

TNF-α is another cytokine that is present in highly elevated levels in arthritic joints,[47] and is the target of a number of biologic therapies. The drugs infliximab and adalimumab are human monoclonal antibodies against TNF-α and are currently used clinically to treat RA and many other inflammatory diseases. Etanercept is a fusion protein consisting of the TNF receptor 2 and the Fc region of human IgG1. The antibody fragment generates a bivalent fragment and gives the construct a greater half-life in vivo than the soluble receptor. It is also approved clinically to treat a large number of inflammatory conditions.[48] However, for effective treatment of RA, subcutaneous injections are required biweekly. A gene therapy method to deliver TNF-α inhibitors would give the advantages of many fewer injections and localization of the transgene to the target area.

Similar results were seen using intra-articular deliveries of AAV[49] and retrovirus[50] in mouse models. When adenovirus was used to deliver a soluble TNF receptor:IgG fusion gene directly to the knees of rabbits with antigen-induced arthritis, moderate reductions in leukocyte infiltration were seen.[40] However, when that vector was given simultaneously with one expressing IL-1 receptor:IgG fusion a synergistic effect was seen, with greater inhibition of leukocyte infiltration and protection from cartilage

breakdown. Also, anti-arthritic effects were observed in the contralateral control knees, indicating that intra-articular delivery, while theoretically local, can be effective at treating joints distal to the injection site.

AAV has also been used in a clinical trial by Targeted Genetics Inc. to deliver the cDNA that encodes etanercept to the joints of RA patients.[51] Serotype 2 capsid was used to package the vector. A phase 1 study was completed in which doses up to 10^{11} DNAse resistant particles (DRP) were injected without serious adverse side effects. During the subsequent phase1/2 in which doses were increased to up to 10^{13} DRP, a woman unfortunately died after receiving a second dose of viral vector. Although the cause of death was linked to histoplasmosis associated with long term use of immune suppressants, the trial was halted until investigations were completed. The patient's final illness began occurring immediately after the second dose, implicating a humoral response to the AAV capsid; however the autopsy revealed no signs of pathology around the injection site. A cell-mediated immune response can not be ruled out as appropriate samples were not kept. Any conclusions about the patient's immune response are complicated by the immunosuppressant therapy the patient was taking. Spread of the vector was not a likely scenario as no co-infection of adenovirus or herpesvirus was detected, and large numbers of AAV genomes outside the injection site were also not found. Although it is also unlikely that the gene product played a role in the pathology, conclusions in this aspect are difficult to make because the company had no reliable or accurate way to measure transgenic etanercept over any other TNF binding agents. Although the timing of vector treatment and appearance of complications appear to link gene transfer to the patient's death, no convincing, detailed

scenario can be identified. The FDA has allowed the study to continue, albeit with stricter guidelines on patient eligibility and increased monitoring of blood cell counts, blood chemistry, vector DNA, TNFR:Fc protein and potential T-cell responses to AAV capsid.

Gene Therapy Vectors

Delivery of protein-based drugs to specific organs faces many difficulties including effective dosage, side effects, and high rate of turnover.[52] Many of these difficulties could be removed with an effective, targeted gene therapy. By delivering the cDNA for therapeutic proteins to the site of disease or injury, local cells could be turned into factories to produce the treatment. As arthritic diseases are chronic in nature, the treatment must likewise have the ability to be long-lived and continuously effective. Advances in gene delivery technology are bringing such a treatment closer to becoming practical.

Nonviral

Nonviral DNA vectors, usually plasmid DNA, are not as efficient as viral vectors, however they are easier and cheaper to produce and have fewer safety issues.[53] Uptake of naked DNA can be improved by using liposomes, nanoparticles, or other synthetic agents.[54-56] Muscle cells are particularly receptive to nonviral DNA transfection, and this can be further enhanced by using electroporation.[57] However, major barriers still exist with intracellular transport to the nucleus, a step that is inefficient and rate-limiting.[58] Naked DNA is also immunostimulatory and inflammatory. Moreover, immune response is elevated when bacterially produced plasmids are used, as the DNA methylation is easily recognized by the innate immune system. Inflammation from nonviral DNA vectors is prevalent when injected into the joint

space.[59,60] As a large, polyanionic molecule, plasmid DNA has little chance of penetrating the cartilage, which is also filled with anionic residues.

Retrovirus and Lentivirus

Retroviruses are enveloped viruses that deliver an RNA based genome to a cell along with the machinery to reverse transcribe the viral genome into DNA and then integrate that genome into the cell's chromosomes. As gene therapy vectors, these viruses provide the advantage that after the initial infection, the transgene will continue to be expressed in all the daughter cells. The major disadvantage they face for use in gene therapy is the possibility of insertional mutagensis leading to carcinogenesis.[61] This limits their potential for use as a therapy for arthritis, as mutagenesis is a risky side effect for treating a nonfatal disease.[62]

Experimentally, retroviruses have been useful. Moloney murine leukemia virus, commonly referred to as "retrovirus," infects dividing mammalian cells with high efficiency.[63] Nondividing cells are not infected by retrovirus as it has no mechanism to actively penetrate the nuclear membrane. Retrovirus has been used for many ex vivo studies, some leading to clinical trials.[64] A more useful retroviral vector has been lentivirus, an HIV based virus pseudotyped with vesicular somatitis virus (VSV) G protein, [65] which infects both dividing and nondividing cells. Apart from its ex vivo uses, lentivirus has been found to infect synovial cells in culture,[66] as well synoviocytes after injection into rats[67] and mice.[68]

Adenovirus

Adenovirus is a double stranded DNA, protein encapsidated virus, with an approximately 36 kb genome encoding thirty viral proteins. It can infect both dividing and nondividing cells, provided they express the coxsackie-adenovirus receptor (CAR).

Wild type adenovirus is also known to cause upper respiratory tract infections in humans. Many serotypes of adenovirus exist, but type 5 is by far the most common for use as a vector. First generation recombinant adenovirus is made replication defective by deletion of the E1 and E3 genes and placing an expression cassette in the place of E1.[69] One function of the E3 product is to suppress MHC presentation of intracellular proteins. When missing E3, low levels of viral gene expression promote immune recognition and elimination of infected cells. This led to the development of second generation adenoviral vectors where E3 is retained while E1 and E4 are deleted.[70] This change still does not eliminate viral protein expression and immunogenicity,[71] and high-capacity adenoviruses, in which all coding regions are deleted were developed as a result.[72] These high capacity vectors are difficult to produce and have reduced transduction efficiency.

Although first and second generation vectors are very efficient for gene transfer, antigenicity and the high prevalence of pre-existing immunity limit their effectiveness as gene therapy vectors.[73] In one survey, neutralizing antibodies (NABs) to adenovirus were found in the synovial fluid of 70% of RA patients tested.[74]

Extensive studies have been conducted using adenovirus to deliver genes to the joint, with most studies reporting inflammatory responses and short-lived transgene expression, although initial transgene expression levels were high.[75] Furthermore, synoviocytes have poor expression of CAR, but modifying the adenoviral capsid has led to increased transduction of synoviocytes.[76,77] Although easy to produce and valuable for experimental study, the inflammation induced by adenovirus in vivo limits its potential for use to treat already inflamed joints.

Adeno-Associated Virus

AAV is a member of the parvovirus family, is a small, non-enveloped single-stranded DNA viruses. It is naturally replication defective, requiring a helper virus to complete its lifecycle: commonly adenovirus or herpes virus. AAV infects both dividing and non-dividing cells and is not associated with any known human disease. With a 4.7 kb genome and only two genes, "gutless" vectors are standard; they retain only the small terminal elements of original viral DNA. With no native viral gene expression, low relative immunogenicity, and long term expression of transgenes, AAV vectors are currently the safest choice for viral gene therapy.

AAV Biology

Genome and Replication

The AAV genome is ~4.7 kb with 145 bp inverted terminal repeats (ITRs). The ITRs are the only cis elements on the viral genome required for replication and packaging. The first 125 nucleotides of the ITR are inverted repeats of nucleotide sequence which folds back onto itself, forming a hairpin structure. The D sequence makes up the remainder of the ITR and remains single-stranded. The ITR is the origin of replication for the viral genome. The first round of DNA synthesis produces a double-stranded genome, called the replicating form monomer. After a second round of DNA synthesis, a replicating form dimer is produced. This entity is then processed into a single-stranded DNA used for packaging and a double-stranded DNA used for transcription.[78] Also contained in the ITR are rep-binding elements (RBE) and a terminal resolution site (TRS) which are critical for proper DNA processing.

Two viral genes, *Rep* and *Cap*, are encoded on the genome. Four Rep proteins are necessary for viral replication: Rep78, Rep68, Rep52 and Rep40. Two promoters

and alternative splicing are responsible for creating the four polypeptides from just the one rep gene. The larger Rep proteins are responsible for DNA binding and processing, while the smaller Rep proteins are used for packaging the genome into the capsid.

Three capsid viral proteins (VP1, VP2, and VP3) are synthesized from the cap gene but differ because of alternative splicing and an alternative start codon. To make a complete capsid, sixty capsid proteins combine in a ratio of 1:1:20 of VP1, VP2, and VP3, respectively. The VP's have nearly identical structure, differing only at their N terminus. At the amino end, VP1 has a phospholipase A2 domain that is required for infectivity.[79]

Entry and Trafficking

The wild type viral life cycle begins when a particle attaches to a cellular receptor and is internalized by endocytosis via clathrin coated pits.[80] Particles make their way towards the nucleus through early and then late endosomes. Endosomal escape is mediated in part by the phospholipase domains on VP1,[81,82] while low pH[83] and the presence of cysteine proteases[84] are also important.

While in the cytoplasm, the viral capsid can become a target of ubiquitination and eventual proteasomal degradation. This process is mediated by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK), through phosphorylation of tyrosine residues on surface of the capsid.[85] The capsid is only ubiquitinated if the surface tyrosines are phosphorylated, making every step of this process a potential target for increasing vector efficiency.

It is unclear whether uncoating occurs before entering the nucleus,[86] but once inside, second strand synthesis must be initiated. AAV does not have its own DNA

polymerase so it must rely upon the cell for initial creation of duplex DNA. EGFR-PTK again enters the lifecyle of AAV at this step. FK506 binding protein (FKBP52) binds to the D sequence of the AAV genome and inhibits second-strand synthesis, but only when phosphorylated, a modification for which EGFR-PTK is responsible.[85]

Once Rep proteins are present, they facilitate stabilization of the viral genome and possible integration into chromosomal DNA. Recombinant AAV vectors lack Rep, and therefore have no active integration mechanism.[87] When an AAV infected cell becomes co-infected with adenovirus or herpesvirus, Rep assists in viral replication and packaging. Wild type viral genomes may also integrate into human chromosome 19 in a loosely site-specific manner,[88] the exact mechanism of which remains unknown.[78]

Tropism and Receptors

Identified by differences in capsid sequence, up to 110 different capsid variants of AAV have been found in primates,[89] although serotypes 1-11 are the most commonly studied. AAV2 is the prototype for most gene transfer research, as it is well-characterized.

Different serotypes have different tropisms within a species. This has been highlighted by work in the brain,[90-92] but this holds true for other tissues as well.[93] For gene therapy purposes, it is important to identify the serotype most likely to infect the cells that are desired to be modified. There can also be differences in tissue tropism between individuals of the same species. This is best exemplified by the variation in results that several groups obtained while working with AAV2 in human CD34+ hematopoietic progenitor cells.[94] Although the donor cells under inquiry shared the CD34+ phenotype, they varied in expressing the surface receptors and co-receptors necessary for efficient transduction. Besides the differences one can see within a

species, it has been documented that the tropism of a single serotype can vary across species. AAV2 and AAV5 showed equivalent transgene expression in human airway epithelial cells, but with mouse cells, transgene expression from AAV5 infected cells was much higher than from AAV2.[95] Species differences in AAV transduction demonstrate that knowledge gained from animal models may not be directly applicable to humans. This has implications for preclinical research, as humans are the final target.

The tropisms of AAV serotypes are the result of expression of cell surface receptors (Table 1-1). Many AAV serotypes require multiple receptors for efficient infection. The receptors for AAV have only been partially characterized.

Immune Response

Although AAV is not known to cause any disease in humans, up to 80% of people worldwide carry antibodies against AAV2.[96] Antibody levels against other serotypes vary, but AAV appears endemic to human populations worldwide. The consequences of this for gene therapy are not clear. Recombinant forms of AAV lack viral open reading frames, and when an immune compatible transgene is used, any immune response should be directed solely against the capsid. There is some evidence that tolerance to foreign transgenes can be induced if hepatocytes are transduced,[97-99] while a systemic injection will likely generate anti-capsid antibodies.[100]

In animal models, the immune response varies by location and load of vector. Several studies have looked at the immune response to AAV vectors in the brain, which is relatively immune privileged.[101] When injected into the brain, low doses of AAV avoided any detectable immune response, and re-administration with the same vector was possible.[102,103] However, high doses of AAV caused inflammation, as measured by

the increased presence of glial fibrillary acidic protein.[104] The presence of pre-existing immunity, when animal serum is positive for NABs, also inhibits transduction of brain tissue,[105] even in the absence of infiltration of immune cells into the brain.[106]

The eye also represents an immune privileged organ, but some response can be seen depending on methods of delivery. Intravitreal delivery induces a humoral response to the capsid,[107] while reports on subretinal delivery have shown both no response[107] or the induction of NABs.[108] While methodology between the experiments could account for the differences, both studies agree that re-administration of the vector after subretinal delivery is still feasible. Furthermore, transduction after a subretinal delivery in one eye is not affected by a previous intravitreal delivery in the other eye,[109] meaning the subretinal space is immune privileged, and can be transduced independent of prior immunity.

Virally infected cells containing viral proteins in the cytoplasm are normally cleared by the CD8+ T-cell mechanism. In a recent clinical trial of AAV2 to deliver the gene for factor IX to the liver, this cell-mediated immune reaction was found to be responsible for clearance of transduced cells.[110] In a patient with low levels of NABs to AAV2 capsid, transgene expression was maintained in the liver at therapeutic levels for four weeks, but then fell to baseline. This elimination of gene expression was determined to be caused by CD8+ T cells,[111] which were also shown to be cytotoxic against AAV2 infected cells in culture.[112]

Methods to avoid immune responses to AAV capsids are complicated by the lack of a suitable animal model. Generation of a T cell response to AAV capsids in mice has been difficult, and when successful, the CD8+ cells failed to clear AAV transduced

hepatocytes in vivo.[113-115] Several strategies for avoiding T cell responses are reviewed by Mingozzi and High,[116] and the most promising solution is to have a low vector dose, which necessitates highly effective vectors.

Optimizing AAV Transduction for Gene Therapy

Throughout the lifecycle of AAV, the virus faces several hurdles to its successful replication, and in the wild, it depends on the help of other viruses. Recombinant viruses used for gene therapy face the same hurdles, yet they are designed not to replicate, and using helper viruses is not practical. Cell entry, intracellular trafficking, double stranded DNA synthesis, and gene expression must still occur, and occur efficiently, for optimal gene therapy. Improvements to the virus have been made to assist at each step of the process.

