

Intra/Extracellular Multi-Drug Delivery for Osteoarthritis

by

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Abstract

Osteoarthritis (OA), the most common form of arthritis, affects hundreds of millions of people worldwide and tens of millions of people within the United States. This disease is typically diagnosed only after extensive and irreparable damage to the joints. There are currently no clinically effective disease modifying drugs that can slow or stop disease progression. While cartilage degeneration is the hallmark of OA, there is increasing recognition that OA is a disease of the whole joint, and multiple joint tissues contribute to disease progression. Due to the complex pathogenic processes that involve interactions between different joint tissues, a successful disease modifying therapy will likely require treatment with multiple drugs, each having a different target.

While several disease modifying drug candidates have shown promise in disease models both *in vitro* and *in vivo*, delivering these drugs effectively and with minimal side effects remains challenging. Cartilage does not have a blood supply, which decreases the efficacy of systemic drug administration methods. In intra-articular injections, drugs are directly injected into the affected joints. But any drugs injected into the joint are rapidly cleared out by the joint capsule over the span of a few hours to a day. As a result, there is limited drug penetration into cartilage. Frequent injections with high drug doses can overcome this challenge, but such a treatment would lead to undesirable systemic side effects and increase the risk of infections within the joint. Recent prior work in our lab has demonstrated that positively charged drug delivery carriers can bind to negatively charged extracellular matrix components in cartilage and thereby improve drug uptake and retention.

In this thesis, we characterized the effect of varying the charge of drug delivery carriers on their uptake and penetration into human and bovine cartilage tissues and cells. We identified optimally charged carriers that can be used to deliver drugs to extracellular or intracellular targets. We successfully used these carriers to provide sustained and targeted delivery of growth factors to full-thickness human cartilage explants *in vitro*, and developed a mathematical model that predicts *in vivo* transport behavior in human knee joints. We further established an *in vitro* cartilage-synovium co-culture model that captures physiologically relevant tissue interactions that contribute to OA progression. We also tested drugs targeting inflammatory pathways in this co-culture model, and the results provide a starting point for developing a combination therapy of growth factors and anti-inflammatory drugs

conjugated to optimally charged carriers.

This thesis is organized as follows:

Chapter 1 provides a broad overview of different diseases affecting cartilage, including osteoarthritis. In Chapter 2, we used engineered green fluorescent proteins (GFPs) with a range of net positive charges and surface charge distributions to characterize the effects of charge on carrier transport in cartilage. In both bovine and human cartilage, the uptake of GFPs into cartilage tissue explants decreased with increasing net charge. In contrast, cellular uptake of GFPs increased with increasing charge. Experiments with three neutrally charged GFP variants demonstrated that the surface charge distribution of the carrier also plays an important role in determining its transport properties. Based on the results of this study, we identified optimally charged GFP carriers for delivering drugs to extracellular matrix or cell-surface targets, as well as to intracellular targets.

In Chapter 3, we tested the delivery of Insulin-like growth factor 1 (IGF-1), a pro-anabolic drug, using engineered GFP carriers. Since the target for IGF-1 is the extracellular domain of a cell-surface receptor, and a cationic GFP variant with a net charge of +9 (abbreviated as +9 GFP) was found to be optimal for extracellular targets, we designed, expressed and purified fusion proteins of IGF-1 with +9 GFP. Five fusion protein variants had flexible or rigid polypeptide linkers of different sizes connecting the IGF-1 and +9 GFP domains. A sixth fusion protein with no linker was also synthesized. Single doses of two of the fusion proteins had sustained IGF-1 bioactivity in both normal and cytokine-treated human cartilage explants for 7 to 10 days. These fusion proteins increased sGAG biosynthesis rates in normal cartilage and rescued the loss of sGAG biosynthesis in cytokine-treated cartilage, but could not rescue the increase in cumulative sGAG loss caused by cytokine treatments. These responses are consistent with the effects of free IGF-1 in human cartilage explant cultures. However, the main difference is that free IGF-1 needs to be continuously replenished to achieve these effects, whereas a single dose at the start of the experiments was sufficient for the fusion proteins.

All of the experimental work in this thesis was performed using *in vitro* cartilage explant cultures. In order to successfully translate these results to preclinical and clinical studies, it is important to predict the transport behavior of GFP carriers and carrier-drug conjugates once they are injected into the joints. In Chapter 4, we developed a mathematical transport model and fit model predictions to data from *in vitro* dynamic uptake experiments to estimate the transport properties of engineered GFPs and GFP-IGF-1 fusion proteins in cartilage. This model was then used to predict the concentration of GFPs and GFP-IGF fusion proteins in synovial fluid and inside human knee cartilage in an intact knee joint as a function of time after intra-articular injection. The model predicted that significant amounts of the injected molecules could quickly penetrate cartilage tissue before being cleared out by the joint capsule. These cartilage concentration predictions will enable the estimation of injection doses that can achieve appropriate drug doses within cartilage. Additionally, the model also predicts the amount of GFPs and GFP-IGF fusion proteins that are cleared out into the systemic circulation by the joint capsule. These predictions will be useful in assessing the likelihood of potential systemic side effects and estimating safe injection doses.

In Chapter 5, we established an *in vitro* model of post-traumatic osteoarthritis (PTOA) in which cartilage and synovium explants were cultured together. In these experiments,

the injury caused by cutting synovium explants triggered the release of large quantities of cytokines. In both human and bovine co-cultures, the cytokine levels were comparable to those observed clinically in the days and weeks following a traumatic joint injury. Cytokine and chemokine levels in co-culture decreased with time, which is also similar to clinical observations. Co-culture with synovium led to significant decreases in the sGAG biosynthesis rate, explant sGAG content, explant metabolic rate and cell viability in cartilage explants. There were no changes in the sGAG (sulfated glycosaminoglycan) biosynthesis rate or explant sGAG content of human cartilage explants that were co-cultured with synovium over the 2-week duration of the experiments.

In chapter 6, we tested toll-like receptor (TLR) inhibitors and MAPK pathway inhibitors in the human cartilage-synovium co-culture system. In experiments with tissues from two human donors, treatment with these inhibitors decreased the levels of cytokines and chemokines released by synovium. Future directions could include further characterization with multiple donor tissues (in order to account for donor-to-donor variability), and conjugating the inhibitors to optimally charged GFPs for combination therapy with GFP-IGF fusion proteins.

Thesis Supervisor: Alan J. Grodzinsky

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Chapter 1

Cartilage Diseases¹

1.1 Introduction: Cartilage Structure and Function

Cartilage is an avascular, aneural, alymphatic connective tissue found in the synovial joints, spine, ribs, external ears, nose, and airways, and in the growth plates of children and adolescents. There are three major types of cartilage found in humans: hyaline, fibrous and elastic [1] (see Figure 1-1). All three types have a low density of cells (chondrocytes) [2] that synthesize and secrete the major components of the extracellular matrix (ECM) [3]. In order to perform the biomechanical functions of providing structural support and resistance to deformation, cartilage ECM contains a unique family of proteoglycans enmeshed within a highly hydrated collagen fibrillar network. Chondrocyte-mediated synthesis and assembly of this matrix is aided, in turn, by synthesis of dozens of additional non-collagenous proteins, proteoglycans and glycoproteins. The abundance, distribution and types of collagens and proteoglycans are different in each of the three types of cartilage, which gives rise to differences in appearance and biomechanical properties.

Hyaline cartilage has a glassy appearance and is the most common form of cartilage in the human body. It is found in the articulating surfaces of bones in synovial joints, and in the ribs, nose, trachea, bronchi, larynx, and growth plates. Articular hyaline cartilage enables joint movements by providing a lubricating surface with an extremely low coefficient

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of friction on the order of 0.001-0.01 [4][5][6][7]. Water accounts for 60 to 85% of the wet weight of the tissue [8][9]. Many different types of collagen molecules are expressed in articular cartilage, but the backbone type II collagen fibrillar network is described by Eyre et al. as a heteropolymeric structure with collagen IX molecules covalently linked to the surface of collagen II and collagen XI forming the inner filamentous template of the fibril as a whole [10]. This network accounts for 60 to 70% of the dry weight [9], with a hydrated spacing of ~ 100 nm between fibrils [11][12], thereby providing the tissue with tensile and shear strength. The basic NC4 domain of collagen IX is thought to enable interactions between other collagen fibrils as well as other matrix macromolecules [13]. Type VI collagen is found in the pericellular matrix and enables chondrocytes to sense changes in the surrounding matrix and respond to them [14][15]. Other collagens found in articular cartilage include type III, type X, type XI, type XII and type XIV[10].

The dominant proteoglycan in hyaline cartilage is aggrecan, comprised of a core protein substituted with over 100 chondroitin sulfate glycosaminoglycan (GAG) chains and a lower number of keratan sulfate chains, forming a bottle-brush structure [16][17][18]. The G1 globular domain at the N-terminus of the core protein binds non-covalently to hyaluronan (stabilized by adjacent binding of link protein) to form large aggrecan aggregates containing over 100 aggrecan monomers [16]. The G1 globular domain at the N-terminus of the core protein binds non-covalently to hyaluronan (stabilized by adjacent binding of link protein) to form large aggrecan aggregates containing over 100 aggrecan monomers [16][19]. These aggregates can achieve molecular weights as high as 350 million Da. The GAG chains of each aggrecan monomer are located between the G2 and G3 globular domains [18], giving each aggrecan a net negative charge of approximately -10000 [20]. Visualized by atomic force microscopy, the protein core of aggrecan in immature and adult cartilage can range from 400-500 nm long, while the GAG chains range from 30-50 nm in length [21][22]. Importantly, with respect to biomechanical and biophysical function of aggrecan, the spacing between GAG chains on the same aggrecan core protein molecule is ~ 3 nm [21], which is on the order of the electrical Debye length under physiological conditions. This close spacing, leads to extremely high electrostatic repulsive interactions between adjacent GAG chains along the same core protein and between adjacent core proteins. Taken

together, these repulsive interactions at the molecular level provide compressive strength to cartilage at the tissue level, enabling it to act as a load bearing tissue in the joints.

Other proteoglycans found in cartilage include versican, perlecan [23](which is found in the hypertrophic chondrocyte regions in articular cartilage and growth plate cartilage), and small leucine-rich proteoglycans (SLRPs [24]) such as decorin, biglycan [25], epiphykan, lumican and fibromodulin [26][15][27]. Decorin, biglycan and epiphykan are dermatan-sulfate proteoglycans [26], while lumican and fibromodulin are keratan-sulfate proteoglycans, which are present in the glycoprotein form devoid of keratan sulfate in adults [26]. SLRPs can interact with collagen, regulating fibril assembly and interactions with additional ECM molecules. SLRPs can also regulate cell-signaling pathways by binding growth factors, cytokines and other ECM components [15]. Additional major non-collagenous proteins in cartilage ECM include cartilage oligomeric matrix protein (COMP), cartilage matrix protein (CMP) or matrilin-1, cartilage intermediate-layer protein (CILP), proline- and arginine-rich end leucine-rich repeat protein (PRELP), fibronectin [28][29][30][31][26] and cell surface heparan sulfate proteoglycans such as syndecan-4 (SDC4) [32]. A most important glycoprotein in the superficial zone of articular cartilage is lubricin, which helps in reducing the friction between articulating surfaces [33]. Finally, ECM macromolecules are also extremely important mediators of cartilage mechanobiology [34], i.e., the ability of physiologic loading to induce cell-signaling responses that may be anabolic or catabolic.

Fibrous cartilage, found primarily in the intervertebral discs and in other locations such as the menisci, bone-tendon interfaces and ligament-tendon interfaces [35][36], has a high density of type I collagen which gives it high tensile strength. This cartilage has type VI collagen in the pericellular matrix and relatively low amounts of type II collagen and proteoglycans throughout the ECM [1]. The extracellular matrix of elastic cartilage is predominantly made up of type II collagen, proteoglycans and elastin fibers [1]. The elastin fibers are responsible for the yellowish appearance of the tissue and also provide it with high elasticity. Elastic cartilage is found in the external ears, larynx and epiglottis.

Diseases affecting cartilage range from extremely common conditions such as osteoarthritis, which is estimated to affect as many as 36.8% of all adults in the United States [37] to

rare genetic disorders such as Spondyloepimetaphyseal dysplasia (SEMD) aggrecan type, which has been reported in three patients worldwide [38]. The etiology and pathogenesis of many cartilage diseases is not fully understood, due to which treatment methods are not optimal in many cases. Damage to cartilage ECM is an important feature of most diseases affecting the tissue. However, since cartilage is aneural, symptoms usually appear only after significant structural destruction of the matrix, which also makes treatment challenging. The dense packing of ECM components also hinders the transport of drug molecules in the tissue, which along with the lack of vascularity, poses an additional challenge to the treatment of cartilage diseases. This review provides an overview of the current knowledge on cartilage diseases and new developments in cartilage-targeted drug delivery strategies.

1.2 The role of cartilage in developmental disorders

Hyaline cartilage plays a crucial role in skeletal growth and development, first during endochondral bone formation in embryonic and fetal life and then in the form of growth plates which lead to the elongation of bones during childhood and adolescence [39][15]. Fractures to the growth plates have the potential to severely disrupt normal growth. Growth plate cartilage is weaker than ligaments and tendons [40][41] and thus, injury patterns which would lead to ligament tears in adults end up causing growth plate fractures in children and adolescents. These fractures are reported to account for approximately 15% of all childhood fractures and also for anywhere between 1% and 30% of sports related injuries [40][41]. The most widely accepted scheme for classifying these fractures based on the pattern of injury was first proposed in 1963 [40]. This scheme includes five types of fractures, and has proven to be important in determining the prognosis for continuation of normal growth. The classification scheme was expanded in 1982 and now includes 9 types of fractures [42].

In addition to external injuries, genetic factors can also affect growth plate cartilage and thereby, disturb normal skeletal growth. The most common form of dwarfism, achondroplasia, is caused by autosomal dominant point mutations in the transmembrane domain of FGFR3 [43][44]. Most of these mutations arise sporadically in the germline cells of older men [44]. A single copy of the mutated gene leads to dwarfism while having two

copies leads to death early in life [45]. The frequency of achondroplasia is estimated to be between 1 in 15,000 and 1 in 28,000 live births [46][47][44]. FGFR3 is expressed by proliferating chondrocytes in the growth plates and FGF signaling through this receptor inhibits proliferation [39]. The mutations in achondroplasia lead to activation of FGFR3 in the absence of a ligand in growth plate proliferating chondrocytes. This in turn leads to reduced growth of endochondral bones without affecting cartilage in other parts of the body. Other autosomal dominant mutations in FGFR3 result in hypochondroplasia, which resembles a mild form of achondroplasia, and thanatotropic dysplasia which results in death in early life [48][49].

Mutations in the Col2A1 gene for type II collagen predominantly affect cartilage and this spectrum of disorders is called type II collagenopathies. In addition to affecting cartilage, they are also typically accompanied by problems with skeletal development, vision and hearing. The most severe of these diseases are achondrogenesis type 2 (also called Langer-Saldino achondrogenesis), hypochondrogenesis and platyspondylic lethal skeletal dysplasia, Torrance type [50][51][52][53]. Together, achondrogenesis type 2 and hypochondrogenesis occur in 1 in 40,000 to 1 in 60,000 newborns [54]. Platyspondylic lethal skeletal dysplasia, Torrance type is far rarer, with reports of only a few individuals in the world [55]. The mutations in these diseases prevent the secretion of type II collagen from chondrocytes, leading to its absence from the ECM. Instead, type I and type III collagen replace the missing type II collagen in the ECM in achondrogenesis type 2 and hypochondrogenesis [56][57][58]. All three diseases lead to severe skeletal underdevelopment and result in death early in life [50][59].

Less severe type II collagenopathies include Spondyloepiphyseal dysplasia congenita (SED congenita), Kniest dysplasia, SED with metatarsal shortening (also Czech dysplasia), Spondyloperipheral dysplasia, Spondyloepimetaphyseal dysplasia (SEMD) Strudwick type, Stickler syndrome type 1, and Mild SED with premature onset arthrosis [60]. In SED congenita and Kniest dysplasia, the collagen II content of cartilage is less than normal since the secretion from chondrocytes is reduced. These two conditions lead to dwarfism in the affected patients. Stickler syndrome type I is the mildest and most common form of type II collagenopathies that is seen in 1 in 10,000 live births [61]. Patients usually grow to a

normal height and the disease is associated with early onset osteoarthritis, vitreous humor abnormalities and congenital myopia. Other variable features of type 1 Stickler syndrome include a flat midface, increased risk for retinal detachment, deafness, slender extremities and joints that are hypermobile [62]. The specific mutations in the Col2A1 gene that lead to type II collagenopathies are discussed in detail in [61].

Type X collagen is expressed by hypertrophic chondrocytes in the growth plates as well as in the deep and calcified zones of articular cartilage [39][1]. Mutations in the Col10A1 gene leads to Schmid-type metaphyseal dysplasia, an autosomal dominant disorder characterized by short stature, reduction in the angle between the femoral head and the shaft and bow legs [63]. Due to the similarity in symptoms, it may be misdiagnosed as rickets (caused by Vitamin D deficiency) [64].

Disorders associated with mutations in aggrecan are extremely rare. It was thought that aggrecan mutations did not cause diseases in humans until it was first identified in 2005 that a mutation in the AGC1 gene caused Spondyloepiphyseal dysplasia (SED) Kimberly type [65]. Patients with SED Kimberly type are typically short in stature and have early onset osteoarthritis (OA) [66]. Since 2005, two other disorders - familial osteochondritis dissecans (OCD) [67] and Spondyloepimetaphyseal dysplasia (SEMD), aggrecan type [38] - have also been identified as being caused by mutations in AGC1. Similar to SED Kimberly type, familial OCD is also associated with dwarfism and early onset OA [68]. SEMD aggrecan type leads to a decrease in height both in homozygous patients and heterozygous carriers, but does not lead to early OA. A thorough review on the current state of knowledge regarding these aggrecanopathies can be found in [69].

1.3 Osteochondritis dissecans

Osteochondritis dissecans (OCD) was first described by König in 1888 as a condition that leads to the formation of loose bodies in the joints without any trauma [70][71]. Most recently, OCD has been defined as “A focal, idiopathic alteration of subchondral bone with risk for instability and disruption of adjacent articular cartilage that may result in premature osteoarthritis” [72]. There are both juvenile and adult forms of the disease, which are

defined based on whether or not the growth plates are open in the patient [73][74]. The joint that is most commonly affected by OCD is the knee, followed by the ankle and the elbow. Within the knee, OCD lesions classically present on the lateral aspect of the medial femoral condyle [75][76]. In one report of disease incidence in femoral condyles, the highest incidence was found in the 10-20 year old age group with 20 in 100,000 women and 30 in 100,000 men affected [77]. A more recent pediatric knee OCD epidemiological study was conducted involving over 1 million people aged between 2 and 19 years [78]. In this study, there was no incidence of knee OCD in the 2-5 year old children, while the incidence in the 6-19 year old age group was 9.5 in 100,000. Within the 6-19 age group, the incidence was much higher for males compared to females (15.4 vs 3.3 in 100,000).

The etiology of OCD has not yet been established conclusively. One proposed mechanism is that repetitive trauma leads to progression of the disease. This is supported by the findings that 55 to 60% of patients are involved regularly in sporting or athletic activities [79][80][81]. Additionally, repetitive contact with the tibial spine has been proposed as the etiology for OCD at the classic location on the medial condyle [75]. Other etiologies that have been proposed include repetitive trauma, inflammation, ischemia, hereditary factors, osteonecrosis and gradual development following an injury to the endochondral epiphyseal growth plate [68][82][81]. There is currently speculation that a single etiology may not explain all cases of OCD, and that the disease may be multifactorial [82].

OCD is diagnosed based on patient history, a physical exam and a radiograph. An MRI or an arthroscopy may also be used to supplement the findings on the radiograph. Once the disease is diagnosed, the treatment options considered depend on the stability of the lesion and the age of the patient. Non-surgical options are usually the first line of treatment for juvenile patients with stable lesions [83][82]. If the stable lesion does not improve with non-surgical management, then surgery is considered. Surgical management is considered as the first line of treatment for cases where the lesion is unstable or when a stable lesion is found in an adult [83]. The various non-surgical and surgical treatment methods are covered in detail in other papers [76][84][85][86], and we refer the reader to them.

1.4 Relapsing polychondritis

Relapsing polychondritis (RP) is a rare inflammatory disease that can affect all forms of cartilage and also organs containing proteoglycans, such as the eye and the heart. Its prevalence is estimated to be 2/million person-years in Hungary [87] and 3.5/million person-years in the United States [88]. The early symptoms of RP include one or more of the following: external ear pain that does not affect the non-cartilaginous lobule, temporary pain in one or more joints, nasal pain, throat pain, hoarse voice, eye involvement in the form of scleritis or episcleritis, vasculitis, skin symptoms, hearing impairment, dizziness and systemic symptoms such as fever and weight loss [89][90]. These symptom(s) can appear as intermittent acute flares, and the disease follows a progressive course that can lead to complete destruction of the affected tissue [89]. For instance, destruction of nasal cartilage leads to the commonly observed “saddle nose” in these patients. The most common cause of death in RP patients is respiratory failure, followed by causes related to cardiovascular involvement. In rare cases, the disease also affects the kidneys and the prognosis is very poor in these patients [91].

The symptoms of RP can overlap with many other diseases and there is currently no specific test that can be used to conclusively diagnose this condition. Biopsy findings cannot be used to make a conclusive diagnosis, and the injury caused by a biopsy can further aggravate the disease. The diagnosis for RP is currently made solely on the basis of clinical presentation and by excluding other diseases based on symptoms or by running specific tests. However, this process leads to frequent misdiagnosis of RP in the initial stages, especially in cases where there is a single presenting symptom. The mean delay between the time a patient first presents with symptoms and the time a diagnosis for RP is made is 2.9 years [89]. The first clinical algorithm for confirming a diagnosis was published in 1976, in which 3 of the following 6 criteria were considered to be sufficient: (1) bilateral chondritis in the ears, (2) polyarthritis that is inflammatory and non-erosive, (3) nasal chondritis, (4) inflammation in the eyes, (5) chondritis involving cartilage in the larynx and/or the trachea and (6) damage to the cochlea or the vestibules in the ears [92]. Other authors have modified these criteria to include biopsy results, response to dapsone and steroid drugs

and seronegative inflammatory arthritis [93][94]. RP frequently occurs in conjunction with other autoimmune diseases, and these should be considered while making a diagnosis.

The etiology of relapsing polychondritis is not fully understood. But there is evidence that autoimmunity against cartilage specific components may play an important role and that there may be a genetic component that may increase susceptibility for the disease [95][96]. Disease progression is measured using the Relapsing Polychondritis Disease Activity Index, which was developed in 2012 and uses 27 variables to generate a score [97]. Clinical progression and RPDAI are used to determine the management strategy for RP. In early stages, NSAIDs, dapsons and steroids are used to manage the symptoms. If these drugs do not induce a response, immunosuppressive drugs such as azathioprine and methotrexate are then administered. In recent years, biologic drugs such as TNF- α antagonists have been used in patients with severe RP that does not respond to other treatments [90]. Additionally, surgical interventions are used to rescue patients who are in respiratory collapse and to improve hearing and/or stability in those with inner ear involvement.

1.5 Chondrocalcinosis

Chondrocalcinosis is the deposition of calcium pyrophosphate dihydrate (CPPD) crystals in joints. This condition can stay asymptomatic, give rise to an acute inflammatory reaction that resembles gout (called pseudogout or acute CPP crystal arthritis) [98][99], or chronic arthritis that may resemble osteoarthritis (OA) or rheumatoid arthritis [100]. In symptomatic patients, finding characteristic CPPD crystals in the synovial fluid confirms the diagnosis. Imaging techniques such as radiographs, ultrasonographs, CT scans and MRI scans are also used to diagnose the disease. The prevalence of chondrocalcinosis increases with age, and estimates range from 7% to 10% after the age of 60 years [101][102][103]. There is also a higher incidence of CPPD crystal deposition in joints with OA, but this has not been found to increase the rate of OA progression [104][105][106].

CPPD crystals are predominantly formed in the pericellular spaces surrounding chondrocytes in cartilaginous tissues [107][100]. Chondrocytes release small amounts of inorganic pyrophosphate as well as ATP into the pericellular space [108][109]. Extracellular

ATP is broken down to form inorganic pyrophosphate by enzymes in the pericellular space [109][110]. Inorganic pyrophosphate reacts with calcium to form CPPD crystals, which can change mechanical properties of cartilage. A transmembrane protein, ANKH, regulates the release of ATP and inorganic pyrophosphate from chondrocytes [111] and mutations in this have been found in genetically inherited cases of chondrocalcinosis [100]. When CPPD crystals are released from cartilage into the joint space, they can induce an acute inflammatory reaction that leads to pseudogout symptoms. The crystals can induce chondrocytes and synoviocytes to upregulate MMP and prostaglandin production and also activate innate immune responses by activating the NLRP3 inflammasome which leads to the release of proinflammatory cytokines [112][113][114][115].

There are currently no treatments that can reduce the quantity of CPPD crystals being formed in the joints. The acute form of the disease affects approximately 25% of patients with chondrocalcinosis [116] and leads to joint pain, swelling and warmth in one or more joints. The knee is the most commonly affected joint in these patients. Current treatment options include intra-articular glucocorticoid injections, oral colchicine (which acts upstream of inflammasome activation) and NSAIDs [117]. The chronic presentation of the disease is more difficult to manage, and in addition to the drugs used for the acute disease, hydroxychloroquine and methotrexate have also been used [118][119][120]. Emerging therapeutics include anti-inflammatory drugs such as IL-1 inhibitors and drugs such as probenecid that can target the ANKH protein and reduce the production of inorganic pyrophosphate in the joint [121][100].

1.6 Cartilaginous tumors

Tumors made of cartilaginous tissue are a major class of bone tumors. The benign cartilaginous tumors are osteochondroma, enchondroma, periosteal chondroma, multiple chondromatosis or enchondromatosis, chondroblastoma, and chondromyxoid fibroma. Enchondroma and osteochondroma are by far the most frequently occurring benign forms, accounting for 34% and 30% of all benign cartilaginous tumors. Malignant cartilaginous tumors (chondrosarcomas (CHS)) include central CHS (both primary and secondary), peripheral

CHS, dedifferentiated CHS, mesenchymal CHS and clear cell CHS. Overall, bone sarcomas account for only about 0.2% of all malignancies in the United States (Frachi 2012) and primary CHS is the third most common primary bone malignancy, accounting for 20 to 27% of all cases. The epidemiology of benign cartilaginous tumors has not been well established, in part because many cases remain undiagnosed due to the absence of symptoms. When they are symptomatic, benign tumors can present with pain, swelling, lesions or a pathological fracture.

In osteochondroma, there is a marrow-containing bony projection which is covered with hyaline cartilage that gets thinner with increasing age. It usually occurs in the metaphyseal region of endochondral bones. In a chondroma, hyaline cartilage can be found either inside the bone (called enchondroma if it is a single lesion or enchondromatosis in the case of multiple lesions) or on its surface (periosteal or juxta-cortical chondroma). Enchondromatosis and periosteal chondroma are far less common than enchondroma. There is emerging evidence that disturbances in the signaling pathways involved in the formation of new bone from growth plate cartilage contribute to the formation of osteochondroma and enchondroma (Bovee 2010). Chondroblastoma is a rare benign tumor (less than 1% of all bone tumors) of unknown etiology that is usually found in the bone epiphysis and is made up of chondroblasts (immature chondrocytes). Chondromyxoid fibroma is an equally rare benign tumors comprising of lobules of chondroid and myxoid tissue with both spindle shaped cells and giant osteoclast-like cells. Benign cartilaginous tumors rarely progress to malignant forms and are usually treated through excision or curettage along with a bone graft if necessary. The radiological and histological features of all these tumors are covered in detail in (WHO Classification of Tumors), and these can be used to confirm the diagnosis. One exception for this is enchondroma, since it is still very challenging to differentiate enchondroma from low grade chondrosarcoma based on histology, imaging and clinical features (Marco 2000, Ferrer-Santacreu 2016). This is an important problem that needs to be solved since chondrosarcoma is a malignant tumor and a misdiagnosis can lead to terminal progression of the disease. Chondrosarcomas may be primary (arising de novo; these account for 85% of all cases) or secondary (arising from a pre-existing tumor) lesions made up of hyaline cartilage [122]. There are 3 grades of CHS and the morphology

of the tissue deviates significantly from normal cartilage in the higher grades [122]. Histological analysis can be used to identify different grades as well as the different types of CHS. Chemotherapy and radiation are not very effective for treating CHS, and surgery is the main form of therapy [123][124][125].

1.7 Inflammatory arthropathies

There are several inflammatory rheumatic diseases that lead to arthritis and can severely damage cartilage tissue. These include rheumatoid arthritis, juvenile idiopathic arthritis, gout, systemic lupus erythematosus, and seronegative spondyloarthropathies.

Rheumatoid arthritis (RA), an autoimmune disease that affects 0.5 to 1% of people worldwide [126][127], is characterized by chronic systemic inflammation and arthritis in multiple synovial joints. Left untreated, the disease leads to progressive destruction of cartilage and synovial joints. Serum markers for RA include rheumatoid factor (RF) and anticitrullinated peptide antibody (ACPA) [127]. Both adaptive and innate immune systems play a role in the progression of the disease and the major cytokines involved are TNF- α , IL-6, IL-1 β and IL-17A [127]. Synthetic disease modifying anti-rheumatic drugs (DMARDs) include methotrexate (which is usually the first line of treatment for RA [128]), leflunomide, hydroxychloroquine and sulfasalazine [126]. Biologic drugs against TNF- α have constituted a major breakthrough for RA treatment, and constitute the first widespread and major success of biologic DMARD era; they are effective in halting the progression of the disease in almost 65% of RA patients. Additional therapeutics, when TNF blockers are not not to be effective, include monoclonal antibodies against CD20 in B cells, IL-1 receptor antagonist and monoclonal antibodies against the IL-6 receptor [126].

Gout is the most common form of inflammatory arthritis [129] that affects approximately 8.3 million people or 4% of the population in the United States [130]. It is caused by the deposition of uric acid crystals in the joint and is managed using non-steroidal anti-inflammatory drugs (NSAIDs), colchicine and lifestyle changes to reduce uric acid levels [131].

Juvenile idiopathic arthritis (JIA) refers to any form of arthritis of unknown cause that

occurs in a person younger than 16 years of age [132]. It is the most common form of arthritis in children and its prevalence is estimated to range from 16 to 150 per 100,000 in the developed world [132]. In the United States, juvenile arthritis and other rheumatological conditions affect approximately 1 in 250 children [133][134]. There are six subtypes: systemic JIA, oligoarticular JIA, polyarticular JIA which may be positive or negative for rheumatoid factor, juvenile psoriatic JIA, enthesitis-related JIA, and undifferentiated arthritis [132].

Systemic lupus erythematosus is an auto-immune disease characterized by the presence of circulating anti-nuclear antibodies (ANA) [135]. Approximately 20% of cases occur in childhood and the childhood onset form of the disease has a worse prognosis compared to the adult onset form [136]. SLE can affect multiple organs in the body including the kidneys, skin, lungs, heart, joints and brain [135]. Clinically, there may be a broad range of signs and symptoms, and the criteria for making a diagnosis were proposed in 1971 and then revised and updated in 1982 and 1997 [137][138]. A majority of patients experience arthropathy [139], and 3 to 6% of patients have Jaccoud's arthropathy which leads to severe deformation of the fingers [140][141].

Seronegative spondyloarthropathies, which comprise of ankylosing spondylitis, psoriatic arthritis, reactive arthritis, arthritis associated with inflammatory bowel disease, and a subtype of juvenile idiopathic arthritis [142]. These conditions are characterized by an absence of rheumatoid factor, a strong correlation with carrying the HLA-B27 gene and clinical features that include inflammatory back pain, inflammation of insertion sites of tendons and ligaments, peripheral arthritis and eye inflammation [143][142]. The overall prevalence of spondyloarthritides in North America is approximately 1% [144] and the most common form of spondyloarthritis is ankylosing spondylitis [142][145]. The prevalence of psoriatic arthritis in the United States is 0.1% [144] and it is seen in 10 to 40% of people with psoriasis [146]. In a majority of patients, the arthritis develops after psoriasis develops [146]. In addition to arthritis and skin lesions, these patients may also experience eye inflammation, tendinitis and pitting of the fingernails [147]. Importantly, anti-TNF biologics have been found to be very effective in treatment of ankylosing spondylitis as well as psoriatic arthritis [148].

1.8 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis, affecting approximately 90 million adults (36.8% of the adult population) in the United States alone [37] and hundreds of millions of people worldwide. The disease primarily affects articular hyaline cartilage in load bearing joints such as the knee, hip and shoulder, and is also commonly found in the hands and feet. Other tissues such as the joint capsule synovium and subchondral bone are also affected and contribute to disease progression. As the disease advances, there is severe degeneration of cartilage, narrowing of the joint space, subchondral bone thickening, formation of osteophytes [149] or bone spurs, and inflammation in the joint accompanied by swelling and pain [150]. While debates continue as to whether disease initiation primarily involves cartilage versus subchondral bone, we focus in this review on processes and mechanisms involving cartilage degeneration.

The risk factors for OA are diverse and include obesity, female gender, age, congenital structural abnormalities in the joint and acute joint trauma[150]. In 2011, the total direct healthcare expenditure associated with OA in the United States was \$ 41.7 billion [151]. Despite the tremendous disease burden worldwide, there are currently no disease modifying drugs to alter or halt disease progression, which usually takes place over multiple years [152]. Early OA diagnosis also remains challenging because cartilage changes involved in the earliest stages do not cause pain, due to the aneural nature of the tissue. Thus, patients may remain symptom-free until advanced stages of the disease occur, with significant and irreparable joint damage [153]. Progress is being made in improving imaging modalities and discovering molecular biomarkers to enable earlier disease diagnosis [154][155][156]. One exception where early intervention is currently possible is post-traumatic osteoarthritis (PTOA), the sub-type of OA that develops in approximately 50% of all patients who experience a traumatic joint injury such as an ACL tear [157]. PTOA accounts for ~12% of all OA cases [158] and typically affects a population that is much younger and otherwise healthier than other forms of OA [157]. Since PTOA usually takes 10-15 years to progress from the time of the joint injury to fully developed OA, effective clinical intervention immediately after the injury could potentially prevent PTOA from developing in

these patients. Even so, developing DMOADs for PTOA remains challenging as there are no approved methods to delivery drugs directly inside of cartilage, either to chondrocytes or ECM targets [159].

The etiopathogenesis of OA is not completely understood. It is known that OA is a multifactorial disease involving the entire joint and not just cartilage [150][160] [161]. Mechanical, biological and structural factors are all known to play a role in disease initiation and progression. For example, in the case of PTOA, the initial joint injury results in damaging mechanical forces on multiple joint tissues. While surgical intervention may enable stabilization of the joint and repair of the initial injury, it is known that surgery does not alter progression to OA. The initial injury can result in permanent structural changes to the joint which can lead to abnormal loads on cartilage and thereby contribute further to PTOA progression [162]. The acute mechanical trauma can also lead to cell death, loss of aggrecan from cartilage and bruising of the subchondral bone [163][164]. In addition to the mechanical damage, there is a sudden increase in the levels of pro-inflammatory cytokines in the joint in the days following the initial injury [165], such as $\text{TNF-}\alpha$, IL-6, and IL-1 β . These cytokines can diffuse into cartilage and cause upregulation of protease activity, leading to degradation of cartilage matrix by the collagenases [166] and aggrecanases [164][165].

Inflammation also plays an important role in other types of OA [167] [168], and there are reports of both innate immune responses (e.g., complement cascade [169], recruitment of macrophages [170], and the activation of pattern recognition receptors (such as toll-like receptors) [171][172][173]) and adaptive immune responses (B cell responses [174] and T cell responses [175]). Inflammation can cause oxidative stress through the action of reactive oxygen species that can damage cartilage tissue and cells [176], also leading to catabolic degradation of ECM and loss of aggrecan and collagen [164] as well as other matrix degradation products [177]. Ultimately, this breakdown compromises the mechanical strength of the tissue and makes it susceptible to further damage and degradation. Since tissue fragments and macromolecules released by mechanical damage can trigger innate immune responses, these factors can amplify each other's effect and form a vicious cycle leading to rapid disease progression [168]. The effects of inflammation on the joint capsule

is also frequently a source of pain for OA patients. Finally, changes in the subchondral bone at early stages contribute to disease progression [178][179].

Based on recent advances in understanding OA pathogenesis, several DMOAD candidates are currently under investigation. Based on evidence that subchondral bone may play a crucial role in OA progression, drugs such as bisphosphonates, calcitonin and strontium ranelate that can reduce bone resorption and increase bone formation are under consideration [152][180][181]. Inflammation and mechanical injury in OA leads to excessive activation of Wnt signaling, which increases transformation of mesenchymal stem cells (MSCs) to bone and induces MMP production, leading to cartilage degeneration. Thus, molecule inhibitors of Wnt signaling, are also being tested [182], and were found to promote in vitro differentiation of MSCs into chondrocytes and to inhibit production of MMPs by chondrocytes treated with cytokines [182][183]. Another potential therapeutic, Kartogenin, induces MSCs to selectively differentiate into chondrocytes [184], and reduces nitric oxide production and GAG loss from bovine cartilage explants in the presence of cytokines [184]. In a rat PTOA model [185], Kartogenin decreased cartilage degeneration and matrix turnover.

To promote cartilage regeneration, growth factors such as IGF-1 and FGF-18 are being tested in-vitro and in-vivo [186][187]. Phase I clinical trials have been completed for FGF18 in advanced OA patients where it was found to be safe for use and also promote cartilage growth while preventing its loss [188]. In a separate phase I trial [189], there were no differences found in the cartilage thickness in the medial femorotibial compartment of the treatment versus placebo groups. However, there was a dose dependent increase in the cartilage thickness and reduction in the joint space narrowing in the lateral femorotibial compartment [189]. However, there was a dose dependent increase in the cartilage thickness and reduction in the joint space narrowing in the lateral femorotibial compartment [189]. A location independent analysis showed that FGF18 reduced cartilage loss and improved cartilage thickness in this trial [190].

To manage OA pain, acetaminophen has been found to be ineffective [191] and NSAIDs (non-steroidal anti-inflammatory drugs) are currently the preferred treatment. β -nerve growth factor (NGF) is a neurotrophin that causes pain and a monoclonal antibody inhibitor

of NGF (tanezumab) has been studied in clinical trials [192]. Phase III clinical trial results showed that tanezumab treatment significantly reduced hip and knee OA pain but caused rapid OA progression in some patients [192][193]. Similar results were found in a phase II-III trial with fasinumab, another anti-NGF monoclonal antibody [192][194]. Further studies are warranted, as increased OA progression could have been due to higher activity and loading of joints in the absence of pain, neuropathic arthropathy or upregulation of TLR (toll-like receptor) inflammatory response [192].

While pain medications may alleviate OA symptoms, they do not resolve the underlying pathological processes. To target the inflammatory and catabolic aspects of OA, MMP inhibitors and aggrecanase inhibitors were developed, but early trials of the MMP inhibitors were not successful and were discontinued [152]. Monoclonal antibody (mAb) inhibitors targeting the aggrecanase ADAMTS-5 are still undergoing preclinical testing [152][195]. Other mAbs targeting the pro-inflammatory cytokines IL-1, TNF- α and IL-6 have yet to be found dramatically effective [152]. But a possible reason for this is that mAbs are too large in size to penetrate cartilage tissue before being cleared from the joint capsule. Emerging targets for anti-inflammatory therapy include reactive oxygen species, senescent chondrocytes and signaling receptors and pathways involved in pro-inflammatory cytokine production (examples: TLR2 and TLR4 receptors, p38 MAPK signaling pathway) [161][196][171][173].

Corticosteroids are currently injected into joints to manage pain, but due to their broad anti-inflammatory effects they are also being tested as potential DMOADs. In an in-vitro bovine cartilage cytokine injury model, dexamethasone was found to rescue cartilage viability and have anti-catabolic effects [186]. On the same study, a combination therapy of Dexamethasone and IGF-1 was found to have both anti-catabolic and pro-anabolic effects, and the combination was superior to either drug alone. A clinical trial to study the efficacy of Dexamethasone in preventing or reducing the severity of PTOA following a wrist fracture was initiated based in part on such data [197]. Another corticosteroid, triamcinolone, was injected into the joints of patients with knee OA in a phase IV clinical trial [198]. Using standard intra-articular injection (with no direct delivery system into cartilage), patients receiving triamcinolone did not have any difference in pain scores compared to controls re-

ceiving saline. Furthermore, the triamcinolone group revealed loss of cartilage volume (by MRI) at the end of two years compared to the controls. In contrast, in a different Phase IIb clinical trial in which triamcinolone was injected with an extended release micro-particle formulation, there was a significant reduction in the pain during the first 11 weeks [199].

The difference in the results of the above trials highlights the challenge of drug delivery to targets inside avascular cartilage. Drugs administered systemically will not reach the tissue in an effective manner due to the lack of blood vessels. A method to overcome this challenge is intra-articular (IA) injection, where drugs are injected directly into the synovial fluid in joints [200]. This has the added advantage of being a localized delivery method that will prevent any systemic side effects associated with the drug. However, drugs injected into the joints are cleared out rapidly by the lymphatics and vessels in the joint capsule, leading to poor cartilage penetration [200]. The dense extracellular matrix of cartilage also provides steric hindrance to the transport of drug molecules, which further reduces drug penetration into the tissue. To achieve therapeutic levels of the drug inside cartilage, repeated injections with high doses would be required. But these are not recommended clinically as they would cause systemic side effects and increase the risk of infection.

To improve the retention time of drugs in the joint, multiple strategies including entrapment of hydrophobic drugs inside liposomes [201], encapsulation inside polymer based micro- or nano-particles [202], and encapsulation in PEGylated single walled carbon nanotubes (PEG SWCNTs) [203] have been studied. A recent development has been the discovery that electrostatic interactions between positively charged nano-carriers and negatively charged aggrecan proteoglycans can dramatically increase both penetration and retention of drugs in cartilage [204][205]. Avidin, a 66 kDa positively charged protein, has been demonstrated to be an effective carrier for dexamethasone, both in-vitro (in cytokine injury models) and in a rabbit PTOA model [206][207]. Increased interfacial partitioning of the cationic nano-carrier, and thus increased diffusive transport into cartilage occurs with increasing positive charge on the nano-carrier. At the same time, highly charged cationic carriers may bind strongly to the negatively charged matrix, which slows transport into cartilage, though increasing retention time [159]. The carrier charge can be fine-tuned to balance these two effects and provide optimal uptake, penetration and retention in the tis-

sue and this is currently a very active area of research. Successful delivery of drugs may potentially transform several of the DMOAD candidates discussed above into effective DMOADs and thereby aid in the development of the first treatment method to slow down or even halt the progression of OA.

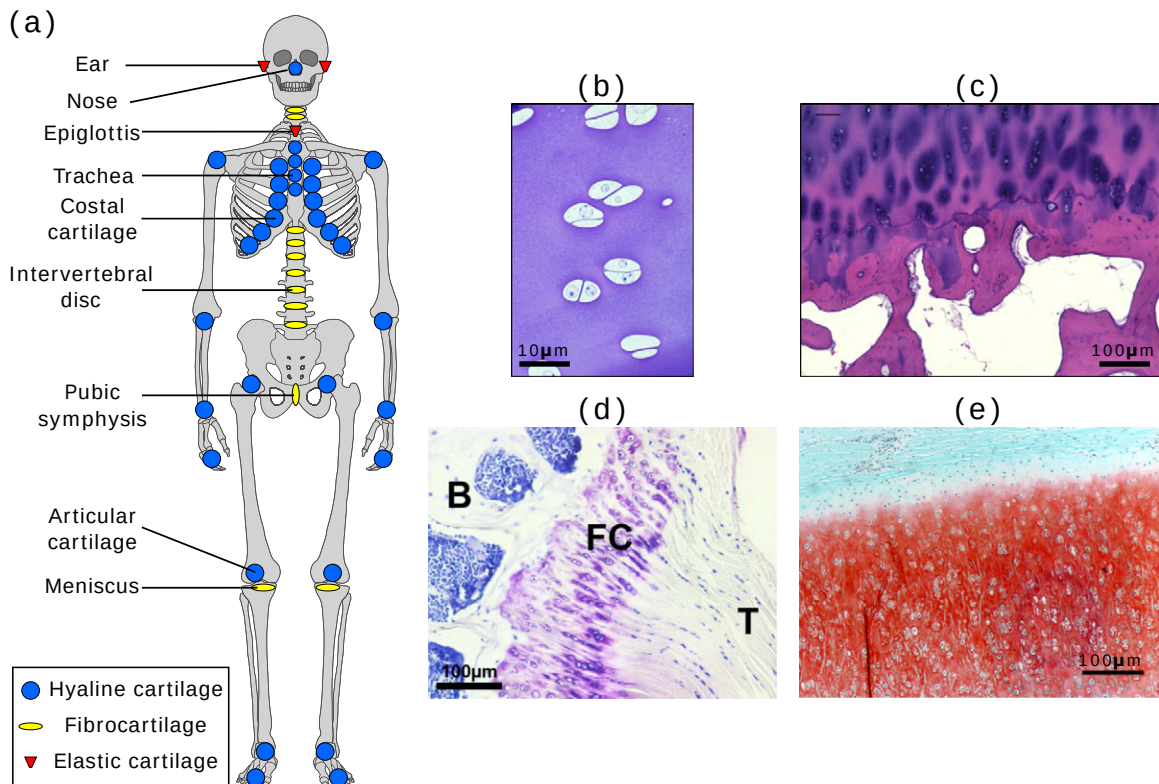
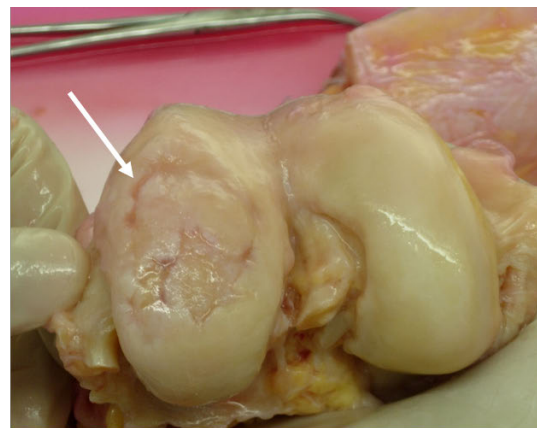


Figure 1-1: Anatomical locations and microscopic structure of cartilage . (a) Locations of hyaline cartilage (blue), fibrocartilage (yellow) and elastic cartilage (red) in the human body; (b) Immature bovine articular cartilage; (c) Human adult articular cartilage (83 year old male donor distal femur) showing chondrocyte clustering near the tidemark, typical of osteoarthritis; (d) Murine fibrous cartilage (FC) at the interface of the supraspinatus tendon (T) and the humeral head of the humerus bone (B); (e) Elastic cartilage of neonatal bovine ear; elastin fibers appear as unstained lines between the safranin-O staining for GAGs



Normal Human Condyle



Grade 3 Osteoarthritis

Figure 1-2: Macroscopic cartilage degradation in human femoral cartilage caused by osteoarthritis

Chapter 2

Green Fluorescent Proteins Engineered for Cartilage-Targeted Drug Delivery: Insights for Transport into Highly Charged Avascular Tissues¹

2.1 Introduction

Osteoarthritis (OA) is a multi-factorial disease with multiple causes and risk factors that leads to degeneration of cartilage and associated joint tissues in ~90 million adults in the United States [37]. There are currently no disease modifying drugs (DMOADs) that slow or reverse the progression of OA [152]. A major challenge to development of DMOADs is that symptoms usually appear late in disease progression when the joint has already been extensively or irreversibly damaged [153]. However, increasing attention is being paid to post traumatic OA (PTOA), caused by the combination of acute mechanical impact and the inflammatory cascade that follows traumatic joint injuries (e.g., rupture of the anterior cruciate ligament). PTOA develops in ~50% of all patients who suffer a traumatic joint

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injury, especially women [157], and accounts for 12% of all OA cases [158]. Since the initiating event that triggers PTOA is known, clinicians can potentially intervene early to prevent the progression of the disease if methods for efficacious DMOAD delivery were available. Indeed, recent clinical trials focusing on treatment after joint injuries have been initiated based on this hypothesis [208][209].

While there are promising DMOAD candidates for PTOA, none have been proven to be effective in slowing or reversing the progression of disease [152][162]. It is now accepted that lack of appropriate delivery to cartilage has been a major impediment to drug discovery [200][204]. Since cartilage is avascular, direct intra-articular (IA) injection of drugs into the joint synovial fluid can minimize potential systemic side effects [200]. However, IA therapy currently remains ineffective due to poor cartilage penetration of drugs and rapid clearance of drugs from the joint via the synovial capillaries and lymphatics [200].

We recently discovered that the cationic nanoparticle Avidin, a 66 kDa positively charged protein, can act as a carrier that dramatically improves both penetration and retention in cartilage, exemplified by delivery of the glucocorticoid, dexamethasone, to cartilage *in vitro* and *in vivo* [204][205][210]. The extracellular matrix (ECM) of cartilage has a high density of negatively charged proteoglycans called aggrecan enmeshed within a collagen network (Figure 2-1). Electrostatic interactions between cationic Avidin and aggrecan, including upward Donnan partitioning, accelerated transport and reversible binding within cartilage matrix, enabling rapid penetration, high uptake and prolonged retention in cartilage. However, while these studies demonstrated that cationic carriers can significantly improve delivery to cartilage, the precise effect of carrier charge density on penetration and transport into cartilage remains unknown.

Proteins can be engineered to carry a wide range of net theoretical charge while maintaining almost identical mass, structure and function. This is achieved by substituting solvent-exposed surface amino acids, which do not play a crucial role in protein folding, with either positively charged or negatively charged amino acids [211]. For example, supercharged green fluorescent proteins (S-GFPs) are engineered by mutating a robust GFP variant called superfolder GFP. Cationic S-GFPs have been reported with net positive charges from +6 to +48 [212][211]. Additional variants of GFP have been engineered to have ap-

proximately zero net charge, but with focal regions of positive and negative surface charge which sum to zero [213]. Proteins engineered in this manner can be used to gain fundamental insights into the role of the net charge as well as the spatial distribution of particle surface charge on transport and delivery into tissues, in isolation of other factors such as molecular weight or protein shape. Engineered GFPs (MW ~ 28 kDa; hydrodynamic diameter ~ 5 nm) are significantly smaller than the upper size limit for penetration into cartilage (~ 15 nm [204]), providing a potential advantage for delivering large biologic drugs in addition to small molecule therapeutics. Additionally, while certain cartilage-targeted carriers (like Avidin) have been shown to be unable to enter cartilage cells [206], supercharged GFPs can be endocytosed into mammalian cells with high efficiency [211]. Thus, these GFPs may be effective carriers for drug delivery to both intracellular and extracellular matrix targets.

In this study, engineered GFPs were used to quantify the effect of net charge and the distribution of surface charge on transport into and through full thickness human and bovine cartilage, independent of other factors such as size and shape that can also affect transport. Specifically, uptake and penetration into cartilage tissue explants was quantified and visualized for four S-GFP variants having net theoretical charges of +9, +15, +25 and +36. In addition, three neutral GFP variants with different surface charge distributions were used as controls for the effects of net charge, and to characterize the effects of local surface charge distribution on transport and binding/retention inside cartilage. The uptake of the four S-GFP variants into chondrocytes within and outside native cartilage explants was also visualized. The results of these extracellular and intracellular uptake studies suggest optimal choices of S-GFP(s) for delivering to intracellular versus extracellular targets. Dose response experiments were carried out to identify any potential adverse or toxic effects of S-GFPs on chondrocyte viability, metabolism, cellular biosynthesis, as well as adverse effects on cartilage extracellular matrix. Finally, dynamic uptake experiments were performed to measure the reaction rate constants for GFP binding in cartilage; the results were used to develop a predictive mathematical model for the transport of GFPs both in *in vitro* and *in vivo* systems.

2.2 Materials and Methods

2.2.1 Structure and Preparation of Cationic and Neutral GFP Variants

The structures and the electrostatic surface potentials of the four cationic supercharged GFP and three neutral GFP variants are shown in Figure 2-2A and B respectively. The surface charge patch distributions of the Janus and HP neutral GFPs are shown in Figures S2.1 and S2.2.

Cationic S-GFPs: BL21 Star (DE3)-competent *E. coli* cells were transformed with plasmids encoding the supercharged GFPs with a His₆ N-terminal purification tag. A single colony was grown overnight in Luria-Bertani (LB) broth containing 50 mg/mL chloramphenicol at 37 °C. The cells were diluted 1:100 into 1 liter of the same media and grown at 37 °C until OD₆₀₀ 0.6-0.7. The cultures were incubated on ice for 60 min before induction of protein expression with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) (GoldBio). Expression was sustained for 15 h with shaking at 16 °C. Cells were collected by centrifugation at 6,000g for 20 min (4 °C) and re-suspended in cell collection buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 1M NaCl, 20% glycerol, 5 mM tris(2-carboxyethyl)phosphine (TCEP; GoldBio), 0.4 mM phenylmethane sulfonyl fluoride (Sigma-Aldrich) and 1 complete, EDTA-free protease inhibitor pellet (Roche) per 50 mL buffer used). Cells were lysed by sonication (5 min total, 3 s on, 5 s off) and the lysate cleared by centrifugation at 22,500g (25 min). The cleared lysate was incubated with His-Pur nickel nitriloacetic acid (nickel-NTA) resin (1.5 mL resin per litre of culture, Thermo Fisher) with rotation at 4 °C for 60 min. The resin was washed with 20 column volumes of cell collection buffer before bound protein was eluted with elution buffer ((100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5mM TCEP (GoldBio), 200mM imidazole). The resulting protein fraction was further purified on a 5 ml Hi-Trap HP SP (GE Healthcare) cation exchange column using an Akta Pure FPLC. An NaCl gradient (from 0 – 1 M) was used to elute the supercharged GFP from the HP SP column; the NaCl concentration required to elute the S-GFP increased with an

increase in protein charge.

Protein-containing fractions were concentrated using a column with a 10 kDa cutoff (Millipore) centrifuged at 3,000g, and the concentrated solution was sterile-filtered through a 22-mm polyvinylidene difluoride (PVDF) membrane (Millipore). After concentration, proteins were resuspended in storage buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5mM TCEP (GoldBio)). After sterile filtration, proteins were quantified with Reducing Agent Compatible Bicinchoninic acid assay (Pierce Biotechnology), snap-frozen in liquid nitrogen and stored in aliquots at 80 °C. SDS-PAGE analysis of the purified proteins is shown in Figure S2.3.

Neutral GFPs: BL21(DE3) cells were transformed with plasmids encoding the neutral GFP variants. For each variant, a well-isolated colony was grown overnight in LB broth containing kanamycin at 37 °C. 5 mL of the overnight culture was diluted in 1 L of LB broth and incubated at 37 °C until OD₆₀₀ 0.8-1. Cultures were then induced with 1 mM IPTG, grown for 20 hours at 37 °C, and harvested. Cells were collected by centrifugation at 4700 rpm for 15 minutes and stored at -80 °C. The frozen cells were homogeneously resuspended in lysis buffer including 10 mM imidazole and then sonicated in an ice bath for 10 minutes. The resulting cell debris and the supernatant were separated by centrifugation at 13000 rpm (4 °C) for 30 minutes. The presence of the expressed protein in the supernatant was confirmed by SDS-PAGE. The collected protein was purified using Ni-NTA metal affinity chromatography and was fully eluted using 250 mM imidazole. Elution fractions containing purified protein were dialyzed into 20mM Tris-Cl, 300mM NaCl, pH 8.0. The purified proteins were analyzed in SDS-PAGE gel as shown in Figure S2.4, quantified using a NanoDrop instrument, sterile-filtered through a 22-mm PVDF membrane and stored at -80 °C. The protein sequences for all the GFP variants are listed in section S2.1 in the Supplementary section.

2.2.2 Bovine cartilage explant and chondrocyte harvest

Cartilage tissue was harvested from the femoropatellar grooves (Figure 2-3A) of 11 knee joints from 11 different freshly slaughtered 1-2 week old bovine calves (Research 87, Boyl-

ston MA) as previously described [186]. Cylindrical cartilage disks (3mm in diameter) that included the intact superficial zone were cut out and washed with Dulbecco's phosphate buffered saline without calcium and magnesium (PBS). They were then trimmed down to 1 mm thick explants and equilibrated for 48 hours at 37 °C and 5% CO₂ in serum-free medium (Low glucose (1 g/L) Dulbecco's Modified Eagle's medium (DMEM), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline and 20 mg/mL ascorbic acid).

To extract live chondrocytes, cartilage tissue slices were first harvested from the femoral condyles of bovine joints. Solutions of collagenase (1.25 mg/mL) and pronase (2 mg/mL) were prepared in low glucose DMEM with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA) and 0.4 mM proline. After washing with PBS, cartilage slices were first incubated in the pronase solution for 1 hour followed by overnight incubation in the collagenase solution at 37 °C and 5% CO₂. The final solution was then passed through a 70 µm filter and a 40 µm filter to remove any undigested cartilage fragments. The filtrate was centrifuged at 400 g for 8 minutes and the pellet was resuspended in 50 mL PBS. Viable cell counts were obtained from aliquots of this suspension using a hemocytometer and Trypan blue. The cells were then centrifuged out of the suspension (400 g, 8 minutes), resuspended in freezing medium (FBS with 5% DMSO) and stored in liquid nitrogen until use.

2.2.3 Human cartilage explant harvest

Two human knee joint distal femurs were obtained through the Gift of Hope Organ and Tissue Donor Network (Itaska, IL). A Collins grade 0 normal knee was from a 36 year male donor and a near-normal grade 1 from a 35 year female. (The Collins visual grading scale grades spans grades 0-4 [214], with grade 0 = normal and grade 4 = end stage osteoarthritis.) All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT. Femoral cartilage plugs (3 mm diam-

eter, Figure 2-3B) were harvested using a biopsy punch, trimmed down to 1 mm thickness and equilibrated for 48 hours (37 °C, 5% CO₂) in high glucose (4.5 g/L) DMEM which was supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline and 20 mg/mL ascorbic acid.

2.2.4 Bovine synovial fluid

Bovine synovial fluid (BSF) was procured from Lampire Biological Laboratories (Pipersville, PA). BSF was collected using sterile syringes from the carpal joints of adult cows within 1 hour of slaughter, pooled and delivered at 10 °C within two days of collection. Some aliquots of BSF were kept frozen at –20 °C (for short-term storage) and the rest were stored at –80 °C.

2.2.5 Quantitative uptake of GFPs into cartilage

Experiments to quantify the uptake of GFPs into cartilage were started after the explants had been pre-equilibrated in culture medium for 48 hours post-harvest. Sterile PBS solutions containing 1% bovine serum albumin (BSA) along with 1 µM concentration of each of the four S-GFP variants (+36, +25, +15 and +9) and the three neutral GFP variants (Janus, NP and HP) were prepared. The BSA was included in the PBS to minimize loss of GFPs in the solution caused by non-specific binding to the plasticware. Since the main function of the albumin in these experiments was to prevent such non-specific binding in the external solution, independent of tissue species, we used BSA for the bovine cartilage as well as the human cartilage experiments (as opposed to BSA with bovine cartilage and human serum albumin (HSA) with human cartilage) to maintain consistency in experimental techniques. Groups of 5 cartilage explants (matched for position along the joint surface) were washed with PBS, and each explant was separately incubated in 250 µL of the GFP solutions in sterile polypropylene vials for 24 hours or for 8 days (37 °C, 5% CO₂). (Standard 96-well cell culture plates were not used because S-GFPs could stick to polystyrene even in the presence of BSA, leading to a loss of protein. In contrast, use of polypropy-

lene with 1% BSA enabled complete recovery of fluorescence). In addition, uptake of the cationic S-GFPs into cartilage explants was also performed in solutions of bovine synovial fluid. On the day of the experiment, BSF was thawed and warmed in a 37 °C water bath with periodic, gentle agitation. At the end of the incubation period in either PBS or BSF, cartilage explants were washed with PBS and transferred to a solution of 10X PBS + 1% BSA for desorption. At the same time, the fluorescence of the absorption bath was measured using a plate reader (Synergy H1, Biotek Instruments Inc.) (excitation: 485 nm, emission: 528 nm). This was used along with a standard curve to obtain the concentration of GFP left behind in the absorption bath. At the end of the desorption in 10X PBS + 1% BSA, cartilage explants were weighed and the fluorescence of the desorption bath was measured. This fluorescence measurement was used with a standard curve and the explant wet weights to calculate the concentration of GFP inside cartilage explants (mol/mg wet weight). The uptake ratio was calculated as the ratio of GFP concentration inside cartilage (mol/mg wet weight) to the final absorption bath concentration (mol/mL).

2.2.6 GFP penetration into cartilage and chondrocytes

Bovine cartilage explants were incubated for either 24 hours or 8 days (37 °C, 5% CO₂) in 1 µM solutions of the cationic S-GFPs dissolved in PBS+1% BSA. Similarly, explants were placed in 1 µM solutions of the neutral GFPs for either 18 hr or 26 hr. In a separate series of experiments, bovine cartilage explants were cultured in ultra-low attachment cell culture plates (Corning) for a period of 8 days in 1 µM solutions of cationic S-GFPs dissolved in low glucose (1 g/L) DMEM (with no phenol red) with 10% fetal bovine serum (FBS) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA) and 0.4 mM proline (37 °C, 5% CO₂). Media changes were performed every 48 hours, and fresh cationic S-GFP were added with each media change. At the end of all such experiments, explants were sectioned longitudinally into ~100 µm slices and washed with PBS. Primary bovine chondrocytes were incubated for 24 hours (37 °C, 5% CO₂) in 100 nM solutions of GFPs dissolved in serum-free medium having the same composition as that

described in the bovine cartilage harvest section. At the end of 24 hours, chondrocytes were washed three times with heparin dissolved in PBS (20 U/mL) to remove non-internalized GFPs, fixed with 4% paraformaldehyde, stained with DAPI and washed with PBS.

Explant slices and fixed chondrocytes were imaged at 10X magnification using a confocal microscope (Olympus FluoView FV1000), and Z-stacks were obtained with a voxel depth of 4.22 μm for cartilage and 1.16 μm for chondrocytes. 3D cartilage Z-stacks were flattened to 2D using the Z project function available in the Fiji software package. During imaging, some cartilage explants were too large to fit into the field of view of the confocal microscope. In these cases, two overlapping Z-stacks were captured and combined using the Stitching plugin available in Fiji [215] before performing 2D Z projections. For chondrocyte Z-stacks, the slice in which the cell images were the sharpest (best focus) were used.

2.2.7 Effects of cationic GFPs on GAG loss, cell viability and metabolism in cartilage explants

10 nM, 100 nM and 1 μM solutions of each of the cationic S-GFP variants were prepared in low glucose (1 g/L) DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM NEAA, 0.4 mM proline and 20 mg/mL ascorbic acid. Groups of 5 bovine cartilage explants were incubated in these solutions in ultra low attachment cell culture plates (Corning) at 37 °C, 5% CO₂. Untreated controls consisted of groups of 5 explants incubated in medium without any GFPs. Medium changes were carried out every 48 hours for the 8-day duration of the experiment. Treatment groups received fresh GFP doses with each medium change. Explant viability by day 8 was measured using the Alamar Blue assay (ThermoFisher). Cartilage explants were incubated for 2.5 hours in a 1X solution of Alamar Blue dissolved in culture medium. Absorbance measurements at 570 nm and 600 nm were performed using a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek) and used to calculate the percentage reduction of Alamar Blue, which is directly proportional to the explant viability. These values were normalized by explant weights. These

weight-normalized readings for the treatment groups were then normalized by the weight-normalized mean value for the untreated controls. To measure chondrocyte biosynthesis rates, explants were cultured in the presence of $5\mu\text{Ci/mL}$ ^{35}S -sulfate radiolabel between days 6 and 8 of culture, and the rate of incorporation of radioactive sulfate into sulfated glycosaminoglycans (sGAGs) was measured [186]. At the end of the experiment, all explants were washed with PBS with non-radioactive sulfate to wash out excess radiolabel, weighed and digested with proteinase K. A liquid scintillation counter (PerkinElmer) was used to measure the amount of radiolabel incorporated within the explant extracellular matrix. Radiolabel incorporation was normalized by the duration of exposure to radiolabel (48 hours) and by the DNA content of the explants (measured using the Hoechst 33258 dye binding assay [216]). To calculate the cumulative loss of GAGs from the explant that may have been caused by the 8-day culture with various cationic S-GFPs, the amount of sGAG lost to the medium and that left behind in the explants at the end of the experiment was measured using the dimethylene blue (DMMB) dye binding assay [217].

2.2.8 Statistical Analysis

Statistical analysis was carried out using MATLAB R2015a software. All quantitative data are plotted as Mean $\pm$ 95% Confidence Interval. Mean values and standard deviations are also reported in tables in the supplementary material. Depending on the experiment, one-way or two way ANOVAs were also performed followed by the post-hoc Tukey's HSD test; p-values less than 0.05 were considered statistically significant.

2.3 Results

All cationic supercharged GFPs used in this study were very similar in size and also had high purity as shown by the gel in Figure S2.3. We therefore used these GFPs to investigate the effect of cationic charge on transport into cartilage tissue independent of any other factors that may affect transport properties. Neutral GFPs (as characterized in Figure S2.4 and Table S2.1) with varying surface charge distributions were used as controls for net charge and to further test whether the surface charge distribution and charge patch size of

net-neutral GFPs might affect cartilage penetration.

2.3.1 Effect of charge on the uptake and transport of cationic carriers into cartilage

Cationic S-GFPs were dissolved in either PBS + 1% BSA or bovine synovial fluid (BSF) and their uptake ratios were quantified in bovine cartilage tissue after 24 hour and 8 day incubations (Figure 2-4A-D and Table S2.2). All the cationic S-GFP variants showed enhanced uptake into cartilage (Figure 2-4) and in every condition tested, the lower charged variants had a significantly higher uptake ratio compared to the higher charged variants. Additionally, it was observed that the uptake of the cationic S-GFPs from PBS + 1% BSA was higher than their uptake from BSF (Figure 2-4, (A) vs (C), (B) vs (D)). Finally, the uptake ratios of S-GFPs at the 8 day time point were higher than their uptake ratios at 24 hours (Figure 2-4, (A) vs (B), (C) vs (D): note differences in y axis scaling).

The higher uptake ratios of cationic S-GFPs at 8 days compared to 24 hours indicated that transport into the cartilage explants had not reached equilibrium in the first 24 hours, i.e., that the GFPs had not penetrated through the full thickness of the cartilage explants within 24 hours. To visualize the extent of penetration, bovine cartilage explants that were incubated for 24 hours in culture medium with 1 μ M concentrations of the cationic S-GFPs were sliced longitudinally and imaged using confocal microscopy (Figure 2-4E). +9 S-GFP penetrated the full 1 mm thickness of cartilage in 24 hours while the extent of penetration decreased as the cationic charge of the GFP variant increased. Similar experiments were performed with continuous doses of cationic S-GFPs that were replenished every 48 hours over an 8 day period and the extent of tissue penetration was visualized every 24 hours from Day 1 to Day 8 (see Figure S2.5).

2.3.2 Effect of surface charge distribution on uptake of net neutral GFPs into cartilage

Uptake ratios of the three neutral GFPs into bovine cartilage after a 24 hour incubation in solutions of the GFPs in 1X PBS + 1% BSA (37 °C and 5% CO₂) are shown in Figure 2-

5 and Table S2.3. Two of the three neutral variants had an uptake ratio of ~ 1 , i.e., the concentration inside the cartilage is the same as that in the bath, and there is no enhanced uptake. However, at the same 24-hour time point, Janus GFP showed an enhanced uptake ratio of 10.1 ± 1.3 (Figure 2-5A, Table S2.3). The 24 hour uptake ratios of each of the cationic S-GFPs from PBS + 1% BSA were at approximately the same level or higher than that of the Janus GFPs, with the highest uptake ratio at approximately 25 for the +9 S-GFP at the same conditions.

Following the 24 hour incubation in GFP solutions, cartilage explants were transferred to either [1X PBS + 1% BSA] or [10X PBS + 1% BSA] solutions for an additional 48 hours, and the percentage of GFP inside the explants that was desorbed was measured (Figure 2-5B). Confocal microscopy images showing the extent of penetration of the neutral GFP variants into cartilage at the 24 hour and 8 day time points can be found in Figures 2-9 and 2-10 respectively.

2.3.3 Uptake of cationic S-GFPs into human cartilage

Tissue from the 35 year old female donor knee was used to obtain the 24 hour and 8 day uptake ratios of cationic S-GFPs dissolved in PBS + 1% BSA (Figure 2-6(A,B) and Table S2.4). The results were consistent with those obtained using bovine tissue in that the uptake ratios increased as the cationic S-GFP charge decreased and the 8 day uptake ratios were much higher than the 24 hour uptake ratios. Tissue from the 36 year old male donor was used to obtain the 24 hour uptake ratios of cationic S-GFPs dissolved either in PBS + 1% BSA or in BSF (Figure 2-6(C,D) and Table S2.4). Similar to bovine cartilage, the uptake from PBS + 1% BSA was higher than the uptake from BSF, and the uptake ratios of +36 and +25 GFPs were significantly lower compared to the other two GFP variants. However, unlike bovine cartilage, +15 GFP (and not +9) had the highest uptake ratio. The difference in the uptake ratios of +15 and +9 GFPs was statistically significant in the case of uptake from BSF (Figure 2-6D), but not in the case of PBS + 1% BSA (Figure 2-6C).

2.3.4 Chondrocyte uptake of cationic S-GFPs increases with net charge

Primary bovine chondrocytes were harvested and incubated in solutions of 100 nM cationic S-GFPs dissolved in culture medium. Control cells were incubated in medium with no GFPs. Confocal microscopy (Figure 2-7), performed with fixed settings for fluorescence imaging, revealed that the cellular uptake of cationic S-GFPs into chondrocytes increased significantly with increasing net charge. These results are consistent with trends observed in other cell types such as HeLa cells [218].

In a separate experiment, the cellular uptake of the three neutral GFP variants was tested using the same method (24 hour incubation in 100 nM solutions followed by fixation and confocal microscopy with fixed settings). In this experiment, a negative control with no GFPs and a positive control with +9 GFP was included. The results shown in Figure 2-8 demonstrate that the cellular uptake of the neutral GFPs is much lower compared to that of +9 GFP.

We also tested the hypothesis that higher cationic charged GFPs could be taken up by chondrocytes within native cartilage tissue. To visualize the extent of intracellular uptake of S-GFPs, bovine cartilage explants that were incubated for 24 hours or 8 days in solutions of either +36 or +9 GFP in PBS + 1% BSA were sliced longitudinally and imaged using confocal microscopy (Figure 2-9 and 2-10). The +9 GFP penetrated the full 1 mm thickness of cartilage in 24 hours while +36 GFP did not (Figure 2-9). However, by day 8, +36 GFPs were found to be localized primarily inside chondrocytes throughout the tissue thickness while the +9 GFPs were found in the extracellular matrix (ECM) of the explant, but not within the cells (Figure 2-10). Thus, the trend of cell uptake versus net cationic charge found within intact cartilage explants was similar to that found with isolated cells Figure 2-7.

2.3.5 Effect of cationic S-GFPs on cartilage matrix sGAG loss, chondrocyte biosynthesis rates and cell viability in native cartilage tissue

Bovine cartilage explants were treated with 1 μ M, 100 nM and 10 nM doses of each of the four cationic S-GFP variants used in this study. An untreated control group was also included. 10 nM and 100 nM doses of cationic S-GFPs did not lead to any changes in the DNA content (Figure 2-11A), cell viability (Figure 2-11B), aggrecan biosynthesis as a measure of cell metabolic activity (Figure 2-11C), or sGAG release from explant (Figure 2-11D). At 1 μ M doses, there was a statistically significant but modest increase in the sGAG loss (Figure 2-11D) from explants and a statistically significant decrease in their biosynthesis rate (Figure 2-11C). But these doses did not lead to significant changes in DNA content (Figure 2-11A) or cell viability (Figure 2-11B). The mean and standard deviations for all the outcome measures are reported in Table S2.5.

2.4 Discussion

The inability to deliver different classes of drugs to cartilage in a sustained manner remains a major challenge in developing disease modifying drugs for OA and PTOA. The use of positively charged delivery carriers [204] that can specifically target cartilage through electrostatic interactions with the highly negatively charged cartilage extracellular matrix (Figure 2-1) is a promising new approach to overcome this challenge. In this study, we demonstrated that while positive charge improves nanocarrier uptake and penetration into cartilage, higher charge does not necessarily lead to better penetration and transport through the full thickness of tissue, and that there are optimal ranges of charge for effective delivery. We used engineered cationic supercharged GFPs (S-GFPs) carrying net charge ranging from +9 to +36 but having similar sizes and shapes (Figure 2-2A). Three additional engineered GFPs with neutral net charge (Figure 2-2B) served as controls and also to study the effects of surface charge distribution on penetration and transport.

S-GFP variants having net charge of +9 and +15 had significantly higher uptake ra-

tios compared to those for higher charged S-GFPs (+25 and +36) or neutral GFPs. These trends were observed with both human (Figure 2-6) and bovine cartilage (Figure 2-4). Additionally, +9 and +15 S-GFPs exhibited a faster transport rate in cartilage and were able to penetrate the full thickness of the tissue at a much earlier time point compared to the +25 and +36 variants (Figures 2-4E, 2-9 and S2.5). Quantitatively, this corresponded to much higher uptake ratios after cartilage incubation with GFPs for 8 days (Figures 2-4(B,D) and 2-6B) compared to 24 hour incubations (Figures 2-4(A,C) and 2-6(A,C)). As the net charge of a delivery carrier increases, upward Donnan partitioning at the bath-cartilage interface increases, which would accelerate transport into the tissue [159]. However, carriers with higher cationic charge can also exhibit stronger binding interactions with the densely packed negatively charged sulfated glycosaminoglycans (sGAGs) of aggrecan proteoglycans in cartilage. A higher coulombic attraction occurs with increasing cationic charge, thus yielding a strong electrostatic driving force for complexation with sGAGs. In addition to leading to a favorable decrease in enthalpy, electrostatic binding of cationic GFP molecules with the negatively charged aggrecan chains can also lead to the release of large numbers of counterions (such as Na⁺) that were previously tightly bound through condensation onto the aggrecan chains. This counterion release leads to a favorable increase in entropy [219], which acts as a strong driving force that stabilizes the GFPs in the bound state to the aggrecans. Additionally, while both arginine and lysine residues have similar contributions to the net charge of cationic S-GFPs, the guanidino group of arginine is known to exhibit much stronger binding with sulfated GAG [220] compared to lysine. The number of arginines in cationic S-GFPs increases monotonically with increasing charge (from 11 in +9 GFP to 20 in +36 GFP). While stronger binding leads to greater retention of the GFPs in cartilage (with the potential for sustained drug release), such binding could slow transport of the GFPs into the tissue, consistent with the decreased uptake and penetration observed with increasing charge (Figures 2-4 and 2-6)

Importantly, we also found that the uptake from synovial fluid (BSF) (Figures 2-4(C,D) and 2-6D) was 20-50% lower than that from PBS + 1% BSA (Figures 2-4(A,B) and 2-6C) for all S-GFPs by 24 hours. Synovial fluid is a dialysate of plasma [221] that contains proteins such as albumin and the negatively charged glycosaminoglycan, hyaluronan (HA),

secreted by joint capsule synovium. These synovial fluid constituents can bind S-GFPs and thereby slow their transport into cartilage. As such, the effective dose of GFP-bound drug would have to be titrated accordingly for clinical translation, since molecules injected into the synovial fluid have a ~ 1 -24 hour half-life [222] in the joint before being cleared by the joint capsule.

While net cationic charge dramatically affected transport into cartilage, we also demonstrated that surface charge distribution of net neutral carriers can significantly affect their transport properties. The net-neutral NP and HP GFPs had an uptake ratio of ~ 1 (Figure 2-5A and Table S2.3), consistent with the absence of Donnan partitioning or electrostatic binding interactions. However, the net-neutral Janus GFPs had a ten-fold higher uptake within 24 hours than the NP and HP GFPs. While the NP and HP GFPs have multiple small patches of both positive and negative charge (Figure 2-2B), the Janus GFP has one large positive and one large negative patch, each encompassing approximately half its surface (Figures 2-2B and S2.1). The positive patch of Janus GFP may enable it to interact more effectively with negatively charged cartilage GAGs, complexing with negative segments and locally displacing counterions (e.g., Na^+), thus driving directional transport into the tissue. While the precise mechanism relating surface charge distribution to transport of net-neutral carriers into cartilage warrants further investigation, this discovery broadens the choice of potential carriers for drug delivery into such avascular tissues.

Potential disease modifying therapeutics for post-traumatic osteoarthritis include anti-catabolic and anti-inflammatory glucocorticoids which act intracellularly, as well as pro-anabolic biologics which act at cell surface receptors. Trends for direct cellular uptake were greatly improved with higher cationic charge (e.g., Figures 2-7 and 2-10), which is opposite to that for extracellular uptake. Taken together, our results demonstrate that +9 GFP exemplifies the optimal carrier for delivering drugs that have targets either in the extracellular matrix or cell surface receptors of cartilage. For drugs with intracellular targets, GFP carriers with higher cationic charges may be more appropriate.

Ongoing studies now focus on conjugation of anti-catabolic and pro-anabolic drugs to these GFP carriers, and quantifying delivery, first using explant organ co-cultures. Since there has been potential concern about the effects of cationic nanocarrier charge on cell

viability [223][224], we first studied the effects of increasing S-GFP dose and increasing S-GFP charge on (a) proteolysis of matrix aggrecan that would lead to release of GAGs (one of the earliest events in PTOA), (b) chondrocyte matrix biosynthesis within living native explants, (c) explant viability and (d) DNA content (Figure 2-11). Cartilage explants were treated continuously for 8 days with cationic S-GFP doses ranging from 10 nM to 1 μ M. S-GFP doses equal to or less than 100 nM had no adverse effects on any of these critical parameters. Even though the bath concentrations were 1 μ M when adverse effects started appearing, the concentration inside the tissue would have been at least an order of magnitude higher in these *in vitro* studies due to enhanced uptake. In a previous study [186], a continuous dosage with a combination of 100 nM dexamethasone and 15 nM insulin-like growth factor 1 (IGF-1) was sufficient to protect human and bovine cartilage viability, degradation of matrix and loss of cell biosynthesis caused by inflammatory mediators that are upregulated immediately after traumatic joint injury. This previous study was performed without a drug carrier at conditions where the concentration of drugs inside cartilage would be approximately the same as the bath concentration. Due to the high uptake in cartilage, 10 to 100 nM doses of S-GFPs conjugated to similar drugs would be sufficient to maintain the requisite drug concentrations inside cartilage and achieve the same efficacy. The required carrier concentrations are therefore at least an order of magnitude lower than the threshold of 1 μ M at which GFPs can lead to adverse effects.

2.5 Conclusions

Recent studies have demonstrated the conceptual advantage of using cationic nanocarriers such as Avidin to achieve enhanced, sustained delivery of low molecular weight drugs (i.e., dexamethasone) to cartilage, both in *in vitro* [206] and *in vivo* systems [207]. Use of such cationic carriers takes advantage of enhanced interfacial partitioning, accelerated transport and increased retention within cartilage due to electrostatic interactions with the tissue's high density of negatively charged aggrecan proteoglycans [204]. The present study has focused on the important need to identify optimal nanocarrier net charge for delivery to both extracellular and intracellular targets in cartilage and other nearby joint tissues

affected by traumatic joint injury. To achieve these aims, we quantified the effect of net charge of carriers on uptake and penetration into cartilage using green fluorescent proteins (GFPs) engineered to carry a wide range of net charge and while retaining nearly identical sizes and shapes. In addition, by using three net-neutral GFPs having different surface charge distributions, we demonstrated that carrier uptake and penetration into cartilage also depends dramatically on the size and distribution of surface charge patches. These engineered GFPs have the advantage of being small enough to deliver both small molecule drugs and large biologics to cartilage. Optimal charge ranges are identified for uptake directly into cartilage cells, which makes these carriers highly effective for drugs with intracellular targets. Results have revealed optimal net charge of GFPs for the effective delivery to intracellular as well as ECM/cell surface receptor targets. For the dose ranges of interest for delivery, we demonstrated that cationic GFPs do not have adverse effects on cartilage matrix turnover, cell viability and metabolic rate.