Self-complementary genomes

To bypass the inefficiency of second-strand synthesis, which limits AAV transduction in certain cell types, McCarty created a self-complementary AAV vector.[117] This virus has had the terminal resolution site (trs) deleted from one of the two ITRs of its genome. Deleting one trs will lead to defective genome replication whereby the Rep protein will cleave at every other trs position, making a double length, inverted repeat AAV genome (Figure 1-1). To package this scAAV DNA inside a capsid, it is necessary for the genome to be only half length, < 2.5 kb. Between functional ITRs, this virus will be wild type length when single-stranded. When it anneals upon itself, this virus will be half length, but fully competent for transcription.

Capsid modification

The AAV capsid is responsible for both the binding of cellular receptors and for contact with the immune system. Modifications of the capsid can be made to alter the

tropism, target or avoid specific tissue types, avoid immune reactions including antibody neutralization, or to enhance intracellular trafficking.

Changes to the AAV capsid have been made by rational design or via a directed evolution procedure, where a large fragmented library of capsid genes are randomly re-assorted and the resulting viruses are screened for transduction efficiency. The engineering of capsid proteins is done generally to solve a specific problem such as adding heparin affinity or removing known antibody epitopes.[118] Specific mutations have been made to the AAV2 capsid to enhance transduction in neurons[119] and muscle cells,[120] whereas directed evolution approaches have created novel viral capsids that infect cells as diverse as airway epithelia,[121] and glial Müller cells of the retina.[122] By inserting a fragment of protein A into the AAV2 capsid, it is possible to link antibodies to allow specific cell targeting.[123] Conjugating biotin to the capsid allows for avidin linked proteins to have targeting functions as well.[124] Polyethylene glycol (PEG) can also be conjugated to the AAV capsid, and within a specific window of polymer size and PEG:lysine conjugation ratio, this protects from antibody recognition, while not decreasing transduction efficiency.[125]

Another type of capsid mutation has nothing to do with cellular binding, but instead helps intracellular trafficking. By replacing certain tyrosine residues on the capsid with phenylalanine, potential sites of ubiquitination are removed without affecting capsid structure or cellular binding.[126] This reduction in intracellular degradation of AAV2 led to a 10-fold increase in transduction of HeLa cells, and a 30-fold increase in transduction of hepatocytes in vivo, at a log lower dose. Tyrosine to phenylalanine mutations in

capsids for serotypes 2, 8, and 9 increased viral transduction to many cell types of the retina after subretinal or intravitreal injection.[127]

Proteasome inhibition

After viral entry, as AAV particles are engaged in the intracellular trafficking machinery, many of the virions can be ubiquitinated, which leads to proteasomal degradation of the virus. The presence of proteasome inhibitors during infection has been shown to decrease the loss of virus due to this form of degradation; the inhibitors are not effective when given several hours after infection.[128]

Several different proteasome inhibitors including N-acetyl-L-leucyl-L-leucyl-L-norleucine (LLnL), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (zLLL), bortezomib, and doxorubicin have shown effectiveness in increasing rAAV transduction in several cell systems. An increase in viral genomes accumulated in the nucleus was measured in airway epithelial cells[129] and endothelial cells[130] following treatment with LLnL or zLLL and viral infection. This held across serotypes 2, 5, and 7. Various intestinal epithelial cell lines also showed increased transduction by AAV2 vectors when pretreated with LLnL.[131] When the inhibitors are given systemically, as when liver tissue is targeted for transduction,[132] or directly onto the lung airway epithelia[133] in mice, increased transduction is also seen.

Table 1-1. Known receptors for several AAV serotypes.

Receptors	AAV Serotypes
HSPG, $\alpha_v\beta_5$ integrin, FGFR-1, HGFR[134-137]	2
PDGFRα[138]	5
Laminin Receptor[139]	2,3,8,9
α2,3 N-linked sialic acid[140,141]	1,5,6
α2,6 N-linked sialic acid[140]	1,6
α2,3 O-linked sialic acid[142]	4

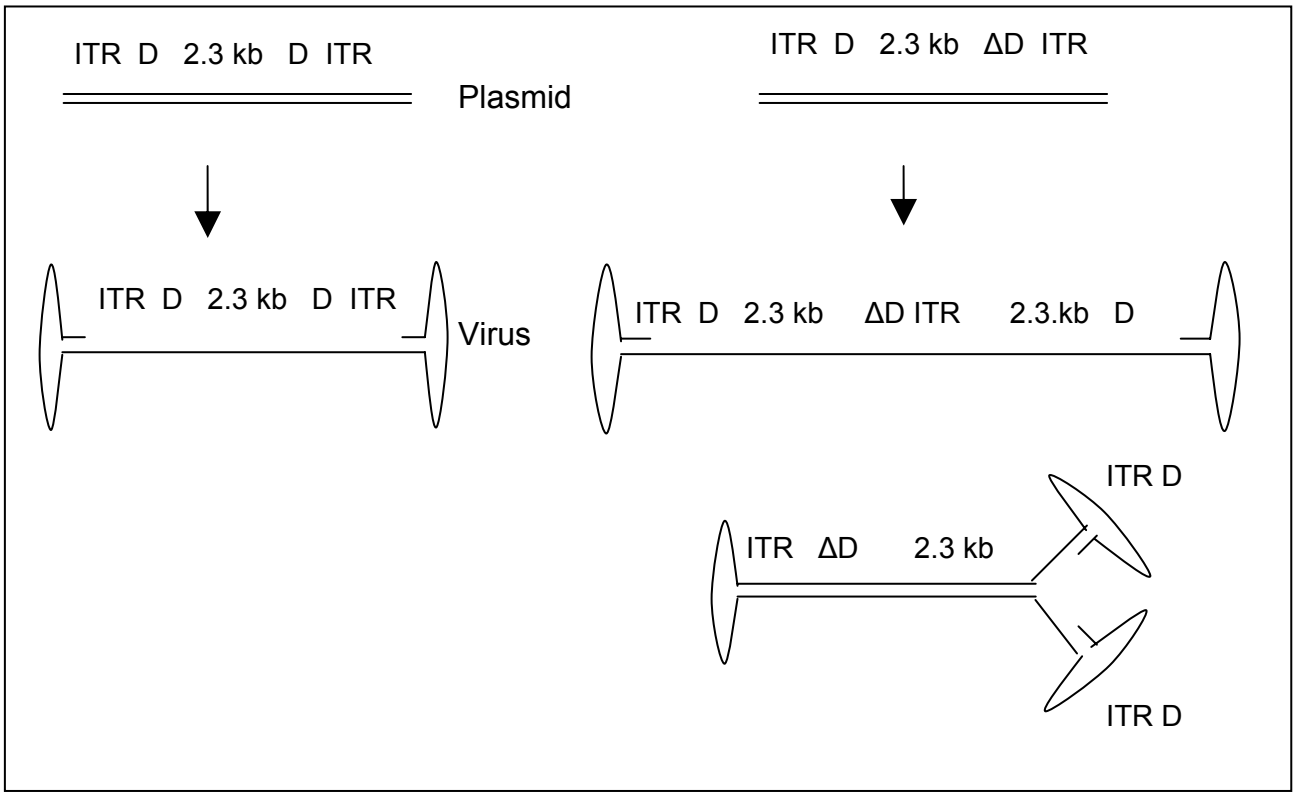

Figure 1-1. The organization of ssAAV and scAAV. A half size AAV genome missing the D sequence on one ITR will make a self-complementary double stranded DNA genome.

CHAPTER 2
INTRA-ARTICULAR GENE DELIVERY AND EXPRESSION OF IL-1RA MEDIATED BY SELF-COMPLEMENTARY ADENO-ASSOCIATED VIRUS

Introduction

Gene transfer has been proposed as a means to improve treatment of the arthritides.[143] By delivering cDNAs encoding anti-arthritic proteins to the cells in the capsular lining of joints, the gene products may be expressed and secreted locally into the joint space and neighboring tissues. Persistent expression of therapeutic gene products may provide long-term benefit in the treatment of chronic joint diseases. Initial studies of the feasibility of this concept employed an ex vivo gene transfer approach.[144,46] While effective, the expense and labor have led to the exploration of methods for delivering exogenous genes directly to joint lining cells in situ. Studies using adenovirus,[145-149] and herpes simplex virus[149,38] as vectors for gene transfer have demonstrated that direct intra-articular gene delivery is feasible. Moreover, ensuing expression of certain therapeutic transgenes is sufficient to inhibit arthritic changes in certain animal models.[148,38,40,150-152] While both vector systems are highly efficient, gene expression from either is transient, in general persisting for no longer than two to three weeks. The loss of transgene expression is frequently accompanied by the onset of an inflammatory response due, at least in part, to the expression of viral proteins that remain encoded by these vector systems.[153]

More recent work by Gouze has shown that direct intra-articular injection of VSV-G pseudotyped, HIV based lentiviral vectors into the knees of rats results in expression of homologous transgene products at relevant levels for greater than six months.[154,155] This demonstrates that certain populations of cells within the synovium and joint capsule are capable of maintaining an exogenous transgene for periods of time

sufficient to treat chronic articular disease. Although lentiviral vectors encode no viral proteins and are powerful gene delivery tools, there may be considerable safety and psychological impediments to the use of HIV-based vectors for in vivo gene delivery for non-fatal articular diseases.

AAV has certain characteristics that may make it more suitable for gene delivery to joint tissues.[156-158] Wild type AAV is non-pathogenic, and recombinant AAV vectors have been engineered that encode no viral proteins. Since the vector infects a variety of dividing and non-dividing cells, in many applications it can achieve significant levels of cellular transduction following delivery in vivo. Advancements in AAV technology, including the capacity to cross-package the vector in alternate capsid serotypes and methods for generating large-scale, high-titer, adenovirus-free preparations,[159,160] have brought wider interest to the use of this vector system including its potential for use in treating the arthritides.

In previous studies, we evaluated conventional, single-stranded AAV2 vectors and found them significantly less effective than herpes simplex virus (HSV), adenovirus or lentivirus for intra-articular gene delivery. The onset of transgenic expression was significantly slower, requiring at least one to two weeks, and the resulting levels of expression were low in culture and borderline detectable following specific intra-articular injection in the knee joints of rats and rabbits (unpublished observations). Despite our poor results, there are literature reports of beneficial effects following local AAV-mediated delivery of anti-arthritic transgenes in the ankles and paws of rodents with experimental arthritis.[161-168] Unfortunately the inflammatory pathology in models such as collagen-induced arthritis and streptococcal-wall induced arthritis, occurs almost

exclusively in the ankles and paws. In rodents these arthrodial, or gliding joints, are extraordinarily small and architecturally complex without a readily identifiable joint space and cannot be reliably targeted for intra-articular injection. Interestingly, consistent with our findings, several studies from independent laboratories indicate that murine and human synovial fibroblasts are inherently resistant to transduction with conventional AAV based vectors.[156,169,170] Indeed, work by Cottard[162] indicates that the primary site of AAV2 transduction following injection in the ankle region is extra-articular muscle. Several groups have shown that certain stimuli, such as UV radiation which increases the production of endogenous DNA repair and synthesis proteins, can significantly enhance intra-articular transgene expression from conventional AAV vectors.[156,169,170] This indirectly indicates that second-strand DNA synthesis is rate-limiting in AAV transduction of joint tissues.

The recent development of double stranded, self-complementary AAV vectors bypasses the need for single strand to double strand genome conversion and has shown dramatically increased transduction efficiency in many tissues compared to conventional AAV vectors.[117,171,] scAAV vectors can be produced either by generation of vector plasmids that are ~half-genome sized combined with selective purification of the infectious double stranded form,[171] or through the use of half-genome sized vector plasmids containing a mutation in one of the terminal resolution sequences of the AAV ITRs.[117] Both strategies generate + and – strand viral genomes that are covalently linked at one terminal repeat. Because the genomes of scAAV are half wild type size (~2.5 kb) the resulting 2x viral construct (~5 kb) can be packaged into the normal AAV capsid.

In the present study, we tested the hypothesis that scAAV vectors provide improved transduction of articular fibroblasts over conventional AAV vectors to enable rapid expression of functional levels of transgene expression in joint tissues.

Materials and Methods

Construction and Generation of AAV Vectors

The cDNA encoding GFP was cloned into the conventional AAV packaging vector pTRUF2 as a Not 1-Sal 1 fragment. For generation of scAAV vector plasmids, the cDNAs for GFP and human IL-1Ra were directionally inserted into the Sac II, Not 1 sites of pHpa-trs-SK plasmid.[117] For all AAV vector constructs transcription was driven by the CMV promoter/enhancer.

AAV vectors were propagated using an adenovirus-free, two plasmid transfection system. Using 10 layer cell factories (Nunc), the respective AAV vector plasmids were co-transfected into 293 cells by $CaPO_4$ precipitation with the pDG packaging/helper plasmid.[160] The pDG plasmid contains the rep and cap genes from AAV2 and complementing adenoviral functions required for amplification and packaging of the AAV genome. Sixty hours post-transfection, cells were harvested with PBS containing 10mM EDTA, pelleted, resuspended in low salt buffer and lysed by three rounds of freeze-thaw. Cellular nucleic acids were digested by incubation with Benzonase (Sigma). Purification of AAV from the crude lysate was performed using iodixanol gradients followed by FPLC affinity chromatography over mono-Q column. The eluate was desalted and concentrated with a Millipore Biomax 100K filter, aliquotted and stored at -80ºC. Viral titers were determined by quantitative competitive PCR assay relative to well-characterized AAV viral reference standards. Each viral preparation was examined for purity by resolution of the viral proteins by SDS PAGE and silver stain.

Vectors were produced in the laboratories of Dr. Steve Ghivizzani and Dr. William Hauswirth.

Isolation and Infection of Primary Articular Fibroblasts

Over the course of our in vitro experiments, four New Zealand white rabbits were euthanized and the capsular tissues from both knee joints were harvested. To isolate fibroblastic cells for experimentation, the non-collagenous soft tissues including the synovial lining and subsynovium were scraped from the dense supporting fibrous tendon and ligamentous tissue of the capsule using a scalpel. Under aseptic conditions, the fresh isolates of rabbit synovial/capsular (articular) tissue were minced with a razor blade and digested in ~30 mL saline solution with 0.2% collagenase for 2hr at 37^{o}C with constant stirring. Afterward, the suspension was passed through a nylon mesh to remove undigested tissue. The cells in the filtrate were then pelleted, washed in saline and plated in DMEM supplemented with 10% FBS with 1% penicillin/streptomycin. Twenty-four hours later, the cultures were washed to remove non-adherent cells and debris; the medium was replaced, and the cultures returned to the incubator.