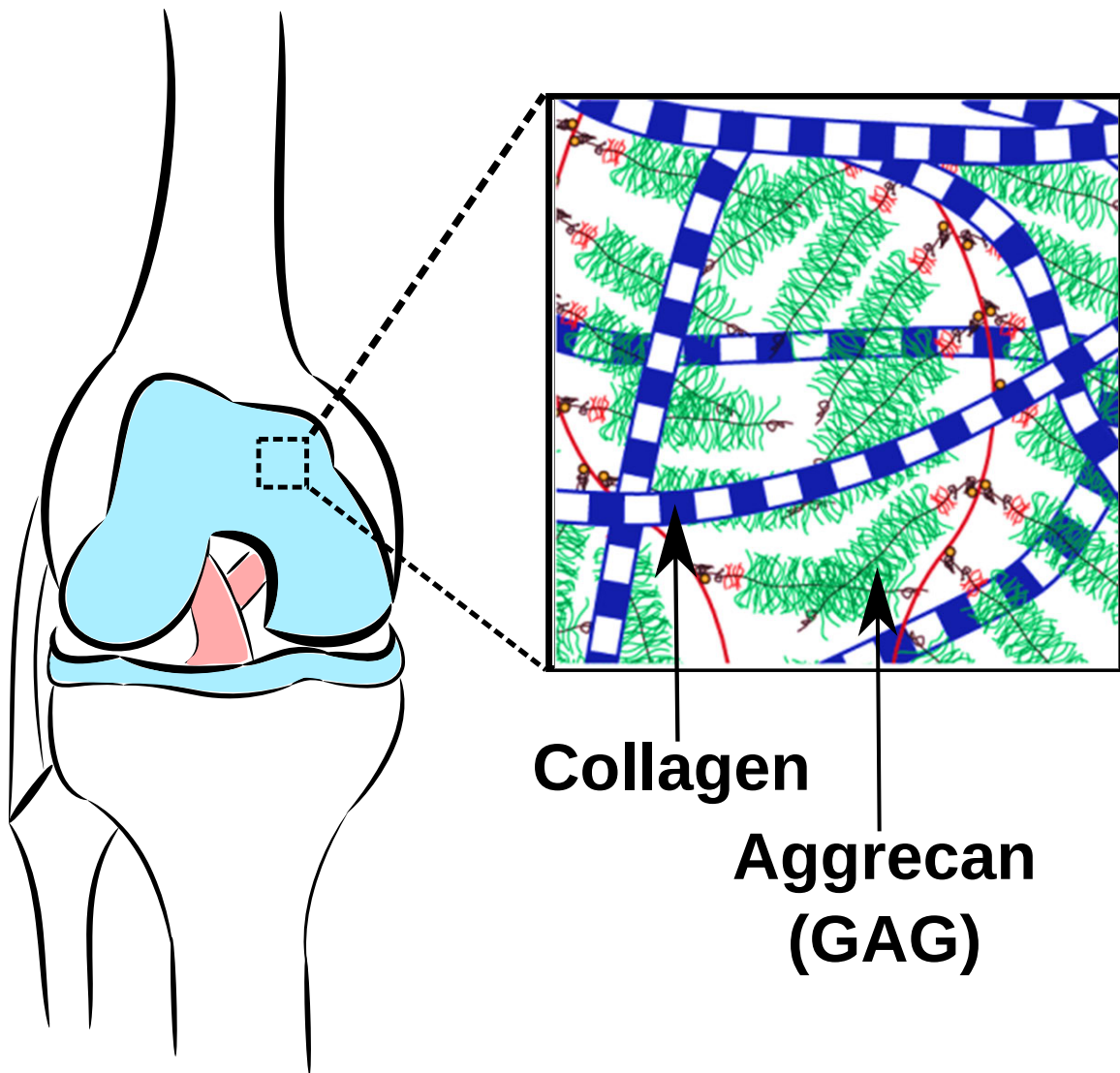


Figure 2-1: The extracellular matrix of cartilage is made up of a collagen II network. The pore spaces are filled with hundreds of proteins and proteoglycans, the most abundant of which is a bottle brush shaped, highly negatively charged proteoglycan called aggrecan. (Adapted from [225])

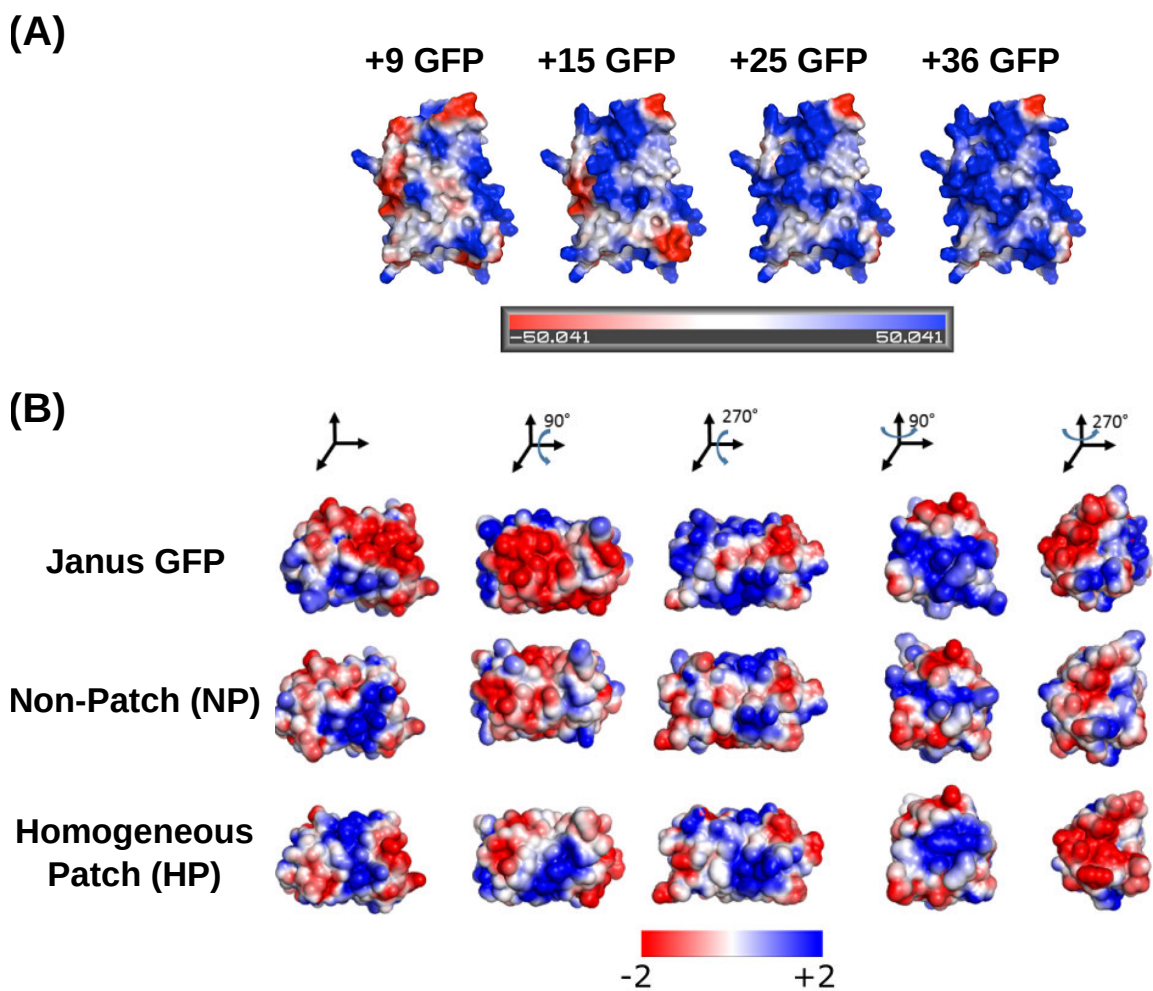


Figure 2-2: (A) Calculated electrostatic surface potentials (units: kT/e) of cationic super-charged GFP variants having a net theoretical charge of +9, +15, +25 and +36 [226].(C) Calculated electrostatic surface potentials (units: kT/e) of neutral GFP variants (Janus, NP and HP) having different surface charge distributions.

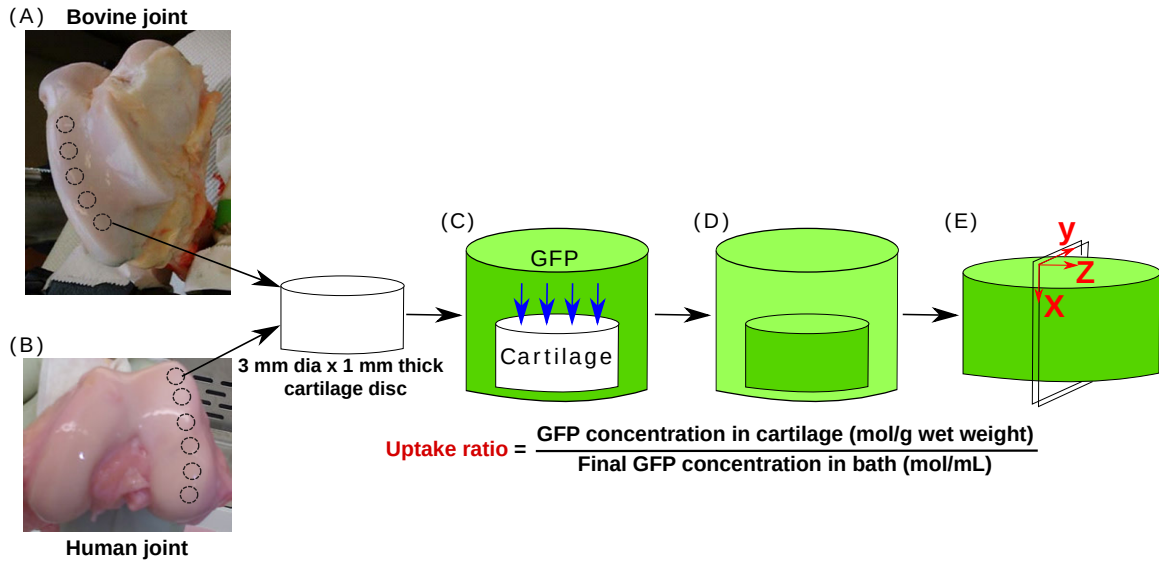


Figure 2-3: Schematic demonstrating the method for quantifying the uptake of GFPs in cartilage. Cartilage explants were harvested from (A) bovine and (B) human knee joints and (C) incubated in GFP solutions. (D) The concentrations of GFPs inside the explants and the final bath at the end of the incubation were quantified using fluorescence measurements, and their ratio is calculated and reported as the uptake ratio. (E) Explant slices were sectioned in the x-y plane, and confocal microscopy was used to visualize transport of GFPs into the explant disks in the x-direction at selected times.

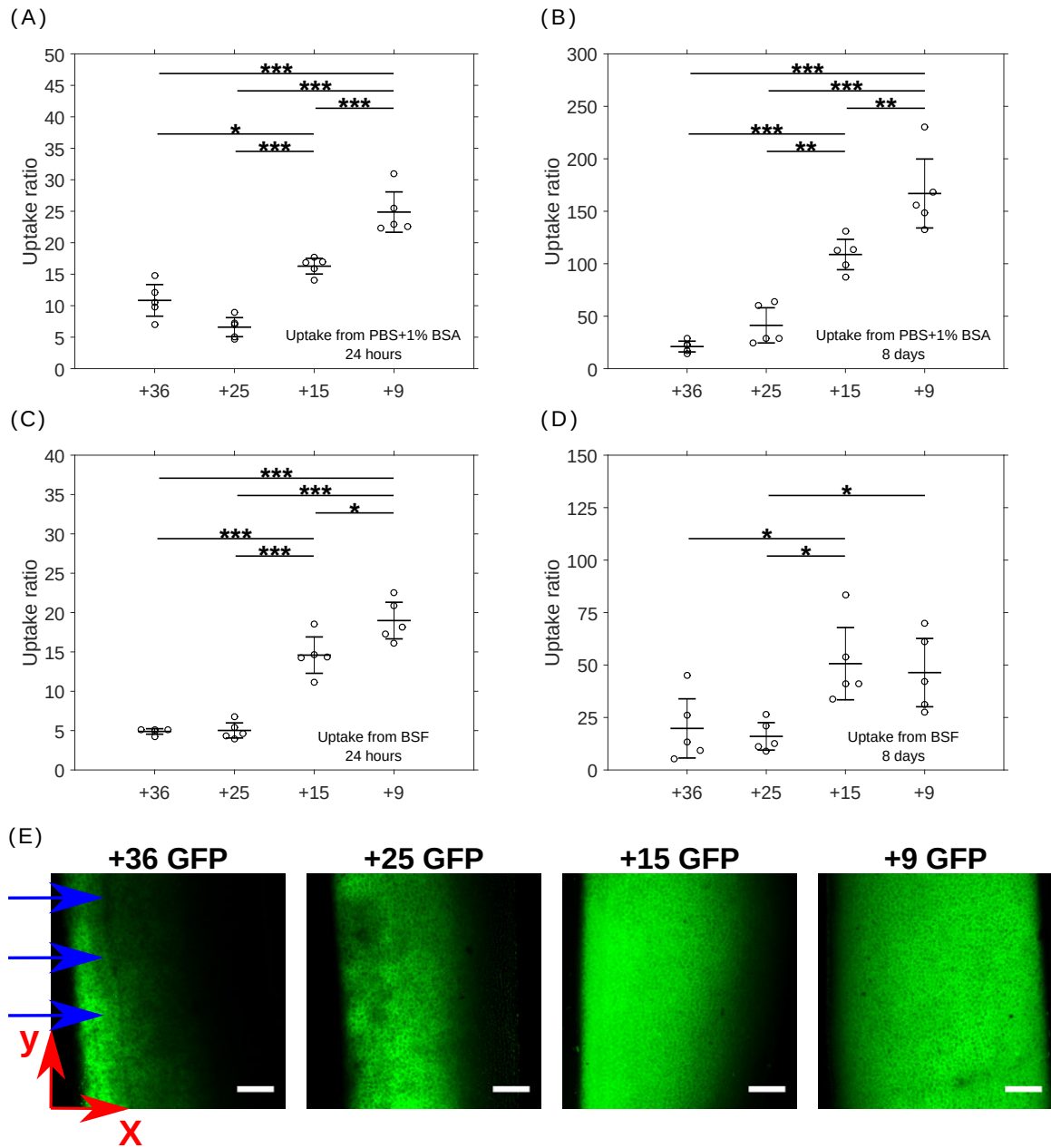


Figure 2-4: The uptake of cationic S-GFPs into bovine cartilage increases as the cationic charge decreases. Bovine cartilage explants were incubated in $1\mu\text{M}$ solutions of cationic S-GFPs dissolved in either PBS + 1% BSA (A,B) or in bovine synovial fluid (C,D). 24 hour uptake ratios are shown in (A,C) and 8-day uptake ratios are reported in (B,D). $n=5$ cartilage explants (that were matched for joint region) from 1 animal were used for each condition and the individual data points, mean and 95% confidence interval are reported; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. (E) Confocal microscopy images show the 24-hour penetration of S-GFPs from medium into bovine cartilage explants. (Scale bar: 200 μm ; Cartilage orientation same as Figure 2-3E.)

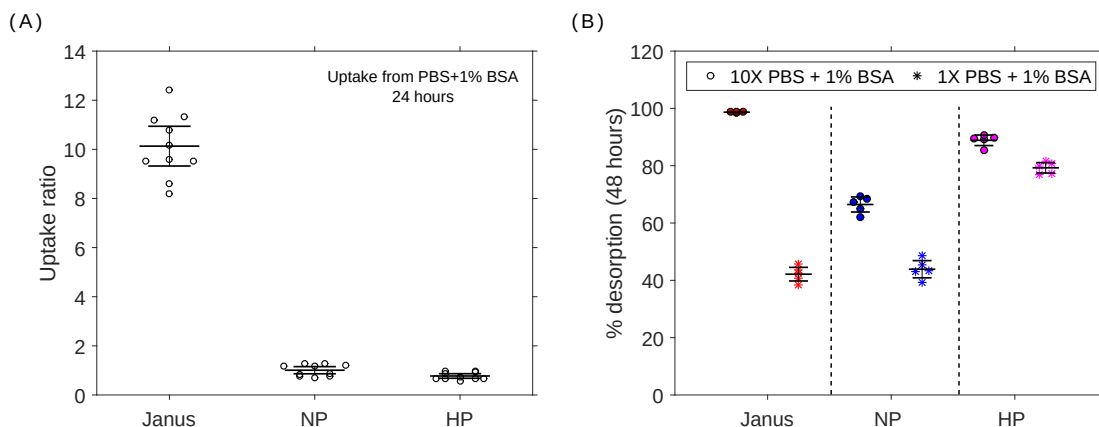


Figure 2-5: (A) 24 hour uptake ratios of three GFPs with net neutral charge and different surface charge distribution. (B) Percentage desorption of neutral GFPs from cartilage into baths of (10X PBS + 1% BSA) and (1X PBS + 1% BSA). $n = 10$ cartilage explants (that were matched for joint region) were used for each condition and the individual explant data points along with mean and 95% confidence interval are shown.

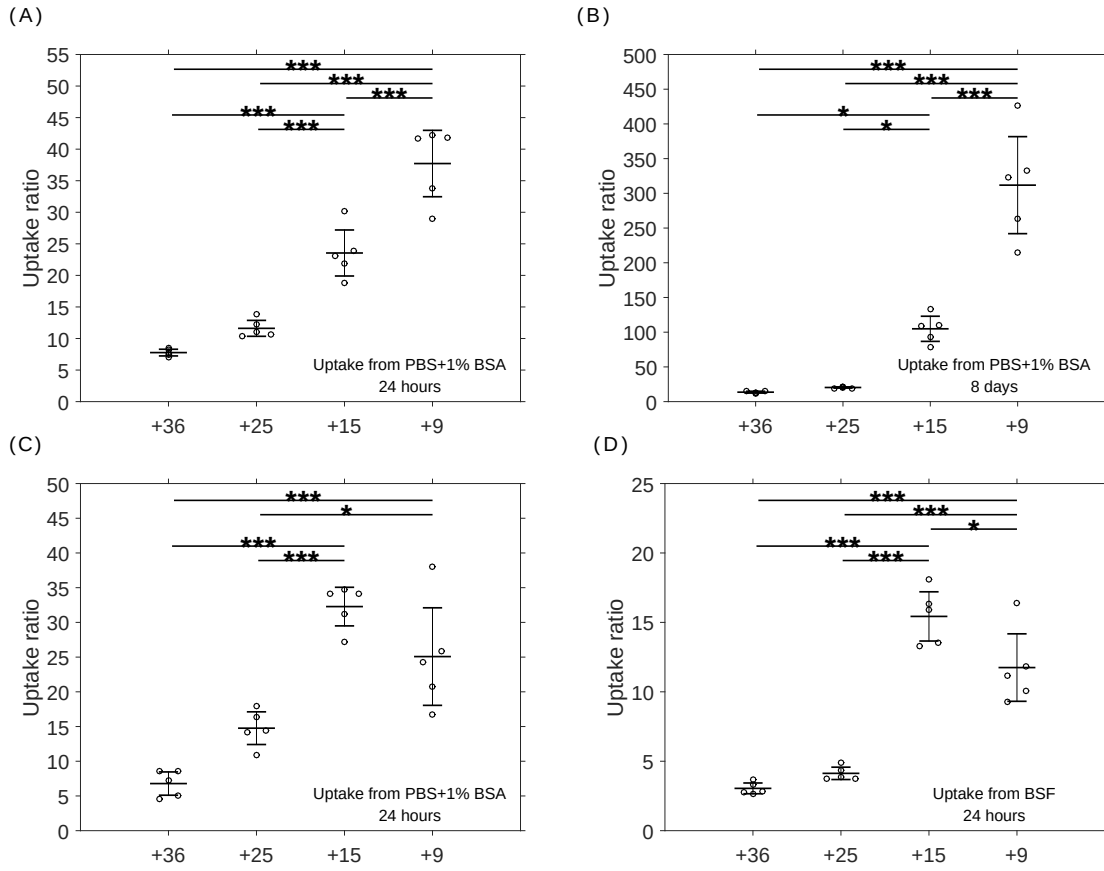


Figure 2-6: 35 year old human female donor knee cartilage (Collins Grade 1, near normal): 24 hour (A) and 8 day (B) uptake ratios for S-GFPs in PBS + 1% BSA. 36 year old human male donor knee cartilage (Collins Grade 1, near normal): 24 hour uptake ratios of S-GFPs dissolved in PBS + 1% BSA (A) and in BSF (B). $n = 5$ cartilage explants (that were matched for joint region) were used for each condition and the individual explant data points along with mean and 95% confidence interval are reported; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

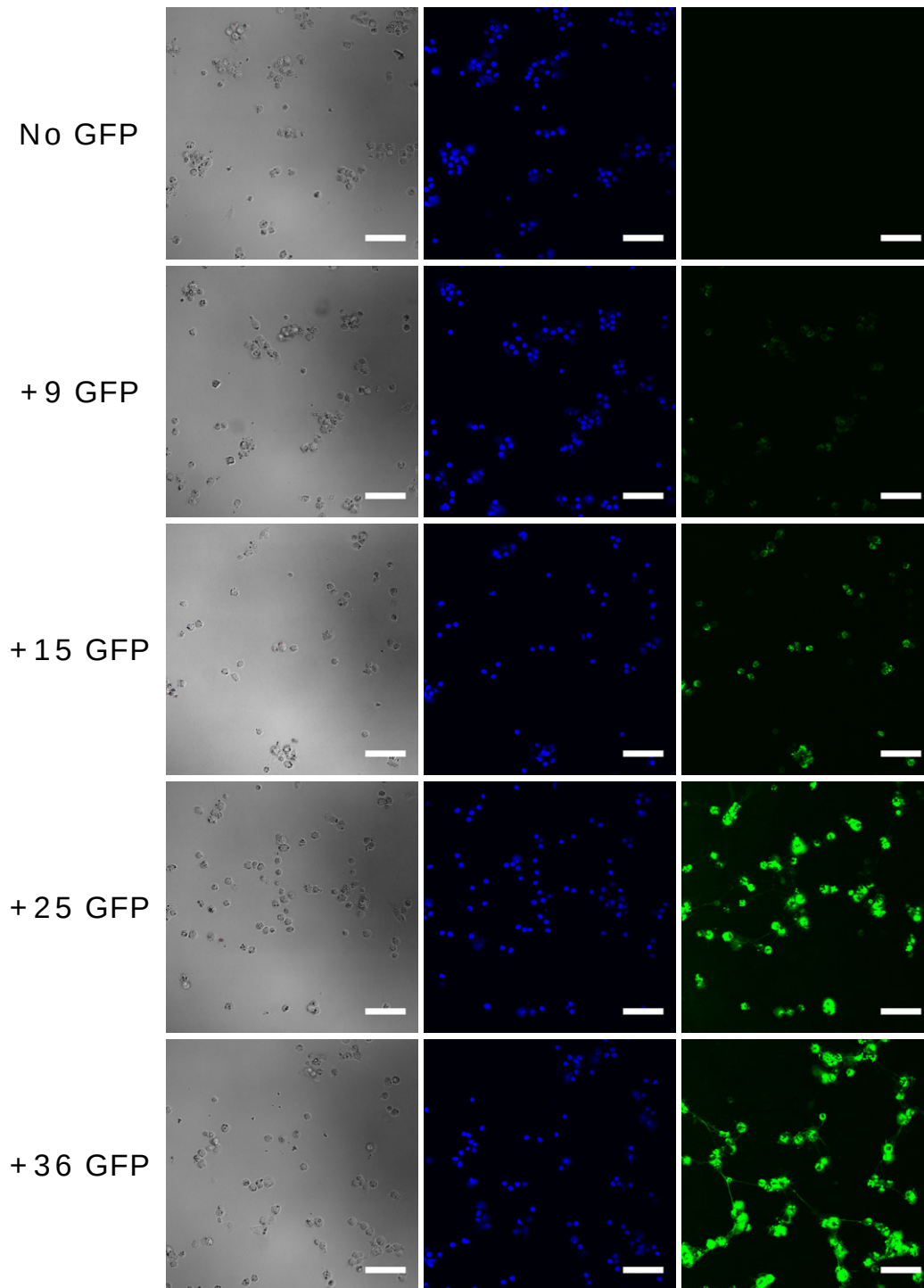


Figure 2-7: Cellular uptake of S-GFPs in primary bovine chondrocytes increases with cationic charge. Cells were incubated in medium with no GFP (controls), +9 GFP, +15 GFP, +25 GFP or +36 GFP. Brightfield images (left column) are shown alongside fluorescence images for DAPI staining (blue, middle column) and cationic S-GFPs (green, right column). Increasing brightness in green fluorescence indicates higher intracellular GFP concentration since the microscope and laser settings were the same for all fluorescence images. Scale bar: $60\mu\text{m}$.

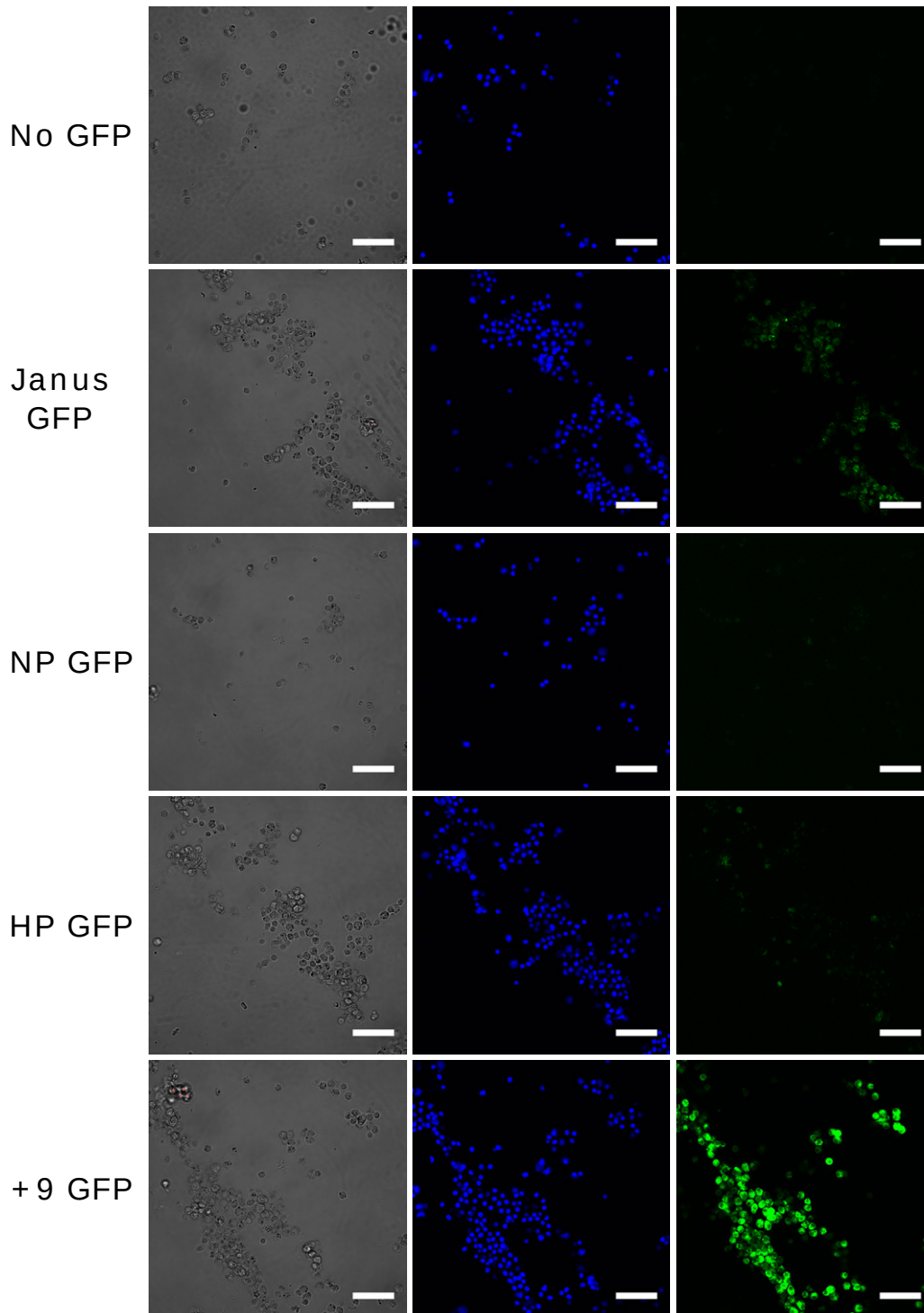


Figure 2-8: Cellular uptake of neutral GFP variants in primary bovine chondrocytes is significantly lower than that of cationic S-GFPs. Cells were incubated in medium with no GFP (controls), Janus GFP, NP GFP, HP GFP and +9 GFP. Brightfield images (left column) are shown alongside fluorescence images for DAPI staining (blue, middle column) and GFPs (green, right column). Increasing brightness in green fluorescence indicates higher intracellular GFP concentration since the microscope and laser settings were the same for all fluorescence images. Scale bar: 60 μ m.

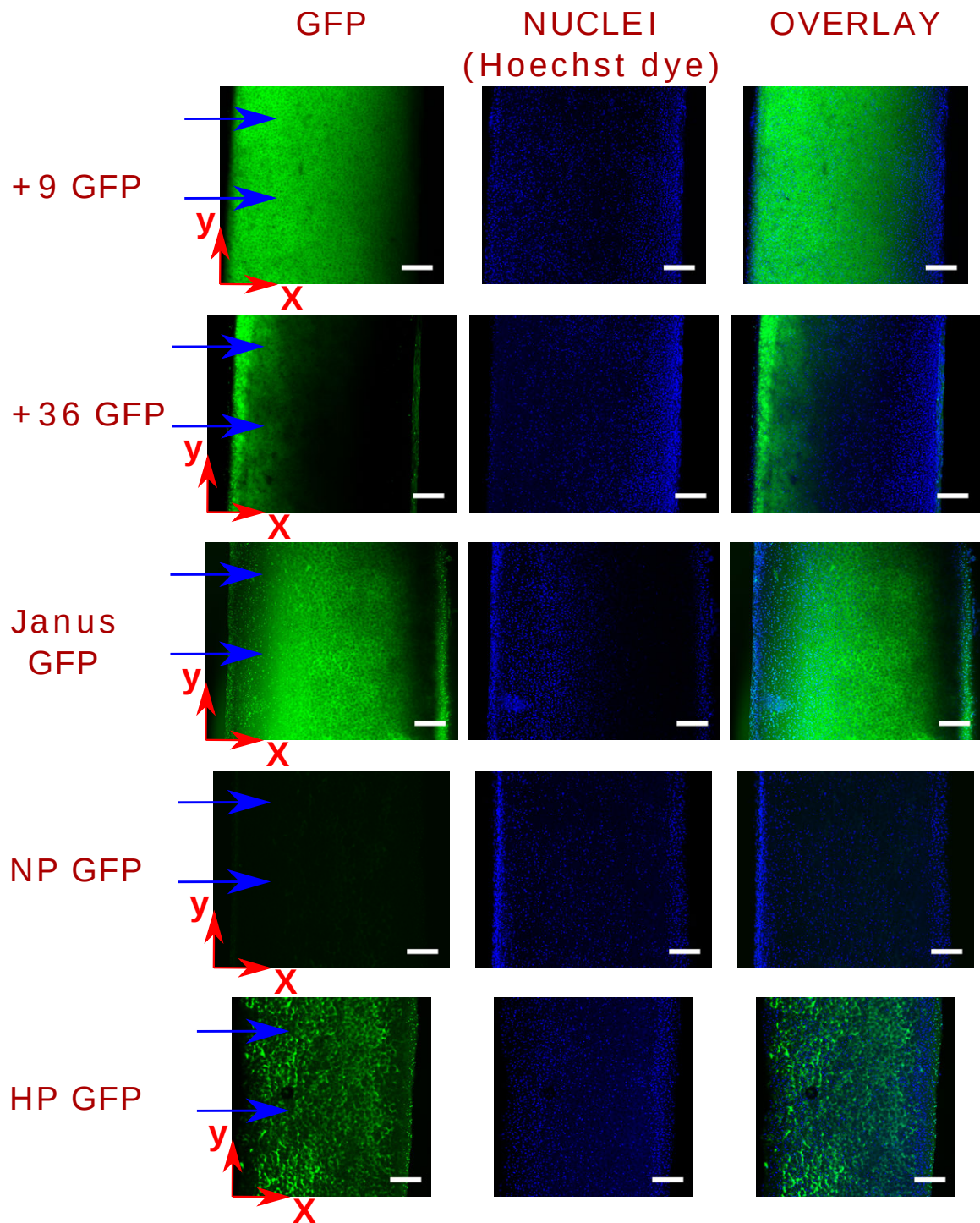


Figure 2-9: Confocal microscopy images showing the 24 hour penetration of cationic and neutral GFPs from PBS + 1% BSA into bovine cartilage explants. The first column shows the fluorescence images for GFPs while the second column shows the location of the cells inside the tissue (nuclei have been stained with Hoechst 33258 dye). The third column is the overlay of the first two columns, and shows the distribution of the GFPs with respect to the cells. Scale bar: 200 μ m.

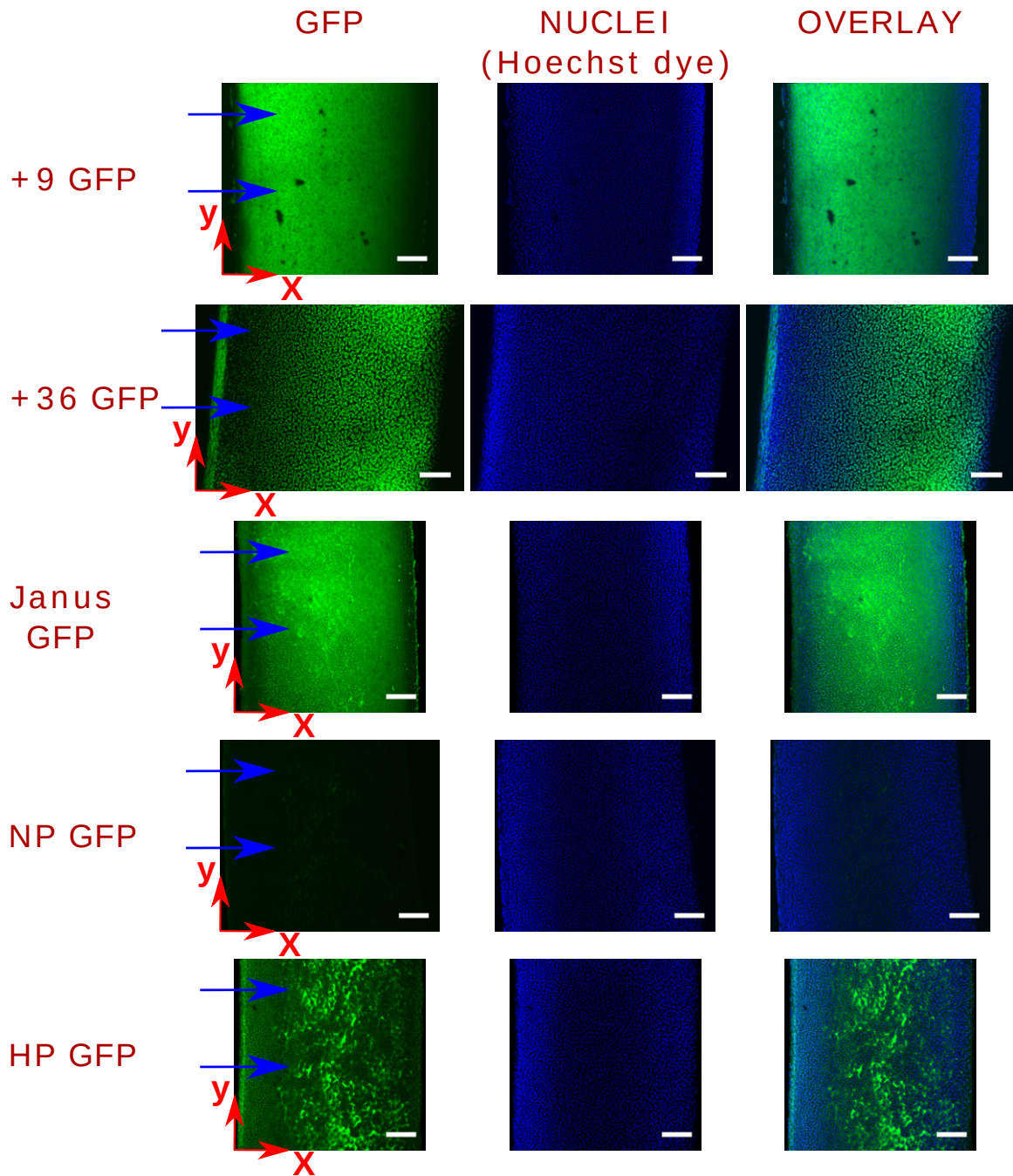


Figure 2-10: Cellular uptake of S-GFPs in bovine chondrocytes inside native tissue increases with cationic charge. Bovine cartilage explants were incubated in solutions of GFPs dissolved in PBS + 1% BSA for 8 days. They were then sectioned (same orientation as Figure 2-3E) and imaged using confocal microscopy. The first column shows the fluorescence images for GFPs while the second column shows the location of the cells inside the tissue (nuclei have been stained with Hoechst 33258 dye). The third column is the overlay of the first two columns, and shows the distribution of the GFPs with respect to the cells. The first two rows show that +9 GFP is localized mostly in the cartilage extracellular matrix, while +36 GFP is predominantly located in the cells. Scale bar: 200 μ m.

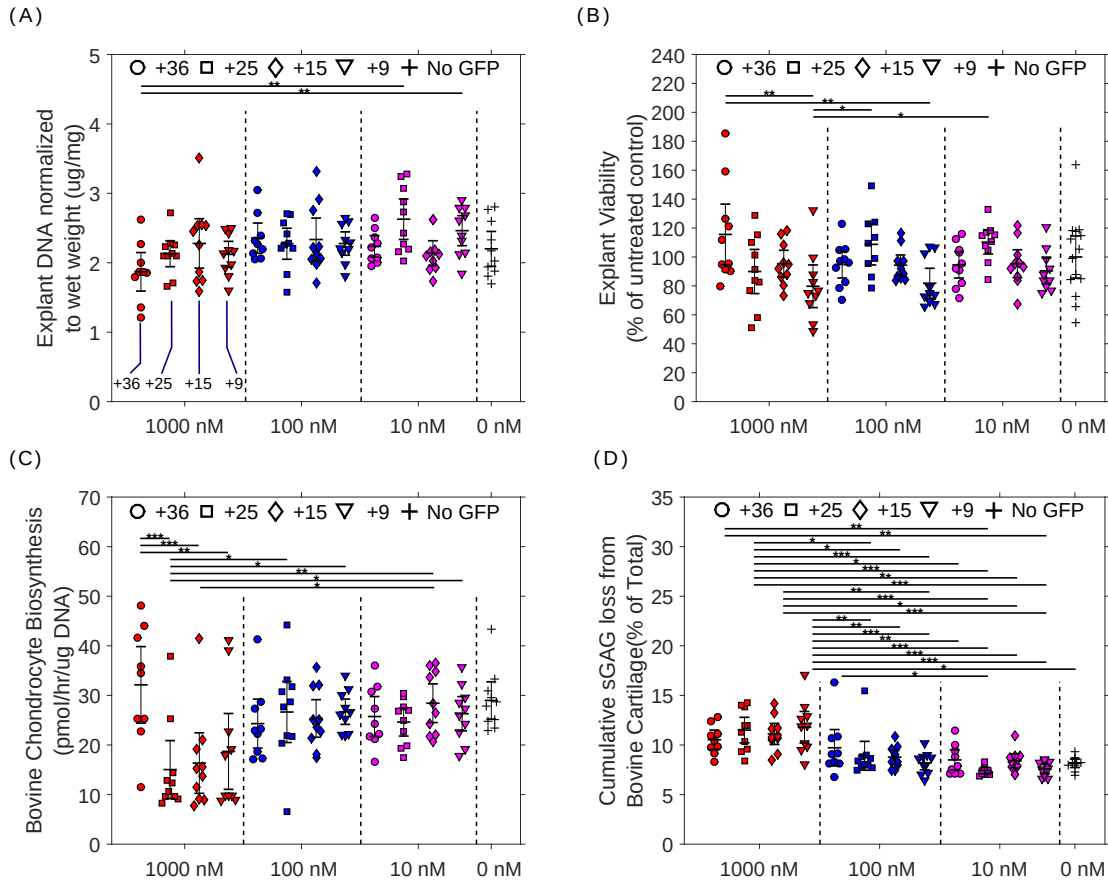


Figure 2-11: 8-day continuous dose response results for bovine cartilage explants treated with 10 nM to 1 μ M doses of S-GFPs. An untreated control group was also included. (A) S-GFPs do not lead to any change in the cellularity (as measured by the DNA content) of cartilage explants. (B) Cartilage explant viability, as measured by the Alamar Blue assay on day 8 of the experiment, remains unaffected by different doses of S-GFP. (C) S-GFPs cause a statistically significant decrease in the chondrocyte biosynthesis rate as measured by the normalized incorporation rate of radioactive ^{35}S -sulfate during days 6-8 of the experiment. (D) S-GFPs cause a slight but statistically significant increase in the cumulative percentage sulfated glycosaminoglycan (sGAG) loss at a dose level of 1 μ M but not at lower doses. $n = 9-10$ cartilage explants (that were matched for joint region) from 2 animals were used for each condition and the individual data points, mean and 95% confidence interval are reported; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

Supplementary Material

S2.1 Protein sequences of GFPs:

+36 GFP

Amino acid sequence:

MGHHHHHHGGASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATR GK
LTLKFICTTGKLPVPWPTLVTTLT YGVQCFSRYPKHMKRHDFFKSAMPKGYVQ
ERTISFKKDGKYKTRAEVKFEGRTL VNRIKLKGRDFKEKGNILGHKLRYNFNS
HKVYITADKRKNGIKAKFKIRHN VKDGSVQLADHYQQNTPIGRGPVLLPRNH Y
LSTRSKLSKDPKEKRDH MVLLEFVTAAGIKHGRDERYK

DNA sequence:

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CGTGGTAAAGTACCGATCTTAGTGGAATTAAAGGGCGACGTGAACGGTCATAAA
TTTAGCGTGCGCGGCAAAGGCAAAGGTGACGCTACCCGTGGTAAATTGACCCTG
AAGTTTATTTGCACAACAGGCAAATTACCCGTTCCGTGGCCACCTTAGTGACC
ACCCTGACCTATGGCGTTCAGTGCTTCAGTCGTTACCCTAAACATATGAAACGT
CACGATTTTTTCAAATCAGCCATGCCTAAAGGATATGTTCAAGAGCGTACAATC
AGCTTCAAGAAGGATGGCAAATATAAAACGCGTGCGGAAGTGAAATTTGAAGGC
CGCACATTAGTAAATCGTATCAAAC TGAAGGTCGTGACTTCAAAGAAAAAGGC
AACATTTTAGGCCATAAACTGCGTTATAACTTTAATTCTCATAAGGTGTATATT
ACGGCCGATAAACGCAAGAATGGTATCAAGGCAAAATTCAA AATTCGCCATAAC
GTGAAAGACGGCAGCGTTCAATTAGCGGATCATTATCAACAAAACACGCCGATT
GGTCGCGGGCCTGTACTGTTACCTCGCAACCACTACCTGAGCACCCGTTCTAAA
CTGAGCAAAGATCCGAAAGAAAAACGCGATCACATGGTTCTGT TAGAATTCGTG
ACCGCTGCAGGCATTAAGCACGGACGCGACGAACGCTACAAG

+25 GFP

Amino acid sequence:

MGHHHHHHGGASKGERLFTGVVPILVELDGDVNGHKFSVRGKGKGDATR GK
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ERTISFKKDGTyKTRAEVKFEGRTLvNRIKLKGRDFKEKGNILGHKLRYNFNS
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LSTRSALS KDPKEKRDH MVLLFVTAAGITHGMDELYK

DNA sequence:

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GACCACCCTGACCTATGGCGTTCAGTGCTTCAGTCGTTACCCTAAACATATGA
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ACCCGTTCAGCACTGAGCAAAGATCCGAAAGAAAAACGCGATCACATGGTTCT
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+15 GFP

Amino acid sequence:

MGHHHHHHGGASKGERLFTGVVPILVELDGDVNGHKFSVRGEGEGDATRGK
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DNA sequence:

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+9 GFP

Amino acid sequence:

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DNA sequence:

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ACCCAGTCAGCACTGAGCAAAGATCCGAACGAAAAACGCGATCACATGGTTCT
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Janus GFP

Amino acid sequence:

MGHHHHHHGSACELMVSKGEELFEGDVPILVELDGDVNGHEFSVRGEGEGD
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NP GFP

Amino acid sequence:

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TGATCACATGGTGCTGCTGGAGTTCGTTACCGCGGCGGGCATTACCCACGGTG
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HP GFP

Amino acid sequence:

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ATNGQLTLKFICTTGELPVPWPTLVTTLTLYGVQCFSAYPNHMKAHDFFKSAMP
NGYVQERTISFKNDGTYKTRAIEVKFAGDTLVNRIALKGIDFKEDGNILGHKLE
YNFNSHNVYITADKQANGIKANFAIRHNVQDGSVQLATHYQQNTPIGNPVL
PDNHYLSTQSALSKDPNETRDHMLLEFVTAAGITHGMNEVYK

DNA sequence:

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AACGGCCAACTGACCCTGAAGTTCATTTGCACCACCGGTGAACTGCCGGTGCC

GTGGCCGACCCTGGTTACCACCCTGACCTACGGTGTGCAGTGCTTTAGCGCGT
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CCCACTACCAGCAAAACACCCCGATCGGCAACGGTCCGGTTCTGCTGCCGGAT
AACCCTATCTGAGCACCCAAAGCGCGCTGAGCAAAGACCCGAACGAGACCCG
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S2.2 Characterization of neutral and supercharged GFPs:

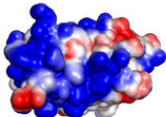
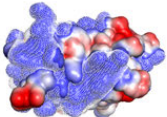
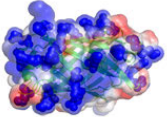
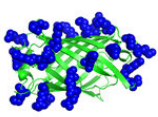
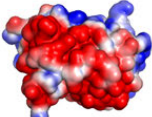
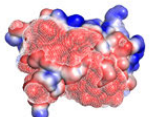
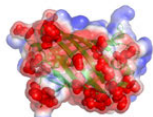
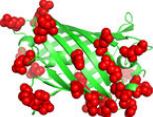
		APBS	Isosurface	Residues	3D	Net charge
(+) Patch 1						21.8
(-) Patch 2						-19.2

Figure S2.1: Surface charge of the positive and negative patches of Janus GFP

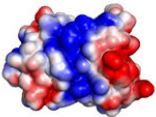
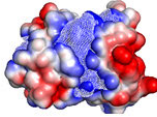
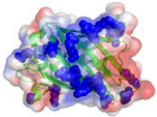
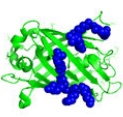
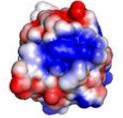
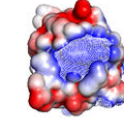
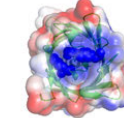
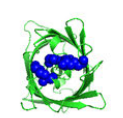
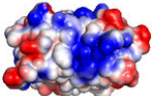
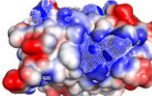
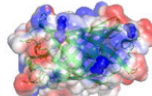
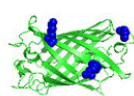
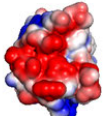
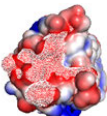
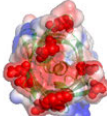
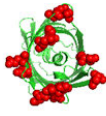
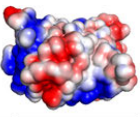
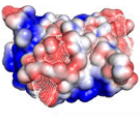
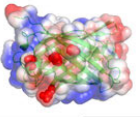
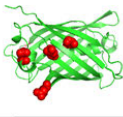
		APBS	Isosurface	Residues	3D	Net charge
(+) Patch 1						8.8
(+) Patch 2						2.8
(+) Patch 3						3.8
(-) Patch 4						-12.2
(-) Patch 5						-3.2

Figure S2.2: Surface charge of the positive and negative patches of HP GFP

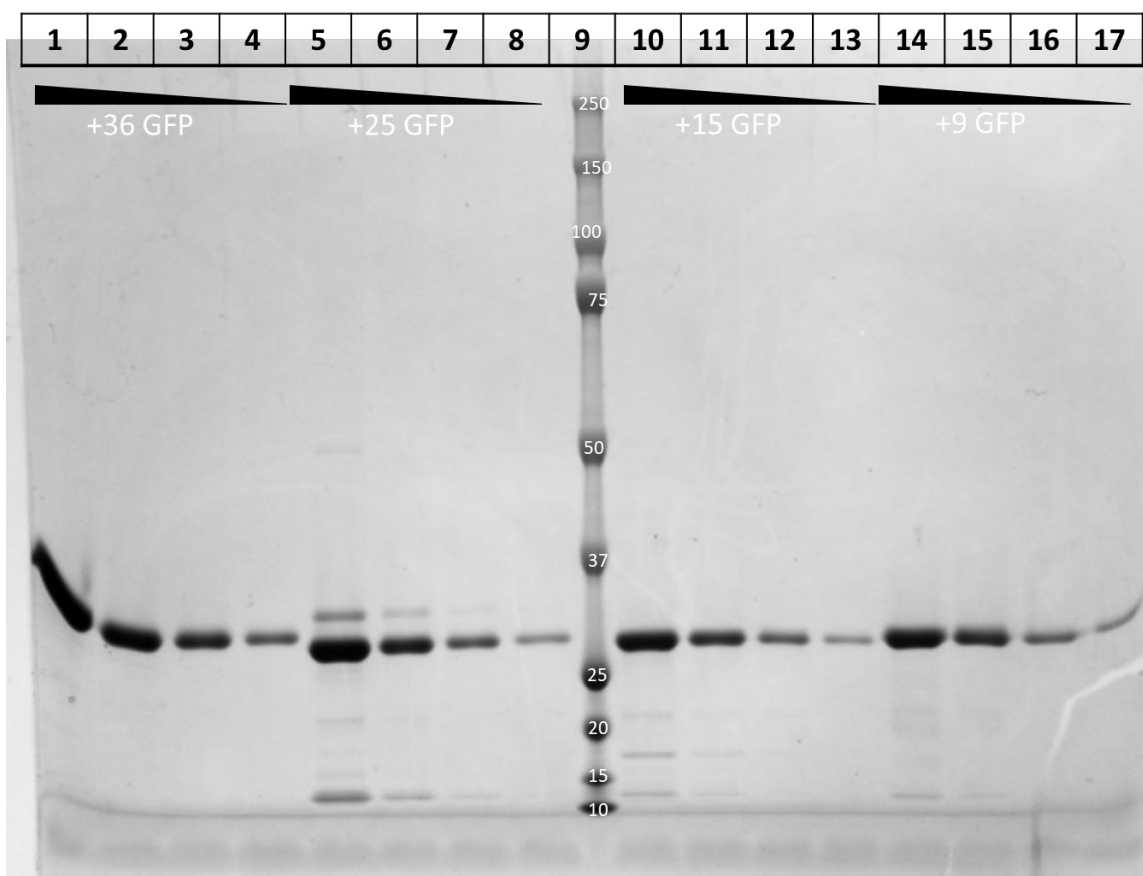


Figure S2.3: 4-12% Bis-tris PAGE BOLT (Thermo Fisher) denaturing gel showing purified supercharged GFPs. After sterile filtration, a two-fold dilution series of each purified S-GFP was made, boiled in NuPAGE LDS sample buffer (Thermo Fisher) dye and run on the gel to assess protein purity. Four samples of each protein were run on the gel, as indicated on the image.

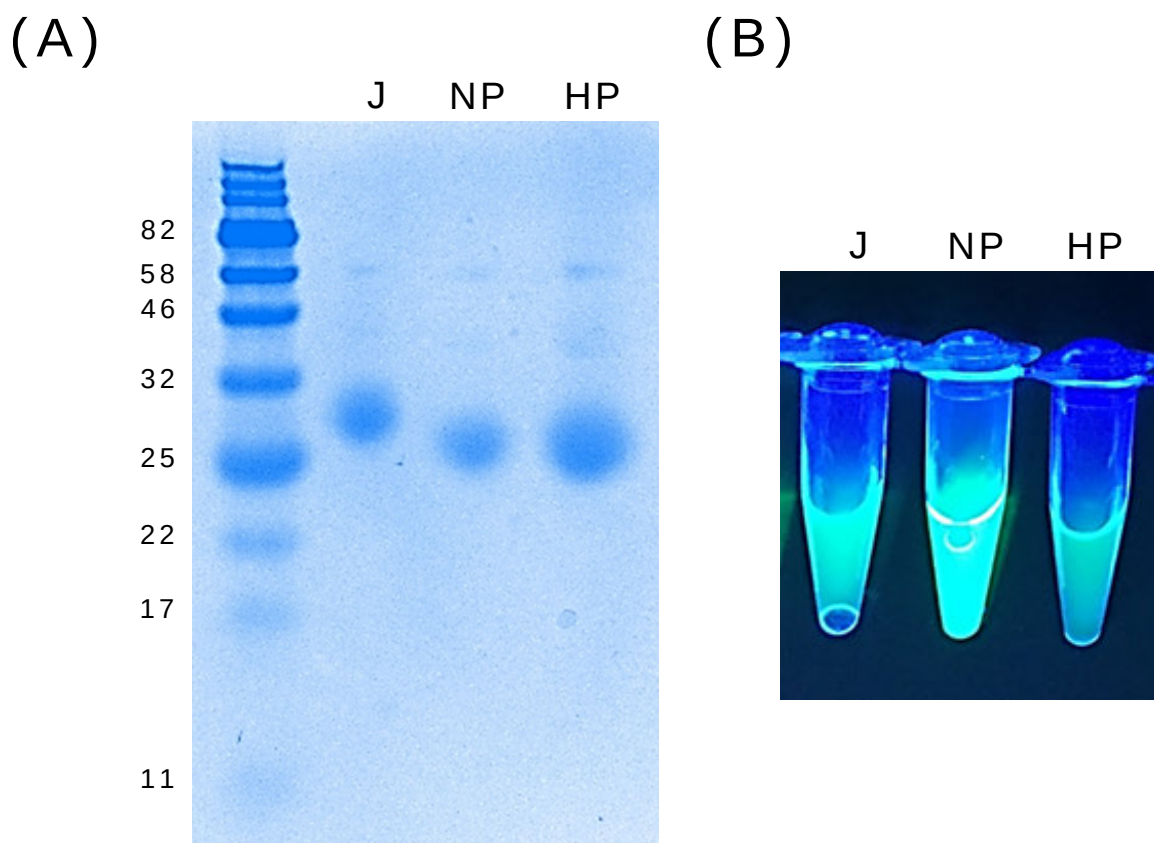


Figure S2.4: (A) SDS PAGE gel showing purified neutral GFPs. (B) UV illuminated samples of purified neutral GFPs. In both images, J is Janus GFP, NP is non-patch GFP and HP is homogeneous-patch GFP.

Sample	MW (kDa)	pI	Positive		Negative		Net charge (pH 7.4)
			# of K	# of R	# of D	# of E	
Janus GFP	28.8	7.11	22	13	16	22	-1.7
NP GFP	28.5	7.11	24	10	19	18	-1.7
HP GFP	27.8	7.09	14	6	12	11	-1.7

Table S2.1: Properties of neutral GFP variants: molecular weights, isoelectric points, number of positive and negative residues and estimated net charge at pH 7.4

S2.3 Data from uptake, dose response, tissue penetration and cell uptake experiments:

GFP charge	PBS + 1% BSA				BSF			
	24 hr		8 days		24 hr		8 days	
	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
+9	24.9	3.6	166.9	37.5	19.0	2.6	46.4	18.6
+15	16.3	1.4	108.8	16.5	14.6	2.6	50.6	19.6
+25	6.6	1.7	41.2	19.1	5.0	1.1	16.0	7.5
+36	10.8	2.9	21.1	5.8	4.9	0.4	19.8	16.1

Table S2.2: Uptake ratios of S-GFPs in bovine cartilage (Mean $\pm$ SD; N=5 ex-plants/condition)

GFP variant	Uptake Ratio	
	Mean	Std. Dev
Janus	10.1	1.3
NP	1.0	0.2
HP	0.8	0.2

Table S2.3: Uptake ratios of neutral GFPs in bovine cartilage (Mean $\pm$ SD; N=10 ex-plants/condition)

GFP charge	35 yo female donor				36 yo male donor			
	24 hr; PBS + 1% BSA		8 days; PBS + 1% BSA		24 hr; PBS + 1% BSA		24 hr; BSF	
	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
+9	37.7	6.0	311.8	79.7	25.1	8.0	11.7	2.8
+15	23.5	4.2	104.9	20.7	32.3	3.2	15.4	2.0
+25	11.6	1.4	20.4	1.0	14.8	2.7	4.1	0.5
+36	7.8	0.6	13.6	1.5	6.8	1.9	3.0	0.4

Table S2.4: Uptake ratios of S-GFPs in human cartilage (Mean $\pm$ SD; N=5 ex-plants/condition)

Condition	sGAG loss		DNA		Biosynthesis rate		Normalized viability	
	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
+36 GFP, 1000 nM	10.5	1.5	1.9	0.4	32.1	11	115.6	33.4
+25 GFP, 1000 nM	11.5	2.1	2.1	0.3	15	9.4	90	24.6
+15 GFP, 1000 nM	11.1	1.7	2.3	0.6	16.4	9.8	95.3	14.9
+9 GFP, 1000 nM	11.8	2.6	2.1	0.3	18.7	12.3	79.7	23.7
+36 GFP, 100 nM	9.7	2.8	2.4	0.3	24.3	7.6	94.8	15.0
+25 GFP, 100 nM	8.9	2.3	2.3	0.4	25.4	6.1	108.7	21.8
+15 GFP, 100 nM	8.8	1.1	2.3	0.5	26.6	9.9	94.3	11.4
+9 GFP, 100 nM	8.2	1.1	2.3	0.3	26.7	4.1	81.8	16.7
+36 GFP, 10 nM	8.5	1.6	2.2	0.2	25.7	6.2	94.6	14.7
+25 GFP, 10 nM	7.4	0.5	2.6	0.5	24.6	4.5	110.3	13.2
+15 GFP, 10 nM	8.4	1.1	2.1	0.3	28.4	6.3	95.3	15.6
+9 GFP, 10 nM	7.6	0.8	2.5	0.3	26.3	5.6	90.4	14.3
+Untreated control	8.2	0.7	2.2	0.4	29.0	6.1	100.0	27.8

Table S2.5: Dose response results in bovine cartilage (Mean $\pm$ SD; N=9 to 10 explants/condition; units same as in Figure 2-11)

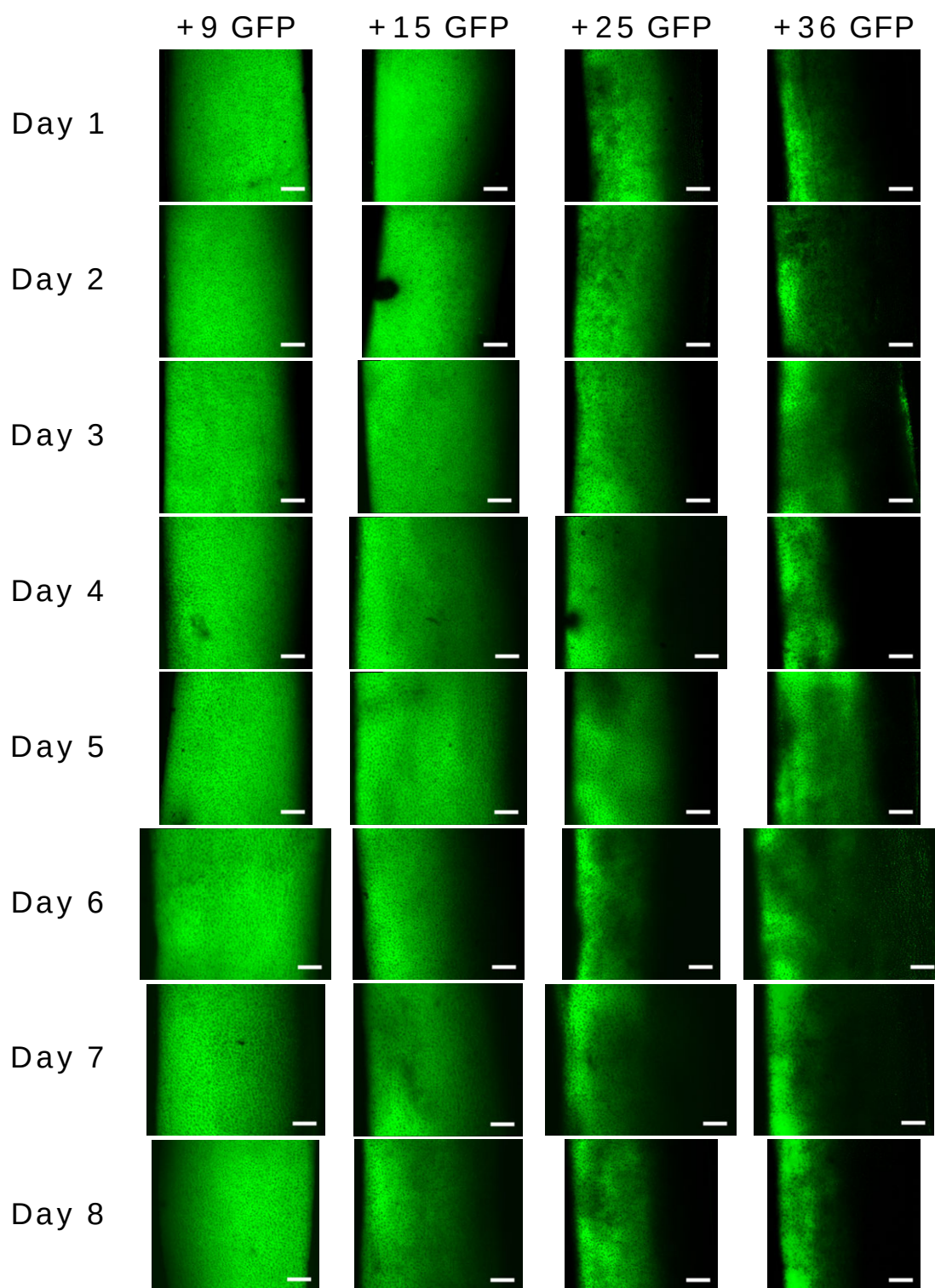


Figure S2.5: S-GFP penetration in cartilage explants treated with continuous doses of S-GFPs ($1\mu\text{M}$; dissolved in culture medium) over an 8 day period. 64 cartilage explants were harvested from 1 animal. Representative images from one of the two cartilage explants used for each of the 32 conditions are shown here.

Chapter 3

Optimally Charged Green Fluorescent Proteins Enable Targeted and Sustained IGF-1 Delivery in Human Cartilage¹

3.1 Introduction

Post-traumatic osteoarthritis (PTOA) is a subtype of osteoarthritis that is triggered by traumatic joint injury and accounts for 12% of all osteoarthritis cases [158]. There are currently no clinically effective disease modifying osteoarthritis drugs (DMOADs) for treating PTOA. Due to the complex pathogenesis of PTOA, treatment of the disease will most likely require combination therapy with multiple drugs that may have intracellular or extracellular targets.

There is also a need for a method to deliver different classes of drugs in a targeted and sustained manner to cartilage in joints affected by OA. Cartilage is an avascular tissue and any drugs that are injected intra-articularly are rapidly cleared out from the joint capsule. Positively charged delivery carriers can rapidly enhance penetration and retention of drugs in cartilage through electrostatic interactions with the negatively charged glycosaminoglycans within the tissue. We have previously demonstrated that cationic supercharged

¹ An edited version of this chapter will be submitted for publication

GFPs can rapidly penetrate cartilage and have high quantitative uptake in bovine and human cartilage [227]. Depending on their net charge, they can also be used to target either extracellular/cell-surface targets or to intracellular targets in cartilage [227]. In this study, we investigate the use of supercharged GFPs to treat full thickness human cartilage with IGF-1 as an example therapeutic.

Previous studies have found that IGF-1 can increase proteoglycan synthesis in bovine cartilage [228][229], decrease proteoglycan degradation and stimulate synthesis in porcine cartilage explants exposed to cytokines [230], and rescue the loading-induced inhibition of proteoglycan synthesis in both bovine and human cartilage explants [231][232]. While IGF-1 has shown both pro-anabolic and anti-catabolic effects in young pig and bovine cartilage, a more recent study [186] has reported that IGF-1 could induce biosynthesis but could not block proteoglycan loss caused by inflammatory cytokines in full thickness human cartilage. In all these studies, continuous doses of IGF-1 were used in explant culture to obtain these beneficial effects.

In recent years, cationic carriers have been used for the sustained delivery of IGF-1 with a single dose in both *in vitro* models and animal models. Heparin binding IGF (HB-IGF), which has a cationic peptide domain attached to IGF-1, was able to sustain extended proteoglycan biosynthesis in bovine cartilage explants [233] and had a retention time of at least 6 days in human cartilage explants [234]. In a rat model of PTOA, dendrimer-IGF-1 conjugates were sustained in the joint space for prolonged times and prevented cartilage degradation [235]. But we know of no studies to date showing the bioactivity of cationic carrier-IGF-1 conjugates in full-thickness human cartilage.

Therefore, our overarching goal in the present study was to develop and test a novel cationic GFP-IGF-1 fusion protein to achieve enhanced penetration and sustained bioactivity inside full thickness adult human articular cartilage. Since the target for IGF-1 is the extracellular domain of a cell-surface receptor, and a supercharged GFP with a net charge of +9 was found to be optimal for extracellular delivery [227], we designed six different fusion proteins of +9 GFP and IGF-1 with different polypeptide linkers. After being expressed and purified, all six fusion proteins were fluorescent and were positive on a Western blot with anti-IGF antibody, indicating that both the GFP and IGF-1 domains

were present in each of them. We found that the fusion proteins had enhanced uptake into human cartilage and were able to penetrate through the full thickness. Two of the fusion proteins had sustained IGF-1 bioactivity in *in vitro* cultures of human cartilage explants: single doses were able to sustain increased proteoglycan biosynthesis for approximately 1 week and rescue the loss of biosynthesis induced by IL-1 β for 7 to 10 days. Neither continuous free IGF-1, nor our bioactive fusion proteins, were able to inhibit aggrecanase activity or reduce sGAG loss from explants treated with IL-1 β , which is consistent with previous findings using free IGF-1 in human cartilage [186].

3.2 Materials and Methods

3.2.1 Fusion Protein Design

Insulin-like growth factor 1 (IGF-1) was fused to +9 GFP via flexible and rigid polypeptide linkers of different sizes. In each case, the C-terminus of the +9 GFP was attached to the linker, which in turn was attached to the N-terminus of IGF-1. The linkers used in this study were: (GGGGS)₄ (flexible), (GGGGS)₃ (flexible), (GGS)₉ (flexible), (EAAAK)₃ (rigid) and A(EAAAK)₄ALEA(EAAAK)₄A (rigid). In addition to five fusion proteins with these linkers, a sixth fusion protein with no linker was also included. Each of the six fusion proteins were designed to have an N-terminus His₆ tag. The codon sequences for the His₆–+9GFP–linker–IGF-1 fusions were optimized for *E. coli* and inserted into the pET-29b(+) vector by Genscript, and purified plasmids were shipped to us.

3.2.2 Protein Expression and Purification

For each fusion protein, BL21 Star (DE3)-competent *E. coli* cells were transformed with the appropriate plasmid and a well-isolated colony was grown overnight in 10 mL Luria-Bertani (LB) broth containing kanamycin at 37 °C (Figure 3.1B). This starter culture was then diluted into 1 liter of LB broth with kanamycin, and grown at 37 °C until OD₆₀₀ 0.8-1. Following this, fusion protein expression was induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG)(GoldBio) and cultures were sustained at 16 °C with shaking

for 20 hours. Cells were collected by centrifugation at 4000 rpm for 15 minutes at 4 °C, and the cell pellets were frozen at - 80 °C.

The cell pellets were defrosted and resuspended in 25 mL lysis buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 8.0, 20% glycerol, 1M NaCl, half tablet of complete EDTA-free protease inhibitor pellet (Roche), 0.4 mM phenylmethane sulfonyl fluoride (PMSF; Sigma-Aldrich) and 5 mM tris(2-carboxyethyl)phosphine (TCEP; GoldBio)). The resuspended cells were lysed by sonication (5 min total time; 3 s on, 2 s off) and centrifuged at 12,500rpm for 60 minutes. The expressed fluorescent proteins were present in the supernatant and this was collected for purification (Figure 3.1B).

The fusion proteins were first purified using immobilized metal affinity chromatography. 3 mL of HisPur™ nickel nitriloacetic acid (nickel-NTA) resin slurry (ThermoFisher; 50 % slurry in 20 % ethanol) was centrifuged for 2 minutes at 700g and the supernatant was discarded. The settled resin (1.5 mL) was washed twice by resuspending in 15 mL lysis buffer, centrifuging at 700g and discarding the supernatant. The supernatant from the cell lysis step (containing the expressed fusion proteins) was added to the washed nickel-NTA resin and mixed on an end-over-end rotator in the cold room for 90 minutes. The resin was washed twice with 15 mL of lysis buffer before bound protein was eluted with elution buffer ((100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 8.0, 20% glycerol, 0.5 M NaCl, 5mM TCEP (GoldBio), 200mM imidazole). The resulting protein fraction was further purified on a 5 ml Hi-Trap HP SP (GE Healthcare) cation exchange column using an Akta Pure FPLC. An NaCl gradient (from 0 – 1 M) was used to elute the fusion protein from the HP SP column. The protein containing fractions were analyzed in an SDS-PAGE gel (NuPAGE™ 4-12% Bis-Tris Protein gel; Invitrogen), and fractions containing pure fusion protein were combined.

Depending on the volume, the combined fraction was concentrated using either a Centricon®-Plus 70 centrifugal unit (10 kDa cutoff; Millipore) or an Amicon®Ultra-15 centrifugal unit (10 kDa cutoff; Millipore) centrifuged at 3,500g. The concentrated proteins were sterile-filtered through a 0.2 µm pore size sterile syringe filter (VWR), quantified with the Reducing Agent Compatible Bicinchoninic acid (BCA) assay (Pierce Biotechnology), snap-frozen in liquid nitrogen and stored in aliquots at -80 °C. The concentrated, sterile filtered

proteins were analyzed in an SDS-PAGE gel followed by a western blot with polyclonal anti-IGF-1 antibody (Abcam, Cambridge, MA).

3.2.3 Human Cartilage Explant and Chondrocyte Harvest

Seven human knee joint distal femurs and twenty seven ankle joints obtained from twenty different donors were obtained from donor banks (Gift of Hope Organ and Tissue Donor Network (Itasca IL), LifeNet Health (Virginia Beach VA), and National Disease Research Interchange (Philadelphia PA)). Eight ankles and one knee were used for harvesting chondrocytes, and explants were harvested from the remaining joints. The age of the donors and the Collins grade of the joints are tabulated in (Table S3.1). All procedures were approved by the Committee on the Use of Humans as Experimental Subjects at MIT. Femoral cartilage plugs (3 mm diameter) were harvested using a biopsy punch. Explants that were thicker than 1 mm were trimmed to 1 mm explants that included the superficial zone. All explants were equilibrated for 48 to 72 hours (37 °C, 5% CO₂) in low glucose (1 g/L) DMEM (Corning Cellgro, Manassas, VA) which was supplemented with 10 mM HEPES buffer (Gibco, Grand Island, NY), 0.4 mM proline (Sigma), 20 µg/mL ascorbic acid (Sigma), 0.1 mM nonessential amino acids (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Sigma).

To extract live chondrocytes, solutions of collagenase (1.25 mg/mL) and pronase (2 mg/mL) were prepared in low glucose DMEM with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA) and 0.4 mM proline. Cartilage tissue slices were harvested from human ankles, washed with PBS and incubated in the pronase solution (37 °C, 5% CO₂) for 1 hour. This was followed by an overnight incubation in the collagenase solution at 37 °C and 5% CO₂. The final solution was then passed through a 70 µm filter and a 40 µm filter to remove any undigested cartilage fragments. The filtrate was centrifuged at 400 g for 8 minutes and the pellet was resuspended in 50 mL PBS. Viable cell counts were obtained from aliquots of this suspension using a hemocytometer and Trypan blue. The cells were then centrifuged out of the suspension (400 g, 8 minutes),

resuspended in freezing medium (FBS with 5% DMSO) and stored in liquid nitrogen until use.

3.2.4 Quantitative uptake of fusion proteins in human cartilage

Cartilage explants that had been equilibrated in culture medium post-harvest were transferred to sterile PBS and allowed to equilibrate for 1 hour in preparation for addition of fusion proteins to measure their uptake into cartilage. Following this, 1 μ M solutions of fusion proteins with (GGGGS)₄, (EAAAK)₃ and (GGGGS)₃ linkers and +9 GFP (the free carrier) were prepared in sterile PBS + 1% bovine serum albumin (BSA). The BSA was included in the PBS to minimize loss of GFPs from the solution caused by non-specific binding to the plasticware. (Since the main function of the albumin in these experiments was only to prevent such non-specific binding in the external solution, we used BSA instead of human serum albumin (HSA) to maintain consistency with previous experimental techniques [227].) Groups of 5 cartilage explants (matched for position along the joint surface) were assigned to each treatment group and to the untreated control group. Each explant was separately incubated in 250 μ L solutions in sterile polypropylene vials for 36 hours or for 4.5 days (37 °C, 5% CO₂), with GFP solutions in PBS + 1% BSA for treatment groups and PBS + 1% BSA for untreated controls. (Standard 96-well cell culture plates were not used because the +9 GFP domain could stick to polystyrene even in the presence of BSA, leading to a loss of protein. In contrast, use of polypropylene with 1% BSA enabled complete recovery of fluorescence).

At the end of the incubation period, cartilage explants were washed with PBS and transferred to a solution of 10X PBS + 1% BSA for desorption. At the same time, the fluorescence of the absorption bath was measured using a plate reader (Synergy H1, Biotek Instruments Inc.) (excitation: 485 nm, emission: 528 nm). This was used along with a standard curve to obtain the concentration of GFP left behind in the absorption bath. At the end of the desorption in 10X PBS + 1% BSA, cartilage explants were weighed and the fluorescence of the desorption bath was measured. This fluorescence measurement was used with a standard curve and the explant wet weights to calculate the concentration of

GFP inside cartilage explants (mol/mg wet weight). The uptake ratio was calculated as the ratio of GFP concentration inside cartilage (mol/mg wet weight) to the final absorption bath concentration (mol/mL).

3.2.5 Fusion protein penetration into cartilage

Human cartilage explants were cultured in ultra-low attachment cell culture plates (Corning) in 1 μ M solutions of fusion proteins dissolved in low glucose (1 g/L) DMEM supplemented with 10 mM HEPES buffer, 0.4 mM proline, 20 μ g/mL ascorbic acid, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin (37 °C, 5% CO₂). Fusion proteins were added only at the start of the experiment, and fresh media without fusion proteins was replenished every 2 to 3 days. An untreated control group and a treatment group with continuous dosage of free IGF-1 were also included in the experiments. Explants were taken out of culture at different time points and washed with PBS for approximately 60 minutes (to wash out any culture medium). These were then sectioned longitudinally into $\sim$ 100 μ m slices and washed once again with PBS.

Explant slices were imaged at 10X magnification using a confocal microscope (Olympus FluoView FV1000), and Z-stacks were obtained with a voxel depth of 4.22 μ m. 3D cartilage Z-stacks were flattened to 2D using the Z project function available in the Fiji software package. During imaging, some cartilage explants were too large to fit into the field of view of the confocal microscope. In these cases, two overlapping Z-stacks were captured and combined using the Stitching plugin available in Fiji [215] before performing 2D Z projections.

3.2.6 Cartilage biosynthesis, ARGS-aggrecan release, cumulative sGAG loss and explant sGAG content

To measure the chondrocyte biosynthesis rate of sulfated glycosaminoglycans (sGAGs), cartilage explants were cultured in the presence of 10 μ Ci/mL ³⁵S-sulfate radiolabel during the final two days of each experiment. Explants were then washed with PBS with non-

radioactive sulfate to wash out excess radiolabel, weighed and digested with proteinase K. A liquid scintillation counter (PerkinElmer) was used to measure the amount of radiolabel incorporated within the extracellular matrix of the tissues. Radiolabel incorporation data were normalized by the duration of exposure to radiolabel and by the wet weight of the explants [186]. The levels of aggrecanase cleavage fragments (ARGS-aggrecan, produced from cleavage at the TEGE³⁹²↓³⁹³ARGS site) released into the culture medium were quantified using an assay from Meso Scale Discovery (MSD) as described in [236]. To calculate the cumulative loss of GAGs from the explants, the amount of sGAG lost to the medium and that left behind in the explants at the end of the experiment were measured using the dimethylene blue (DMMB) dye binding assay [217]. The final sGAG contents of the explants were normalized with their wet weights.

3.2.7 Biological response to GFP-IGF-1 fusion proteins

Cartilage explants that were equilibrated in culture medium post-harvest were transferred to ultra-low attachment cell culture plates (Corning) for fusion protein dose response experiments, carried out in low glucose, serum free medium (same composition as equilibration medium). Previous studies of stimulation of sGAG biosynthesis in cartilage explants by free IGF-1 on cartilage explants have used doses ranging from 10 ng/ml to 300 ng/ml (*Insert references*); therefore, we chose 300 ng/ml (equivalent to 39.2 nM) of recombinant human IGF-1 (PeproTech, Rocky Hill, NJ) as the positive control. Since each fusion protein had one IGF-1 domain, we chose doses of 39.2 nM (to match free IGF-1 concentration of 300ng/mL), as well as 500 nM, 1 μ M and 2 μ M. In all cases, only a single dose of the fusion proteins was added at the start of the experiment, and media changes were carried out without fusion proteins once every 2 to 3 days thereafter. In contrast, continuous treatment with free IGF-1 were used for the positive control, and an untreated control group was also included. Experiments were terminated at 3, 7, and 15 day time points. Outcome measures included the biosynthesis rate of sulfated glycosaminoglycans (sGAG), sGAG loss to the culture medium and the explant sGAG content at the end of the experiment.

3.2.8 Biological response to GFP-IGF-1 fusion proteins in the presence of IL-1 β

We first tested the effects of human IL-1 β (PeproTech, Rocky Hill, NJ) on human ankle cartilage explants from two different donors, cultured in serum-free low glucose medium (same composition as equilibration medium) and treated with continuous IL-1 β doses ranging from 10 pg/ml to 1 ng/ml for 8 days, $\pm$ 300 ng/ml free IGF-1 at each dose of IL-1 β . Based on the results, 100 pg/ml IL-1 β was chosen as the concentration for the fusion protein dose response experiments. Ankle and knee cartilage explants (equilibrated in culture medium post-harvest) were treated continuously with IL-1 β . Single doses of fusion proteins (ranging from 39.2 nM to 2 μ M) were added to treatment groups at the start of the experiments. Media changes with IL-1 β but without fusion proteins were performed once every 2 to 3 days. An untreated control group, a control with continuous IL-1 β alone, and a control with IL-1 β combined with continuous free IGF-1 (300 ng/ml or 39.2 nM) were included. Experiments were terminated at 7 and 10 days, and the sGAG biosynthesis rate, sGAG loss to culture medium and sGAG content of the explants were measured.

3.2.9 Chondrocyte Akt signaling studies

Human chondrocytes from 4 different donors (stored in liquid nitrogen) were resuspended in DMEM/Ham's F12 supplemented with 10 % fetal bovine serum (FBS). Monolayer cultures were plated on tissue culture treated 24 well plates. The target seeding density was 250,000 cells/cm², but due to the limited number of cells obtained from each donor and the requirement that cells from each donor are included in every treatment group, the actual seeding density varied from 114,000 cells/cm² to 234,000 cells/cm² depending on the donor. Media changes were performed with DMEM/Ham's F12 supplemented with 10 % fetal bovine serum (FBS) every two days. The cells reached approximately 90 % confluence after 5 to 6 days in culture.

Cells were then washed with serum-free DMEM/Ham's F12, and incubated in serum-free DMEM/Ham's F12 for a day before being treated with the fusion proteins. For each donor, treatment groups with all six fusion proteins, an untreated control group, a positive

control with free IGF-1, and a carrier control with free +9 GFP were included. Following these treatments, the cell culture plates were placed on ice, culture medium was removed from all the wells, and each well was washed with 1 mL ice-cold heparin dissolved in PBS (20 U/mL) (to remove any +9 GFP in the fusion proteins and the free-carrier control that may have been bound to cell surface proteoglycans). The untreated control and the free IGF-1 control were also washed with heparin dissolved in PBS for consistency. Cells in each well were then washed with 1 mL ice-cold plain PBS. After this, 100 μ L cell lysis buffer supplemented with protease and phosphatase inhibitors (provided by Meso Scale Discovery) was added to each well, the cells were scraped using sterile cell-scrapers, and the suspension was transferred to microcentrifuge tubes. These tubes were rotated end-over-end for 30 minutes in the cold room. The tubes were then centrifuged at 13,000g for 10 minutes at 4 °C, and the supernatant was collected. In some experiments, the supernatants were immediately used for downstream assays. In other experiments, the supernatants were snap-frozen in a dry ice-ethanol bath, stored at -80 °C, and the downstream assays were performed at a later date. The total protein content in the supernatants were measured using the micro BCA assay. The levels of phosphorylated Akt and total Akt were measured using multiplex ELISA assay (Phospho(Ser473)/Total Akt Whole Cell Lysate Kit; Meso Scale Discovery).

3.2.10 Statistical Analysis

Statistical analysis was carried out using MATLAB and JMP Pro 14 software packages. All quantitative data are plotted as Mean $\pm$ 95% Confidence Interval. For experiments with a single human donor and multiple location-matched explants per condition, a one-way ANOVA followed by the post-hoc Tukey's HSD test was performed. Data from experiments with multiple human donors were analyzed using the linear mixed effects model with human donor as a random factor followed by the post-hoc Tukey's HSD test. In both cases, p-values less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Characterization of purified fusion proteins

During the FPLC step of protein purification, the fusion proteins with linkers were eluted as a single sharp peak which was collected in several fractions (a representative FPLC trace is shown in Figure S3.1A). Fractions containing pure protein (as characterized by SDS PAGE) were pooled. The fusion protein with no linker was eluted as two peaks (FPLC trace is shown in Figure S3.1B). Once again, fractions containing pure protein were pooled, but the fractions from each peak were pooled separately. After these pooled samples were concentrated and sterile filtered, the purity of the protein was confirmed through SDS PAGE (Figure 3.1C).

All the fusion proteins were fluorescent, indicating that the +9 GFP domain was present and had folded properly. To confirm that they also had the IGF-1 domain, a western blot with polyclonal anti-IGF-1 antibody was performed. A control with free IGF-1 and a control with +9 GFP (free carrier control) were also included in the blot. Highly intense bands were found in the lanes with the free IGF-1, the five fusion proteins with linkers, and one of the two elution peaks of the fusion protein without a linker (Figure S3.2). The lane with the other peak for the fusion protein without a linker had a faint band, and no band was found in the lane with +9 GFP. This confirmed that all the fusion proteins had the IGF-1 domain.

The lanes for three of the fusion proteins had bands with molecular weights that were approximately twice and three times the theoretical molecular weights of the proteins, indicating that these correspond to dimers and trimers. The IGF-1 lane also had a second band which most likely corresponds to a dimer.

3.3.2 Uptake and penetration of fusion proteins in human cartilage

1 μ M solutions of +9 GFP, two fusion proteins with flexible linkers ((GGGGS)₄, (GGGGS)₃) and one fusion protein with a rigid linker ((EAAAK)₃) were prepared in PBS + 1% BSA. The uptake ratios of these 4 proteins were quantified in human ankle cartilage after 36 and

108 hour incubations (37 °C, 5% CO₂). At 36 hours, the concentrations of all three fusion proteins was approximately 10 to 20 times higher in cartilage compared to the final bath (Figure 3.2A and Table S3.2), demonstrating greatly enhanced uptake of the fusion proteins into cartilage. At the same time, these values were significantly lower than the uptake ratio of +9 GFP.

Similar trends were observed at the 108 hour time point as well (Figure 3.2B and Table S3.2). Additionally, the uptake ratios of each fusion protein was higher after a 108 hour incubation compared to a 36 hour incubation; however, the increase observed in +9 GFP uptake ratio from 36 to 108 hours was much higher than that observed for any of the fusion proteins.

To visualize the extent of penetration of the fusion proteins in cartilage, human knee and ankle cartilage explants treated with a single dose (1 µM) of the proteins and were cultured in serum-free, low glucose medium for 7 to 8 days. Media changes were performed every 2 to 3 days. Subsets of explants were terminated at different time points, washed with PBS, sliced longitudinally and imaged using confocal microscopy. These images as well as the intensity profiles (Figure 3.3) show that all three fusion proteins penetrated through the full ~1.5mm thickness of cartilage within the first 1 to 2 days. *(Write a sentence or two for the Day 7 and Day 8 images.)*

3.3.3 Single doses of two fusion proteins sustain elevated sGAG biosynthesis in human cartilage for at least 1 week

Treatment with IGF-1 increases the sGAG biosynthesis rates in cartilage, but maintaining this increase requires continuous addition of IGF-1 to the medium (Figure S3.3). Four of the fusion proteins used in the present study (the protein with no linker, and the proteins with the (GGGGS)₃, (GGS)₉ and the A(EAAAK)₄ALEA(EAAAK)₄A linkers) did not increase the sGAG biosynthesis rates compared to the untreated controls at any concentration (Figure S3.5). 39.2 nM doses of the remaining two fusion proteins (equivalent to 300 ng/ml free IGF-1) also did not increase the biosynthesis rate of explants compared to the untreated control (Figure S3.4). However, 500 nM, 1 µM and 2 µM doses of these fusion proteins

(with the (GGGGS)₄ linker and the (EAAAK)₃ linker) led to a significant increase in the sGAG biosynthesis rates in both ankle and knee cartilage as measured between Day 0 and Day 3 of the culture (Figure 3.5). This increase was comparable to the biosynthesis increase caused by treatment with free IGF-1 during the same time period.