For viral infection, unless otherwise indicated, cells were plated in 12 well plates and grown to ~70% confluence. Prior to infection, cells were washed two times with serum free media (DMEM). AAV vector from stock solutions was mixed with serum free media to produce working solutions containing appropriate DNAse resistant viral genomes/per cell and placed on cell cultures. For experiments involving the GFP transgene and intracellular trafficking, viral doses of 10^4 viral genomes per cell were used. For those involving IL-1Ra, viral doses ranged from 10^3-10^5 viral genomes per cell. After incubation with virus for 2 hrs, complete media was added to each well and the cells returned to the incubator. For quantitation of IL-1Ra, at 24 hr intervals following

infection, the media from each well were harvested, and replaced with fresh media. Harvested media from selected days was stored frozen at –80^{o}C. Each viral dose was added to four individual wells and supernatant from each well was tested individually by ELISA (R & D Systems).

Quantitation of Viral Genomes in Cytoplasmic and Nuclear Cell Fractions

Viral DNA from fractionated cells was isolated from a procedure adapted from Zhao et al.[172] Briefly, lapine articular fibroblasts were seeded at 10^5 cells per well of a 6-well dish, allowed to attach, and then infected with either single stranded or scAAV at 10^4 viral genomes/cell as described above. After 24 hours, the cells were trypsinized, incubated in hypotonic buffer for 5 minutes on ice, and lysed in non-ionic detergent. Centrifugation of the lysate allowed the nuclear fraction to be collected as the pellet, while the supernatant was reserved as the cytoplasmic fraction. Low molecular weight DNA from each fraction was isolated by Hirt extraction[173] and then used for quantitative PCR. The Hirt extraction is performed by first adding 2 volumes of Hirt's solution (0.6% SDS, 10mM EDTA), mixing, then adding one quarter volume of 150 mL NaCl and incubating overnight at 4°C. Following centrifugation for 1 hr at 15,000 x g, the supernatant is retained and DNA is extracted by ethanol precipitation. Primer pairs (forward 5'-CACGCTGTTTTGACCTCCATAGAAGACACCGGG, reverse 5'-TTCTTTGATTTGCACCACCACCGGATCCGGG) were designed to anneal to sequences within the CMV promoter sequence. Viral genomes were detected using SYBR Green dye in an Eppendorf Mastercycler Realplex2. The results were standardized to a dilution series of vector plasmid DNA of known copy number. Three independent experiments were performed, yielding similar results. Values were then expressed as the mean of these experiments.

Animal Models

All animal experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering. Rabbits used in the study were housed separately in metal cages and maintained on commercial food and water ad libitum. The cages were kept at a constant temperature (22-25°C) and relative humidity (50-55%).

A retroviral vector, DFG-hIL-1β-neo encoding both the mature form of human IL-1β fused to the secretory polypeptide sequence from human parathyroid hormone, and the neomycin phosphotransferase genes[174] was used to transduce HIG-82 cells. Following infection, transduced cells were positively selected by culture in DMEM containing 10% FBS and 0.5 mg/mL G418. After selection, this cell line was found to produce over 200 ng of IL-1β per 10^6 cells per 48 hrs. To induce arthritis in the rabbit knee, the HIG-82-IL-1β+ cells were first trypsinized from culture plates, washed twice in Gey's balanced salts solution (GBSS) and counted using a haemocytometer. Approximately 5 x 10^4 of the cells were resuspended in GBSS in a 0.25 ml volume and injected via the parapatellar approach into both knees of 3-4 kg New Zealand White rabbits.

Joint lavage was utilized to monitor intra-articular transgene expression and leukocytic infiltration. For this procedure, rabbits were first anesthetized by subcutaneous injection with a cocktail of xylazine, ketamine and acepromazine. The rabbit knee joints were then lavaged, first by direct intra-articular injection of 1 mL GBSS. The joints were then put through several ranges of motion. The needle was re-inserted, and the fluid aspirated using the syringe. Leukocytes in recovered fluids were counted using a haemocytometer. Lavage fluids were centrifuged to pellet cells and

debris, and the supernatant was aliquotted and stored at –80ºC. For sacrifice of the animals, the rabbits were anesthetized by subcutaneous injection with the xylazine, ketamine, acepromazine cocktail as above followed by intravenous overdose of Nembutal via the ear vein. IL-1Ra levels in recovered fluids were measured using an ELISA kit from R & D Systems as directed.

Statistical Analysis

A pooled two-sample t-test was used to determine the significance of the differences in leukocytic infiltration in inflamed knees that were injected with scAAV.IL-1Ra and untreated controls. Values of $p<0.05$ were considered statistically significant.

Results

scAAV Transduces Rabbit Synovial Fibroblasts with High Efficiency

To determine the relative transduction efficiency in articular fibroblasts of the double stranded, self-complementary (sc) AAV vector and the conventional single stranded form, we first inserted the cDNA for green fluorescent protein (GFP) into the respective vector plasmids and packaged each into AAV capsid serotype 2. Articular fibroblasts isolated from synovial and capsular tissues of the joints of rabbits were cultured in multi-well plates and infected with ~10^4 viral genomes per cell of either AAV.GFP or scAAV.GFP. At periodic intervals post infection individual cultures were analyzed by microscopy and flow cytometry for numbers of GFP+ cells and the levels of fluorescence. As shown in Figure 2-1 A and B, the scAAV.GFP vector provided ~25-fold greater transduction than the conventional single-stranded vector, with onset of GFP expression within 24 hours. Fluorescence was noted to diminish somewhat by day 7; however, this was attributed to the loss of the episomal AAV genomes from the cells of the rapidly dividing line in culture.

To determine if the enhanced transduction of the scAAV vector might be partially attributable to variation in intracellular trafficking and nuclear entry, we infected parallel cultures of cells and with both vector types and determined the viral genomes present in the cytoplasmic and nuclear fractions using quantitative PCR. As shown in Figure 2-1C, no significant differences were observed between the different AAV vector types; both entered the cells with similar efficiency, and similar proportions of viral DNA entered the nucleus (~18% and 14% for conventional and scAAV, respectively). These data indicate that at least within this cell type, there are no appreciable differences between the two vectors with regard to viral entry into the cell, as well as entrance into the nucleus. Consistent with previous reports, these results show that transduction of articular fibroblasts with conventional AAV vectors is severely limited by the inability of these cells to effectively achieve second strand DNA synthesis.

To determine the levels of therapeutic protein synthesis provided by the scAAV vector, the cDNA for human interleukin-1 receptor antagonist (IL-1Ra)[175] was inserted into the pHpa-trs-SK, scAAV vector (scAAV.hIL-1Ra) and packaged into AAV serotype 2. IL-1Ra is a secreted protein that serves as a competitive inhibitor of interleukin-1 (IL-1) by binding to available type I IL-1 receptors and preventing subsequent interaction with IL-1 ligand and IL-1 receptor accessory protein.[176] IL-1Ra is useful as a reporter gene because it has no known agonist activity and can be measured in biological fluids by ELISA that distinguishes between the human form and the endogenous IL-1Ra of the experimental animal.

Cultures of primary rabbit articular fibroblasts were infected with increasing amounts of scAAV.IL-1Ra ranging from 10^3 to 10^5 particles per cell. To follow the

pattern of gene expression over time, the media were collected every 24 hrs post-infection, the cells washed and fresh culture media added. IL-1Ra levels in the media collected at days 1, 3 and 7 post-infection were measured by ELISA. As shown in Figure 2-2, the lapine articular fibroblasts were amenable to transduction with scAAV.hIL-1Ra, and expressed the transgene in a dose-dependent manner. Interestingly, for all doses the greatest level of expression was measured at 24 hrs post infection. As with expression of GFP, we noted that IL-1Ra production gradually diminished over the week-long experiment, which is consistent with the loss of the episomal viral genomes from cell division.

Altogether the results above demonstrated that the scAAV.IL-1Ra vector was infectious for rabbit articular fibroblasts and thus suitable for evaluation in the rabbit knee in vivo. The levels of hIL-1Ra synthesis were comparable to those achieved previously with recombinant adenoviral and lentiviral vectors.

Intra-Articular Expression of AAV.IL-1Ra in Normal and Arthritic Rabbit Knee Joints

Having established that the scAAV.IL-1Ra vector was able to efficiently transduce articular cells in culture, we wanted to determine and compare the patterns of intra-articular gene expression after injection of the vector into normal and inflamed joints. To establish an inflammatory environment in the rabbit knee, approximately 5 x10^4 cells of a rabbit synovial fibroblast line retrovirally transduced to constitutively express human IL-1β (HIG-82-IL-1β-neo) were injected into both knees of 10 rabbits. This procedure has been shown to induce an acute inflammatory response in the joint that mimics many of the pathologies associated with rheumatoid arthritis in humans and persists for about 10-14 days.[174] Three days after delivery of the IL-1β+ cells, approximately 5 x10^{11}

particles of scAAV.hIL-1Ra were injected into both knees of 5 rabbits receiving the IL-1+ cells and into both knees of 5 normal rabbits. For negative controls, an equivalent volume of saline solution was injected into both knees of the remaining IL-1+ rabbits as well as 5 additional normal rabbits. The knees of all four groups of rabbits were initially lavaged at 3 and 7 days post-injection of the vector and then weekly thereafter for 28 days. Recovered lavage fluids from each knee were analyzed individually for levels of human IL-1Ra by ELISA as well as for numbers of infiltrating leukocytes.

As shown in Figure 2-3 A, scAAV.IL-1Ra-mediated intra-articular gene delivery resulted in approximately the same level and duration of IL-1Ra expression for both inflamed and naïve joints. At days 3 and 7, mean levels of about 1 ng of IL-1Ra per ml of recovered lavage fluid were detected in both inflamed and normal joints. By day 14, mean IL-1Ra levels had decreased by 60-80%, and by day 21 human IL-1Ra was undetectable in lavage fluids of any animals. IL-1Ra expression was not detected in the normal and inflamed control rabbits beyond normal background levels for this procedure.

A significant decrease in leukocytosis of the synovial fluid was observed at days 3 and 7 in the arthritic (inflamed) joints receiving the scAAV.IL-1Ra, relative to the saline-injected arthritic controls ($p<0.05$) (Figure 2-3 B). This is consistent with previously observed anti-inflammatory effects associated with intra-articular delivery and overexpression of the IL-1Ra cDNA in this model system.[38] Different from the intra-articular injection of adenoviral vectors, no detectable increase in leukocyte levels in synovial fluids was observed in the normal rabbits receiving the scAAV vector at any time point during the four week experiment (Figure 2-3 B).

To determine if the onset of a second inflammatory response would re-stimulate intra-articular IL-1Ra transgene expression, HIG-82-IL-1β-neo cells were injected into the knees of the previously inflamed and normal rabbits that had received the AAV.IL-1Ra five weeks earlier. The knees of the rabbits were then lavaged 3 days later and again, following sacrifice, at day 7. The recovered fluids were analyzed individually for IL-1Ra levels by ELISA. In contrast to previous reports indicating that once extinguished, AAV-mediated transgene expression could be re-established by a subsequent inflammatory stimulus[42,177] no IL-1Ra was detected in lavage fluids from animals of either group. PCR analyses of recovered synovial tissues did not detect the presence of scAAV.IL-1Ra genomes (data not shown).

Repeat Dose of AAV.IL-1Ra does not Restore Transgene Expression

Having found no difference in levels of IL-1Ra expression following AAV-mediated gene delivery between normal and inflamed joints, patterns of transgene expression were determined following repeat dosing. As above, 5 x 10^4 HIG-82-IL-1β+ cells were injected into both knees of 10 rabbits. Three days later, approximately 5x10^{11} particles of scAAV.IL-1Ra were injected into both knees of 5 of the rabbits receiving the IL-1 cells and into both knees of 5 normal rabbits. The knees of all rabbits were lavaged weekly until IL-1Ra expression had completely diminished. As shown in Figure 2-4, similar to the results of the previous experiment, both normal and inflamed joints injected with the scAAV.IL-1Ra expressed approximately 1 ng of IL-1Ra per ml of recovered lavage fluid at day 7, and IL-1Ra production gradually diminished thereafter. Somewhat different from the results obtained in Figure 2-3, at day 21 one rabbit each from the inflamed and the normal groups still expressed IL-1Ra above background levels in both joints. By day 28, IL-1Ra expression persisted only in the knees of one animal in the inflamed group.

At day 35, no IL-1Ra was present in the joints of any of the rabbits. Two weeks after IL-1Ra expression was undetectable in all the animals, a second dose of AAV.IL-1Ra was injected intra-articularly. Lavage fluids recovered at day 3 and 7 following the second injection showed no significant IL-1Ra expression at either time point.

Discussion

In this study we evaluated patterns of transgene expression in articular cells following infection with scAAV vectors, first in vitro, and then in vivo in normal and inflamed joints. We found that in culture the scAAV vector represented a significant technical advance over conventional single-stranded vectors with regard to cellular transduction, providing ~25-fold enhancement in transgenic expression. The comparatively poor performance of the conventional vector indicates that second-strand DNA synthesis can be a major impediment to effective transduction of joint tissues. Concerning normal and inflamed articular environments, we found no significant difference in the levels or duration of expression of the IL-1Ra transgene following delivery of the self-complementary vector. Generally, following injection of about 5 x 10^{11} particles, sufficient levels of IL-1Ra transgene product were generated to cause a reduction in the leukocytic infiltration in joints inflamed by constitutive IL-1 production. Despite published reports to the contrary,[42,177] we found that following the loss of IL-1Ra transgene expression, neither re-injection of the scAAV.IL-1Ra vector nor the induction of a second inflammatory response could generate detectable levels of IL-1Ra expression intra-articularly.

In animal studies of intra-articular transgene expression we found several advantages to the rabbit knee as a model system. Being approximately the same size as the metacarpophalangeal joints of the human hand, a frequent site of RA, it offers a

reasonable simulation of the process of gene delivery in the treatment of human joint disease. Given that the studies published to date have been performed in rats and mice[156,161-168,178,179] with joints 1-2 log orders smaller than those in humans, we believe the data here are among the first to report intra-articular transgene expression from an AAV vector in articular tissues on a clinically size-relevant scale. As shown by the ability of the IL-1Ra expression to alleviate leukocytosis in inflamed joints of rabbits, the efficiency of scAAV-mediated gene delivery and ensuing expression is sufficient to induce a beneficial biological response in this context.

The patterns of transgene expression observed with the self-complementary vector in rabbits differ somewhat from those observed in the joints of mice injected with conventional AAV. In the murine system, AAV mediated transgene expression was found to initiate significantly earlier in arthritic joints, and levels of expression were greater than in normal joints.[156] This was primarily attributed to differences in the synthesis of the second DNA strand of the AAV vector within the infected cell, and increased production of DNA synthesis/repair enzymes in cells receiving inflammatory stimuli. With the self-complementary vector, AAV-mediated transgene expression from normal rabbit synovial fibroblasts in culture as well as in vivo in normal joints had a rapid onset, with no evidence of delay relative to arthritic joints. Thus, the self-complementary vector bypasses the variability associated with conventional AAV vectors in the arthritic environment and, thereby, provides a more predictable gene delivery reagent. The observation that cells within both normal and inflamed joints of the rabbit are equally capable of being transduced by an scAAV-based vector indicates that this system may have application in a spectrum of articular ailments. These include inflammatory

conditions, such as RA, as well as those not directly associated with chronic inflammation, such as osteoarthritis, and repair of joint tissues such as meniscus and ligament.

Although intra-articular gene transfer accompanied by limited inflammation has been previously reported with AAV, the capacity of this vector to enable persistent transgenic expression in joint tissues has yet to be fully assessed. In the present report, we found that within the rabbit knee, expression of the human IL-1Ra transgene was gradually lost over a period of a few weeks and that re-administration of the vector could not restore expression. Unfortunately, the human IL-1Ra transgene product used in these experiments, while extremely useful as a secretable marker, is at the same time immunogenic when administered across species boundaries to the joints of normal, immunocompetent animals.[154] We have found similar patterns of abbreviated intra-articular expression following the use of other xenogenic transgene products, regardless of whether they are secreted or intracellular.[37] Recent experiments have shown that in the absence of specific T-cell mediated immunity directed against non-self proteins of transgenic or viral vector origin, cells within fibrous tissues of the joint can support long-term (>6 months) transgenic expression.[154,155] The capacity with which AAV vectors can infect and transduce these particular cell types is currently unknown, but is an area of ongoing study within our group, as well as others.

AAV-mediated transduction of the target cell involves several key steps that broadly include, viral attachment and entry,[134,135] intracellular trafficking to the nucleus,[180-182] nuclear entry and uncoating,[183,184] and conversion of the single-stranded genome into a double stranded form.[185,186] In attempting to improve the efficiency of

AAV transduction, the development of methods to cross-package vector genomes in alternate capsid serotypes has dramatically expanded the host cell range of the widely-used, AAV serotype 2-based vectors. Further, the development of scAAV vectors bypasses the limitations associated with second-strand DNA synthesis. As shown here and by others, important barriers to transduction of articular fibroblasts appear to still remain at the level of intracellular trafficking.[170,187,188] Relative to other viral vectors, high numbers of viral particles are required to achieve transduction of human, rat and rabbit synovial fibroblasts in culture, typically in the range of 10^4-10^5 viral particles per cell, and as shown here only between 10-20% of AAV genomes enter the nucleus. Several lines of evidence implicate the ubiquitin-proteasome pathway as a key hurdle to efficient intracellular trafficking by AAV2-based vectors and other serotypes. Jennings et al. showed that the addition of proteasome inhibitors, such as carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (zLLL), dramatically enhanced nuclear uptake of AAV genomes in human synovial fibroblasts and was accompanied by a proportional increase in transgenic expression.[188] More recently, Zhong demonstrated that cellular phosphorylation of specific tyrosine residues on the AAV capsid surface led to increased ubiquitination of the viral particle and enhanced proteasome degradation.[189] Site directed mutagenesis of these tyrosines to phenylalanine blocked ubiquitination and led to ~10-fold enhancement of transduction efficiency.[190] The relative utility of these modified capsids in articular cells has not been investigated, but like the self-complementary vectors they have the potential to significantly enhance the efficiency of AAV-mediated gene transfer.

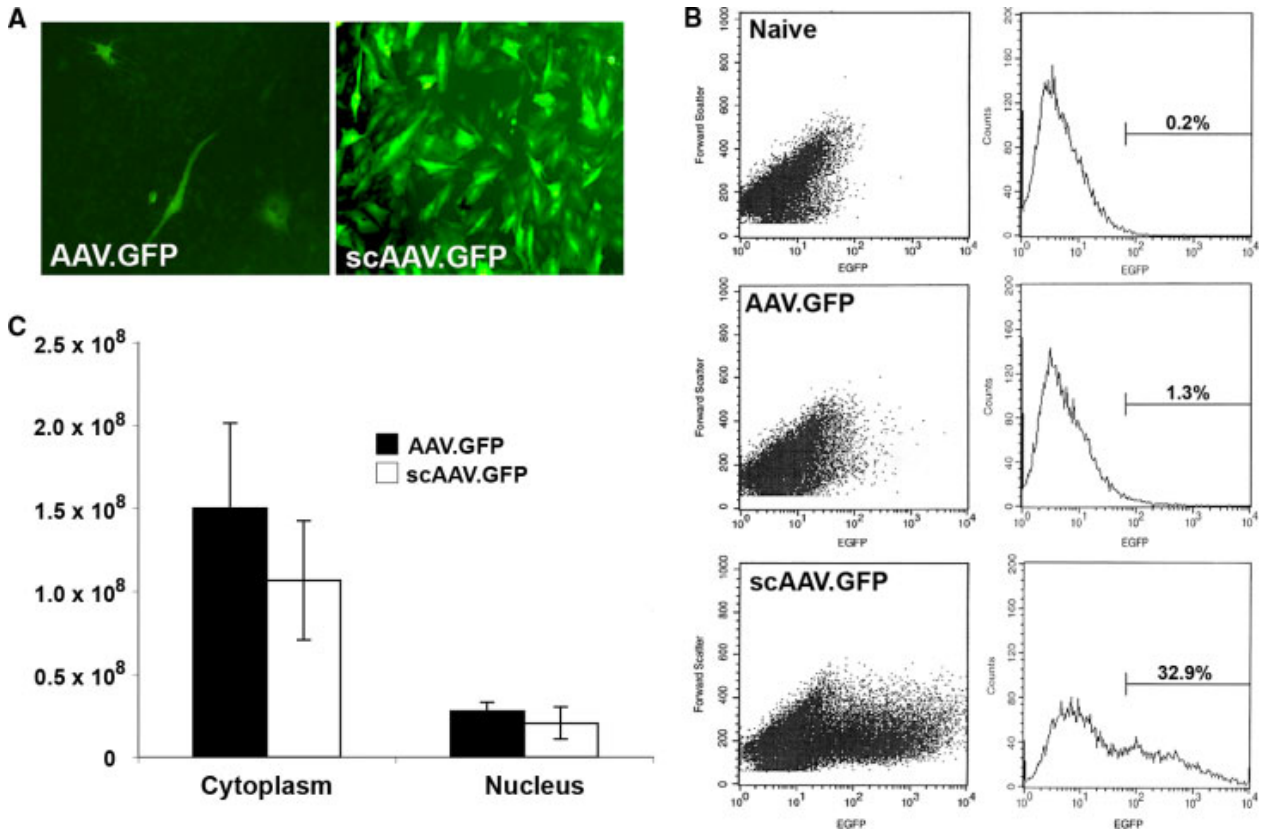

Figure 2-1. scAAV-mediated gene transfer to rabbit articular fibroblasts in vitro. Cultures of primary articular fibroblasts isolated from the joints of rabbits were infected with 104 viral genomes per cell of either conventional AAV.GFP or double-stranded, self-complementary AAV (scAAV.GFP). Parallel cultures of uninfected cells (Naive) were used as negative controls. Both vectors were packaged in AAV serotype 2 capsid. (A) Three days later, the cultures were examined visually by fluorescence microscopy.(B) Fluorescence was then quantified using flow cytometry. In these assays, scAAV provided an approximately 25-fold greater transduction than the conventional AAV vector. For each scatter plot shown on the left, levels of fluorescence are represented on the horizontal axes, and cell size is indicated on the vertical axes. For graphs on the right, fluorescence is indicated on the horizontal axes and cell number on the vertical axes. (C) To track the intracellular migration of the respective viral genomes, cultures of rabbit fibroblasts were infected with either AAV.GFP or scAAV.GFP. Twenty-four hours later, the cells were harvested, and the nuclear and cytoplasmic fractions were isolated. Viral genomes in the respective fractions were determined using quantitative PCR. Values plotted for each vector and compartment represents the means of four replicates. Error bars represent one standard deviation. For both types of vectors, less than 20% of the viral genomes entered the nucleus at 24 h post-infection. For the cytoplasm and nuclear fractions, separate two-sample t-tests were conducted; using $p < 0.05$, there was no significant difference between the respective samples in each group

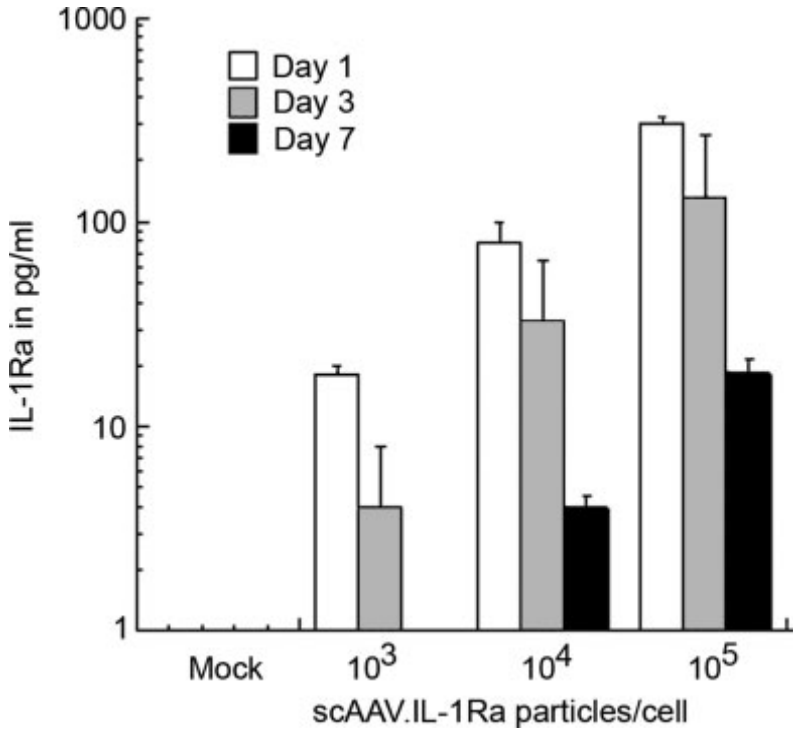

Figure 2-2. Infection of primary synovial fibroblasts with scAAV.IL-1Ra results in high level expression of the transgene. To test the function of the scAAV.IL-1Ra, cultures of primary, lapine, articular fibroblasts were plated and allowed to grow to approximately 70% confluence. The cells were then incubated with increasing amounts of the scAAV.IL-1Ra vector as indicated. At 24-h intervals post-infection, the culture media were removed and stored. The cells were washed with saline and the media replaced. IL-1Ra levels in culture supernatants from days 1, 3 and 7 were determined using ELISA. Each viral dose was tested in quadruplicate, and the bars represent the mean ± SD. For observations made on days 1, 3 and 7, IL-1Ra levels and scAAV.Il-1Ra doses were transformed using a base-10 logarithm, and a simple linear regression was fit to the transformed data. Using $p < 0.05$ for each test, a significant trend was detected for days 1, 3 and 7.

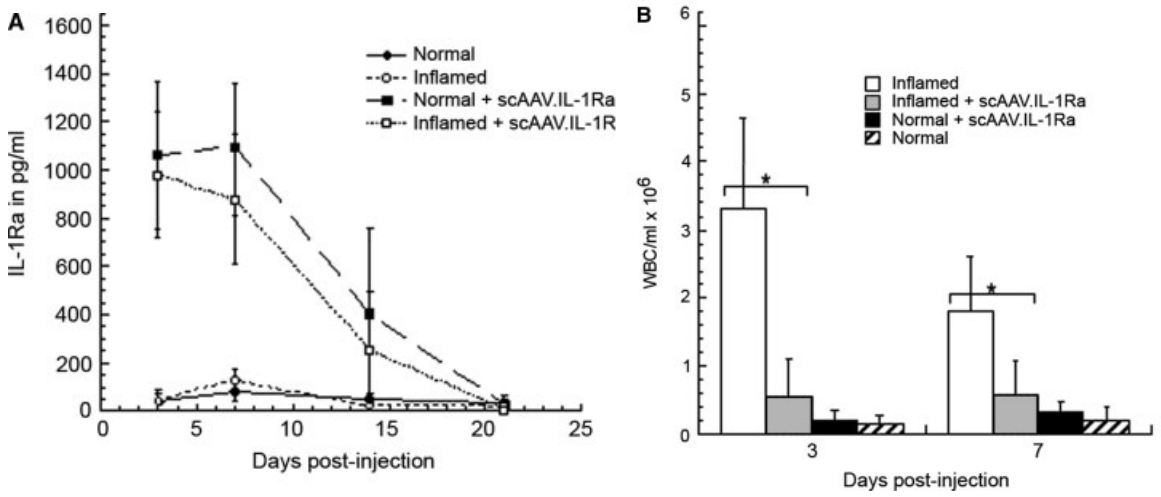

Figure 2-3. Intra-articular expression of scAAV.IL-1Ra after direct injection into normal and inflamed rabbit knee joints. Ten rabbits were initially injected in both knees with 5×10^4 HIG-82-IL-1β-neo cells, which stimulates an immediate, persistent inflammatory state. Three days later, 5×10^{11} particles of scAAV.IL-1Ra were injected into both knees of five of the rabbits with inflamed knees and five normal rabbits. An equivalent volume of saline was injected into the remaining five inflamed rabbits and an additional five normal rabbits. (A) Periodically, the knees of all rabbits were lavaged with saline and the IL-1Ra content in recovered fluids measured by ELISA. Data are shown as the mean ± SD. For rabbits receiving scAAV-IL-1Ra, using a two-sample t-test and $p < 0.05$, no significant differences were observed between inflamed and normal knees for all days post-injection. The same test conducted for rabbits injected with saline showed no significant differences between normal and inflamed as well. (B) Infiltrating leukocytes in lavage fluids recovered at days 3 and 7 for each group were quantified using a hemocytometer. Data are shown as the mean ± SD. A pooled two-sample t-test was used to determine the significance of the differences in leukocytic infiltration in inflamed knees that were injected with scAAV.IL-1Ra and untreated controls.

$*p < 0.05$

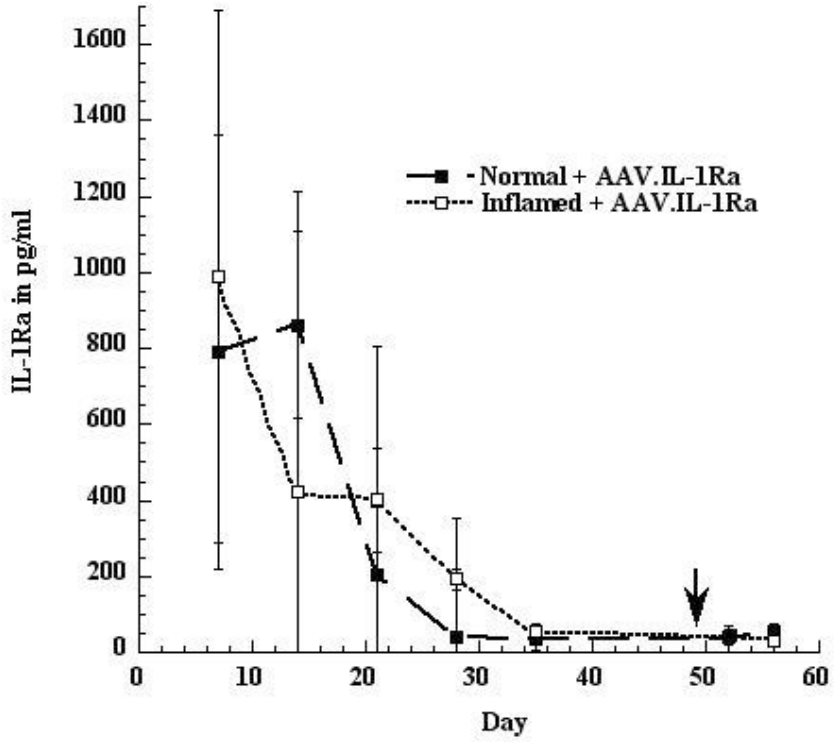

Figure 2-4. Repeat injection of scAAV.IL-1Ra does not result in rescue of transgene expression. Similar to the procedure described in Figure 2-3, five rabbits were injected in both knees with HIG-82-IL-1β-neo cells to establish an inflammatory state. Three days later, the inflamed rabbits and five normal rabbits were then injected with scAAV.IL-1Ra in both knees. At weekly intervals, the knees of the rabbits were lavaged with saline, and IL-1Ra levels measured using ELISA. At 49 days after the initial scAAV.IL-1Ra injection and 14 days after IL-1Ra expression had diminished in all joints, a second intra-articular injection of scAAV.IL-1Ra was administered to all rabbit knees (indicated by an arrow). The knees of all rabbits were lavaged at 3 and 7 days after the second vector injection. Values shown are the mean ± SD IL-1Ra levels at specific time points. A two-sample t-test was conducted for IL-1Ra levels between groups for each day post-injection; using $p < 0.05$, the only significant difference detected was at day 14 post-injection.