While fusion proteins were only added on day 0 of culture, elevated biosynthesis induced by the (GGGGS)₄ and the (EAAAK)₃ groups was sustained up to Day 7 of the culture. However, at Day 15, biosynthesis rates were not different than that in controls. The increase in biosynthesis caused by continuous 39.2 nM doses of free IGF-1 at Days 3, 7 and 15 are shown for comparison (Figure 3.5).

In one of the experiments, a free carrier control (500 nM, 1 μ M and 2 μ M of +9 GFP) was included to confirm whether or not the drug delivery carrier had an effect on the sGAG biosynthesis rate. There was no statistically significant difference in sGAG biosynthesis rates with any +9 GFP concentrations compared to untreated controls (Figure S3.7).

3.3.4 Effect of single doses of fusion proteins on final explant sGAG content and cumulative sGAG loss to medium

For the explants used in Figure 3.5, treatment with a single dose of fusion proteins did not affect the final explant sGAG content (normalized to explant wet weight) at any of the three time points (Figure 3.6). The cumulative percent sGAG loss to the culture medium was also comparable to that in untreated controls at day 3 and 7. However, some of the fusion protein groups elicited a modest but statistically significant increase in the cumulative GAG loss compared to the untreated controls by day 15 (statistical comparisons shown in Table 3.3). Explants treated with free IGF-1 did not show any change in the final explant GAG content or cumulative GAG loss compared to untreated controls at any of the three time points.

3.3.5 Biosynthetic response of human ankle cartilage to different doses of IL-1 β with and without free human IGF-1

In preliminary experiments, fusion proteins were tested in a cytokine injury model using 10 ng/ml mouse IL-1 β . In these experiments, continuous treatment with 39.2 nM doses

(equivalent to 300 ng/ml) of recombinant IGF-1 rescued the biosynthesis loss caused by IL-1, but equivalent single doses (39.2 nM) of the fusion proteins did not (Figure S3.6). Upon switching to human IL-1 β (instead of mouse IL-1 β), even the continuous free IGF-1 treatment could not rescue biosynthesis loss even though the IL-1 dose was reduced from 10 ng/ml to 1 ng/ml (data not shown). Before testing the fusion proteins further in this injury model, we performed dose response experiments to determine optimal human IL-1 β dose levels such that the injury caused could be rescued by IGF-1.

Ankle cartilage explants from two donors were treated continuously for 8 days with doses of recombinant human IL-1 β (rhIL-1 β) ranging from 10 pg/ml to 1 ng/ml. At each dose, half the explants were also treated with continuous 300 ng/ml doses of IGF-1. Each experiment included an untreated control group. sGAG biosynthesis rates measured during the last two days of the experiment decreased with increasing doses of IL-1 β . Adding IGF-1 partially rescued this decreased biosynthesis, but this rescue was weaker with increasing IL-1 concentrations.

Treatment with IGF-1 in the presence of 10 pg/ml IL-1 β resulted in a biosynthesis rate that surpassed the untreated control biosynthesis in both donors. On the other hand, the explants receiving IGF-1 and 500 pg/ml or 1 ng/ml IL-1 β in the 58 year old male donor had significantly lower biosynthesis compared to the untreated control. In both donors, a 100 pg/ml dose of IL-1 β resulted in a significant loss of biosynthesis and adding IGF-1 brought back the biosynthesis rate to a level that was not statistically significantly different from the untreated control. Based on these results, 100 pg/ml rhIL-1 β was used for all other cytokine challenge experiments.

3.3.6 Single doses of the two bioactive fusion proteins rescued decreased biosynthesis induced by IL-1 β in human ankle cartilage

Human ankle cartilage explants from six different donors were treated continuously with 100 pg/ml IL-1 β for 7-10 days, with media changes performed every 2-3 days. The treatment groups received single doses (500 nM, 1 μ M or 2 μ M) of the fusion proteins with the (GGGGS)₄ and (EAAAK)₃ linkers (which were bioactive in the experiments in Figure 3.5).

An untreated control, a control with 100 pg/ml IL-1 β , and a group with 100 pg/ml IL-1 β + 300 ng/ml IGF-1 were included in each experiment. Explants from four donors were terminated on day 7 of the experiment, while the experiments from the other two donors were terminated on day 10.

In both the 7 day and 10 day experiments, biosynthesis rates in the IL-1 β groups are significantly lower than that of untreated controls (Figure 3.8A,B). Continuous treatment with free IGF-1 leads to a complete rescue of biosynthesis loss, and the free IGF-1 groups in both experiments are significantly higher than the IL-1 β group and not significantly different compared to the untreated control.

At day 7 (Figure 3.8A), a single 2 μ M dose of the (GGGGS)₄ linker protein could completely rescue the loss of biosynthesis caused by IL-1 β ; biosynthesis was significantly higher than in the IL-1 β group and not significantly different than untreated controls. Additionally, a single 1 μ M dose of the (EAAAK)₃ linker protein achieved a partial rescue of biosynthesis loss: biosynthesis was significantly higher than the IL-1 β group but also significantly lower than the untreated control.

By day 10 (Figure 3.8B), single doses of the (GGGGS)₄ linker protein (1 μ M and 2 μ M) and the (EAAAK)₃ linker protein (2 μ M) partially rescued the biosynthesis loss caused by IL-1 β . All three groups were significantly higher than the IL-1 β group but also significantly lower than the untreated control.

In both the 7 day and 10 day experiments, there were no differences in the final sGAG content of the explants (Figure 3.8D). However, at the 7 day time point, all the fusion protein groups and the free IGF-1 group had higher cumulative sGAG loss to the medium compared to the untreated control (Figure 3.8C). At day 10, the IL-1 β group and the groups receiving 500 nM (GGGGS)₄ linker protein and 2 μ M (EAAAK)₃ linker protein had higher cumulative sGAG loss compared to the untreated control.

The ARGS-aggrecan concentrations in the culture medium of the explants in Figure 3.8 were measured at days 3, 5 and 7 (Figure 3.9). Most of the concentrations measured for the untreated control group were below the lowest assay standard, and all of them were below the LLOQ. Treatment with IL-1 β caused an increase in ARGS-aggrecan concentration at all three time points. At each time point, the concentrations in the free IGF-1 and the fusion

protein groups were similar to the concentrations in the IL-1 β group (Figure 3.9). These data are consistent with the increased cumulative sGAG loss seen in (Figure 3.8C).

3.3.7 Effects of fusion protein and free IGF-1 in an IL-1 β challenge model with human knee cartilage

Human knee cartilage explants from two donors were treated continuously with 100 pg/ml rhIL-1 β , with media changes every 2-3 days. One donor was an 80 year old male (Collins grade 2, early OA). The second donor was a 69 year old female with Collins grade 1 cartilage (near normal). Treatment groups received single doses (500 nM, 1 μ M or 2 μ M) of the fusion proteins with the (GGGGS)₄ and (EAAAK)₃ linkers (which were bioactive in the experiments in Figure 3.5). An untreated control, a control with 100 pg/ml IL-1 β , and a group with 100 pg/ml IL-1 β + 300 ng/ml IGF-1 were included in each experiment. Explants from the 80 year old male donor were terminated on day 7 while those of the female donor was terminated on day 10.

At day 7 (Figure 3.10A), the biosynthesis rate for the IL-1 β group was not significantly different from that of the untreated control group. A potential reason for this could be that since the cartilage was from a grade 2 joint, the cartilage had already been exposed to an inflammatory/osteoarthritic environment *in vivo*, and further treatment with IL-1 β *in vitro* did not have a significant effect. However, since the injury was not effective, we could not make any conclusions regarding the rescuing capacity of the fusion proteins or IGF-1 in this case.

In the 10 day experiment (Figure 3.10B), treatment with 100 pg/ml IL-1 β caused a significant loss in sGAG biosynthesis compared to control. However none of the treatment groups, including continuous free IGF-1 doses, could rescue the biosynthesis loss caused by IL-1. The IL-1 dose level (100 pg/ml) was chosen based on dose response experiments with ankle cartilage (Figure 3.7). Previous studies have indicated that while inflammatory cytokines have similar qualitative effects on ankle and knee cartilage, the quantitative effects are significantly different and the deleterious effects on knee cartilage are more severe than the effects seen in ankle cartilage (*insert citations*).

In order to test the effects of the fusion proteins in a cytokine challenge model with knee cartilage, we would need to repeat the cytokine dose response experiments with knee cartilage from multiple donors belonging to different age groups and different stages of OA progression. There may also be differences depending on gender, and these would also need to be investigated. Future steps for this project could include completing these dose response studies, determining dose(s) of IL-1 β at which free IGF-1 can rescue biosynthesis, and testing the fusion proteins at these optimal cytokine doses.

3.3.8 Bioactivity of fusion proteins in primary human chondrocytes

Primary human chondrocytes from four different donors were cultured in high density monolayers until they reached 80 to 90% confluence. Cells from each donor were treated with fusion proteins, +9 GFP, or free IGF-1 after serum starvation. An untreated control group was also included for each donor.

Both the 40 nM and 1 μ M doses of free IGF-1 led to a significant increase in the levels of phosphorylated Akt, consistent with previous studies (*Insert citation*). Treatment with +9 GFP (40 nM or 1 μ M) did not cause any changes in pAkt levels compared to untreated controls. At the 40 nM dose level, all the fusion protein treatment groups had pAkt levels that were not significantly different from the untreated control (Figure S3.8). At the 1 μ M dose level, treatment with four fusion protein variants (with the (GGGGS)₃ linker, (GGS)₉ linker, A(EAAAK)₃ALEA(EAAAK)₃A linker and with no linker) did not cause any changes in the pAkt levels compared to the untreated control.

For each of the four donors, chondrocytes treated with 1 μ M doses of fusion proteins having the (GGGGS)₄ and the (EAAAK)₃ linkers had pAkt levels higher than the untreated control (Figure 3.11). These changes were much more modest than the increases induced by free IGF-1, and were not statistically significant. However, these are the same fusion proteins that increased the sGAG biosynthesis rate in cartilage explants (Figure 3.5) and rescued the biosynthesis loss caused by IL-1 β (Figure 3.8). This indicates that the increase in pAkt phosphorylation is a real effect despite not being statistically significant. Additionally, since the +9 GFP by itself does not cause an increase in pAkt, we infer that the

increase in sGAG biosynthesis was caused by the IGF-1 domain in these fusion proteins.

3.4 Discussion

In this work, we fused a pro-anabolic drug, Insulin-like growth factor 1 (IGF-1), to a supercharged GFP with a theoretical net charge of +9 via polypeptide linkers. Once a drug is attached to the carrier, the conjugate needs to penetrate the full depth of human cartilage which has an average thickness of 1 to 3 mm [237][238]. The rate of diffusion is proportional to the inverse square of cartilage thickness. In commonly used small animal models such as mouse or rat models, the average cartilage thickness is approximately 100 μm and 200 μm respectively [238], which is an order of magnitude lower than the thickness of human cartilage. Thus, the rate of transport of drugs or drug-carrier-conjugates into human cartilage will be ~ 100 times slower than that into rodent cartilage. This means that even if a particular drug or drug-carrier conjugate of interest shows efficacy in small animal models by being able to penetrate cartilage before being cleared out, it may still get cleared from human joints before cartilage penetration. To avoid transport-related confounding factors and to obtain data that is more relevant for clinical translation to human subjects, we chose to test the +9GFP-IGF-1 conjugates in an *in vitro* model incorporating full thickness human cartilage.

The linker used to connect the two protein domains in a fusion protein can affect properties such as fusion protein expression, stability and importantly the biological activity of the two protein domains [239]. We designed, expressed and purified five fusion protein variants with different flexible and rigid linkers, and one fusion protein with no linker (Figure 3.1A). In each fusion protein, the N-terminus of the IGF-1 domain was connected to the C-terminus of the +9 GFP domain. This was done to block the IGFBP binding site that is located near the N-terminus of IGF-1, thereby minimizing off-target binding of IGF-1. All fusion proteins were fluorescent and showed up positive on a western blot with anti-IGF antibody (Figure S3.2). This demonstrated that they all had both the GFP and IGF-1 domains present, and the GFP domain had folded properly.

The fusion proteins had a lower quantitative uptake into human cartilage compared to

the carrier (+9 GFP) alone. Since the IGF-1 domain and the linkers are all close to net neutral charge, the lower uptake is potentially due to the larger size of the fusion proteins which slows down their transport. However, at the 36 hour time point, the concentration of the fusion proteins within cartilage was 10 to 20 times higher than their concentration in the bath (Figure 3.2). They were also able to penetrate through the full thickness of human cartilage within the first two days of incubation.

Four fusion proteins (with no linker and with the (GGGS)₃, (GGS)₉ and A(EAAAK)₄ALFA(EAAAK)₄A) did not lead to elevated sGAG biosynthesis in cartilage, and did not stimulate Akt signaling in chondrocytes. This indicated that the IGF-1 domain was perhaps not bioactive in these proteins, at least at concentrations less than or equal to 2 μ M.

The remaining two fusion proteins (with the (GGGS)₄ and with the (EAAAK)₃ linkers) were found to have IGF-1 bioactivity. A single dose of free IGF-1 was not able to sustain elevated sGAG biosynthesis in human cartilage, and continuous doses were required to achieve this effect (Figure S3.3). In contrast, single doses of the two bioactive fusion proteins could sustain elevated sGAG biosynthesis in human knee and ankle cartilage explants upto 1 week (Figure 3.5). However, these effects were only seen when the fusion protein doses were an order of magnitude higher than the dose of free IGF-1 that increased sGAG biosynthesis. A potential reason for this could be that the +9 GFP domain might restrict the access of IGF-1 to its cell surface receptor (IGF-1R) and thus receptor binding may be less efficient. Another possibility is that a large proportion of the fusion protein molecules are bound to extracellular matrix of cartilage explants due to electrostatic interactions between matrix glycosaminoglycans (GAGs) and +9 GFP. While such bound fusion proteins cannot access the cells immediately, the binding creates a drug depot which can result in slow release over a prolonged period as the molecules closer to cells get internalized.

The binding interactions with glycosaminoglycans (GAGs) could also explain the results of the monolayer chondrocyte experiments in which 1 μ M concentrations of the bioactive fusion proteins caused modest but non-statistically significant increases in pAkt levels that were much lower than the increases caused by free IGF-1. In these experiments, fusion proteins were added only after 5 to 7 days of culture. Chondrocytes in monolayer cultures

continue to synthesize and deposit extracellular matrix components including collagen II and aggrecan. It is possible that the +9 domain of the fusion proteins could bind to the aggrecan/GAGs deposited by the chondrocytes, thereby preventing the IGF-1 domain from accessing cell-surface receptors and causing less Akt pathway stimulation compared to free IGF-1.

Single doses of the (GGGS)₄ and (EAAAK)₃ fusion proteins were also able to either partially to completely rescue the IL-1 β induced biosynthesis loss in ankle cartilage after 7 to 10 days of culture. This was consistent with prolonged bioactivity seen in Figure 3.5.

Similar effects were not observed in knee cartilage (Figure 3.10). But these experiments failed primarily because the controls did not work as expected. In one donor, IL-1 β group was not statistically significantly different from untreated control. This modified Collins grade for this joint was 2, meaning that the donor already had OA, and the tissue was potentially exposed to pro-inflammatory cytokines *in vivo*. Due to this, the baseline biosynthesis levels of the explants could have been quite low to begin with (Figure 3.10A), and the IL-1 β added to the culture medium did not have a further deleterious effect.

In the second donor with near normal cartilage (modified Collins grade 1), IL-1 β led to a significant reduction in the biosynthesis rate. But even the free drug (free IGF-1) could not rescue the loss in biosynthesis. The IL-1 β dose (100 pg/ml) used in the experiments with knee cartilage explants was chosen based on dose response experiments that used ankle cartilage. There is prior evidence that knee cartilage may be more susceptible to cytokine damage compared to ankle cartilage (insert citation). Future work could possibly include performing careful dose response experiments of IL-1 β in knee cartilage cultures before repeating the fusion protein experiments.

Neither free IGF-1 nor the fusion proteins could rescue the elevated sGAG loss or aggrecanase cleavage fragment release caused by IL-1 β . This is consistent with previous studies where IGF-1 alone could not rescue GAG loss, and an anti-catabolic drug such as Dexamethasone was required to achieve this. Ongoing studies now focus on conjugating Dexamethasone to an appropriately charged GFP carrier, and testing it both individually and in combination with +9GFP-IGF-1 fusion proteins.

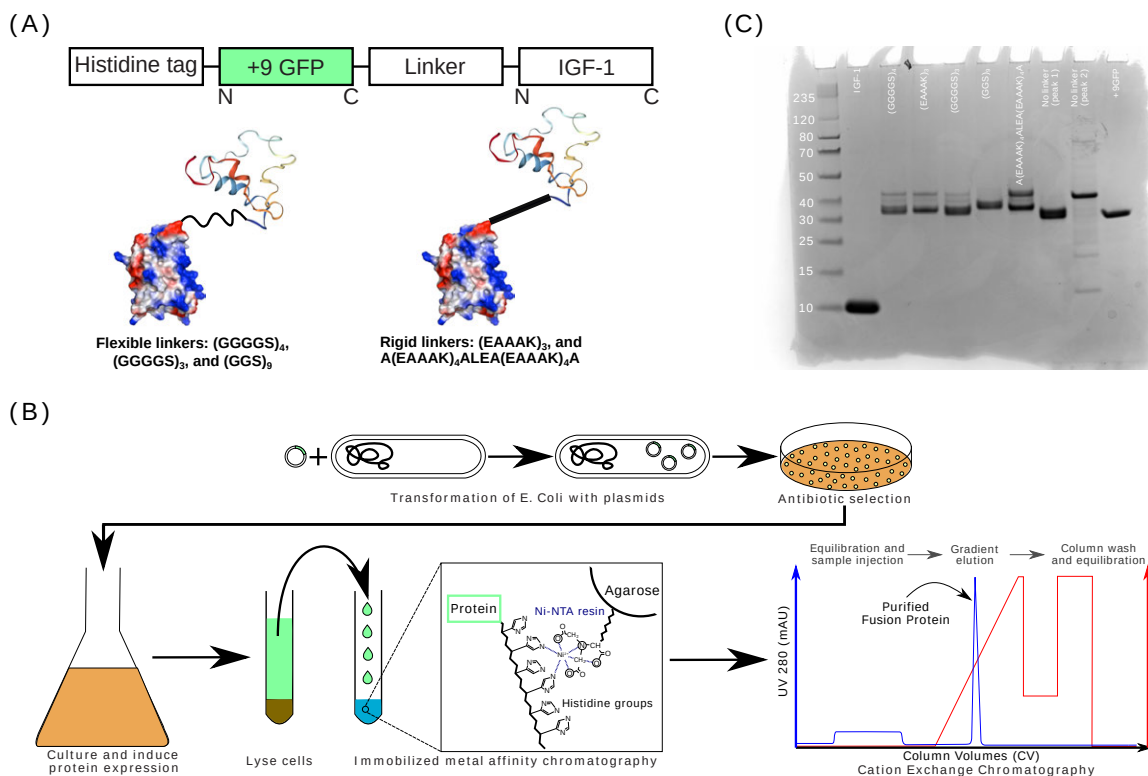


Figure 3.1: Design, synthesis and characterization of +9GFP-IGF-1 fusion proteins. (A) Fusion proteins were designed with +9 GFP at the N-terminus and IGF-1 at the C-terminus. Five different variants were designed with different flexible and rigid linkers. A sixth variant with no linker was also included. All six fusion proteins have an N-terminus 6xHis-tag. (B) Fusion proteins were expressed in *E. coli*, and purified using immobilized metal affinity chromatography (with Nickel NTA resin) followed by ion-exchange chromatography. (C) 4-12% Bis-Tris Protein gel showing recombinant human IGF-1, purified +9GFP-IGF-1 fusion proteins, and purified +9GFP. The western blot analysis for these proteins is shown in Figure S3.2.

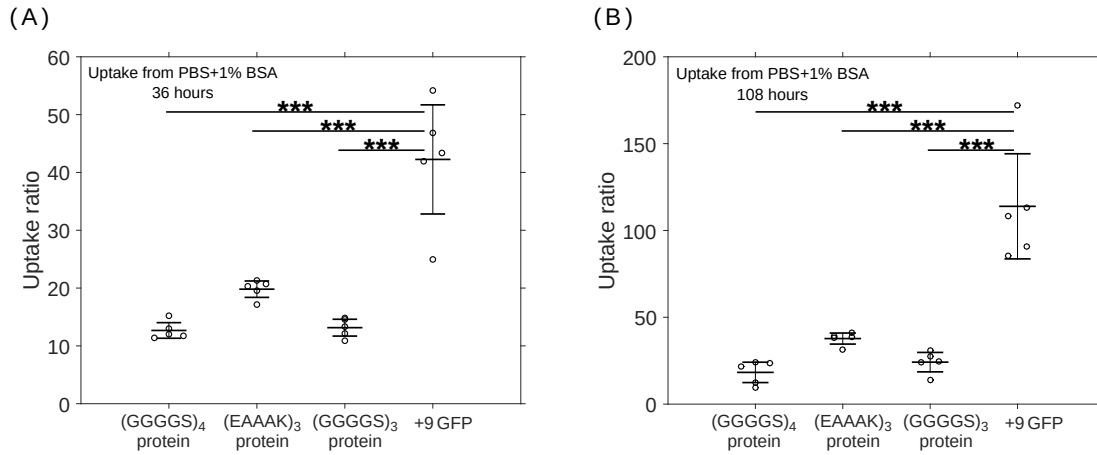


Figure 3.2: Uptake ratio of +9GFP-IGF-1 fusion proteins in human cartilage. Human ankle cartilage explants were incubated in 1 μ M solutions of +9GFP and +9GFP-IGF-1 fusion proteins dissolved in PBS + 1% BSA. 36 hour uptake ratios are shown in (A) and 108 hour uptake ratios are shown in (B). N=5 cartilage explants (matched for joint region) from 1 human donor were used for each condition and the individual data points, mean and 95% confidence interval are reported; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

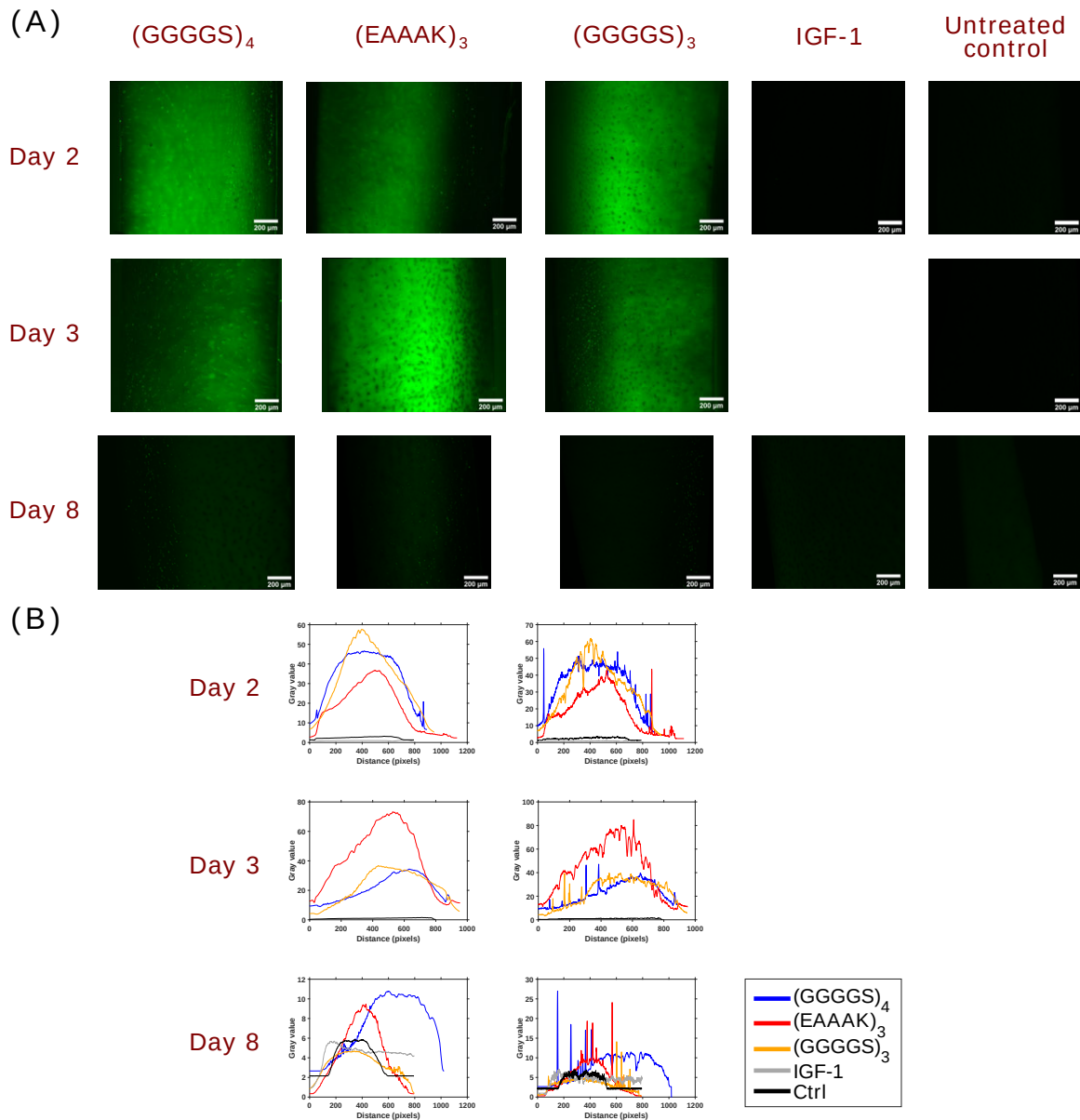


Figure 3.3: Fusion protein penetration in human knee cartilage explants (23 year old male donor). (A) Confocal microscopy images of cartilage explants treated with single 1 μ M doses of three different +9GFP-IGF-1 fusion protein variants. An untreated control and a group with continuous free IGF-1 dosage are included for comparison. (Scale bar: 200 μ m.) (B) Fluorescence intensities of the images in (A) are shown here. The X-axis is the distance in pixels from left to right of each image. The figures in the second column show the fluorescence intensities along a line within each image in (A). The fluorescence intensities were also vertically averaged over the entire height of each image and these are shown as a function of distance along the X-axis in the figures in the first column

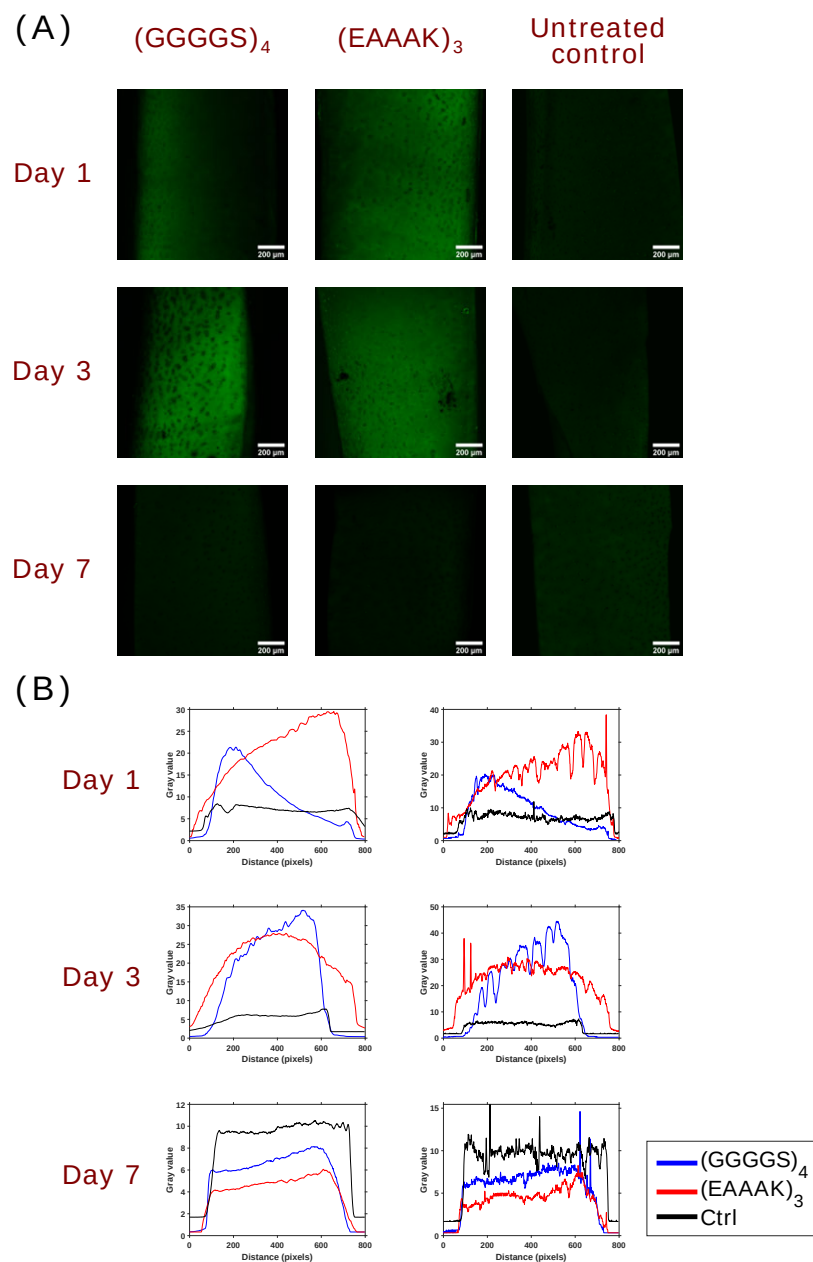


Figure 3.4: Fusion protein penetration in human ankle cartilage explants (36 year old female donor). (A) Confocal microscopy images of cartilage explants treated with single 1 μ M doses of two +9GFP-IGF-1 fusion protein variants. An untreated control is shown for comparison. (Scale bar: 200 μ m.) (B) Fluorescence intensities of the images in (A) are shown here. The X-axis is the distance in pixels from left to right of each image. The figures in the second column show the fluorescence intensities along a line within each image in (A). The fluorescence intensities were also vertically averaged over the entire height of each image and these are shown as a function of distance along the X-axis in the figures in the first column

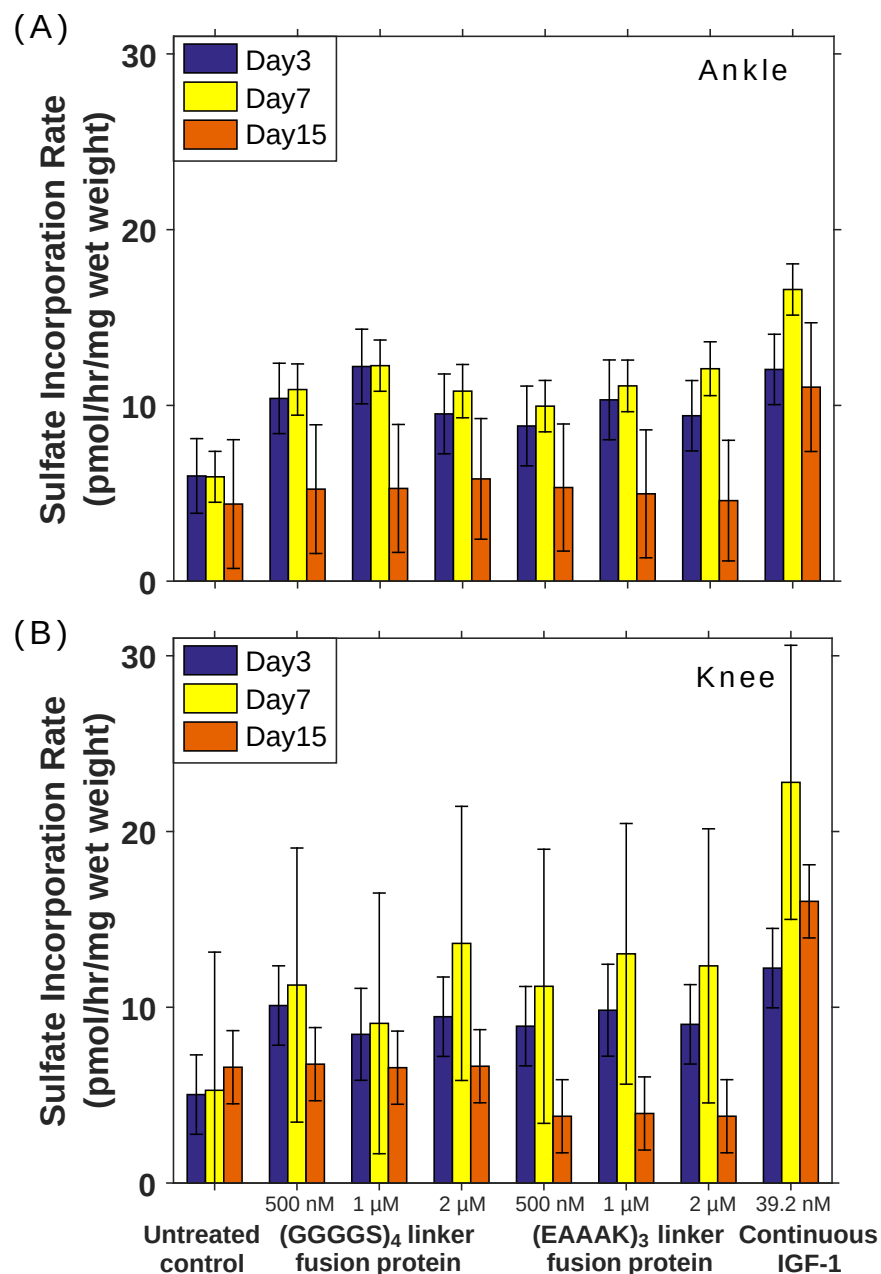


Figure 3.5: Explant biosynthesis, as measured by ^{35}S -sulfate incorporation rate in dose-response experiments using +9GFP-IGF-1 fusion protein variants. Biosynthesis rates during the last 48 to 72 hours of each experiment were normalized to explant wet weights. The results for human ankle cartilage are shown in (A). Explants from 1 donor ($N = 7$ to 9 explants/condition) were used for the 3 day experiment, 6 donors ($N = 6$ to 12 explants/condition/donor) for the 7 day experiment and 3 donors ($N = 6$ to 8 explants/condition/donor) for the 15 day experiment. The results for human knee cartilage are shown in (B). Explants from 2 donors ($N = 5$ explants/condition/donor) were used for the 3 day experiment, 3 donors ($N = 5$ to 6 explants/condition/donor) for the 7 day experiment and 1 donor ($N = 6$ explants/condition) for the 15 day experiment. All bars represent least squares means with 95% confidence intervals. Statistical comparisons are shown in Tables 3.1 and 3.2.

(A) 3 day experiment; ankle cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.0677	0.0027	0.3249	0.6014	0.1182	0.2852	0.0026
(GGGGS) ₄ protein	500 nM		0.9139	0.999	0.9665	1	0.9968	0.938
	1 μ M			0.6634	0.3787	0.9216	0.5418	1
	2 μ M				0.9999	0.9996	1	0.7039
(EAAAK) ₃ protein	500 nM					0.982	0.9999	0.4099
	1 μ M						0.9988	0.9436
	2 μ M							0.5809

(B) 7 day experiment; ankle cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
(GGGGS) ₄ protein	500 nM		0.522	1	0.8818	1	0.7609	$<10^{-4}$
	1 μ M			0.5095	0.0252*	0.7328	1	$<10^{-4}$
	2 μ M				0.9453	0.9999	0.7194	$<10^{-4}$
(EAAAK) ₃ protein	500 nM					0.7368	0.0911	$<10^{-4}$
	1 μ M						0.9008	$<10^{-4}$
	2 μ M							$<10^{-4}$

(C) 15 day experiment; ankle cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.879	0.8587	0.5099	0.8299	0.9845	1	<10⁻⁴
(GGGGS) ₄ protein	500 nM		1	0.9929	1	0.9999	0.9861	<10⁻⁴
	1 μ M			0.9956	1	0.9997	0.981	<10⁻⁴
	2 μ M				0.9977	0.9421	0.7712	<10⁻⁴
(EAAAK) ₃ protein	500 nM					0.9994	0.9727	<10⁻⁴
	1 μ M						0.9995	<10⁻⁴
	2 μ M							<10⁻⁴

Table 3.1: Post hoc comparisons (using Tukey's HSD) for the experiment in Figure 3.5A. The tables show p-values for the 3-day long experiment (Table A), 7-day long experiment (Table B), and 15-day long experiment (Table C). Significant p-values (less than 0.05) are shown in bold font.

(A) 3 day experiment; knee cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.0002	0.1715	0.0014	0.0074	0.0117	0.0054	<10⁻⁴
(GGGGS) ₄ protein	500 nM		0.9137	0.9984	0.9428	1	0.9651	0.4372
	1 μ M			0.9943	1	0.9797	0.9999	0.0968
	2 μ M				0.9995	1	0.9999	0.1409
(EAAAK) ₃ protein	500 nM					0.9969	1	0.039
	1 μ M						0.9986	0.6053
	2 μ M							0.0511

(B) 7 day experiment; knee cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.0073	0.4272	$<10^{-4}$	0.0084	0.0011	0.0006	$<10^{-4}$
(GGGGS) ₄ protein	500 nM		0.9338	0.8296	1	0.9779	0.9976	$<10^{-4}$
	1 μ M			0.2151	0.944	0.479	0.6326	$<10^{-4}$
	2 μ M				0.8076	1	0.9936	$<10^{-4}$
(EAAAK) ₃ protein	500 nM					0.9726	0.9965	$<10^{-4}$
	1 μ M						0.9999	$<10^{-4}$
	2 μ M							$<10^{-4}$

(C) 15 day experiment; knee cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		1	1	1	0.5483	0.6187	0.5488	$<10^{-4}$
(GGGGS) ₄ protein	500 nM		1	1	0.4718	0.5412	0.4723	$<10^{-4}$
	1 μ M			1	0.5597	0.6301	0.5602	$<10^{-4}$
	2 μ M				0.5232	0.5936	0.5237	$<10^{-4}$
(EAAAK) ₃ protein	500 nM					1	1	$<10^{-4}$
	1 μ M						1	$<10^{-4}$
	2 μ M							$<10^{-4}$

Table 3.2: Post hoc comparisons (using Tukey's HSD) for the experiment in Figure 3.5B. The tables show p-values for the 3-day long experiment (Table A), 7-day long experiment (Table B), and 15-day long experiment (Table C). Significant p-values (less than 0.05) are shown in bold font.

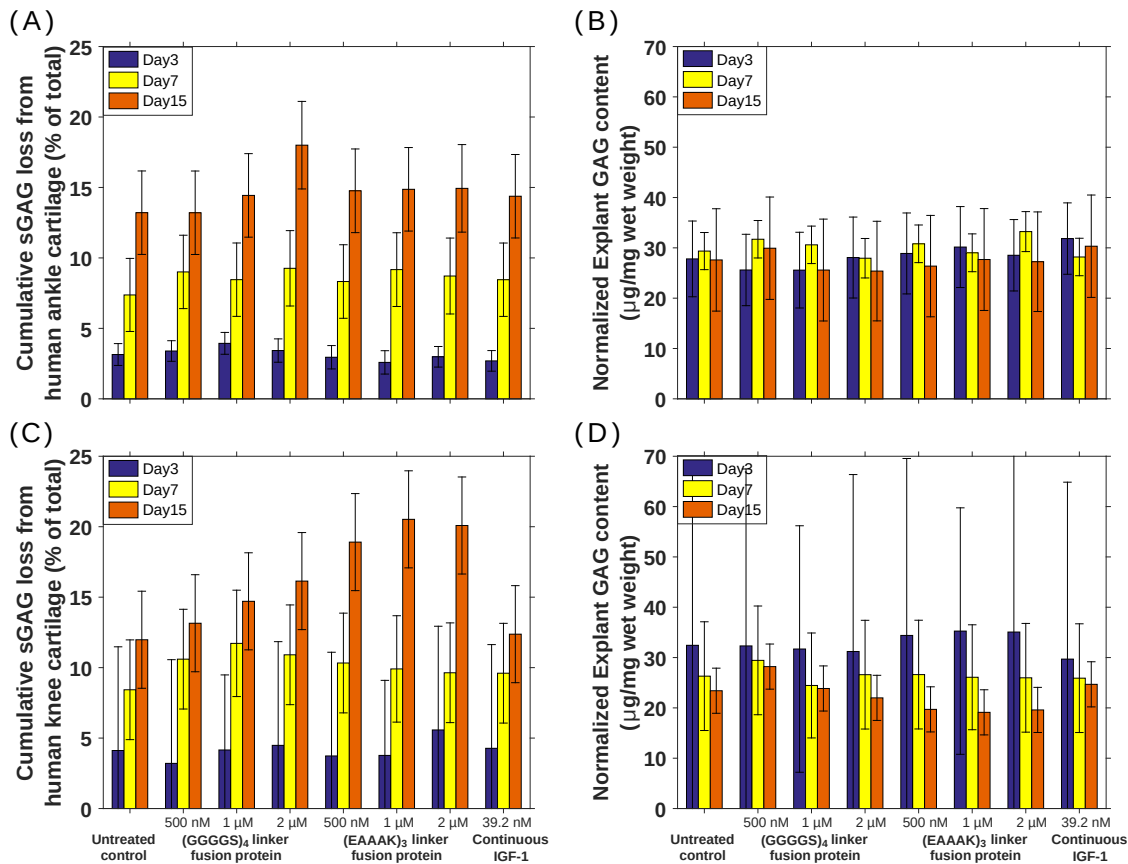


Figure 3.6: (A,B)Cumulative sGAG loss as a percent of total sGAG and final explant sGAG content (normalized to wet weight) for the ankle cartilage in the dose reponse experiments of Figure 3.5A. (C,D)Cumulative sGAG loss and final explant sGAG content (normalized to wet weight) for the knee cartilage dose response experiments of Figure 3.5B. All bars represent least squares means with 95% confidence intervals.