CHAPTER 3
SELF-COMPLEMENTARY ADENO-ASSOCIATED VIRUS MEDIATED DELIVERY TO RAT AND EQUINE JOINT TISSUES

Introduction

The degenerate joint disease osteoarthritis affects humans and many large mammals alike, severely decreasing quality of life. Inflammatory events trigger an imbalance between the anabolic and catabolic processes that maintain cartilage health which favors degradation of cartilage over promotion of new cartilage growth. The inflammation is associated with an increase in the expression of cytokines such as IL-1β and TNFα.

Until recently, treatment of these conditions has been mostly palliative, either with analgesics or anti-inflammatory corticosteroids. However, novel biologically active agents such as interleukin-1 receptor antagonist (IL-1Ra) and a soluble form of the TNFα receptor have been tested and shown to be effective at treating the underlying inflammation and damage of the cartilage. These emerging biologics have created a new therapeutic potential, but they also have a unique set of drawbacks. As proteins, these agents are inherently more unstable than pharmaceutical compounds and are subject to any number of processes present in mammals that eliminate or degrade protein products. Anakinra, a formulation of the anti-inflammatory protein IL-1Ra, for example, has an in vivo half-life of six hours. Although its activity is very desirable as a treatment option, its delivery, requiring daily injections, is not ideal.

One solution to this lack of persistence is gene transfer. By introducing the gene or cDNA for the therapeutic protein to a local population of cells near the target of treatment, these cells could manufacture a continuous supply of the therapeutic protein. Local production of the protein also has the benefit of keeping the concentrations higher

where their action is desired, therefore reducing the potential for off target effects. Delivery of the genetic material to the cells is not without its difficulties or dangers. Viral gene delivery to joints has been attempted numerous times, with some satisfactory results.[148,38,155,163-165,43,44] Recombinant adenovirus constructed to overexpress Il-1Ra has been used in equine joints, but immune reaction to the virus limited the duration of expression.[40]

Adeno-associated virus (AAV) vectors may be better suited towards these needs. AAV has a favorable safety profile, compared to other viral vector systems, and several recent advances have considerably increased its value as a vector, namely the introduction of the self complementary genome[117], which effectively makes the viral genome double stranded, increasing the levels of transgene production with a more rapid onset of gene expression.[171]

Effective gene delivery approaches to treating arthritic conditions have been met with several challenges, including suitable vector and model systems. Rodent models, including mouse, rat, and rabbit have been used in dozens of published experiments, but all use some form of induced arthritis, either by injection of collagen or adjuvant, or by delivering cells that have been genetically modified to overexpress an inflammatory agent. These models produce an induced arthritis that is characterized by a rapid onset and severe inflammation, which is more akin to rheumatoid arthritis than osteoarthritis. However, many of these studies have produced encouraging results, namely that several vectors can effectively deliver a variety of transgenes that can positively affect the arthritic condition. For instance, our group has successfully used self

complementary AAV (scAAV) to deliver IL-1Ra to the knee joints of rabbit with the result of reducing measurable symptoms of an induced arthritis.

To move beyond the rodent systems, it is necessary to use a model with more clinical relevance to the eventual human patient. In this regard the horse offers considerable potential. Horses naturally develop osteoarthritis through traumatic injury or repetitive athletic stress, much like humans. Furthermore, the size and architecture of equine limb joints are similar to human knees, both being encased in large quantities of connective tissue as a necessity of being used frequently for load bearing movement, and are both common sites of OA.

In this study we begin to explore the utility of the equine system as a model for gene delivery to joints in humans.

Materials and Methods

scAAV Vector Production

For generation of scAAV vector plasmids, the cDNAs encoding green fluorescent protein (GFP) and human interleukin (IL)-1 receptor antagonist (Ra) were directionally inserted into the SacII and NotI sites of pHpa-trs-SK plasmid. For all AAV vector constructs, transcription was driven by the cytomegalovirus (CMV) promoter/enhancer.

AAV vectors were propagated using an adenovirus-free, two or three plasmid transfection system. Using ten-layer, cell factories (Nunc, Rochester, NY, USA), the respective AAV vector plasmids were co-transfected into 293 cells by polyethyleneimine with a packaging/helper plasmid(s). For normal capsid production, the helper plasmid based on pDG-2 contained the AAV2 rep gene and cap gene for each serotype needed, as well as complementing adenoviral functions required for amplification and packaging of the AAV genome. The AAV5 Y719F capsid viruses were produced from pACG2R5C-

Y719F (created by Li Zhong, University of Florida) which contains the necessary mutations for the AAV5 capsid as well as rep from AAV2, and was complemented with pXX6 which contains the adenovirus helper genes. Sixty hours post-transfection, cells were harvested with phosphate-buffered saline containing 10 mM ethylenediaminetetraacetic acid (EDTA), pelleted, resuspended in low salt buffer and lysed by three rounds of freeze-thaw. Cellular nucleic acids were digested by incubation with Benzonase (Sigma, St Louis, MO, USA). Purification of AAV from the crude lysate was performed using iodixanol gradients followed by fast protein liquid chromatography affinity chromatography over a mono-Q column. The eluate was desalted and concentrated with a Millipore Biomax 100K filter (Millipore, Billerica, MA, USA), aliquotted and stored at -80°C. Viral titers were determined by quantitative competitive polymerase chain reaction (PCR) assay relative to well-characterized AAV viral reference standards. Each viral preparation was examined for purity by resolution of the viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stain.

Equine Tissue Collection

Thoroughbred horses were euthanized and tissues from diarthroidal joints of the forelimb were isolated. To isolate fibroblastic synovial cells for experimentation, the soft tissues, including the synovial lining and subsynovium, were scraped from the dense supporting fibrous tendon and ligamentous tissue of the capsule using a scalpel. Fragments of ligament and tendon tissues were harvested separately. Cartilage fragments were obtained by shaving with a scalpel. Under aseptic conditions, the fresh isolates of equine articular tissue were minced with a razor blade and digested in approximately 30 mL of saline solution with 0.2% collagenase for 2 h at 37°C with

constant stirring. Afterward, the suspension was passed through a nylon mesh to remove undigested tissue. The cells in the filtrate were then pelleted, washed in saline and plated in complete medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin). Twenty-four hours later, the cultures were washed to remove non-adherent cells and debris; the medium was replaced, and the cultures returned to the incubator. The cartilage derived cells were isolated much like the other cells, except that minced, enzymatically digested cartilage was placed directly on culture plates without passing through a screen.

Human cell cultures, derived from tissue surrounding the ankle, were isolated in the same manner as those from the horse. Human tissue was taken in a manner approved by the University of Florida Institutional Review Board, IRB# 59-03.

In Vitro Viral Infections

Unless otherwise indicated, cells were plated in 12-well plates and grown to approximately 70% confluence. Prior to infection, cells were washed two times with serum free media (DMEM). AAV vector from stock solutions was mixed with serum free media to produce working solutions containing appropriate DNAse resistant viral genomes per cell and placed on cell cultures. For experiments involving the GFP transgene and intracellular trafficking, viral doses of 10^4 viral genomes per cell were used. For those involving IL-1Ra, viral doses ranged from 10^3 to 10^5 viral genomes per cell. After incubation with virus for 2 hours, complete media was added to each well and the cells returned to the incubator. For quantification of IL-1Ra, at 24 h intervals after infection, the media from each well was harvested, and replaced with fresh media. Harvested media from selected days was stored frozen at -80°C. Each viral dose was

added to four individual wells and supernatant from each well was tested individually by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA).

Cell Fractionation and Quantification of Viral Genomes

Viral DNA from fractionated cells was isolated according to a procedure adapted from Zhao.[172] Briefly, cells were seeded at a 10^5 cells per well of a six-well dish, allowed to attach, and then infected with virus at 10^4 viral genomes/cell. After 24 hours, the cells were trypsinized, incubated in hypotonic buffer for 5 min on ice, and lysed in a non-ionic detergent. Centrifugation of the lysate allowed the nuclear fraction to be collected as the pellet whereas the supernatant was reserved as the cytoplasmic fraction. Low molecular weight DNA from each fraction was isolated by Hirt extraction and then used for quantitative PCR. Primer pairs (forward 5'-CACGCTGTTTTGACCTCCATAGAAGACACCGGG, reverse 5'-TTCTTTGATTTGCACCACCACCGGATCCGGG) were designed to anneal to sequences within the CMV promoter sequence. Viral genomes were detected using SYBR Green dye in an Eppendorf Mastercycler Realplex2 (Hamburg, Germany). The results were quantified by comparison to a well characterized viral reference standard.

Neuraminidase Treatment

Sialic acid residues on cells in culture were removed with neuraminidase to test AAV dependence on them for infectivity. Equine synovial fibroblasts were incubated with serum free medium containing 50mU/mL neuraminidase (Sigma) for 2 hours at 37°C. The medium was changed three times to remove the enzyme and was replaced with serum free medium containing either type 2 or type 5 scAAV.GFP at 10^4 viral genomes per cell. Cells were incubated with virus for 1 hour, followed by another three

washes, and then the addition of complete medium. Cells were trypsinized after five days and fluorescence was measured by flow cytometry.

In Vivo scAAV Delivery

$1x10^{12}$ vg of scAAV.hIL-1Ra packaged in type 2 and type 5 capsids were directly injected into four carpal and metacarpophalangeal (fetlock) joints of thoroughbred racehorses. At days 7, 21 and 35 post injection, synovial fluid was collected by arthrocentesis and analyzed for hIL-1Ra by ELISA.

Self-complementary AAV vectors with GFP or hIL-1Ra cDNAs were packaged into type 2 and type 5 capsids. A mixture of recombinant viruses with each transgene and of the same titer, $2.5x10^{9}$ vg, and serotype were injected intra-articularly into both knee joint of two Wistar rats for each serotypes, with 4 joints total per serotype. Five days post injection, the rats were sacrificed, and the knees were removed, dissected, and placed under an inverted fluorescent microscope to view the presence of GFP. The dissected joints were then placed into individual wells of a 12-well tissue culture plate. 1 mL of serum free DMEM was added, and the tissue was incubated in a humidified cell culture chamber at 37ºC and 5% CO_2 for 24 hours. The medium was then used for a hIL-1Ra ELISA.

Results

Equine Cells are Highly Receptive to AAV Transduction

Multiple serotypes of AAV are available for use in producing recombinant viruses for gene transfer, and each serotype can have a different tropism which is cell type and species dependent. The first step to determine AAV's viability in a horse model was to test the tropism of several available serotypes by infecting cells in culture.

scAAV.GFP packaged in serotypes 2,3,5, and 8 was used to transduce primary equine cells derived from synovium, cartilage, tendon, and ligament at 10^3 vg/cell. A composite of fluorescent micrographs taken 5 days post infection (Figure 3-1) illustrate the differences in serotype tropism. Chondrocytes were not highly receptive to transduction by any of the serotypes tested, nor was serotype 8 effective at transducing any of the cell types. The synovium derived fibroblasts, however, were transduced by the remaining serotypes, with serotype 5 transducing the remaining three cell types: synovium, tendon, and ligament derived cells. This correlates with published observations that rodent joints are also highly receptive to serotype 5 infections.[163,179] To quantify positively transduced cells, flow cytometry was performed on synovium derived cells infected with serotypes 2 or 5. Three days post infection, over 98% of cells were positive (Figure 3-4, A and B).

Transduced equine synovial cells were further examined for their capacity to secrete a therapeutic transgene. Another compilation of scAAV packaged in capsids 2,5, and 8 containing a transgene cassette for IL-1Ra was used to infect synovium derived fibroblasts at 10^4 vg/cell. IL-1Ra ELISA was performed on medium collected from the infected cells at days 3, 7, 14, and 21 (Figure 3-3). Cells infected with types 2 or 5 remained positive for the transgene protein product over the course of the experiment, with onset occurring at day 3 and peaking at day 7. Cells infected with serotypes 7 and 8 did not produce measurable transgene product after day 3. In summary, through all tests, serotypes 2 and 5 consistently expressed the highest levels of hIL-1Ra.

Transduction Patterns Vary by Species

Cells derived from rat and human joint tissues were also tested for their transducibility by several AAV vector serotypes. scAAV.GFP packaged into serotypes 1, 2, 5, and 8 were used to infect isolated primary cells from rat and human synovium, cartilage, tendon, and ligament. Fluorescence micrographs recorded GFP expression while flow cytometry was used to quantify the number of transduced cells. Three days post-infection, many rat cell types show minimal GFP expression (Figure 3-3), although the chondrocytes seem receptive across all serotypes tested, with 13-67% of the cells being GFP positive as measured by flow cytometry. In contrast, the human cells show a quite different transduction pattern (Figure 3-4), with no one cell type being dominantly receptive, but all tested cell types are most receptive to AAV2 transduction. These results indicate that between species, AAV receptor expression, or other factors, can vary by cell type.

AAV2 and AAV5 Transgene Expression in Equine Synovial Fibroblasts

The high level of transduction with AAV2 and AAV5 in equine cells warranted further investigation. Comparable infections of human derived synovial fibroblasts with scAAV5.GFP showed a maximum of 30% of cells positively transduced (Figure 3-2 C). To investigate the mechanistic difference, equine and human synovial fibroblasts were infected with 10^4 vg/cell of either scAAV2.GFP or scAAV5.GFP and incubated for 24 hours. Cells were fractionated into cytoplasmic and nuclear components and small DNA molecules were isolated with a Hirt extraction. These samples were used for a qPCR using primers that reside in the CMV promoter region of the vector. The results of the qPCR were used to directly compare the number genomes detected in each fraction (Figure 3-6 and 3-7). In the type 5 infected cells, roughly 10-fold more viral

genomes were detected from the equine samples than the human, although the relative ratios of DNA in the nuclear and cytoplasmic fractions remained the same. A similar pattern was seen with serotype 2 vectors. This indicates that cell entry, rather any intracellular localization process is responsible for the enhanced transduction of the equine cells. Also of note is that in both experiments, the vast majority of viral genomes were detected in the cytoplasm, even after 24 hours. Although entry into equine cells appears to be an efficient process, intracellular trafficking could be limiting step.