(A) 15 day experiment; ankle cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		1	0.9725	0.0168	0.9115	0.8716	0.9185	0.9777
(GGGGS) ₄ protein	500 nM		0.972	0.0167	0.9104	0.8702	0.9175	0.9772
	1 μ M				1	1	1	1
	2 μ M				0.3019	0.3362	0.4625	0.1636
(EAAAK) ₃ protein	500 nM					1	1	1
	1 μ M						1	0.9999
	2 μ M							0.9999

(B) 15 day experiment; knee cartilage

		(GGGGS) ₄ protein			(EAAAK) ₃ protein			IGF-1
		500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.9997	0.9457	0.6701	0.1051	0.0206	0.0329	1
(GGGGS) ₄ protein	500 nM		0.9979	0.9144	0.2742	0.0692	0.1044	1
	1 μ M			0.9988	0.6597	0.2625	0.355	0.9765
	2 μ M				0.9415	0.6115	0.7254	0.7686
(EAAAK) ₃ protein	500 nM					0.9973	0.9997	0.1489
	1 μ M						1	0.0315
	2 μ M							0.0495

Table 3.3: Post hoc comparisons (using Tukey's HSD) for the data in Figure 3.6A and C. p-values for the 15-day cumulative sGAG loss data in Figure 3.6A are shown in (Table A). p-values for 15-day cumulative sGAG loss data in Figure 3.6C are shown in (Table B). Significant p-values (less than 0.05) are shown in bold font.

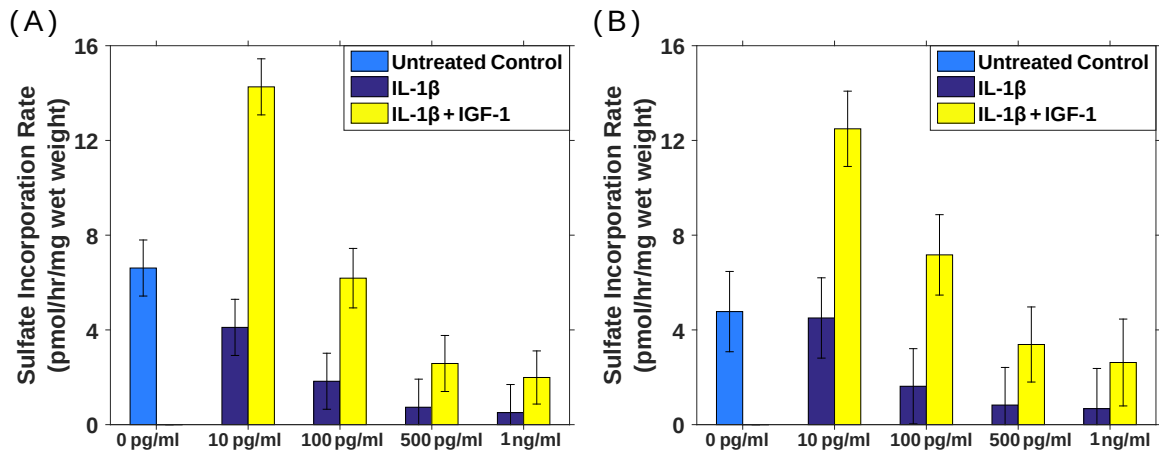


Figure 3.7: Normalized sulfate incorporation rate measured during day 6 to 8 of an 8-day dose response experiment. Human ankle cartilage explants from a 58 year old male donor (A) and a 68 year old male donor (B) were treated with 10 pg/ml to 1 ng/ml doses of human IL-1 β either individually or in combination with 300 ng/ml IGF-1. Untreated control groups were included in both experiments. All bars represent least squares means with 95% confidence intervals. N = 9 to 10 explants/condition in (A) and N = 6 to 8 explants/condition in (B).

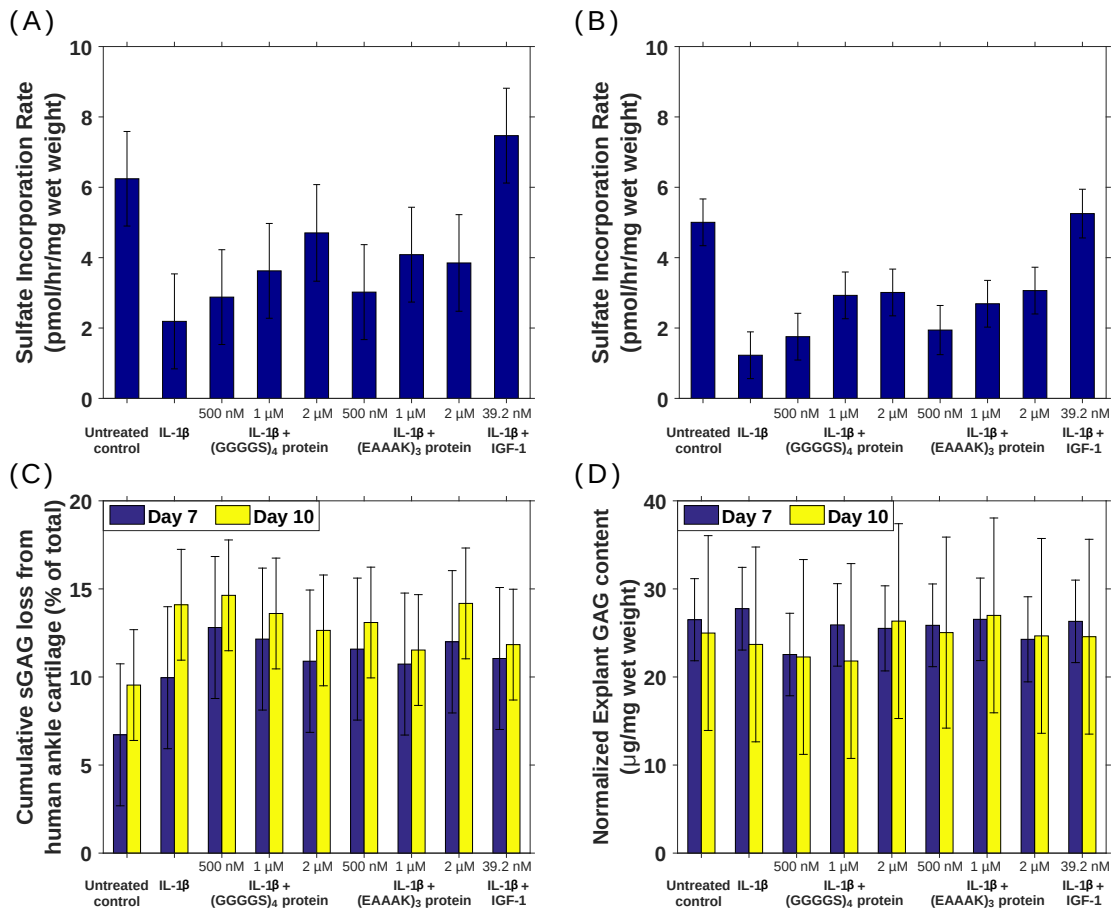


Figure 3.8: Dose response of +9GFP-IGF-1 fusion proteins in a cytokine challenge model of PTOA. Human ankle cartilage explants were treated with a continuous 100 pg/ml dose of IL-1 β and single doses of fusion proteins. An untreated control, a group with continuous IL-1 β , and a group with continuous IGF-1 + IL-1 β were included. (A) Normalized sulfate incorporation rate measured during day 5 to 7 of a 7 day long experiment with explants from five ankle donors (N= 6 to 10 explants/condition/donor). (B) Normalized sulfate incorporation rate measured during day 7 to 10 of a 10 day long experiment with explants from two ankle donors (N= 8 to 11 explants/condition/donor). (C) Cumulative percent sGAG loss from the explants in (A) and (B). (D) Total explant sGAG content normalized to wet weight at the end of the experiments in (A) and (B).

(A) 7 day experiment; ankle cartilage

		IL-1 β	IL-1 β + (GGGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		<10 ⁻⁴	<10 ⁻⁴	<10 ⁻⁴	0.079	<10 ⁻⁴	0.0004	0.0002	0.2262
IL-1 β			0.9002	0.0921	<10 ⁻⁴	0.7649	0.0048	0.045	<10 ⁻⁴
IL-1 β + (GGGGS) ₄ protein	500 nM			0.8471	0.0161	1	0.2592	0.6461	<10 ⁻⁴
	1 μ M				0.503	0.9516	0.9908	1	<10 ⁻⁴
	2 μ M					0.0403	0.9607	0.8246	<10 ⁻⁴
IL-1 β + (EAAAK) ₃ protein	500 nM						0.4403	0.8187	<10 ⁻⁴
	1 μ M							1	<10 ⁻⁴
	2 μ M								<10 ⁻⁴

(B) 10 day experiment; ankle cartilage

		IL-1 β	IL-1 β + (GGGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		<10 ⁻⁴	<10 ⁻⁴	0.0014	0.0026	<10 ⁻⁴	0.0002	0.0038	0.9999
IL-1 β			0.9778	0.0199	0.0115	0.8855	0.081	0.008	<10 ⁻⁴
IL-1 β + (GGGGS) ₄ protein	500 nM			0.3021	0.2175	1	0.6168	0.1727	<10 ⁻⁴
	1 μ M				1	0.5678	0.9999	1	0.0003
	2 μ M					0.4545	0.9993	1	0.0007
IL-1 β + (EAAAK) ₃ protein	500 nM						0.8575	0.3858	<10 ⁻⁴
	1 μ M							0.9978	<10 ⁻⁴
	2 μ M								0.001

Table 3.4: Post hoc comparisons (using Tukey's HSD) for the data in Figure 3.8A and B. p-values for the sGAG biosynthesis rate data in Figure 3.8A are shown in (Table A), and p-values for the data in Figure 3.8B are shown in (Table B). Significant p-values (less than 0.05) are shown in bold font.

(A) 7 day experiment; ankle cartilage

		IL-1 β	IL-1 β + (GGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.054	< 10⁻⁴	< 10⁻⁴	0.0063	0.0002	0.0042	0.0001	0.0013
IL-1 β			0.155	0.4957	0.9958	0.8436	0.9983	0.6744	0.9828
IL-1 β + (GGGS) ₄ protein	500 nM			0.9995	0.7423	0.9647	0.5645	0.9984	0.7636
	1 μ M				0.971	0.9998	0.9163	1	0.9809
	2 μ M					0.9996	1	0.9903	1
IL-1 β + (EAAAK) ₃ protein	500 nM						0.9968	1	0.9999
	1 μ M							0.97	1
	2 μ M								0.9953

(B) 10 day experiment; ankle cartilage

		IL-1 β	IL-1 β + (GGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.0372	0.0112	0.098	0.4045	0.2421	0.8905	0.0316	0.7847
IL-1 β			1	1	0.9818	0.9986	0.6627	1	0.7978
IL-1 β + (GGGS) ₄ protein	500 nM			0.9982	0.8903	0.9759	0.4042	1	0.5517
	1 μ M				0.9989	1	0.8649	1	0.9418
	2 μ M					1	0.9969	0.9749	0.9997
IL-1 β + (EAAAK) ₃ protein	500 nM						0.9739	0.9977	0.9937
	1 μ M							0.6255	1
	2 μ M								0.7664

Table 3.5: Post hoc comparisons (using Tukey's HSD) for the data in Figure 3.8C. p-values for the cumulative sGAG loss data in the 7-day experiment are shown in (Table A). p-values for the cumulative sGAG biosynthesis loss data in the 10-day experiment are shown in (Table B). Significant p-values (less than 0.05) are shown in bold font.

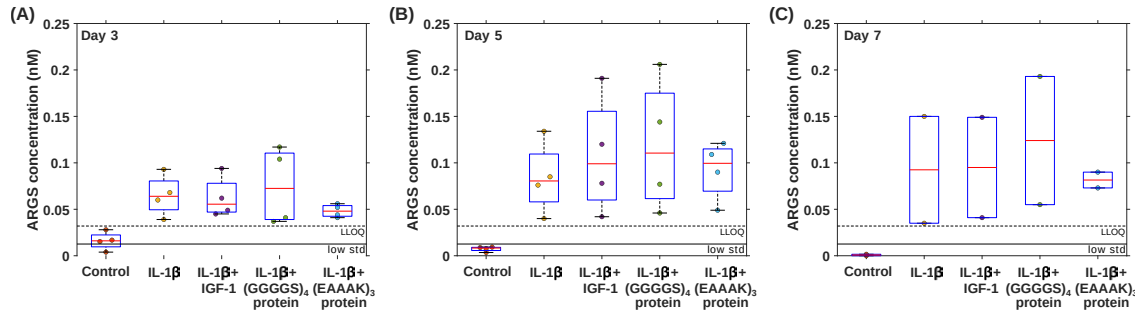


Figure 3.9: Release of aggrecanase cleavage fragments from ankle cartilage explants in the cytokine challenge experiments of Figure 3.8. Human ankle cartilage explants were treated with a continuous 100 pg/ml dose of IL-1 β and single doses of fusion proteins. An untreated control, a group with continuous IL-1 β , and a group with continuous IGF-1 + IL-1 β were included. The ARGs-aggrecan concentration in the culture medium for each treatment and control group was measured at Day 3 (shown in A), Day 5 (shown in B), and Day 7 (shown in C). In each graph, the solid black line is the concentration of the lowest assay standard and the dotted black line is the LLOQ. Individual data points, box plots with mean values along with 25th and 75th percentile, and the whiskers showing the range are plotted. N = 4 donors for day 3 and day 5 time points, and N = 2 donors for the day 7 timepoint.

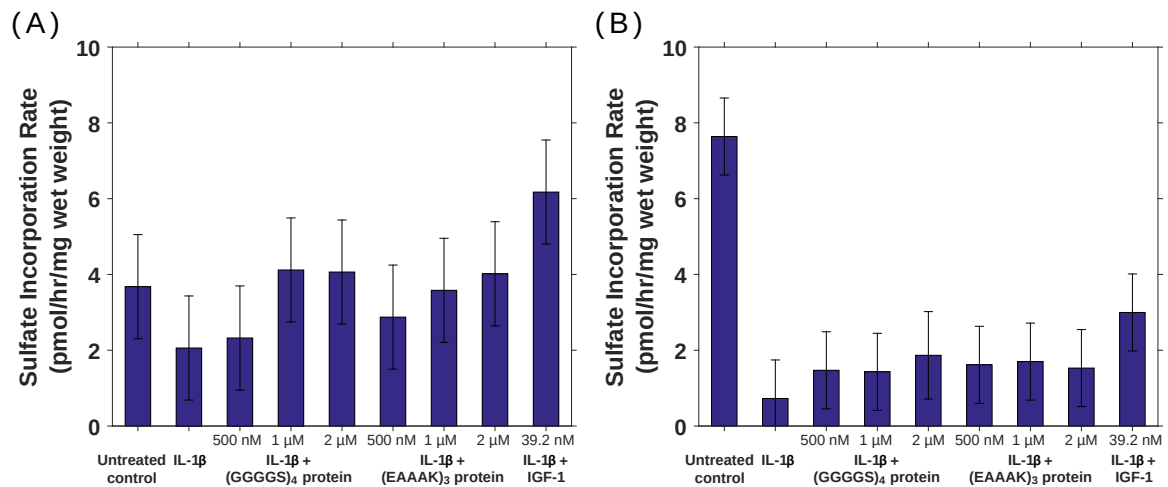


Figure 3.10: Dose response of +9GFP-IGF-1 fusion proteins in a cytokine challenge model of PTOA. Human knee cartilage explants were treated with a continuous 100 pg/ml dose of IL-1 β and single doses of fusion proteins. An untreated control and a group with continuous IGF-1 + IL-1 β were included. (A) Normalized sulfate incorporation rate measured during day 5 to 7 of a 7 day long experiment with explants from an 80 year old male donor (Collins grade 2) (N= 9 to 10 explants/condition). (B) Normalized sulfate incorporation rate measured during day 7 to 10 of a 10 day long experiment with explants from a 69 year old female donor (Collins grade 1) (N= 7 to 9 explants/condition).

(A) 7 day experiment; knee cartilage

		IL-1 β	IL-1 β + (GGGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		0.7697	0.8995	0.9999	1	0.9958	1	1	0.2216
IL-1 β			1	0.4748	0.5119	0.9954	0.8237	0.5437	0.0020
IL-1 β + (GGGGS) ₄ protein	500 nM			0.6571	0.6932	0.9997	0.9320	0.7230	0.0051
	1 μ M				1	0.9360	0.9998	1	0.4769
	2 μ M					0.9501	0.9999	1	0.4406
IL-1 β + (EAAAK) ₃ protein	500 nM						0.9983	0.9602	0.0293
	1 μ M							1	0.1808
	2 μ M								0.4105

(B) 10 day experiment; knee cartilage

		IL-1 β	IL-1 β + (GGGGS) ₄ protein			IL-1 β + (EAAAK) ₃ protein			IL-1 β + IGF-1
			500 nM	1 μ M	2 μ M	500 nM	1 μ M	2 μ M	
Untreated control		<10⁻⁴	<10⁻⁴	<10⁻⁴	<10⁻⁴	<10⁻⁴	<10⁻⁴	<10⁻⁴	<10⁻⁴
IL-1 β			0.9813	0.9869	0.8613	0.9463	0.9118	0.9703	0.0573
IL-1 β + (GGGGS) ₄ protein	500 nM			1	0.9999	1	1	1	0.4709
	1 μ M				0.9997	1	1	1	0.4344
	2 μ M					1	1	1	0.8668
IL-1 β + (EAAAK) ₃ protein	500 nM						1	1	0.605
	1 μ M							1	0.6831
	2 μ M								0.5246

Table 3.6: Post hoc comparisons (using Tukey's HSD) for the data in Figure 3.10. p-values for the sGAG biosynthesis rate data in Figure 3.10A are shown in (Table A), and p-values for the data in Figure 3.10B are shown in (Table B). Significant p-values (less than 0.05) are shown in bold font.

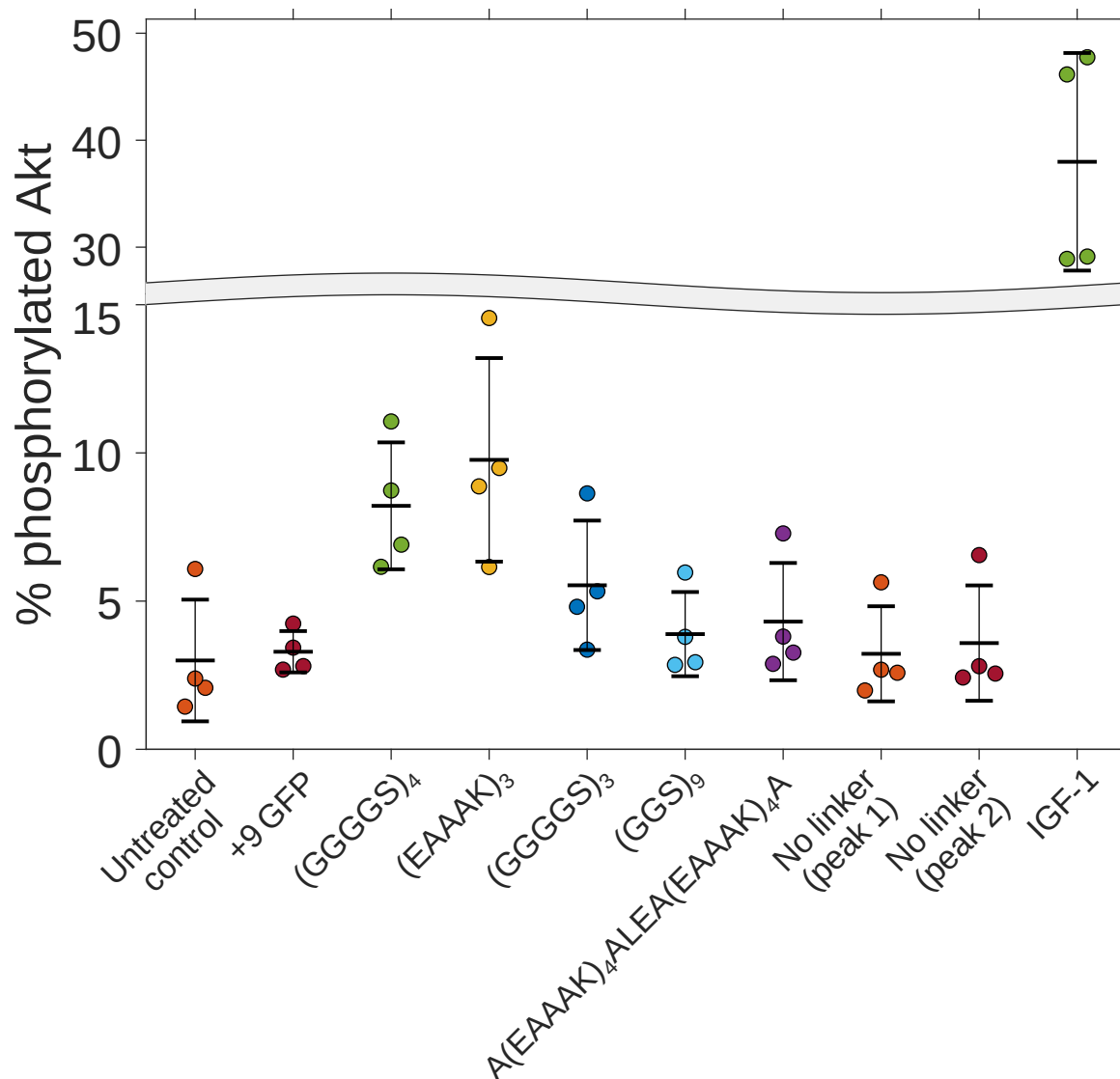


Figure 3.11: Effect of fusion protein treatment on Akt signaling in primary human chondrocytes. Ankle and knee chondrocytes that were grown to ~90% confluence in monolayer cultures were treated for 15 minutes with 1 μ M doses +9GFP-IGF-1 fusion proteins, +9 GFP or free recombinant human IGF-1. An untreated control group was also included. The levels of phosphorylated Akt and total Akt were measured using a multiplex ELISA, and the percent phosphorylation was quantified. Individual data points, mean values and 95% confidence intervals are reported.

	+9GFP	(GGGGS) ₄	(EAAAK) ₃	(GGGGS) ₃	(GGS) ₉	A(EAAAK) ₄ - ALEA- (EAAAK) ₄ A	No linker (peak 1)	No linker (peak 2)	IGF-1
Untreated control	1	0.5286	0.1868	0.9915	1	1	1	1	<10⁻⁴
+9GFP		0.6957	0.3356	0.9971	1	1	1	1	<10⁻⁴
(GGGGS) ₄			0.9998	0.9894	0.8224	0.8926	0.6793	0.7612	<10⁻⁴
(EAAAK) ₃				0.84	0.4655	0.5669	0.3218	0.3968	<10⁻⁴
(GGGGS) ₃					0.9997	1	0.9963	0.999	<10⁻⁴
(GGS) ₉						1	1	1	<10⁻⁴
A(EAAAK) ₄ - ALEA- (EAAAK) ₄ A							1	1	<10⁻⁴
No linker (peak 1)								1	<10⁻⁴
No linker (peak 2)									<10⁻⁴

Table 3.7: Post hoc comparisons (using Tukey's HSD) for the data in Figure 3.11. Significant p-values (less than 0.05) are shown in bold font.

Supplementary Material

S3.1 Protein sequences of GFPs:

+9GFP-IGF-1

Amino acid sequence:

HHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTG
KLPVPWPTLVTTLTLYGVQCFSRYPDHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAE
VKFEGRTLNVNRIKLKGRDFKEKGNILGHKLRYNFNNSHKVYITADKQKNGIKANFKIRHNV
EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITH
GMDELYKGPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSSRRAPQTGIVDECCFRSCD
LRRLEMYCAPLKPAKSA

DNA sequence:

CATCATCATCATCATCATGGCGGTGCGAGCAAGGGCGAAGAAGTGTTTACCGGCGTT
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GAGGGCGAAGGTGATGCGACCAACGGCAAGCTGACCCTGAAATTTATCTGCACCACCGGT
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AGCAGCCGTCGTGCGCCGACAGACCGGTATTGTGGACGAGTGCTGCTTCCGTAGCTGCGAC
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+9GFP-(GGGGS)4-IGF-1

Amino acid sequence:

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VKFEGRTLNVNRIKLKGRDFKEKGNILGHKLRYNFNHSHKVYITADKQKNGIKANFKIRHNV
EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITH
GMDELYKGGGSGGGGSGGGGSGGGGSGPETLCGAELVDALQFVCGDRGFYFNKPTGYGS
SSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA

DNA sequence:

CATCATCATCATCATCATGGCGGTGCGAGCAAGGGCGAAGAAGTGTGTTACCGGCGTT
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AACTGCCGGTGCCGTGGCCGACCCTGGTTACCACCCTGACCTACGGTGTGCAGTGCTTC
AGCCGTTATCCGGACCACATGAAGCGTCACGATTTCTTTAAGAGCGCGATGCCGAAAGGC
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GAGAAAGGCAACATTCTGGGTCACAAGCTGCGTTACAACCTTTAACAGCCACAAAGTGTAT
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+9GFP-(GGGGS)3-IGF-1

Amino acid sequence:

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DNA sequence:

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+9GFP-(GGS)9-IGF-1

Amino acid sequence:

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+9GFP-(EAAAK)3-IGF-1

Amino acid sequence:

HHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFICTTG
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DNA sequence:

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+9GFP-A(EAAAK)4ALEA(EAAAK)4A-IGF-1

Amino acid sequence:

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Joint #	Sex	Age	Joint type	Modified Collins Grade	Figures
1.	Female	58	Ankle	1	Fig. S3.3
2.	Male	57	Knee	(NDRI)	Fig. 3.11, S3.8
3.	Female	53	Ankle	1	Fig. S3.4, S3.6
4.			Ankle	1	Fig. S3.4, S3.6
5.	Male	66	Ankle	1	Fig. S3.6
6.	Male	77	Ankle	2	Fig. S3.5A
7.	Male	23	Knee	(LNH)	Fig. 3.3, 3.5B
8.	Male	66	Ankle	0	Fig. 3.2
9.			Ankle	0	Fig. 3.5A
10.	Male	55	Ankle	1	Fig. 3.11, S3.8
11.			Ankle	1	Fig. 3.11, S3.8
12.	Male	38	Knee	(LNH)	Fig. 3.5B
13.	Male	58	Ankle	1	Fig. 3.7
14.			Ankle	1	Fig. 3.5A
15.	Male	68	Ankle	1	Fig. 3.7
16.			Ankle	1	Fig. 3.8A, 3.8C, 3.8D
17.	Female	60	Ankle	0	Fig. 3.5A
18.			Ankle	0	Fig. 3.8B, 3.8C, 3.8D, 3.9
19.	Female	69	Ankle	1	Fig. 3.8B, 3.8C, 3.8D, 3.9
20.	Male	67	Ankle	1	Fig. 3.11, S3.8
21.			Ankle	1	Fig. 3.11, S3.8
22.	Female	69	Knee	1	Fig. 3.5B
23.			Knee	1	Fig. 3.10B
24.	Male	59	Ankle	1	Fig. 3.11, S3.8
25.			Ankle	1	Fig. 3.11, S3.8
26.	Female	36	Ankle	0	Fig. 3.5A, 3.4
27.			Ankle	0	Fig. 3.8A, 3.8C, 3.8D
28.	Male	70	Ankle	1	Fig. 3.8A, 3.8C, 3.8D, 3.9
29.			Ankle	2	Fig. 3.5A
30.	Male	80	Ankle	1	Fig. 3.5A
31.			Ankle	1	Fig. 3.8A, 3.8C, 3.8D, 3.9
32.			Knee	2	Fig. 3.10A
33.			Knee	2	Fig. 3.10A
34.	Male	63	Ankle	1	Fig. 3.8A, 3.8C, 3.8D

Table S3.1: List of human joints used in this study.

S3.2 Characterization of +9GFP-IGF-1 fusion proteins:

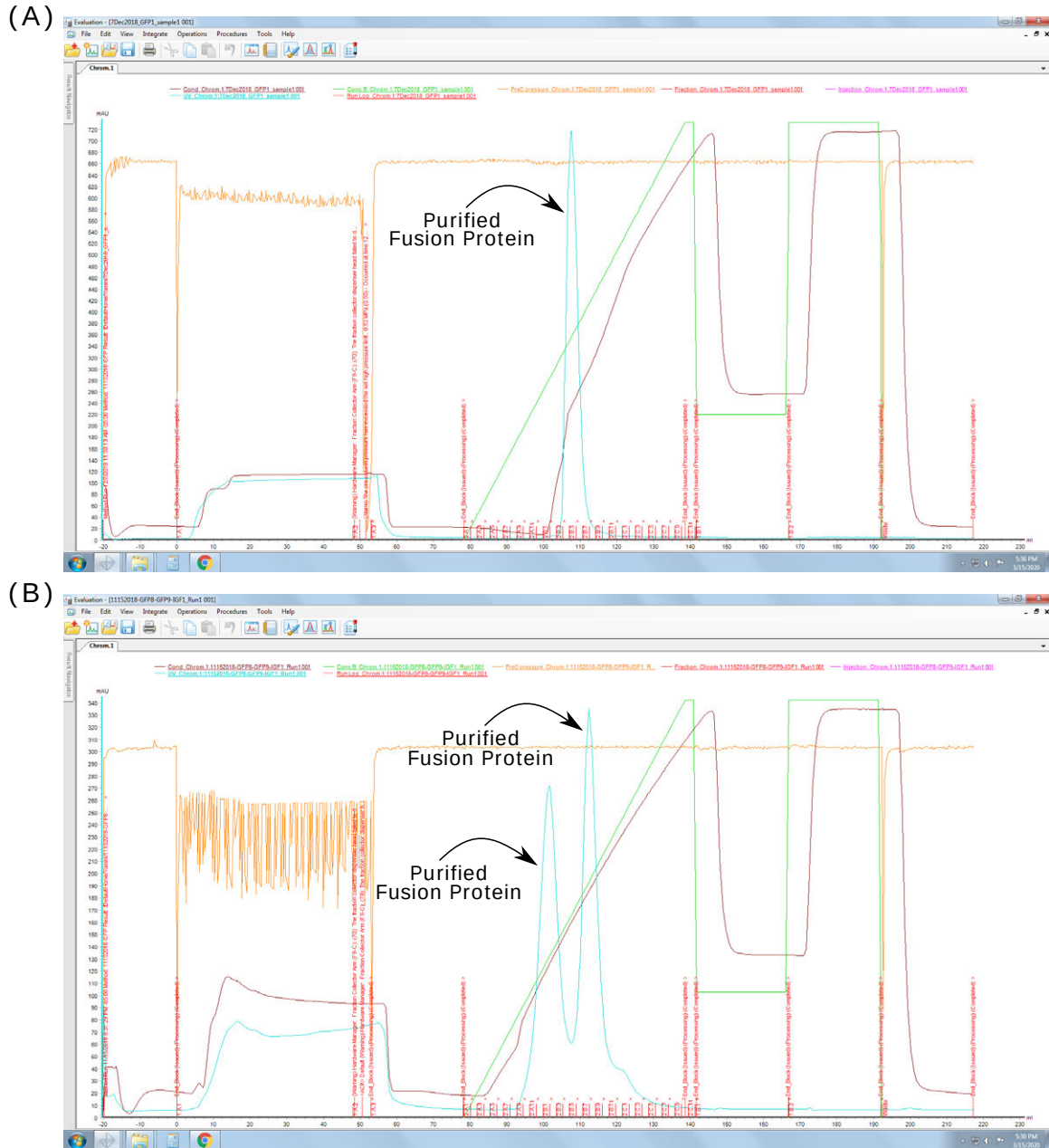


Figure S3.1: Representative FPLC cation exchange chromatography elution profiles. Elution was performed with a linear NaCl gradient (0 to 1 M; shown in green). UV readings at 280 nm are shown in blue.

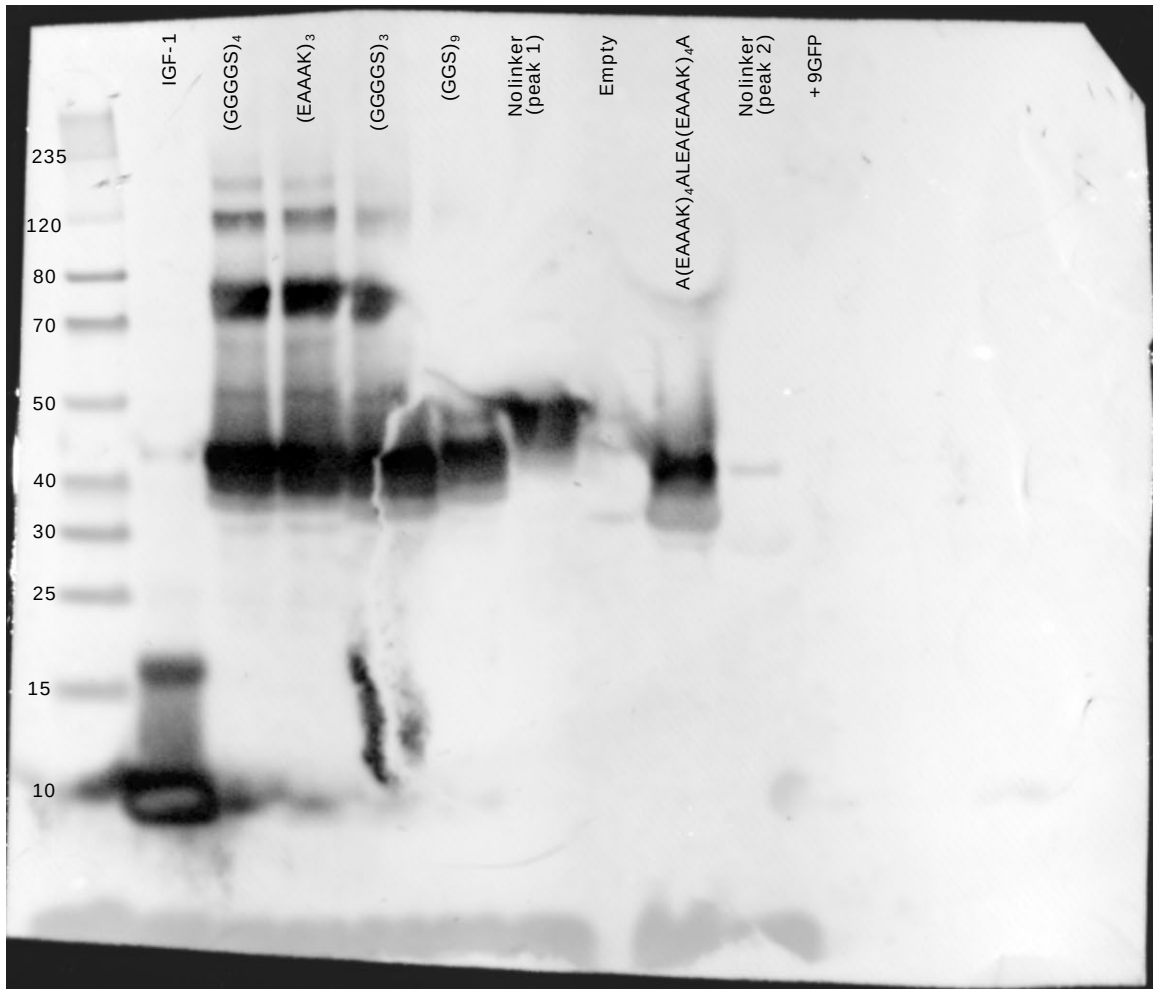


Figure S3.2: Western blot analysis of purified and concentrated +9GFP-IGF-1 fusion proteins. Recombinant human IGF-1 (1st lane) and +9GFP (free carrier; last lane) were included as controls.

S3.3 Data from uptake, tissue penetration and dose response experiments:

Protein	PBS + 1% BSA			
	36 hr		108 hr	
	Mean	Std. Dev	Mean	Std. Dev
(GGGGS) ₄	12.7	1.5	18.3	6.7
(EAAAK) ₃	19.8	1.6	37.8	3.6
(GGGGS) ₃	13.1	1.7	24.2	6.4
+9GFP	42.3	10.8	113.9	34.5

Table S3.2: Uptake ratios of fusion proteins in human cartilage (Mean $\pm$ Standard Deviation; N=5 explants/condition)

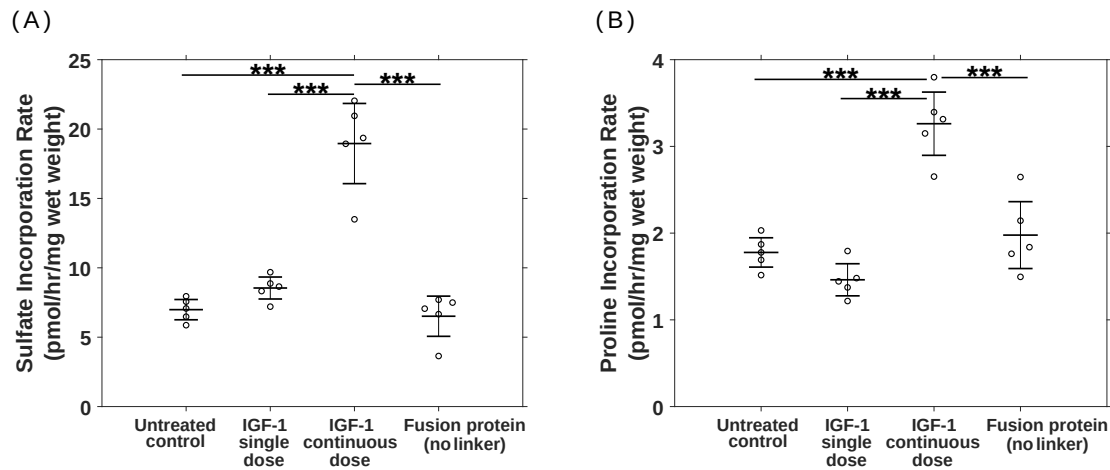


Figure S3.3: Normalized sulfate and proline incorporation rate measured during days 6 to 8 of an 8-day dose response experiment. Groups of 5 ankle cartilage explants were treated with a single 300 ng/ml (39.2 nM) dose of IGF-1, continuous doses (39.2 nM) of IGF-1 or a single dose (39.2 nM) of the +9GFP-IGF-1 fusion protein with no linker. An untreated control group with 5 explants was also included in the experiment. Normalized sulfate incorporation rates are shown in (A) and normalized proline incorporation rates are shown in (B). The individual data points, mean values and 95% confidence intervals are reported; *: p<0.05; **: p<0.01; ***: p<0.001.

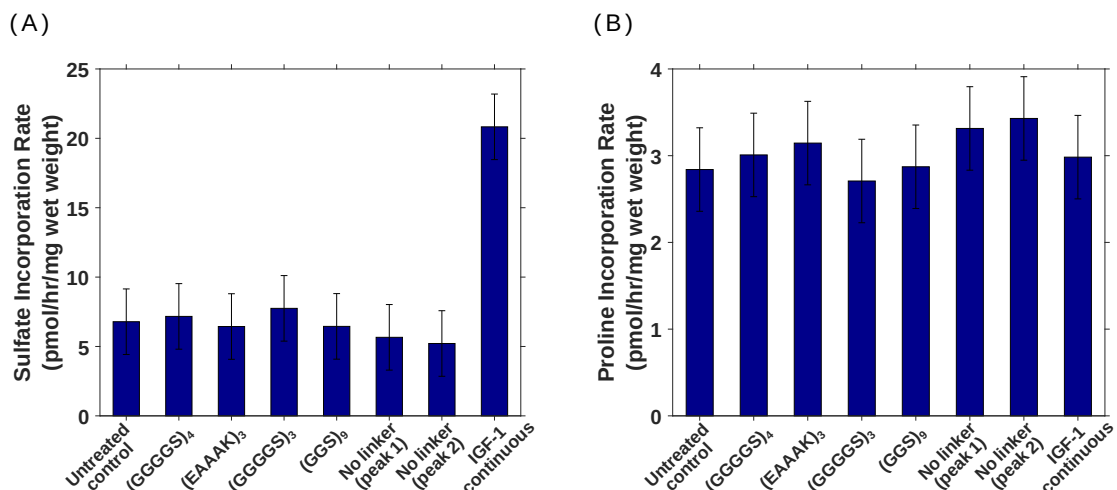


Figure S3.4: Testing the effect of fusion proteins on the normalized sulfate and proline incorporation rates of human ankle cartilage. Groups of 7 explants from one human donor were treated with single doses (39.2 nM) of +9GFP-IGF-1 fusion proteins. An untreated control group and a group with continuous 39.2 nM doses of free IGF-1 were also included in the experiment. (A) Only the free IGF-1 group had a significantly increased sulfate incorporation rate compared to the untreated control. (B) There were no differences between the proline incorporation rates of any of the groups. Bars represent least squares mean values with 95% confidence interval.