Sialic Acid is Primarily Responsible for AAV5 Transduction of Equine Synovial Cells

Sialic acid is a known receptor for AAV5 and its enzymatic removal with neuraminidase would inhibit AAV5 infection, if it is the only mechanism available for cell entry. Low passage equine synovial cells were treated with 50U/mL of neuraminidase for two hours and then infected with 10^4 vg/cell of scAAV5.GFP. Fluorescent micrographs were taken on days 1, 3 and 5 post-transfection and followed by flow cytometry to monitor GFP expression. Neuraminidase treatment had a drastic effect on transduction. Five days post infection, only 6.8% of equine synovial cells were positive for GFP transgene expression, compared to 92.8% of control untreated equine cells (Figure 3-8). Neuraminidase treatment showed no effect on AAV2 infectivity, as expected (data not shown). Therefore the enhanced transduction displayed by AAV5 on equine cells, it appears to be dependent on the presence of sialic acid.

Intra-Articular Delivery of scAAV Serotypes 5 and 8 to the Rat Knee Joint

The tissues that make up a joint are particularly fibrous and the cells of some tissues, especially the cartilage, are embedded in a thick extracellular matrix. Delivery of the virus directly to these tissues is necessary to determine if transduction patterns

observed *in vitro* are relevant *in vivo*. To determine the identity of transduced tissues, a GFP reporter was used, and to determine if these tissues could support secreted protein production, hIL-1Ra was used as a reporter. Preliminary experiments determined that vectors containing each of these transgenes could be injected simultaneously with now adverse effects on expression of either reporter. In this manner, both reporter cassettes were packaged into either AAV serotypes 5 or 8 and injected intra-articularly to the knee joints of Wistar rats, with a dose of $2.5x10^9$ vg of each reporter gene, giving total load of $5x10^9$ AAV vector genomes injected per joint per serotype. After five days, the knee joints were harvested, dissected, and examined under a fluorescent microscope for GFP expression (Figure 3-9). Contrary to what the above cell culture results would indicate, no GFP was observed in the cartilage or the adjacent synovium. Weak GFP expression was seen in the fat pad, a tissue that contains adipocytes and some synovial cells. Most surprising was the high GFP expression seen in the extra articular muscle. This indicates that the AAV vectors left the synovial space either due to diffusion or some unknown transport mechanism. This data contrasts with previous work where adenoviral and lentiviral vectors only transduced tissue immediately adjacent to the synovial space.

After examination, knee joints were placed in 12-well tissue culture plates and incubated overnight in 1 mL of serum free DMEM in a cell culture incubator. The medium from this explant culture was used in an ELISA to test for hIL-1Ra expression, which resulted in over 20 ng/mL for tissue from both AAV5 and AAV8 injections. Although it is unknown how much of this transgene product would enter the synovial

fluid, these results would indicate that the muscle surrounding the joint is extremely capable of supporting therapeutic protein production.

Equine Joints Express High Levels of AAV2 and AAV5 Delivered Transgene

The rat knee joint is much smaller than a human knee joint, and it does not bear as much proportional body weight as a human knee. These differences are further exemplified in the architecture of the joint, with the human knee joint containing proportionately much denser fibrous tissue. This could contribute to the transduction patterns of AAV vectors as well as the potential of the virus to escape the joint space after intra-articular delivery. The forelimbs of a horse are much more analogous to human knees, both in size, function, and architecture, and were selected for further *in vivo* testing.

Based on in vitro data above, serotypes 2 and 5 were selected for use in vivo as they had the most potential for high levels of transgene expression. CMV-hIL-1Ra expression cassettes were used instead of a GFP reporter. The secreted transgene allows for measurements to be taken from synovial fluids, and the due to the large size of the equine joint and small amount of fluid needed, the same joint can be repeatedly sampled. Using a human cDNA as opposed to an autologous horse cDNA will allow for accurate detection of transgene in the presence of the native proteins.

Either vector was directly injected into the carpal and midcarpal joints of thoroughbred race horses at a dose of $1x10^{12}$ vg. These joints are frequently the site of equine osteoarthritis and are homologous to human knees for the impact of athletic activity.

Synovial fluid was collected periodically from each treated joint for five weeks and analyzed by ELISA to detect hIL-1Ra (Figure 3-10). Transgenic human IL-1Ra was

detectable at measurable levels for the entire length of the five week study, with synovial fluid from type 5 infected joints averaging 1.5 ng/mL and type 2 joints averaging 0.5 ng/mL.

Capsid Modifications of Tyrosine to Phenylalanine Enhances Effectiveness

Newly generated tyrosine to phenylalanine capsid mutants of AAV vectors allow for enhanced gene expression through bypassing some of the ubiquitination/proteasome degradation pathway that can inhibit a large fraction of AAV virions from ever reaching the nucleus.[74] When these mutations are in place, intracellular trafficking to the nucleus is enhanced, leading to increased levels of transduction. An AAV5 based capsid mutant, Y719F, was used to generate new scAAV.GFP vectors. These Y719F capsid viruses then used to infect equine synovial fibroblasts at three different log doses: 10^4, 10^3, and 10^2 vg/cell. The experiment was replicated with wild type AAV5 encapsidated vectors for comparison. The cells were monitored for GFP expression, as captured by fluorescent photomicrographs, and quantified on days 1, 3, and 5 post-infection by flow cytometry. The results at day 3 are shown in Figure 3-11. No difference between the capsid types is seen at the standard dose of 10^4 vg/cell, but as the dose is lowered, the Y719F mutant supports a much higher transduction level. At the 10^2 vg/cell dose, 56% of Y719F cells are GFP positive while this is true for only 7% of standard AAV5 infected cells. This indicates 10^4 vg/cell is a saturating dose of virus on equine cells, and Y719F vectors remain quite effective at lower doses.

Discussion

In the present study, we evaluated AAV as a gene therapy vector for use in equine models. AAV has established itself as the viral gene therapy vector of choice due to its

unmatched safety profile, although much basic research still remains to be done, especially for use in non-human systems. The virus is naturally replication defective and has no known link to any human disease. Recombinant AAV vectors are made even safer as nearly the entire viral genome can be replaced by an expression cassette. Advances in AAV technology, such as development of self complementary vector genomes, are beginning to bring its efficacy in line with its safety. Further advances, such as the tyrosine to phenylalanine replacements on the viral capsid, are increasing efficacy even more. We believe that AAV is reaching a point where it can be successfully adapted for use in gene therapy based medicine for joint diseases.

Osteoarthritis is a particularly good disease candidate for gene therapy. Palliative treatments can reduce pain and inflammation, but offer no chance to halt progression or reverse damage already incurred. Biologic agents can affect these changes, and local, sustained expression of these proteins is the first step towards a working therapy. To further develop this therapy, rational models are needed, ones that closely mimic the human condition so treatment optimization can be undertaken.

The equine joint provides just such a model. Weight bearing and encapsulated with strong connective tissue and muscle, it is by far a better match in structure and function to the human knee than that of any rodent joint. Athletic horses also develop osteoarthritis themselves, and could potentially benefit from of any therapy being developed for humans.

scAAV Differentially Transduces Joint Cells from Different Species

This study contains the first survey of the transducibility of joint cells by various AAV serotypes. Cells from two model species, rats and horses, were compared to cells from humans to both collect expression data and to ascertain the usefulness of the

models. As to expression patterns, differences between species are clearly present. In culture, only the cartilage derived cells of the rats appeared to be receptive to AAV transduction, while the human cells were only receptive to AAV type 2. In contrast, the equine cells were extremely receptive to multiple serotypes. Taken alone, this data offers a difficult interpretation of how data from the animal models can be related to humans. The rat data suggest a completely different transduction pattern from humans, while a model using the equine data could lead to over-optimistic expectations of performance in human tissues.

scAAV Transduction of Equine Synovial Cells

Synovium is often the target of gene delivery to the joint, and we have found that equine synovium derived cells are highly receptive to several serotypes of AAV, especially types 2 and 5, as measured by expression either of GFP or the secreted gene product IL-1Ra. In cell culture, a dose of 10^4 viral genomes per cell was sufficient to transduce nearly 98% of cells. For comparison, in our hands, when AAV2 was used to infect 293 cells only 55% transduction occurred. Though not quantitatively measured, AAV5 also showed a high degree of transduction in ligament and tendon cells.

Further investigations into the particularly effective combination of AAV5 and equine synovial cells revealed that nearly 10-fold more virus was entering equine cells than those of comparable human synovium-derived fibroblasts. However, even within these cells, a vast majority of the virus remained in the cytoplasm and was not trafficked to the nucleus. This was true when AAV2 vectors were used as well.

It appears that surface receptors, namely sialic acid, on equine cells facilitate efficient viral entry. AAV5 vector entry appears to be primarily mediated through sialic

acid residues, as enzymatic removal of sialic acid drastically curtailed transduction levels, thus reducing the likelihood of an alternative receptor for AAV in this setting. This interpretation would mean that AAV5 transduction of joint tissues is dependent on available sialic acid surface residues, and since receptor density appears to vary among species, being much greater in horses than in humans, it remains to be determined whether there will be a more effective serotype than AAV5 for transducing human synovial cells.

Capsid Modification of AAV5 Enhances Transduction

The idea of capsid modification of viral vectors has been researched for years, and one particular type of enhancement is now available for AAV is particularly promising. A paper by Zhong initially showed that replacement of tyrosine residues with phenylalanine on the surface of the AAV capsid removes a site of ubiquitination, which targets the virus for degradation in the proteasome.[190] By removing some of the intracellular degradation which normally occurs in AAV infections, these minor capsid changes serve to enhance nuclear trafficking of the virus, leading to higher transgene production without affecting the tropism or other viral functions. As noted above, much of the viral genomes detected after subcellular fractionation following infection were found to be in the cytoplasm, rather than the nucleus. Improving trafficking to the nucleus could improve overall transduction efficiency. Using a single amino acid mutation on the AAV5 capsid, Y719F, we found lower doses of virus would work more efficiently than the conventional AAV5 capsid. The highest dose we routinely give to cells in culture, 10^4 vg/cell, appears to be saturating and no improvements were seen. Significantly, the Y719F capsid appears to be less affected by dose reduction than wild type AAV5 capsid. For each ten-fold reduction in dose, the Y719F capsid only loses

roughly a quarter of its transduction ability, while the wild type capsid loses three-quarters or more. Tyrosine to phenylalanine AAV capsid mutants will add a greater degree of efficiency as AAV gene therapy progresses.

scAAV Mediated Gene Transfer to Rat and Equine Joints

A survey of AAV vectors was completed on cells in culture; however conditions in the joint could affect these transduction patterns. The cellular density and much great amounts of extracellular matrix, especially in the cartilage, could affect AAV's infectivity when delivered into the joint space.

The knee joints of rats are the largest accessible joint of the rodent that can be reliably used for intra-articular delivery of viral vectors. scAAV.GFP packaged in serotypes 5 and 8 were injected into the knees of rats. Upon examination of the joint tissues, the bulk of GFP expression was not seen in the immediate synovial space, the site of the injection, but in the muscles surrounding the joint. This would suggest that the AAV particles are able to leave the joint space, although the mechanism for this remains unresolved. It is possible that the small size of the AAV capsid, 20 nm, allows it to diffuse out through the joint capsule. Active transport through the synovial cell layer or the lymphatic system are also possibilities. This high mobility of AAV after intra-articular injection has not been observed before, and is not seen with other viral vectors such as adenovirus or lentivirus. As the surrounding muscle was shown to be capable of supporting a high level of therapeutic protein secretion, intra-articular delivery may still be a suitable choice for AAV delivery. However, alternative delivery techniques, such as delivering the virus directly to the extra-articular muscle should be investigated as it may offer equal effectiveness with fewer complications. Infection at injection sites

is a possible side effect of any gene delivery method, and an intra-articular infection of an arthritic joint in a human would be detrimental.

Transduction of equine joints differs could differ greatly from those of a rat. The increased size and thickness structural components could impact AAV transduction. The large volume of the equine synovial fluid also allows for taking of fluid for direct measurement of transgenic proteins, whereas no such measurements are reliable in rodents. When scAAV5.IL-1Ra was injected into the joints of horses, transgene product in the synovium was measured to be near 1 ng/mL for five weeks. This concentration of IL1-Ra to is remarkable; in view of the above rabbit study in which 1 ng/mL of IL-1Ra was the threshold for significant reduction in measurable arthritic markers. To test if this level is therapeutic in horses, experiments will need to be conducted on arthritic horses, with improvements in swelling and lameness being markers for improvement. Direct measurement of inflammatory markers in the synovial fluid will also be possible in a horse model, and could be compared to transgene production over time.

Implications from Animal Models

After 5 weeks, the study was curtailed, but the transgene level was on a decreasing trend. The transgene was of human origin and thus we expect that immune incompatibly may be responsible for the loss of transgene expression. Further tests using homologous transgenes that are immune compatible, but still allow for differential detection over endogenous gene expression would be needed to clarify the impact of the immune system's elimination of transduced cells.

Of equal importance to immune compatibility is the longevity of the transduced cells. When an integrating lentivirus was injected into rat knee joints, the synovial cells surrounding the joint cavity were lost over several weeks and were not replaced by

mitotic division, while transduction of the connective tissue was stable over the life of the animal.[154] Since recombinant AAV is generally maintained as an episome, its productive transduction longevity will be diluted by cell division as well as cell loss. Further investigation into the location of the transduced cells in the equine joint is needed for these issues to be addressed. This aspect of the equine model should be directly translatable for any proposed AAV based gene therapy to large human joints, as the joint architecture and injection methods will be similar. However, the in vitro data suggest that AAV transduction between species can be highly variable, and must be taken into account before any translation into a human system can take place.

We believe this study provides solid evidence of the utility of scAAV to deliver therapeutic transgenes to the joints of horses.