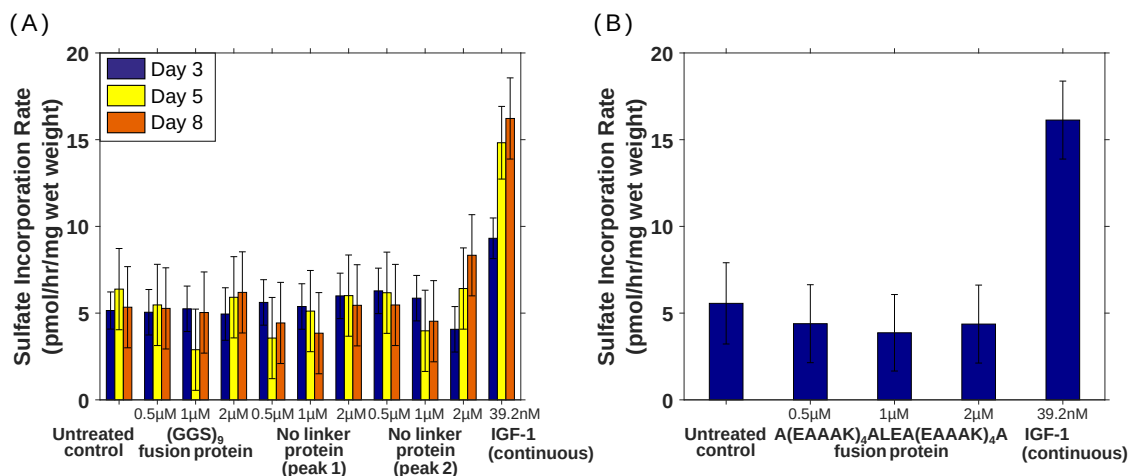


Figure S3.5: Dose response results for +9GFP-IGF-1 fusion proteins with the (GGS)₉ linker, A(EAAAK)₄ALEA(EAAAK)₄A linker, and no linker. (A) Normalized sulfate incorporation rate of human ankle cartilage explants treated with single doses (500 nM to 2 μM) of fusion protein variants with the (GGS)₉ linker and with no linker. Treatment with the fusion proteins did not lead to any changes in the normalized sulfate incorporation rate compared to the untreated control at day 3, 5 or 8 of culture. (N = 1 donor; 4 to 6 explants/condition. Bars are mean values with 95% confidence intervals.) (B) Normalized sulfate incorporation rate of human ankle cartilage explants treated with single doses (500 nM to 2 μM) of the A(EAAAK)₄ALEA(EAAAK)₄A linker fusion protein. Explants were cultured for 7 days, and the normalized sulfate incorporation rate of the fusion protein treatment groups were not significantly different from the incorporation rate of the untreated control group. (N = 2 donors; 7 to 12 explants/condition/donor. Bars are least squares means with 95% confidence intervals.)

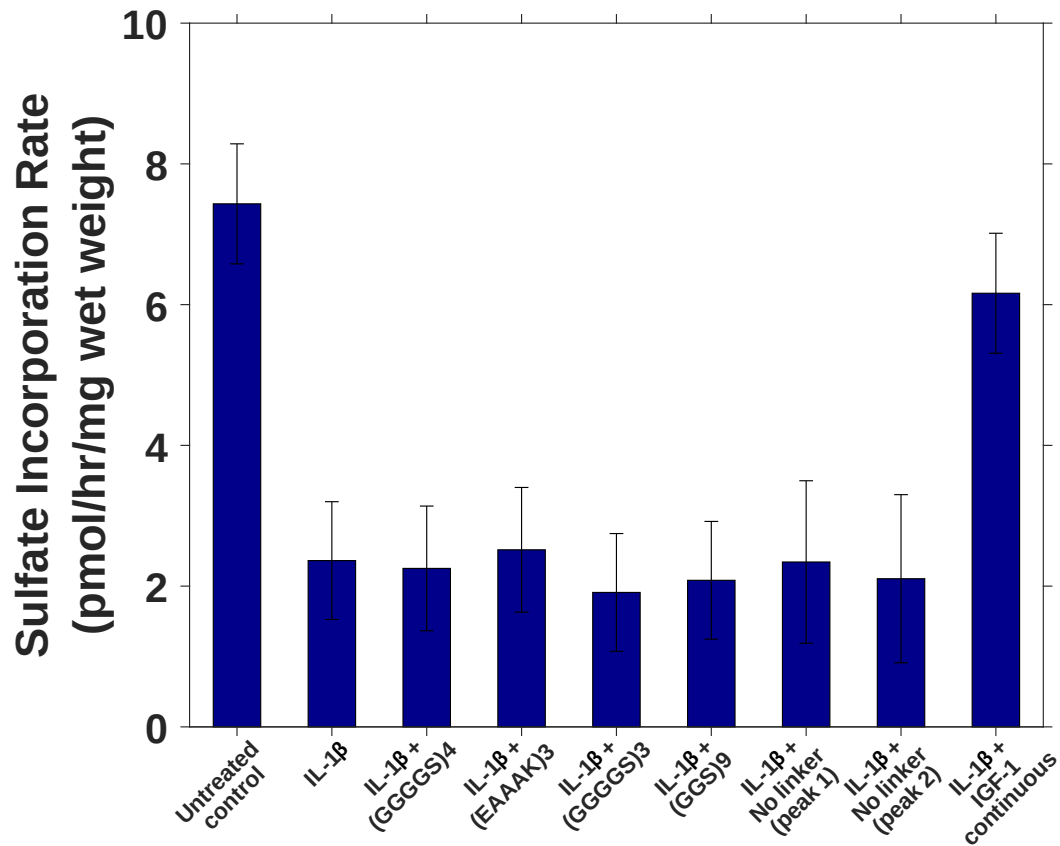


Figure S3.6: Testing the effect of fusion proteins in a cytokine challenge model of PTOA. Groups of explants from two human donors were treated continuously with 10 ng/ml mouse IL-1 β . Treatment groups received single doses (39.2 nM) of +9GFP-IGF-1 fusion proteins or continuous doses (39.2 nM) of IGF-1. An untreated control group was also included in the experiment. The sGAG biosynthesis rate of the explants was measured between day 7 and day 9 of the experiment and normalized using the wet weights. Only the continuous free IGF-1 treatment could rescue the biosynthesis loss caused by IL-1 β treatment.(N = 2 donors; 3 to 8 explants/condition/donor; Bars represent least squares mean values with 95% confidence intervals.)

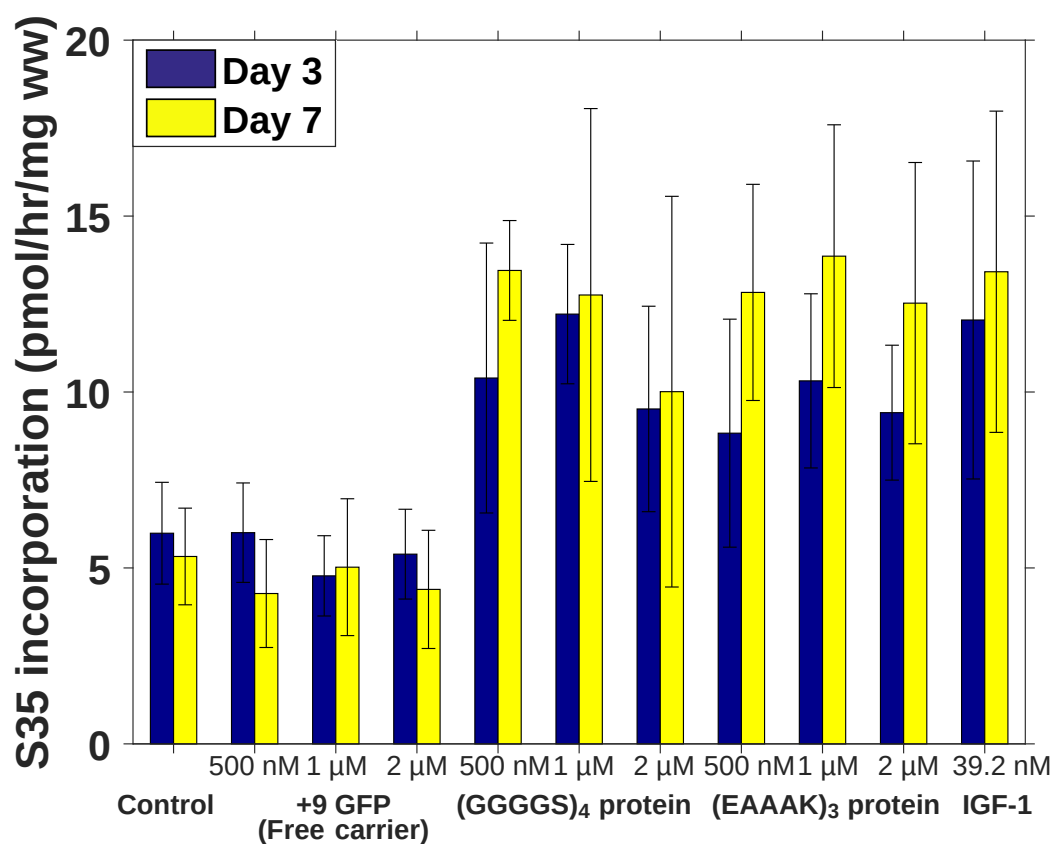


Figure S3.7: Normalized sulfate incorporation rate of human ankle cartilage explants (66 year old male donor; Collins grade 0) in a dose response experiment with +9GFP-IGF-1 fusion protein variants. Groups of explants were treated with 500 nM, 1 μM and 2 μM doses of fusion protein variants with the (GGGGS)₄ and (EAAAK)₃ linkers. Groups of explants were also treated with equivalent doses of +9GFP (the drug delivery carrier). An untreated control group and a group with continuous dosage of free IGF-1 were also included in the experiment. All the fusion protein groups had sulfate incorporation rates comparable to the IGF-1 group, while the +9GFP groups were not significantly different from the untreated control. Bars represent mean values $\pm$ standard deviations; N = 7 to 9 explants/condition.

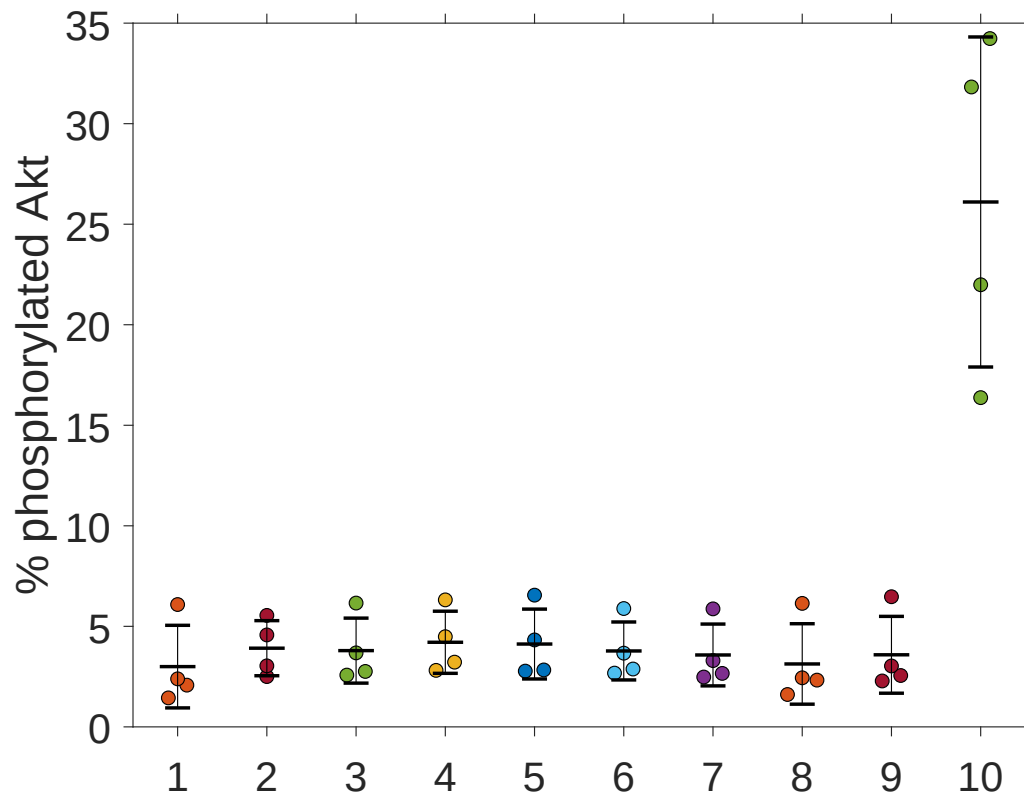


Figure S3.8: Effect of fusion protein treatment on Akt signaling in primary human chondrocytes. Ankle and knee chondrocytes that were grown to ~90% confluence in monolayer cultures were treated for 15 minutes with 40 nM doses +9GFP-IGF-1 fusion proteins, +9 GFP or free recombinant human IGF-1. An untreated control group was also included. The levels of phosphorylated Akt and total Akt were measured using a multiplex ELISA, and the percent phosphorylation was quantified. Individual data points, mean values and 95% confidence intervals are reported.

Chapter 4

Transport Kinetics of Engineered Green Fluorescent Protein Carriers and Fusion Proteins: Experiments and Mathematical Modeling¹

4.1 Introduction

In chapters 2 and 3 of this thesis, we quantified the uptake of engineered GFP carriers and GFP-IGF-1 fusion proteins in *in vitro* cartilage explant cultures. We also demonstrated that single doses of two GFP-IGF-1 fusion proteins could provide sustained IGF-1 bioactivity for 7 to 10 days in full thickness human cartilage explants. While these *in vitro* experiments provide useful insights into the behavior of our drug delivery system, the quantitative findings are not directly translatable to *in vivo* systems. There is a continuous turnover of synovial fluid in human and animal joints whereas media changes in *in vitro* systems are performed only once in two or three days. Additionally, the size scales involved in the two systems are very different: the cartilage surface area to synovial fluid volume ratio in

¹Some parts of this chapter have been published as part of: **Krishnan, Y.**, Rees, H.A., Rossitto, C.P., Kim, S.E., Hung H.K., Frank, E.H., Olsen, B.D., Liu, D.R., Hammond, P.T. and Grodzinsky, A. J.; “Green fluorescent proteins engineered for cartilage-targeted drug delivery: Insights for transport into highly charged avascular tissues.,” *Biomaterials* 2018, Vol. 183, 218-233.

human knee joints is over an order of magnitude greater than the cartilage surface area to bath volume ratios used in the experiments of chapters 2 and 3. Due to these factors, the kinetics of GFP transport within the human knee joint after intra-articular injection would be very different compared to the transport kinetics observed in our *in vitro* experiments.

In order to predict *in vivo* transport kinetics and set the stage for successful scale-up to preclinical and clinical studies, we developed a mathematical model for the transport of engineered GFPs and GFP-IGF-1 fusion proteins. By fitting the model predictions to dynamic uptake experiments, we obtained estimates for the transport properties of these proteins such as their partition coefficients in cartilage and the rate constant for electrostatic binding interactions. We confirmed that the model was able to accurately predict transport behavior in an independent set of *in vitro* experiments in which the surface area to volume ratio was an order of magnitude higher than the dynamic transport experiments used to obtain model parameters. We finally used the model to predict the concentration of GFPs and GFP-IGF fusion proteins in synovial fluid and inside human knee cartilage in an intact knee joint as a function of time after intra-articular injection. These cartilage concentration predictions will enable the estimation of injection doses that can achieve appropriate drug doses within cartilage. Additionally, the model also predicts the amount of GFPs and GFP-IGF fusion proteins that are cleared out into the systemic circulation by the joint capsule. These predictions will be useful in assessing the likelihood of potential systemic side effects and estimating safe injection doses.

4.2 Materials and Methods

4.2.1 Structure and Preparation of Cationic and Neutral GFP Variants

The structures and the electrostatic surface potentials of the four cationic supercharged GFP and three neutral GFP variants are shown in Chapter 2.

Cationic S-GFPs: BL21 Star (DE3)-competent *E. coli* cells were transformed with plasmids encoding the supercharged GFPs with a His₆ N-terminal purification tag. A single

colony was grown overnight in Luria-Bertani (LB) broth containing 50 mg/mL chloramphenicol at 37 °C. The cells were diluted 1:100 into 1 liter of the same media and grown at 37 °C until OD₆₀₀ 0.6-0.7. The cultures were incubated on ice for 60 min before induction of protein expression with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) (GoldBio). Expression was sustained for 15 h with shaking at 16 °C. Cells were collected by centrifugation at 6,000g for 20 min (4 °C) and re-suspended in cell collection buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 1M NaCl, 20% glycerol, 5 mM tris(2-carboxyethyl)phosphine (TCEP; GoldBio), 0.4 mM phenylmethane sulfonyl fluoride (Sigma-Aldrich) and 1 complete, EDTA-free protease inhibitor pellet (Roche) per 50 mL buffer used). Cells were lysed by sonication (5 min total, 3 s on, 5 s off) and the lysate cleared by centrifugation at 22,500g (25 min). The cleared lysate was incubated with His-Pur nickel nitriloacetic acid (nickel-NTA) resin (1.5 mL resin per litre of culture, Thermo Fisher) with rotation at 4 °C for 60 min. The resin was washed with 20 column volumes of cell collection buffer before bound protein was eluted with elution buffer ((100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5mM TCEP (GoldBio), 200mM imidazole). The resulting protein fraction was further purified on a 5 ml Hi-Trap HP SP (GE Healthcare) cation exchange column using an Akta Pure FPLC. An NaCl gradient (from 0 – 1 M) was used to elute the supercharged GFP from the HP SP column; the NaCl concentration required to elute the S-GFP increased with an increase in protein charge.

Protein-containing fractions were concentrated using a column with a 10 kDa cutoff (Millipore) centrifuged at 3,000g, and the concentrated solution was sterile-filtered through a 22-mm polyvinylidene difluoride (PVDF) membrane (Millipore). After concentration, proteins were resuspended in storage buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5mM TCEP (GoldBio)). After sterile filtration, proteins were quantified with Reducing Agent Compatible Bicinchoninic acid assay (Pierce Biotechnology), snap-frozen in liquid nitrogen and stored in aliquots at 80 °C.

Neutral GFPs: BL21(DE3) cells were transformed with plasmids encoding the neutral GFP variants. For each variant, a well-isolated colony was grown overnight in LB broth

containing kanamycin at 37 °C. 5 mL of the overnight culture was diluted in 1 L of LB broth and incubated at 37 °C until OD₆₀₀ 0.8-1. Cultures were then induced with 1 mM IPTG, grown for 20 hours at 37 °C, and harvested. Cells were collected by centrifugation at 4700 rpm for 15 minutes and stored at -80 °C. The frozen cells were homogeneously resuspended in lysis buffer including 10 mM imidazole and then sonicated in an ice bath for 10 minutes. The resulting cell debris and the supernatant were separated by centrifugation at 13000 rpm (4 °C) for 30 minutes. The presence of the expressed protein in the supernatant was confirmed by SDS-PAGE. The collected protein was purified using Ni-NTA metal affinity chromatography and was fully eluted using 250 mM imidazole. Elution fractions containing purified protein were dialyzed into 20mM Tris-Cl, 300mM NaCl, pH 8.0. The purified proteins were analyzed in SDS-PAGE gel, quantified using a NanoDrop instrument, sterile-filtered through a 22-mm PVDF membrane and stored at -80 °C. The protein sequences for all the GFP variants were previously listed in Chapter 2.

4.2.2 Fusion Protein Design, Expression and Purification

Insulin-like growth factor 1 (IGF-1) was fused to +9 GFP via flexible and rigid polypeptide linkers of different sizes. In each case, the C-terminus of the +9 GFP was attached to the linker, which in turn was attached to the N-terminus of IGF-1. The linkers used in this study were: (GGGGS)₄ (flexible), (GGGGS)₃ (flexible), (GGS)₉ (flexible), (EAAAK)₃ (rigid) and A(EAAAK)₄ALEA(EAAAK)₄A (rigid). In addition to five fusion proteins with these linkers, a sixth fusion protein with no linker was also included. Each of the six fusion proteins were designed to have an N-terminus His₆ tag. The codon sequences for the His₆+9GFP-linker-IGF-1 fusions were optimized for *E. coli* and inserted into the pET-29b(+) vector by Genscript, and purified plasmids were shipped to us.

For each fusion protein, BL21 Star (DE3)-competent *E. coli* cells were transformed with the appropriate plasmid and a well-isolated colony was grown overnight in 10 mL Luria-Bertani (LB) broth containing kanamycin at 37 °C. This starter culture was then diluted into 1 liter of LB broth with kanamycin, and grown at 37 °C until OD₆₀₀ 0.8-1. Following this, fusion protein expression was induced with 1 mM isopropyl- β -D-1-

thiogalactopyranoside (IPTG)(GoldBio) and cultures were sustained at 16 °C with shaking for 20 hours. Cells were collected by centrifugation at 4000 rpm for 15 minutes at 4 °C, and the cell pellets were frozen at - 80 °C.

The cell pellets were defrosted and resuspended in 25 mL lysis buffer (100mM tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 8.0, 20% glycerol, 1M NaCl, half tablet of complete EDTA-free protease inhibitor pellet (Roche), 0.4 mM phenylmethane sulfonyl fluoride (PMSF; Sigma-Aldrich) and 5 mM tris(2-carboxyethyl)phosphine (TCEP; GoldBio)). The resuspended cells were lysed by sonication (5 min total time; 3 s on, 2 s off) and centrifuged at 12,500rpm for 60 minutes. The expressed fluorescent proteins were present in the supernatant and this was collected for purification.

The fusion proteins were first purified using immobilized metal affinity chromatography. 3 mL of HisPur™ nickel nitriloacetic acid (nickel-NTA) resin slurry (ThermoFisher; 50 % slurry in 20 % ethanol) was centrifuged for 2 minutes at 700g and the supernatant was discarded. The settled resin (1.5 mL) was washed twice by resuspending in 15 mL lysis buffer, centrifuging at 700g and discarding the supernatant. The supernatant from the cell lysis step (containing the expressed fusion proteins) was added to the washed nickel-NTA resin and mixed on an end-over-end rotator in the cold room for 90 minutes. The resin was washed twice with 15 mL of lysis buffer before bound protein was eluted with elution buffer ((100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 8.0, 20% glycerol, 0.5 M NaCl, 5mM TCEP (GoldBio), 200mM imidazole). The resulting protein fraction was further purified on a 5 ml Hi-Trap HP SP (GE Healthcare) cation exchange column using an Akta Pure FPLC. An NaCl gradient (from 0 – 1 M) was used to elute the fusion protein from the HP SP column. The protein containing fractions were analyzed in an SDS-PAGE gel (NuPAGE™ 4-12% Bis-Tris Protein gel; Invitrogen), and fractions containing pure fusion protein were combined.

Depending on the volume, the combined fraction was concentrated using either a Centricon®-Plus 70 centrifugal unit (10 kDa cutoff; Millipore) or an Amicon®Ultra-15 centrifugal unit (10 kDa cutoff; Millipore) centrifuged at 3,500g. The concentrated proteins were sterile-filtered through a 0.2 µm pore size sterile syringe filter (VWR), quantified with the Reducing Agent Compatible Bicinchoninic acid (BCA) assay (Pierce Biotechnology), snap-

frozen in liquid nitrogen and stored in aliquots at -80 °C. The concentrated, sterile filtered proteins were analyzed in an SDS-PAGE gel followed by a western blot with polyclonal anti-IGF-1 antibody (Abcam, Cambridge, MA).

4.2.3 Human cartilage explant harvest

Human ankle joints and one knee joint distal femur were obtained through the Gift of Hope Organ and Tissue Donor Network (Itaska, IL). The list of human donor joints used in this study is shown in the table below along with the Collins grade of the cartilage. (The Collins visual grading scale grades spans grades 0-4 [214], with grade 0 = normal and grade 4 = end stage osteoarthritis.) All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT. Cartilage explants (6 mm diameter) were harvested using a biopsy punch, trimmed down to 1 mm thickness and equilibrated for 48 hours (37 °C, 5% CO₂) in low glucose (1 g/L) DMEM which was supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline and 20 µg/mL ascorbic acid. Explants were maintained in culture with media changes every 2 to 3 days. Prior to setting up each dynamic uptake experiment, an explant was equilibrated in PBS + 1% BSA and sliced to ~ 500 µm thickness.

Joint #	Sex	Age	Joint type	Modified Collins Grade
1.	Female	35	Knee	1
2.	Male	60	Ankle	1
3.	Male	66	Ankle	1
4.	Male	70	Ankle	1
5.	Female	75	Ankle	1
6.	Female	58	Ankle	1

Table 4.1: List of human joints used in this study.

4.2.4 Dynamic Uptake: Transport Experiments

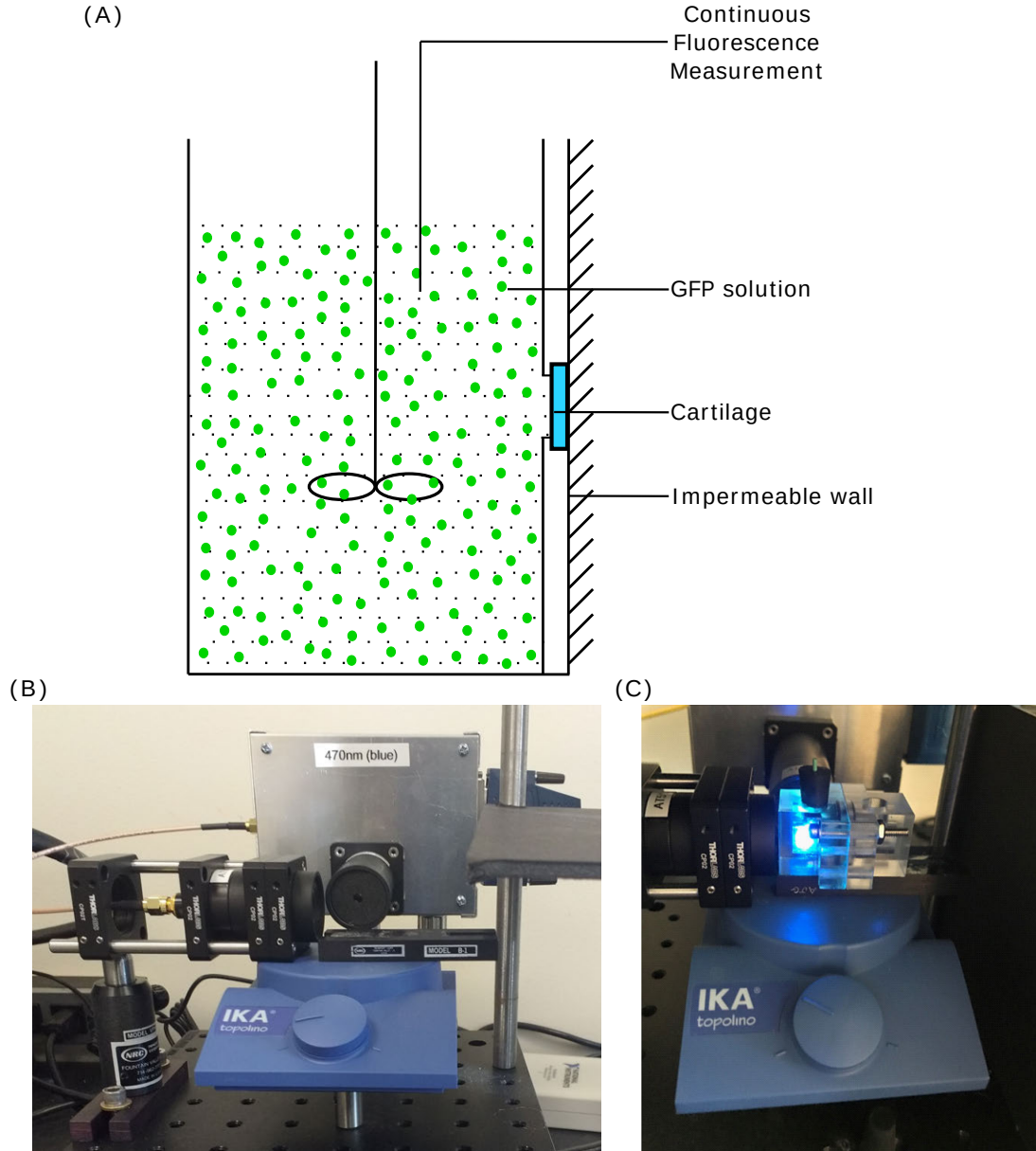


Figure 4.1: Dynamic transport experiments for quantifying transport properties of delivery carriers in cartilage (A) Schematic for dynamic uptake transport experiments: Human cartilage is clamped to one side of the transport chamber with one side facing an impermeable wall and the opposite face exposed to a well mixed solution of GFPs in PBS+1%BSA. The concentration of GFPs in the bath is measured continuously through fluorescence measurements.(B)Monochromatic light emitter ($\lambda = 470$ nm: excitation wavelength for GFP) and fluorescence detector (C)Transport chamber with dynamic uptake experiment in progress

To measure the kinetics of the uptake of cationic S-GFPs into cartilage, dynamic uptake experiments were performed. Cartilage disks (6 mm diameter) were clamped to one end of a transport chamber in such a way that one face was in contact with an impermeable wall and the other face had a circular area with a 4 mm diameter exposed to a well-mixed bath (Figure 4.1). The initial bath was a 1X PBS + 1% BSA solution (total volume = 2 mL) and the explant was allowed to equilibrate in this. After this, cationic GFP carriers or GFP-IGF-1 fusion proteins were added to the bath (considered to be “time 0” for the experiment), and the bath concentration was monitored continuously through fluorescence measurements. Experiments were also set up without cartilage explants to measure the bleaching curves for each GFP carrier and fusion protein. A mono-exponential or biexponential equation was fitted to the experimental bleaching curves. The bath concentration data for experiments with cartilage explants was corrected for bleaching using these equations [240].

A mathematical diffusion-reaction model was formulated and used to predict the changes in bath concentration as a function of time. By fitting the model to bleaching-corrected experimental bath concentrations, diffusion-reaction parameters were obtained. These parameters were then used in the model to predict the transport of GFPs in *in vitro* uptake experiments (described in Chapter 2 of this thesis) and these predictions were compared to experimental data. The model was also used to predict the transport behavior of cationic GFP carriers and GFP-IGF-1 fusion proteins upon intra-articular injection into human knee joints.

4.3 Mathematical model for dynamic uptake of GFPs into cartilage

In this section, the abbreviation “GFP” will be used to refer to cationic and neutral engineered GFP variants, as well as the +9GFP-IGF-1 fusion proteins.

With reference to the schematic of Figure 4.1A, mass balance of GFP in the bath is

governed by:

$$\frac{d(V_b c_b)}{dt} + \iint_S \vec{N} \cdot d\vec{A} = 0 \quad (4.1)$$

where V_b is the volume of the bath, c_b is the bath concentration of +9 GFP, $\vec{N}$ is the flux of +9 GFP into cartilage and $\vec{A}$ is the surface area vector of cartilage.

Initial condition in bath:

$$c_b|_{t=0} = c_{b0} \quad (4.2)$$

where c_{b0} is the initial concentration of GFP in the bath.

Assuming a simple 1-D case where there is only diffusive flux at the interface,

$$\frac{d(V_b c_b)}{dt} - A_{cart} D_{cart} \frac{\partial c_{cart}}{\partial x} \Big|_{x=0,t} = 0 \quad (4.3)$$

Here, c_{cart} is the concentration of GFP inside cartilage and x is the spatial coordinate in cartilage ranging from $x=0$ at the interface with the bath to $x=L$ (where L is the thickness of cartilage) at the location of the impermeable wall.

Since the bath volume is a constant:

$$V_b \frac{dc_b}{dt} = A_{cart} D_{cart} \frac{\partial c_{cart}}{\partial x} \Big|_{x=0,t} \quad (4.4)$$

From Equation 4.2, the initial condition becomes:

$$c_b(t = 0) = c_{b0} \quad (4.5)$$

and the diffusion-reaction equation for the concentration of free GFP inside cartilage is

$$\frac{\partial c_{cart}}{\partial t} = D_{cart} \frac{\partial^2 c_{cart}}{\partial x^2} + R \quad (4.6)$$

where D is the diffusion coefficient of GFP in cartilage, and R is the net rate of generation

or formation of free GFP inside cartilage associated with its binding in cartilage. For GFP variants that are internalized by chondrocytes, R includes the rate at which GFPs are taken up by the cells.

For the case of a reversible binding reaction in the matrix,



$$R = -k_f c_{cart} c_S + k_b c_B \quad (4.8)$$

$$c_S + c_B = N_T \quad (4.9)$$

where F represents the free GFP molecules inside cartilage, S represents the free binding sites and B represents the bound GFP-binding site complex. In Equation 4.8, we have assumed that the forward reaction in Equation 4.7 has an overall order of 2 (first order with respect to F and also with respect to S), while the backward reaction is a first order reaction. The forward reaction (rate constant k_f) here is the binding of free GFP to free binding sites (concentration c_S), while the backward reaction (rate constant k_b) is the unbinding of GFP from bound sites (c_B). The total initial binding site concentration (N_T) in the tissue is then defined in equation (4.9).

The concentration of negatively charged groups in cartilage is on the order of 100 mM ([241]) while GFP concentrations in the tissue are on the order of 1 to 100 μ M. Therefore, it is reasonable to assume that $c_B \ll N_T$, which means:

$$c_S \sim N_T \quad (4.10)$$

Early in the process, the forward reaction will dominate the reverse reaction, and the second term in Equation 4.8 will be negligible compared to the first term. Alternately, in addition to $c_B \ll N_T$, if $k_b c_B \ll k_f c_F c_S$ (which will be true if the dissociation constant (equal to k_b/k_f) is very small), then the second term in equation (4.8) can be ignored. Therefore the

equations (4.6), (4.8) and (4.9) become:

$$\frac{\partial c_{cart}}{\partial t} = D_{cart} \frac{\partial^2 c_{cart}}{\partial x^2} - k c_{cart} \quad (4.11)$$

$$R = -k_f N_T c_{cart} = -k c_{cart} \quad (4.12)$$

$$k = k_F N_T \quad (4.13)$$

Note: An alternative way of obtaining Equation 4.12 is to assume that the binding reaction of GFP to cartilage is irreversible.

Note 2: In cases where GFPs are internalized by cells, the internalization rate can be approximated with a first order rate term: $R_{int} = -k_{int} c_{cart}$. The overall reaction term can still be expressed as $R = -k c_{cart}$ where k is the sum of the rate constant for matrix binding and the rate constant for cell internalization.

The boundary conditions at $x=0$ involves partitioning at interface with bath:

$$c_{cart}(x = 0, t) = K_p c_b(t) \quad (4.14)$$

Here, K_p is the partition coefficient of GFP in cartilage. At $x=L$, the no flux boundary condition is:

$$\left. \frac{\partial c_{cart}}{\partial x} \right|_{x=L, t} = 0 \quad (4.15)$$

There is no GFP inside cartilage at the start of the experiment. Therefore, the initial condition is:

$$c_{cart}(x, t = 0) = 0 \quad (4.16)$$

The model equations were non-dimensionalized to obtain:

$$\frac{d\tilde{c}_b}{d\tilde{t}} = \frac{A_{cart}L}{V_b} \frac{\partial \tilde{c}_{cart}}{\partial \tilde{x}} \bigg|_{\tilde{x}=0, \tilde{t}} \quad (4.17)$$

$$\tilde{c}_b(\tilde{t} = 0) = 1 \quad (4.18)$$

$$\frac{\partial \tilde{c}_{cart}}{\partial \tilde{t}} = \frac{\partial^2 \tilde{c}_{cart}}{\partial \tilde{x}^2} - \frac{kL^2}{D_{cart}} \tilde{c}_{cart} \quad (4.19)$$

$$\tilde{c}_{cart}(\tilde{x} = 0, \tilde{t}) = K_p \tilde{c}_b(\tilde{t}) \quad (4.20)$$

$$\frac{\partial \tilde{c}_{cart}}{\partial \tilde{x}} \bigg|_{\tilde{x}=1, \tilde{t}} = 0 \quad (4.21)$$

$$\tilde{c}_{cart}(\tilde{x}, \tilde{t} = 0) = 0 \quad (4.22)$$

where $\tilde{x} = x/L$, $\tilde{t} = D_{cart}t/L^2$, $\tilde{c}_b = c_b/c_{b0}$ and $\tilde{c}_{cart} = c_{cart}/c_{b0}$.

In these equations, the bath volume, cartilage thickness and cartilage surface area are known from the experimental setup. The unknowns are the diffusion coefficient (D), the partition coefficient (K_p) and the reaction rate constant (k).

For the engineered GFP variants, the diffusivity in cartilage (D_{cart}) was estimated to be $2.7 * 10^{-11} m^2/s$ based on the size of GFP reported in literature [242] and the empirical model developed in [243]. For the fusion proteins, we knew the molecular weight but not the length in nanometers. For globular proteins, the ratio of diffusivities can be estimated well by the formula below [244], where D is diffusivity and M is molecular weight:

$$\frac{D_1}{D_2} = \sqrt[3]{\frac{M_2}{M_1}} \quad (4.23)$$

We used this equation to obtain one estimate for fusion protein diffusivity. However, the fusion proteins have polypeptide linkers and their shape may not be necessarily globular. For rod-like or cylindrical proteins, a better estimate of diffusivity ratios is obtained by using the length (L) instead of molecular weight [244][245] :

$$\frac{D_1}{D_2} = \frac{L_2}{L_1} \quad (4.24)$$

While we did not know the precise length of the fusion proteins, other groups have

estimated linker length using a peptide unit length of 0.38 nm [246]. We estimated fusion protein linker lengths using this value of the peptide unit length, added the linker length to GFP length and used this to get a second estimate of fusion protein diffusivity.

For each fusion protein, the model simulations were fit to experimental data twice to obtain estimates for K_p and k : once with the diffusivity value based on length and for a second time with diffusivity based on molecular weight. This was done by spatial discretization of partial differential equations to obtain a set of ordinary differential equations (ODEs) in time followed by the use of ODE solvers and the optimization toolbox in MATLAB.

While numerical methods were used for solving the system of equations in the current work, analytical solutions can be attempted in future work. One of these methods could involve replacing the PDE with a boundary integral over the flux at the interface. In the boundary integral form with the transient bath boundary condition, the problem would reduce into an ordinary integro-differential equation. In case this integro-differential equation is not easily solvable analytically, analytical approximations could potentially be derived. Another way in which the model equations could be solved would be to use Laplace transforms. In case the inversion is not tractable, the Laplace transforms can be used to get the asymptotic behavior at early and late times. Since we were interested in getting concentration profiles at all times and all spatial locations, we chose to go ahead with the numerical simulations. We used the data from uptake ratio experiments in chapters 2 and 3 to validate the model (this validation is described further in later sections of this chapter). Any analytical approximations derived in the future could be compared with numerical simulation results to further validate the current work.

4.3.1 Model predictions for uptake experiments

The equations for transport in the transient uptake experiments shown schematically in Figure 3 of Chapter 2 would be identical to those in the dynamic uptake experiments detailed above. While the cartilage in the transient uptake experiment has the lateral surface of the disk exposed to the bath in addition to the top surface, the confocal microscopy images in Figures 2-4 and S2.5 demonstrate that GFP transport into cartilage was mainly taking place

through the top surface. Therefore a 1-dimensional transport equation for cartilage transport was considered to be a reasonable assumption, and the change in bath concentration in the transient uptake experiments can be modeled by equations (4.17)-(4.22). The main difference between the dynamic uptake system (this chapter) and the transient uptake system (Chapters 2 and 3) is that the bath volume (V_b) and the dimensions of the cartilage (A_{cart} and L) are different. The diffusivity of GFPs in cartilage (D_{cart}), the partition coefficient (K_p) and the reaction rate constant (k) would be the same in both systems. Therefore, the partition coefficient and the reaction rate constant that were estimated by fitting dynamic uptake data can be used along with known parameters to predict the bath concentration in uptake experiments. These predictions were compared to experimental data to validate whether or not the model could provide accurate predictions at different size scales.

It is to be noted that a more accurate way to model the transport of GFPs in the transient uptake experiments would be to use a 2-dimensional diffusion reaction equation to account for the transport occurring in the radial direction through the lateral surface of the cartilage explants. The same transport parameters (diffusivity (D_{cart}), the partition coefficient (K_p) and the reaction rate constant (k)) could be used in the 2D model, and the results can be compared with the 1D model results. Since our 1D model results matched well with experimental data (shown in the Results and Discussion section), we did not repeat the calculations with a 2D model. However, the 2D model can be formulated and used for analysis as part of future work.

4.4 Predictions for transport inside the human joint

The 1-D transport model described in equations 4.17 to 4.22 can be used to obtain an estimate for the time scale for the uptake into cartilage of GFPs injected intra-articularly into the human knee joint. The values for diffusivity (D_{cart}), partition coefficient (K_p) and reaction rate constant (k) were assumed to be the same as those obtained from fitting the model to dynamic uptake experiments. From values reported in literature, the values for the synovial fluid (SF), cartilage surface area and cartilage thickness were assumed to be 3 mL [247], 6000 mm² [248] and 2 mm [237][238] respectively. The coupled differ-

ential equations were converted into a system of ordinary differential equations (ODEs) by discretizing the spatial derivatives . This system of ODEs was solved using an ODE solver in MATLAB, and the concentration profile was obtained as a function of time in the bath (SF) and as a function of time and space inside cartilage. The concentration of the free and bound GFP inside cartilage was averaged over the thickness of cartilage and was normalized with the initial SF concentration.

The transport equations used to obtain these concentration profiles do not account for the clearance of molecules from the joint space due to the constant turnover of SF. The SF volume in the human knee joint is turned over in approximately 1 hour [249] and the resulting protein clearance rate is approximately 0.05 mL/min [250][251]. Multiplying this volumetric clearance rate with the SF concentration of GFP will give the molar clearance rate of the GFPs from the joint, and the modified differential equation for the SF concentration will be:

$$V_b \frac{dc_b}{dt} = A_{cart} D_{cart} \frac{\partial c_{cart}}{\partial x} \Big|_{x=0,t} - R_v c_b \quad (4.25)$$

where R_v is the volumetric clearance rate from literature.

This new system of equations was solved numerically using MATLAB to obtain the concentration of GFPs in the synovial fluid and knee cartilage as well as the moles of GFPs entering systemic circulation as a function of time.