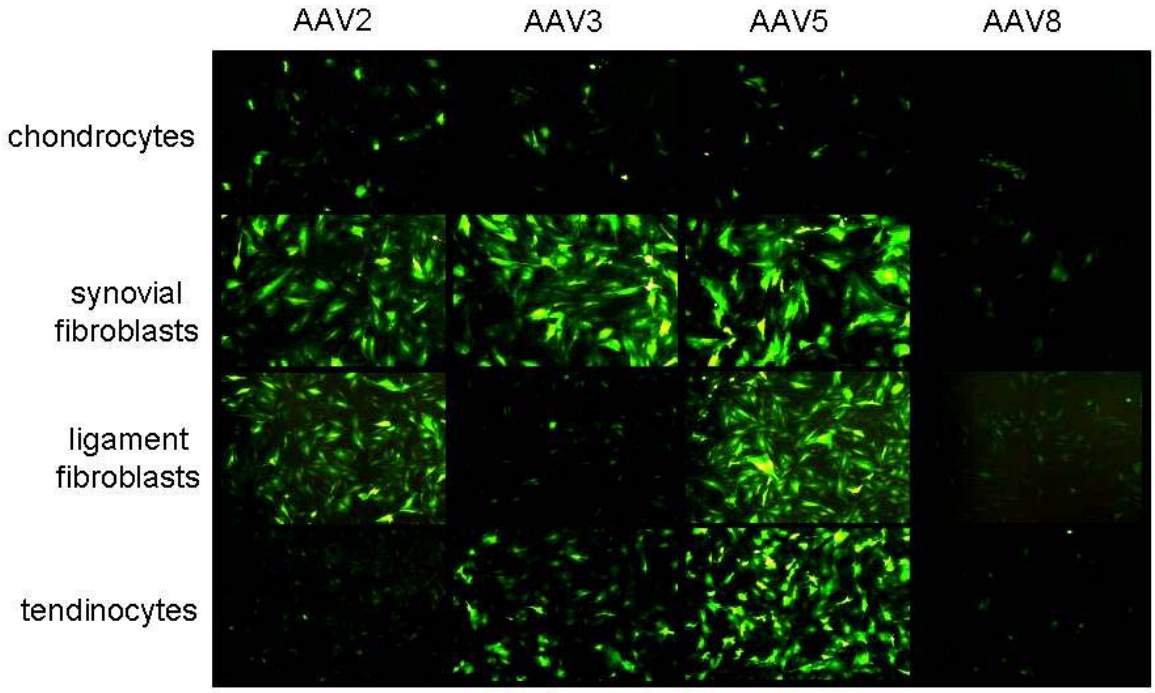

Figure 3-1. Differential transducibility across serotypes and cell types of the equine joint. Cells derived from equine joint tissue were cultured and infected with 10^3 vg/cell of scAAV.eGFP vectors. Fluorescence photomicrographs are from day 5 post infection cells.

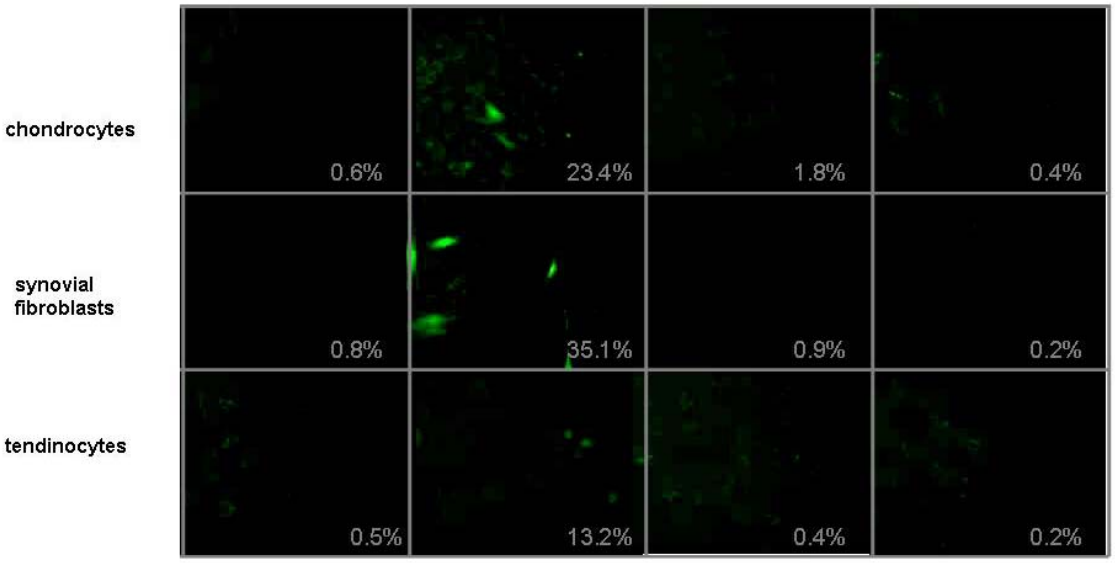

Figure 3-2. Differential transducibility across serotypes and cell types of the human joint. Primary cells derived from human joint tissue were cultured and infected with 10^4 vg/cell of scAAV.eGFP vectors. Fluorescence photomicrographs are from day 3 post infection cells.

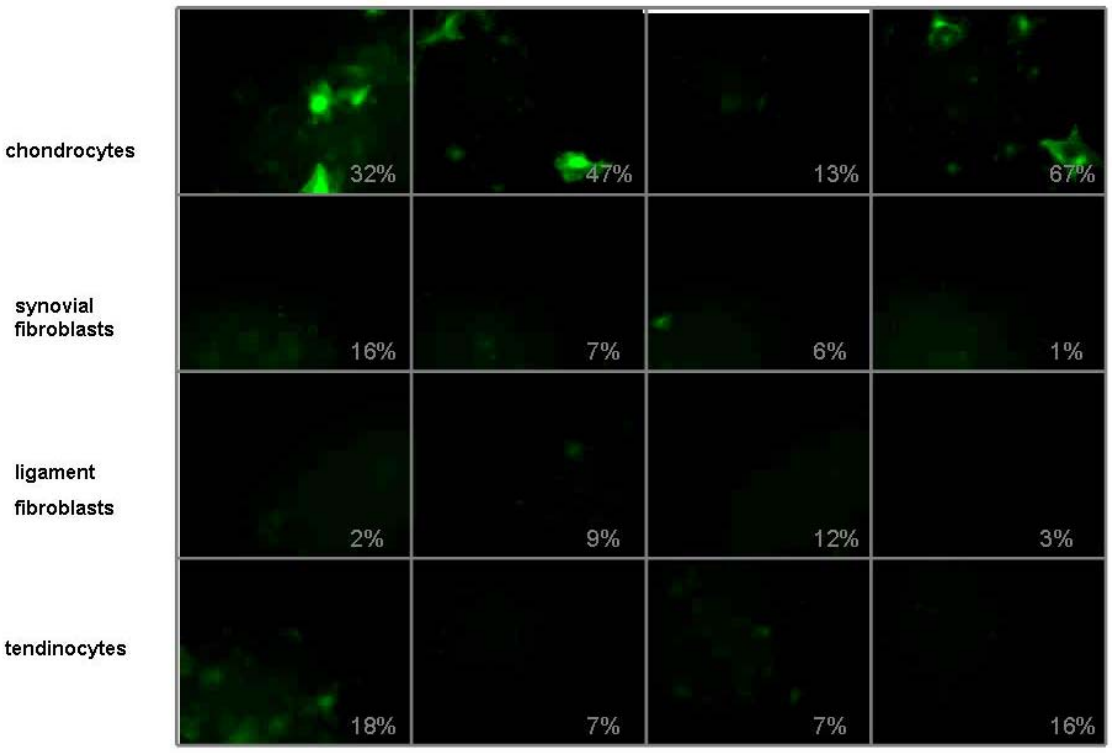

Figure 3-3. Differential transducibility across serotypes and cell types of the rat joint. Cells derived from rat joint tissue were cultured and infected with 10^4 vg/cell of scAAV.eGFP vectors. Fluorescence photomicrographs are from day 5 post infection cells.

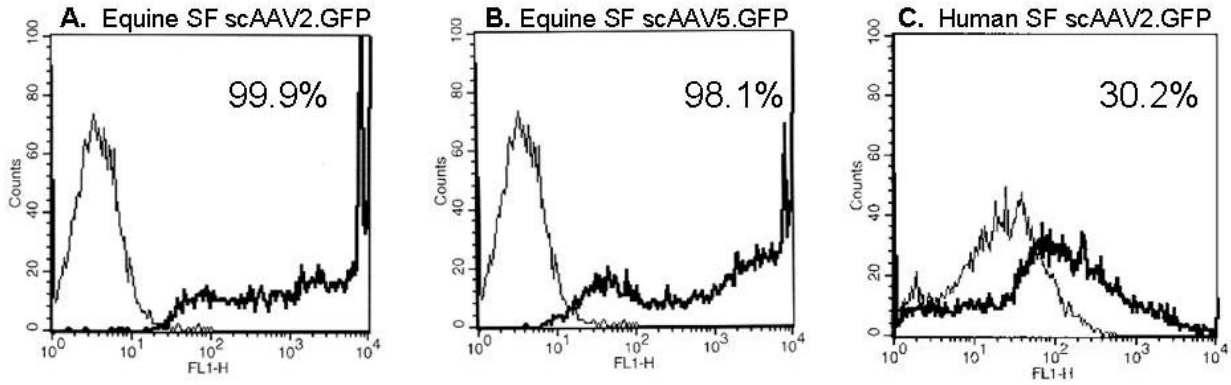

Figure 3-4. Transduction of equine and human synovial fibroblasts with scAAV. Low passage equine and human cells were infected with $1x10^4$ vg/cell of scAAV2.GFP or scAAV5.GFP and incubated 3 days before fluorescence was measured with flow cytometry. Gated against uninfected controls, GFP was detected in 98-99% of equine cells (A and B) and 30.2% of human cells (C).

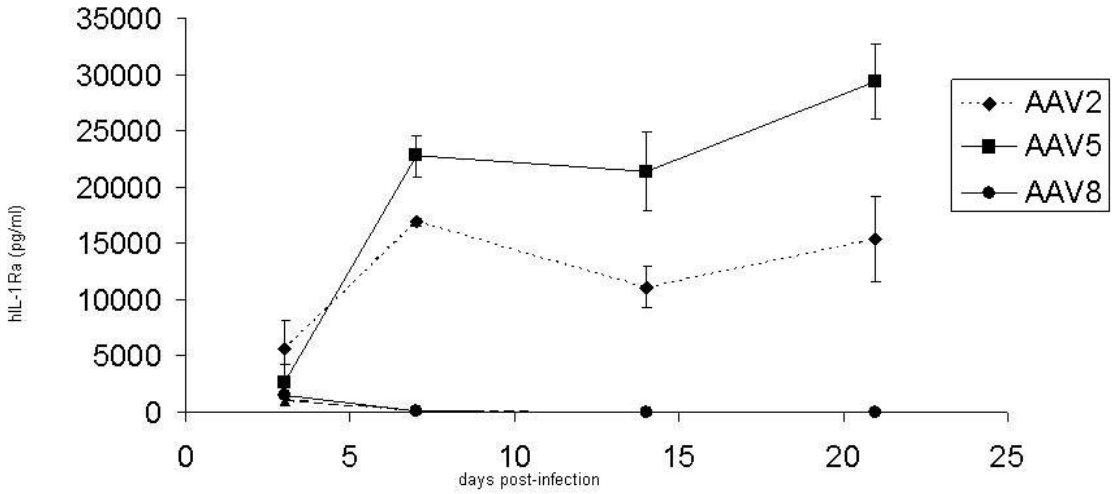

Figure 3-5. Transgene expression following infection of equine synovial cells with AAV viruses encoding human IL-1Ra. Following isolation of the cells from equine synovium, cells were grown in monolayer and infected with $3x10^9$ vg of AAV2, 5, or 8 encoding hIL-1Ra. At day 2, 6, 13 and 20, medium was replaced by 1 mL of fresh medium. Twenty-four hours later, conditioned media were harvested and hIL-1Ra contents measured by specific ELISA. Results are expressed in pg/mL.

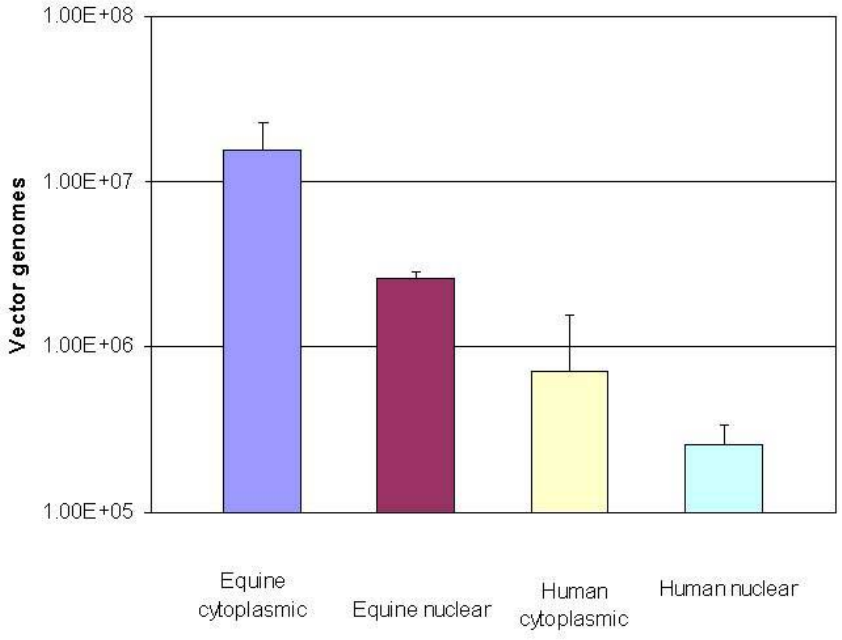

Figure 3-6. Viral genomes detected in equine and human cell fractions 24 hours post infection with scAAV5. In order to track intracellular migration of AAV genomes in equine and human cells, fractions of each cell type were taken 24 hours after infection with 10^4 vg/cell. Quantitative real time PCR was performed to determine the number of viral genomes in each sample. Results were corrected for dilution; the mean of three replicates are presented.

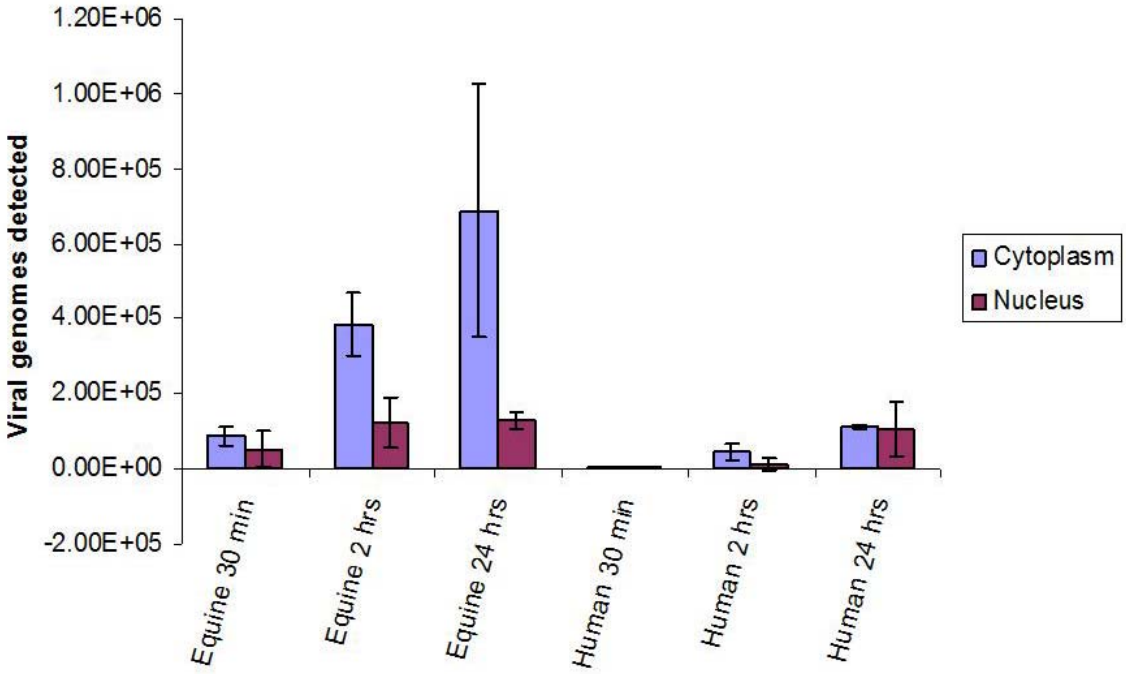

Figure 3-7. Viral genomes detected in equine and human cell fractions 30 minutes, 2 hours, and 24 hours post infection with scAAV2. In order to track intracellular migration of AAV genomes in equine and human cells, fractions of each cell type were taken 24 hours after infection with 10^4 vg/cell. Quantitative real time PCR was performed to determine the number of viral genomes in each sample. Results were corrected for dilution; the mean of three replicates are presented.

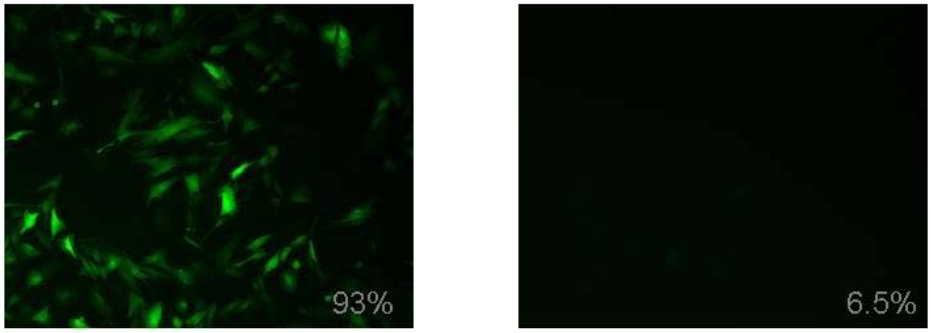

Figure 3-8. Neuraminidase treatment inhibits AAV5 transduction of equine fibroblasts. To determine if sialic acid was responsible for increased AAV5 transduction efficiency, neuraminidase was used to remove sialic acid residues from the cell surface. Cells were treated with 50 U/mL of neuraminidase for 2 hours, washed 3 times, and then infected with 10^4 vg/cell scAAV.GFP. Fluorescence photomicrographs and flow cytometry was performed 24 hours later. The percent of GFP-positive cells is indicated

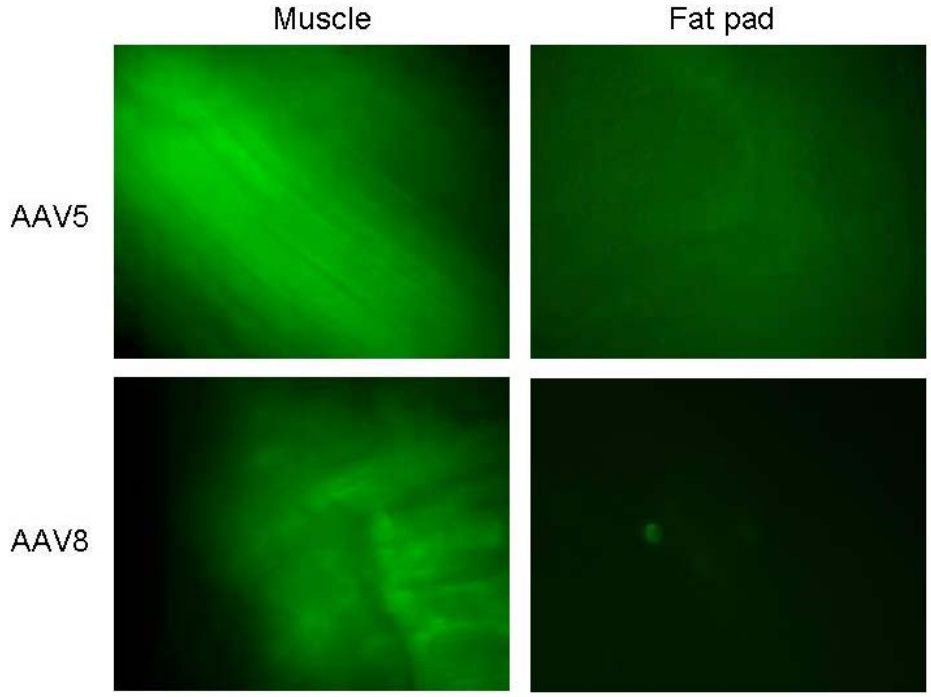

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Figure 3-9. Fluorescence images of rat joint tissues five days after intra-articular injection with scAAV.GFP vectors. $2.5x10^9$ vg of scAAV.GFP packaged in serotypes 5 or 8 capsids were injected directly into the synovial space of the rat knee. Images are representative views of dissected tissues adjacent to the joint space.

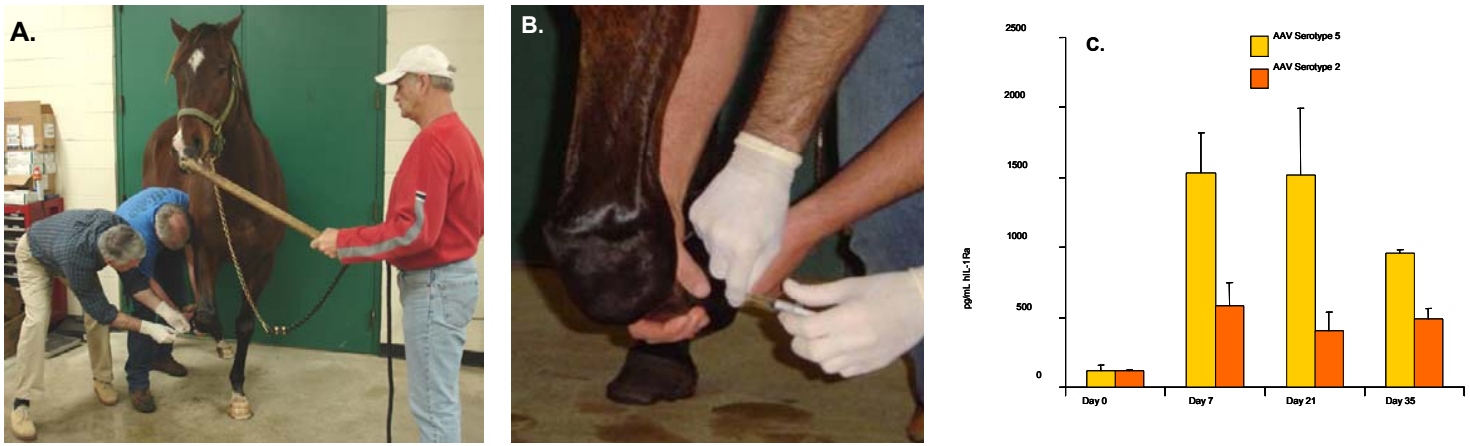

Figure 3-10. scAAV-mediated gene delivery to the joints of horses. Intra-articular injection of scAAV-IL-1Ra into the carpal joint of a thoroughbred horse (A). Aspiration of synovial fluid from the metacarpophalangeal joint (fetlock) (B). Mean expression levels over time of hIL-1Ra in the joints of horses following injection of scAAV-IL-1Ra packaged in serotype 5 or serotype 2. Expression is shown as pg/mL of synovial fluid aspirate (C).

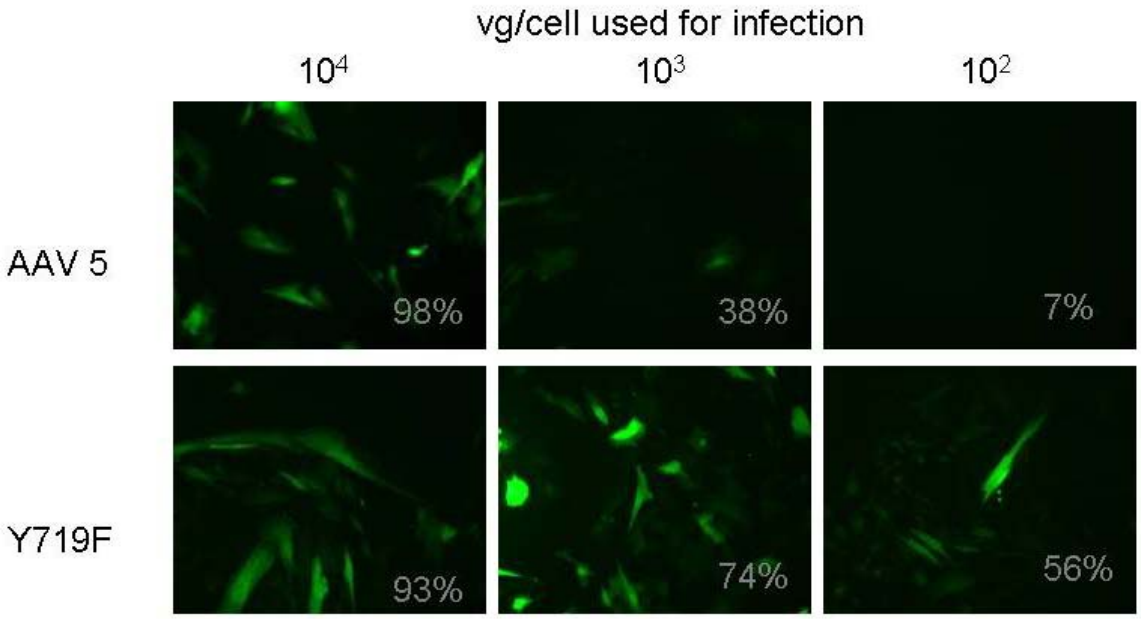

Figure 3-11. AAV5 Y719F capsid mutation increases transduction efficiency in equine synovium derived fibroblasts. Self-complementary AAV genomes with identical CMV-GFP expression cassettes were packaged in either wild type AAV5 capsid or Y719F capsid and used to infect equine synovial cells at 10^4, 10^3, or 10^2 vg/cell. After 72 hours, fluorescent micrographs were taken and GFP expressing cells were counted by flow cytometry. The percentage of GFP positive cells indicated in each panel.

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

Tens of millions of Americans are currently suffering from inflammatory joint diseases known collectively as arthritis. The pain, restriction of motion, and reduction in quality of life costs are not only personal, but cost society tens of billions of dollars in medical care and lost wages.[13] These costs, and the attendant pain and suffering of individuals, will rise as our population ages. Prevention of these conditions is not likely as they are a function of normal activity and aging. Current common treatments are only palliative and make no headway against the underlying causes of the painful inflammation.

Gene therapy provides an opportunity to directly affect these debilitating aspects of arthritis by modifying local cells of the joint to become a source of anti-inflammatory proteins. In the case of OA, a sustained, long term suppression of inflammation would give the joint tissues an opportunity for self repair, which may ultimately reduce the cause of inflammation. Nonviral vectors have been shown to be highly inflammatory when introduced into the joint and are not suitable for use. Likewise, adenovirus, although highly effective at transducing human joint tissues, can not evade immune stimulation and subsequent elimination. Integrating retroviruses are not suitable to treat arthritis as any risk of oncogenesis is presently unknown and potentially finite. Adeno-associated virus has none of the drawbacks of these other possible vectors, save that transduction efficiency has yet to be optimized.

Self-complementary AAV vectors is shown here to have improved gene expression to the point where therapeutic levels of transgenic protein can be obtained in a model of inflammatory arthritis in rabbits. Knowledge gained in rodent models will not

necessarily transfer to human systems, so investigations using equine tissues and joints have been initiated. Arthritic horses can provide necessary data which is unattainable in rodent models. The size, structure, and disease progression of OA in humans and horses are a very close match, and these similarities are what is needed for accurate predictions of how AAV will react when eventually used in human joints. However, differences in the levels of viral infection may overestimate the efficiency of treatment. Thus the validity of animal models of gene therapy remains a challenge.

Once effective vectors are established, these can be used to answer remaining questions that stand between AAV and a useful clinical treatment: longevity and re-administration. In mitotic cells, episomal AAV genomes will be lost through dilution. Transduction of long-lived cells is desirable, although these cells appear to reside in the tendons and muscles surrounding the joint, and it is unknown if this will reduce the therapeutic efficacy of the transgene compared to transduction of synoviocytes.

If re-administration becomes necessary, it is unknown what, if any, immune response would be mounted against the vector. Pre-existing immunity is also a factor as up to 80% of people globally are seropostive for antibodies against AAV2.[96] Interestingly, that same study indicates that some individuals had been infected with multiple serotypes, presumably at different times, meaning that antibodies to one serotype may not confer protection from another. In a gene therapy situation, this may appear to be useful, as a different serotype could be used for each treatment, however this may not be practical in the near term. There are no more than a dozen well characterized serotypes that could be easily modified and used in vector production, although this could change. More importantly, the tropism of the virus is dependent on

its capsid protein, which determines the serotype. Changing the serotype of the vector would potentially alter the transduction pattern and efficacy of the treatment, which may or may not be desirable. Lowering the titer of the vector used would seem to solve these problems, but again, this will necessitate the use of highly efficient vectors and our study here of Y-F mutant capsid vectors is a start in this direction. There are numerous Y-F mutant capsid vectors available on several serotypes, and they would need to be evaluated alone and in combination to test their efficacy.

Overall, this work lays down a solid foundation for these future investigations. There are many challenges that face AAV before it will be ready for use as a gene therapy vector, but many of the basic questions have been answered. Self-complementary vectors with current expression cassettes are sufficient to produce therapeutic amounts of protein, contingent on infection of joint tissues. The longevity of transgene expression ultimately depends on what cells are infected, and what immune response occurs. Highly sensitive assays need to be developed to test these conditions concurrently, as they are tightly linked.

The state of the art for AAV vectors is improving month after month, and the challenges of transduction longevity and re-administration will not remain unsolved for long.

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BIOGRAPHICAL SKETCH

Jesse Douglas Kay was born on Fort Lewis, Washington, outside of Tacoma. He graduated valedictorian of Jacksonville High School's class of 1999 in the city Jacksonville, Arkansas. His undergraduate studies began the following fall at Vanderbilt University, Nashville, Tennessee, where he majored in biological sciences. During his junior year, Jesse began working as a research assistant in the lab of Dr. John S. Penn, where he contributed to the understanding and treatment of the retinopathy of prematurity. After receiving his Bachelor of Science in May 2003, he continued to work for Dr. Penn as a research assistant until joining the Interdisciplinary Research Program at the University of Florida, College of Medicine in August of 2004. In May of 2005, Jesse joined the lab of Dr. Steven C. Ghivizzani, where he investigated the use of gene therapy applications for orthopaedic conditions and received his Ph.D. in the fall of 2009.