4.5 Results and Discussion

The bleaching corrected bath concentrations for dynamic uptake experiments using cationic GFP variants are shown in Figure 4.2. The x-axis is the non-dimensional time ($\tilde{t} = D_{cart}t/L^2$) and the y-axis is the non-dimensional concentration ($\tilde{c}_b = c_b/c_{b0}$).

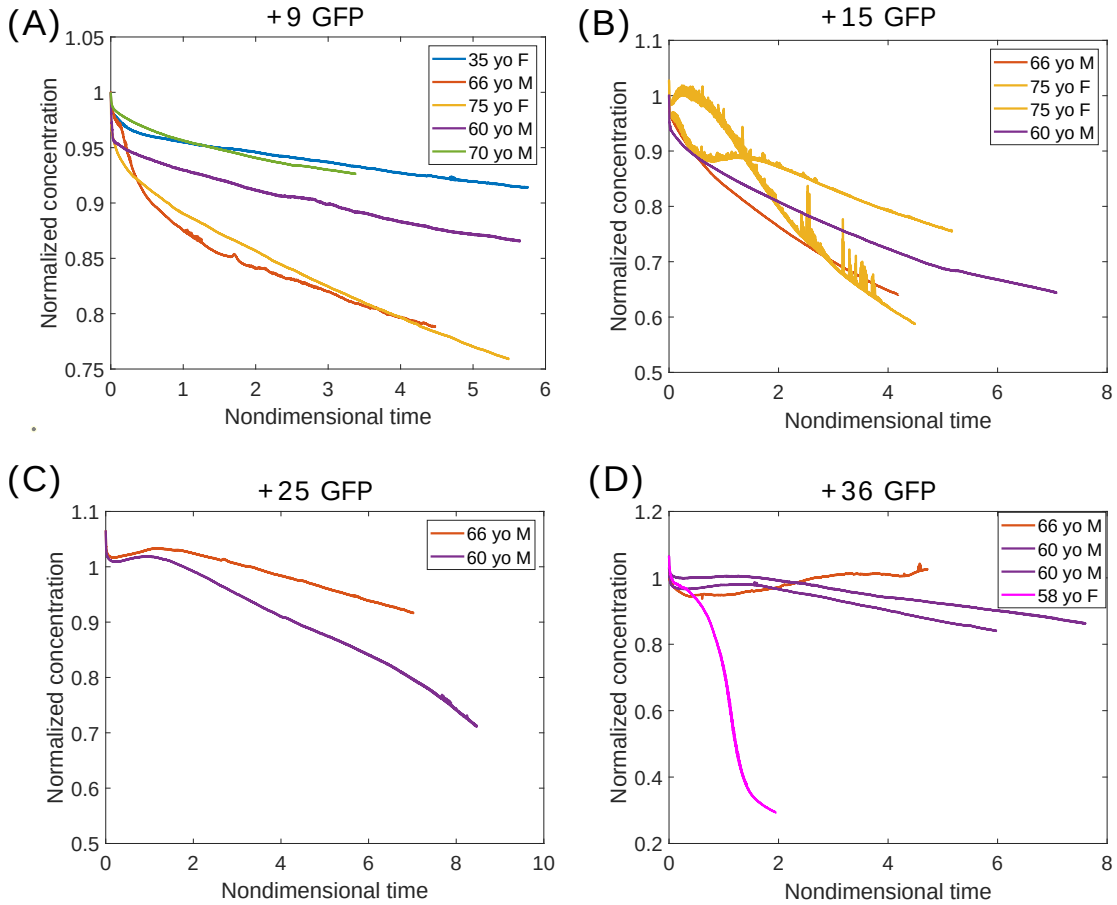


Figure 4.2: Dynamic uptake data for cationic GFP carriers in human cartilage. Bleaching corrected bath concentrations (normalized to initial bath concentration) for +9, +15, +25 and +36 GFP are shown in (A), (B), (C) and (D) respectively. In the x-axis, time has been non-dimensionalized using the diffusion time scale (L^2/D).

Bleaching corrected bath concentration curves for experiments with GFP-IGF fusion proteins are shown in Figures 4.3 to 4.5. In Figure 4.3, the x-axis is time in hours. In Figures 4.4 and 4.5, the x-axes represent time that has been non-dimensionalized using diffusivity based on protein length and diffusivity based on protein molecular weight respectively.

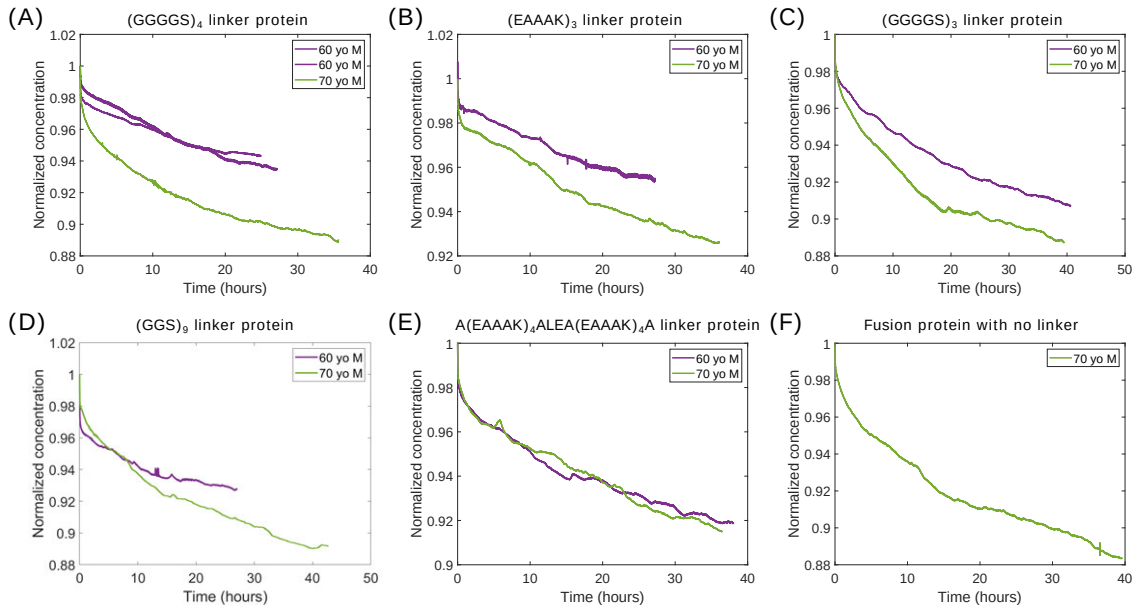


Figure 4.3: Dynamic uptake data for +9GFP-IGF-1 fusion proteins in human cartilage. Bleaching corrected bath concentrations (normalized to initial bath concentration) for fusion proteins with the (GGGGS)₄, (EAAAK)₃, (GGGGS)₃, (GGG)₉, A(EAAAK)₄ALEA(EAAAK)₄ linkers and with no linker are shown in (A), (B), (C), (D), (E) and (F) respectively. The x-axis is time in hours.

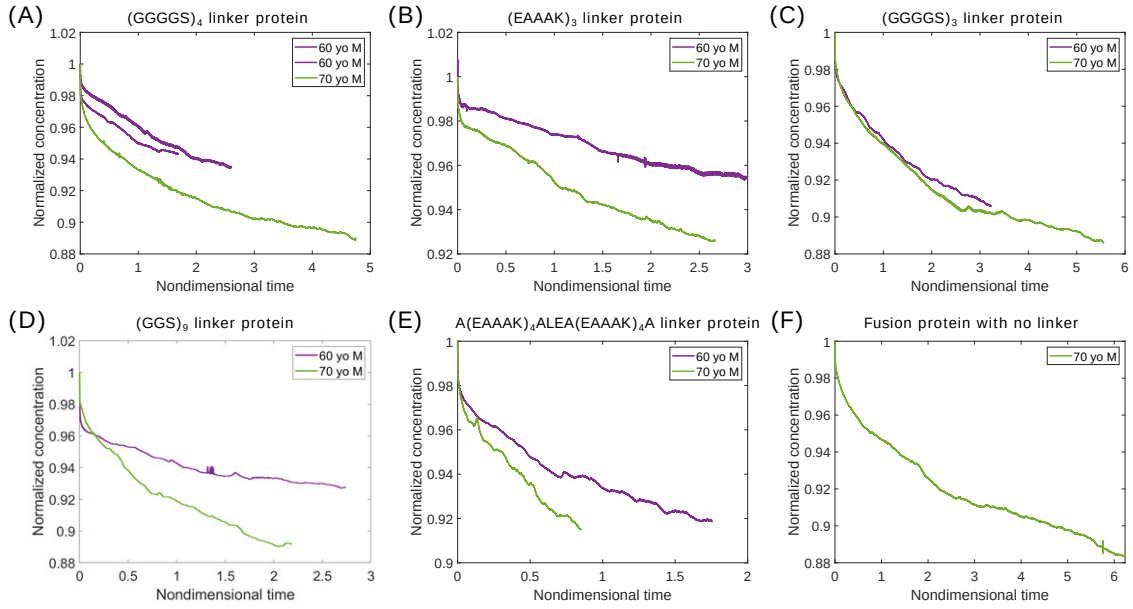


Figure 4.4: Dynamic uptake data for +9GFP-IGF-1 fusion proteins in human cartilage. Bleaching corrected bath concentrations (normalized to initial bath concentration) for fusion proteins with the (GGGGS)₄, (EAAAK)₃, (GGGGS)₃, (GGS)₉, A(EAAAK)₄ALEA(EAAAK)₄ linkers and with no linker are shown in (A), (B), (C), (D), (E) and (F) respectively. In the x-axis, time has been non-dimensionalized using the diffusion time scale (L^2/D_{len}) in which diffusivity has been estimated based on the length of the fusion proteins.

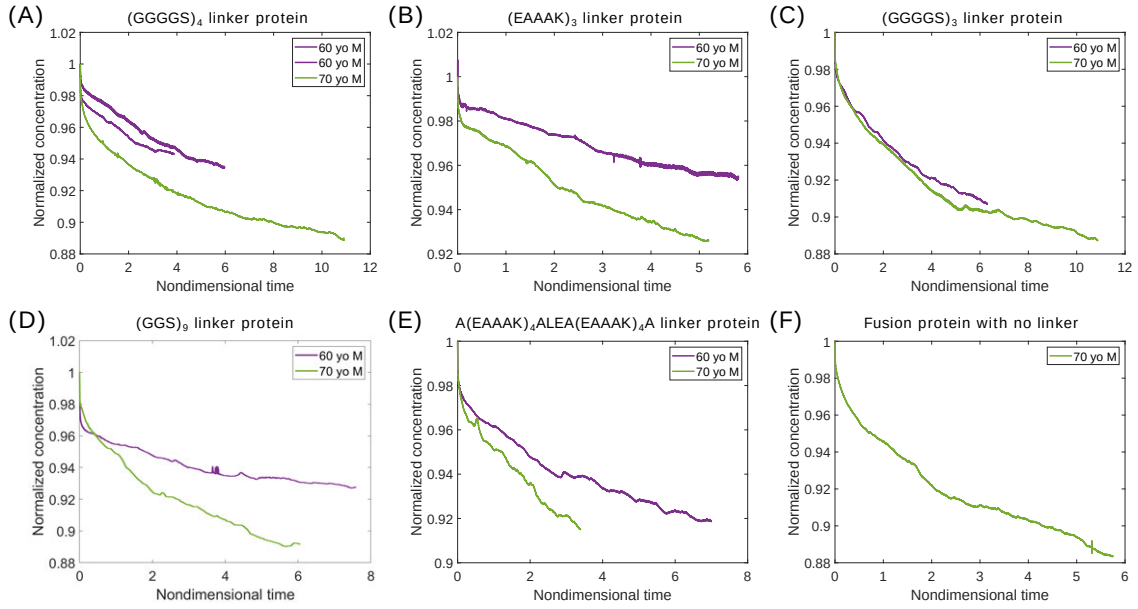


Figure 4.5: Dynamic uptake data for +9GFP-IGF-1 fusion proteins in human cartilage. Bleaching corrected bath concentrations (normalized to initial bath concentration) for fusion proteins with the (GGGGS)₄, (EAAAK)₃, (GGGGS)₃, (GGS)₉, A(EAAAK)₄ALEA(EAAAK)₄ linkers and with no linker are shown in (A), (B), (C), (D), (E) and (F) respectively. In the x-axis, time has been non-dimensionalized using the diffusion time scale (L^2/D_{MW}) in which diffusivity has been estimated based on the molecular weight of the fusion proteins.

The mathematical model fits for +9 GFP dynamic uptake data are shown by the dashed black lines in Figure 4.6, and the corresponding values of K_p and k are shown in Table 4.2. The scalability of the model was validated by making predictions for the *in vitro* uptake experiments described in Chapter 2. The model predictions matched well with experimental data as shown in Table 4.3.

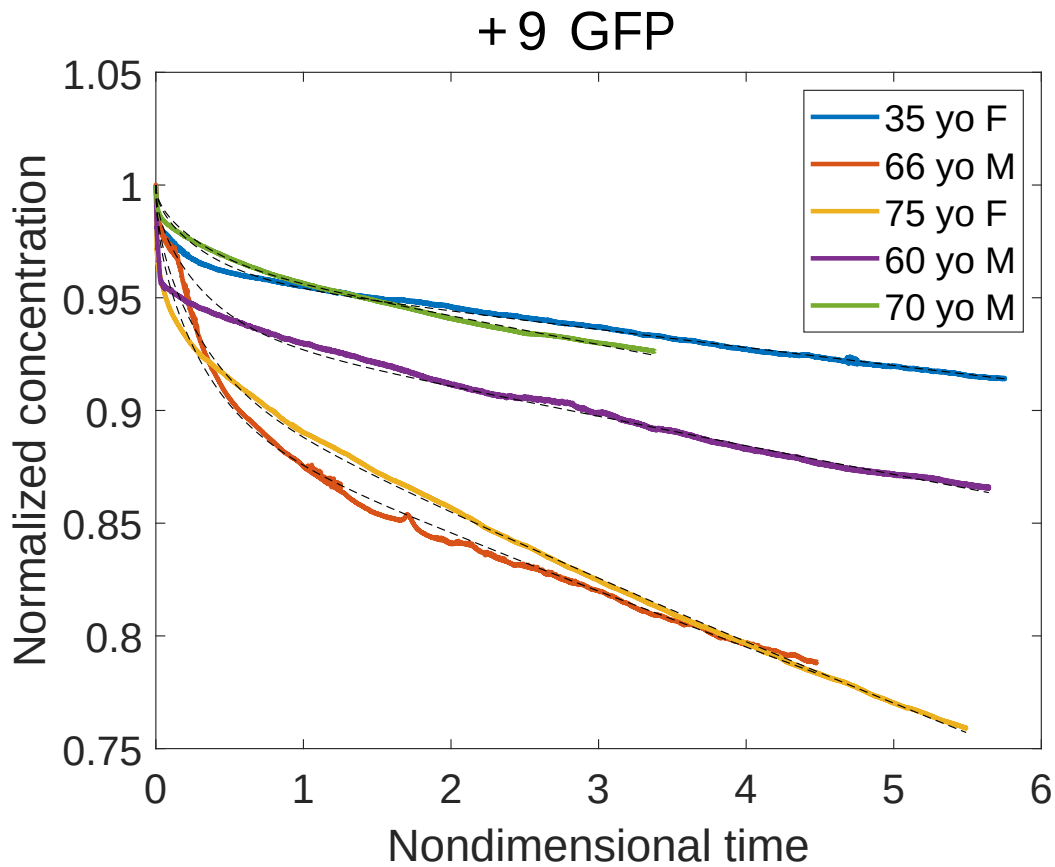


Figure 4.6: Experimental data (solid lines) and mathematical model fits (dashed lines) for dynamic uptake of +9 GFP in human cartilage from five donors.

Donor	Partition coefficient (K_p)	Reaction rate constant (k)
35 yo F	10.9	1.2×10^{-5}
66 yo M	22.3	8.6×10^{-6}
75 yo F	21.8	1.5×10^{-5}
60 yo M	17.6	1.3×10^{-5}
70 yo M	15	5.2×10^{-5}

Table 4.2: Best fit values for partition coefficient and reaction rate constant for the transport of +9 GFP in human cartilage from five donors.

Normalized bath concentration		Human cartilage	
		24 hr	8 day
Predicted		0.46 ± 0.12	0.06 ± 0.05
Experimental data	35 yo F	0.46 ± 0.04	0.08 ± 0.02
	36 yo M	0.51 ± 0.07	NA

Table 4.3: Predicted and experimental normalized uptake bath concentrations for +9 GFP in human cartilage

For three of the +9GFP-IGF-1 fusion proteins (with the (GGGGS)₄, (EAAAK)₃ and (GGGGS)₃ linkers), the model simulations were fit to experimental data twice: once with diffusivity estimates based on protein length (Figure 4.7, top row) and for a second time with diffusivities estimated using molecular weight (Figure 4.7, bottom row).

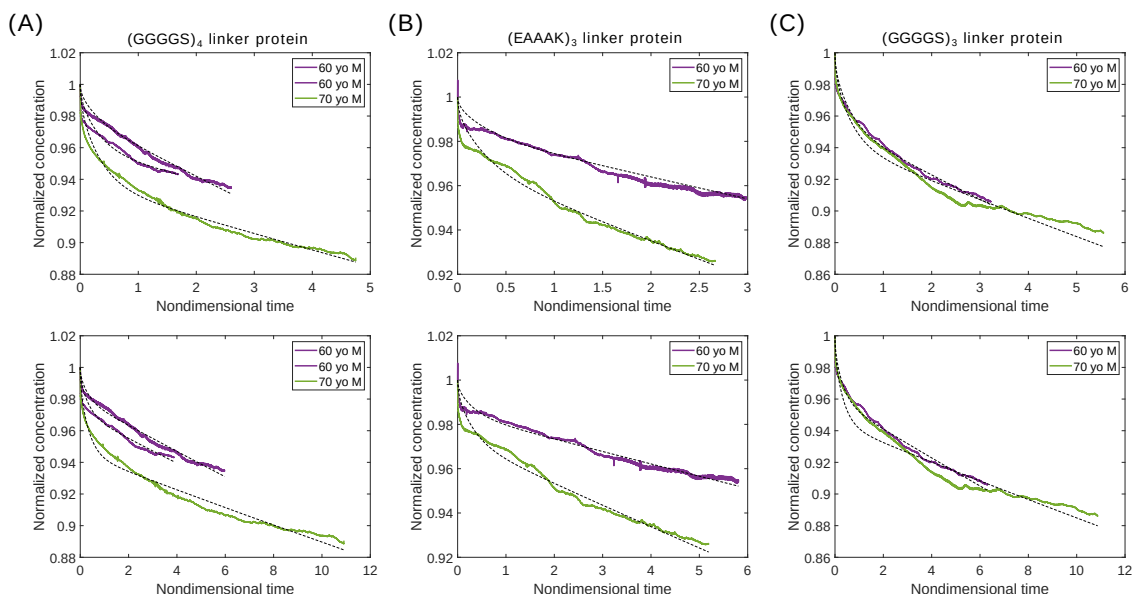


Figure 4.7: Experimental data (solid lines) and mathematical model fits (dashed lines) for dynamic uptake of fusion proteins with (GGGGS)₄, (EAAAK)₃ and (GGGGS)₃ linkers. Diffusivity coefficients estimated based on fusion protein lengths were used in the graphs in the top row, and the bottom row shows graphs in which diffusivities were estimated based on molecular weights.

The K_p and k values estimated from these simulations are shown in Table 4.4 for the (GGGGS)₄ linker protein, Table 4.5 for the (EAAAK)₃ linker protein, and Table 4.6 for the (GGGGS)₃ linker protein. The two estimates of diffusivity for each protein were different by approximately a factor of 2, and the difference between the corresponding values of K_p and k was less than this in most cases (Tables 4.4, 4.5 and 4.6).

Donor	Diffusivity estimated from protein length		Diffusivity estimated from protein molecular weight	
	Partition coeff. (K_p)	Rxn rate constant (k)	Partition coeff. (K_p)	Rxn rate constant (k)
60 yo M	8	$2.1 \cdot 10^{-5}$	6.5	$2.3 \cdot 10^{-5}$
60 yo M	10.6	$4.0 \cdot 10^{-6}$	7.4	$1.2 \cdot 10^{-5}$
70 yo M	22	$6.5 \cdot 10^{-6}$	18.3	$9.3 \cdot 10^{-6}$

Table 4.4: Best fit values for partition coefficient and reaction rate constant for the transport of +9GFP-IGF-1 fusion protein with (GGGGS)₄ linker in three human cartilage explants from two donors

	Diffusivity estimated from protein length		Diffusivity estimated from protein molecular weight	
Donor	Partition coeff. (K_p)	Rxn rate constant (k)	Partition coeff. (K_p)	Rxn rate constant (k)
60 yo M	5.5	1.7×10^{-5}	4.7	2.0×10^{-5}
70 yo M	8.7	1.0×10^{-5}	6.9	1.4×10^{-5}

Table 4.5: Best fit values for partition coefficient and reaction rate constant for the transport of +9GFP-IGF-1 fusion protein with (EAAAK)₃ linker in two donors

	Diffusivity estimated from protein length		Diffusivity estimated from protein molecular weight	
Donor	Partition coeff. (K_p)	Rxn rate constant (k)	Partition coeff. (K_p)	Rxn rate constant (k)
60 yo M	12.4	7.3×10^{-6}	10.3	9.7×10^{-6}
70 yo M	19.3	8.5×10^{-6}	17.7	8.7×10^{-6}

Table 4.6: Best fit values for partition coefficient and reaction rate constant for the transport of +9GFP-IGF-1 fusion protein with (GGGGS)₃ linker in three human cartilage explants from two donors

For each fusion protein, the two sets of D , K_p and k values were used to predict the results of the *in vitro* uptake experiments described in Chapter 3. The comparisons of the model predictions and experimental values are shown in Table 4.7 for the (GGGGS)₄ linker protein, Table 4.8 for the (EAAAK)₃ linker protein, and Table 4.9 for the (GGGGS)₃ linker protein. For each fusion protein, model predictions using both sets of transport parameters were a good match with experimental data at the 36 hour time point. At the 4.5 day time-point, the predicted values with error estimates overlapped with experimental values with error estimates for parameters that were estimated based on diffusivity calculated from protein length. The predictions from the model with diffusivity calculated from protein molecular weight did not overlap with experimental values at the 4.5 day timepoint, but they were very similar to the predictions from the protein length based diffusivity model.

(A) Diffusivity estimated from fusion protein length

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.62 ± 0.11	0.39 ± 0.16
Experimental data	66 yo M	0.67 ± 0.03	0.62 ± 0.16

(B) Diffusivity estimated from fusion protein molecular weight

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.59 ± 0.11	0.31 ± 0.10
Experimental data	66 yo M	0.67 ± 0.03	0.62 ± 0.16

Table 4.7: Predicted and experimental normalized uptake bath concentrations for (GGGS)₄ linker fusion protein in human cartilage

(A) Diffusivity estimated from fusion protein length

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.71 ± 0.04	0.46 ± 0.03
Experimental data	66 yo M	0.65 ± 0.01	0.51 ± 0.06

(B) Diffusivity estimated from fusion protein molecular weight

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.69 ± 0.03	0.41 ± 0.02
Experimental data	66 yo M	0.65 ± 0.01	0.51 ± 0.06

Table 4.8: Predicted and experimental normalized uptake bath concentrations for (EAAK)₃ linker fusion protein in human cartilage

(A) Diffusivity estimated from fusion protein length

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.56 ± 0.10	0.33 ± 0.11
Experimental data	66 yo M	0.65 ± 0.05	0.54 ± 0.12

(B) Diffusivity estimated from fusion protein molecular weight

Normalized bath concentration		Human cartilage	
		36 hr	4.5 day
Predicted		0.56 ± 0.09	0.30 ± 0.09
Experimental data	66 yo M	0.65 ± 0.05	0.54 ± 0.12

Table 4.9: Predicted and experimental normalized uptake bath concentrations for (GGGGS)₃ linker fusion protein in human cartilage

The surface area to volume ratio in the *in vitro* uptake experiments was an order of magnitude larger than the ratio in the dynamic uptake experiments, and the mathematical model was still able to provide reasonably accurate predictions of the transport behavior of +9 GFP and +9GFP-IGF-1 fusion proteins. These results provided confirmation that the mathematical model performed well at different size scales. We then used it to predict the transport behavior of these proteins in human knee joints, in which the surface area to volume ratio is two orders of magnitude larger than the dynamic uptake experiments.

We assumed a uniform initial concentration of the proteins in synovial fluid after intra-articular injection, and used the model to predict the protein concentrations in cartilage tissue and in synovial fluid as a function of time. The model was also modified to incorporate protein clearance from the joint capsule, and the simulations were repeated. In this second set of simulations, we also obtained predictions for the amount of proteins in the systemic circulation as a function of time. The simulation results with and without joint capsule clearance are shown in Figure 4.8 for +9 GFP, Figure 4.9 for the (GGGGS)₄ linker protein, Figure 4.10 for the (EAAAK)₃ linker protein, and Figure 4.11 for (GGGGS)₃ linker protein. In Figures 4.9 to 4.11, the left column shows predictions from the model

with protein-length based diffusivities, and the right column graphs are predictions from the model with diffusivities estimated using molecular weights.

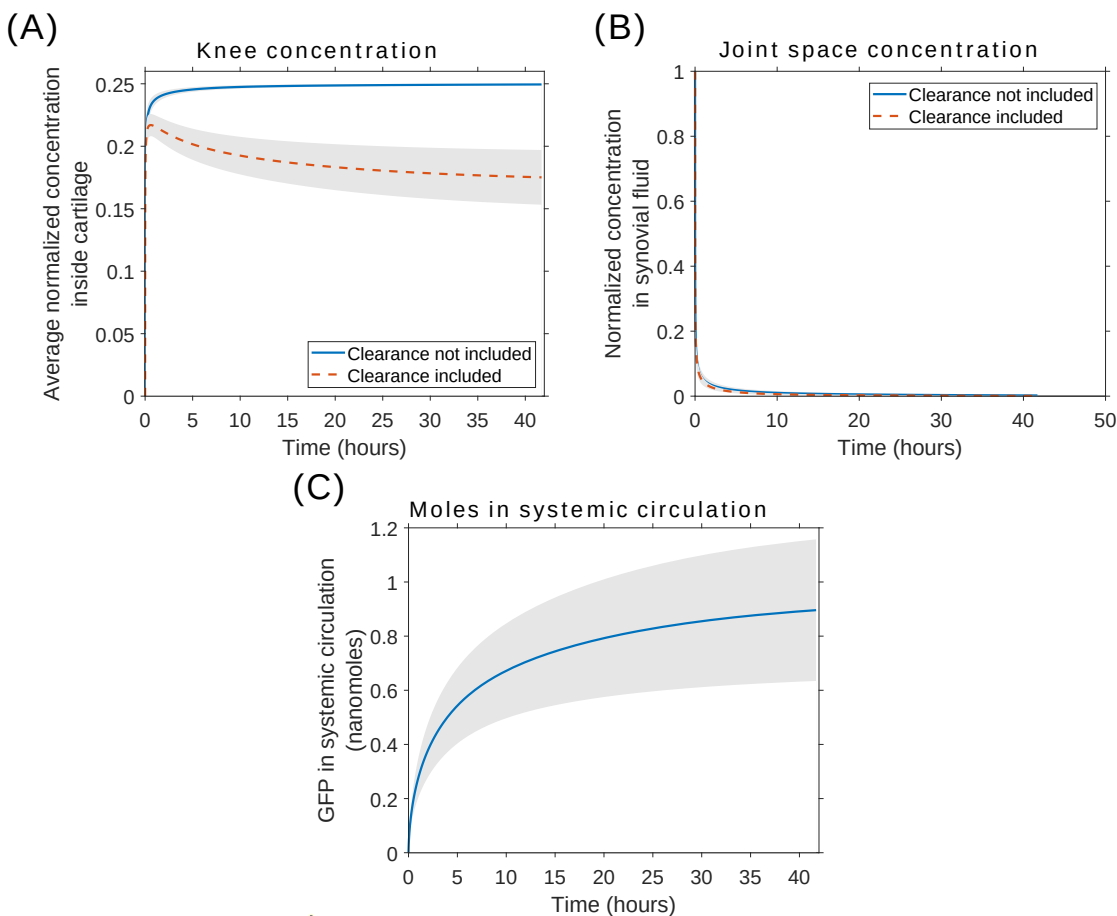


Figure 4.8: Model prediction for concentration in knee cartilage, concentration in synovial fluid and systemic levels of intra-articularly injected +9 GFP. Mean predictions based on parameters from 5 donors are shown (dashed and solid lines) along with the standard deviation (shaded gray region).

In each case, significant fractions of the total injected proteins are able to penetrate cartilage within the first few hours of intra-articular injection. Accounting for joint capsule clearance led to lower concentrations of proteins in cartilage. But even with clearance included, the steady state average concentration inside cartilage was 0.1 to 0.2 times the initial protein concentration in the synovial fluid. This means that if we want to achieve a particular drug concentration inside cartilage to ensure efficacy, the injection dose should be such that the synovial fluid concentration is approximately 10 times higher than the target cartilage concentration.

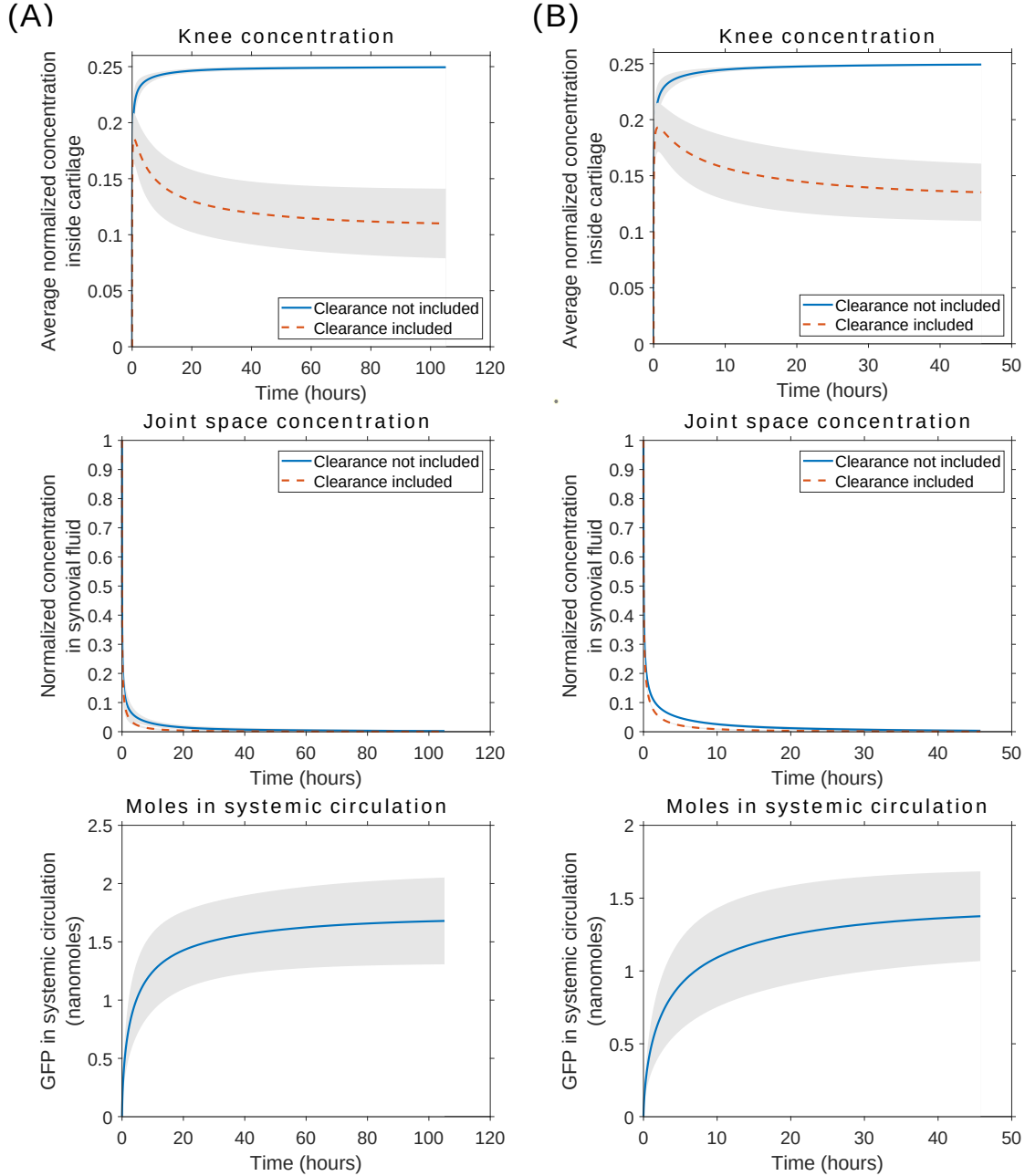


Figure 4.9: Model prediction for concentration in knee cartilage, concentration in synovial fluid and systemic levels of intra-articularly injected +9GFP-IGF-1 fusion protein with (GGGGS)₄ linker. Mean predictions based on parameters from 2 donors are shown (dashed and solid lines) along with the standard deviation (shaded gray region). Diffusivity coefficients estimated based on fusion protein lengths were used in the graphs in the left column, and the right column shows graphs in which diffusivities were estimated based on molecular weights.

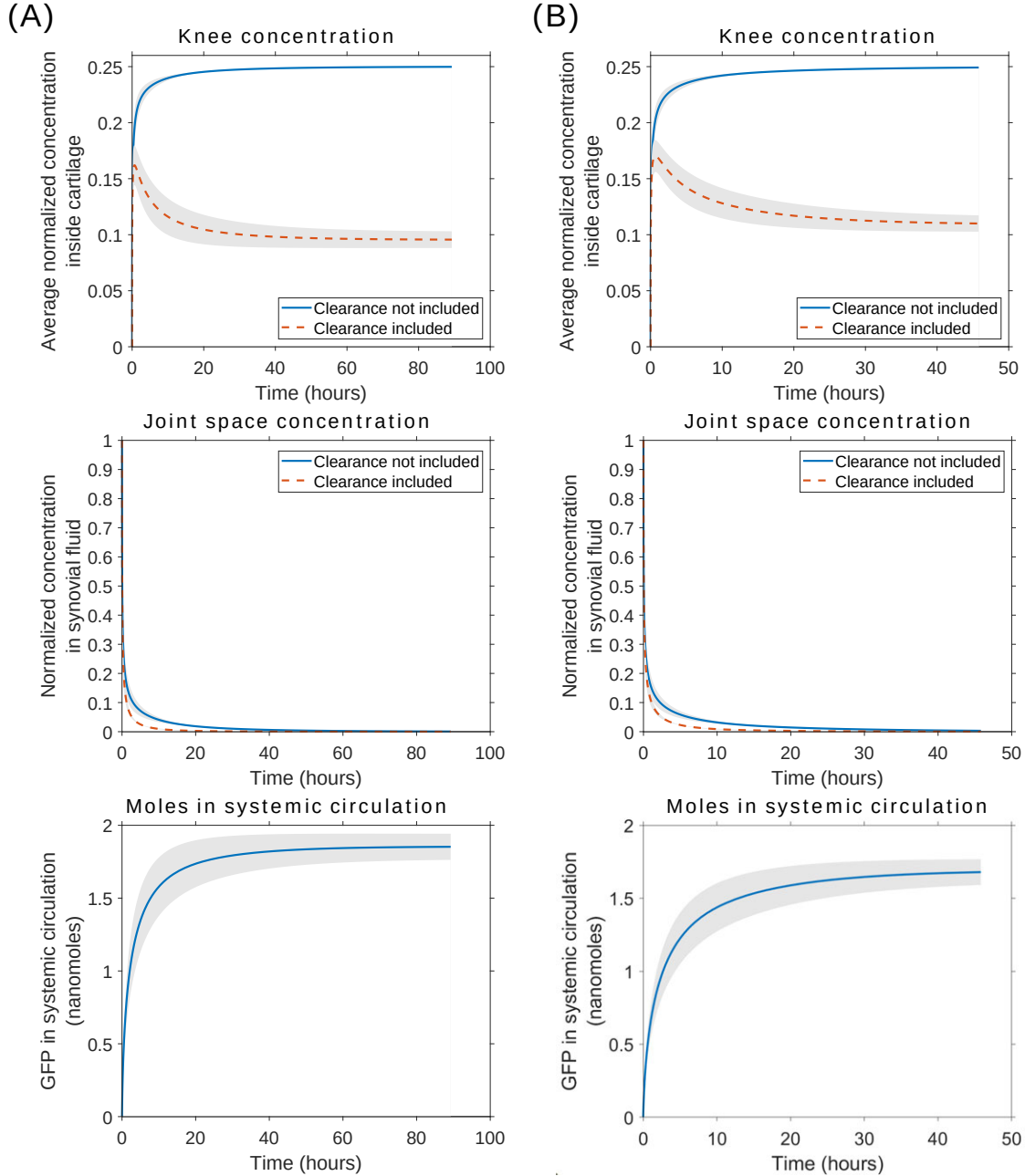


Figure 4.10: Model prediction for concentration in knee cartilage, concentration in synovial fluid and systemic levels of intra-articularly injected +9GFP-IGF-1 fusion protein with (EAAAK)₃ linker. Mean predictions based on parameters from 2 donors are shown (dashed and solid lines) along with the standard deviation (shaded gray region). Diffusivity coefficients estimated based on fusion protein lengths were used in the graphs in the left column, and the right column shows graphs in which diffusivities were estimated based on molecular weights.

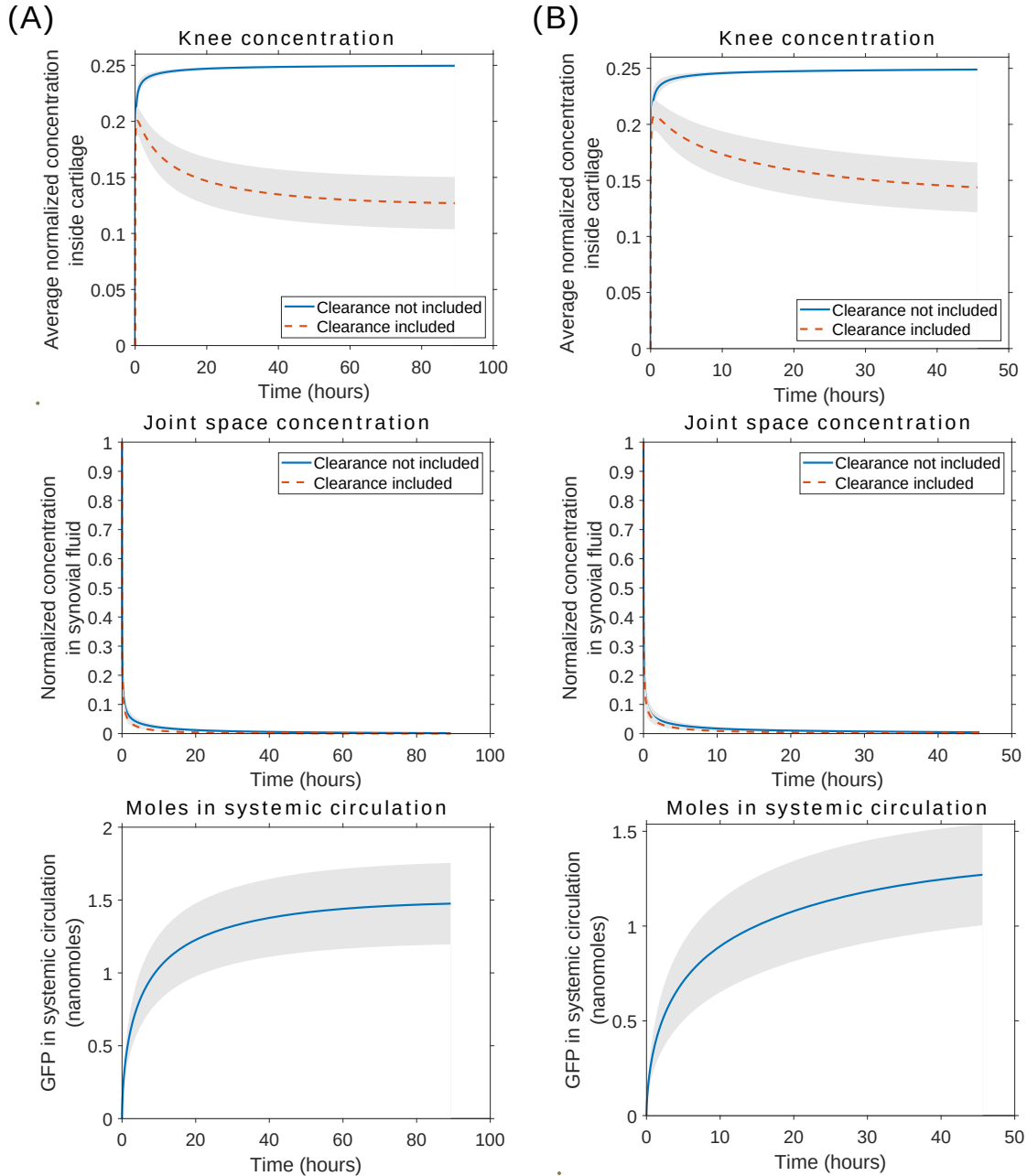


Figure 4.11: Model prediction for concentration in knee cartilage, concentration in synovial fluid and systemic levels of intra-articularly injected +9GFP-IGF-1 fusion protein with (GGGGS)₃ linker. Mean predictions based on parameters from 2 donors are shown (dashed and solid lines) along with the standard deviation (shaded gray region). Diffusivity coefficients estimated based on fusion protein lengths were used in the graphs in the left column, and the right column shows graphs in which diffusivities were estimated based on molecular weights.

To make the predictions for the number of nanomoles of proteins in systemic circulation, we assumed that the initial protein concentration in synovial fluid was 1 μM . If a different initial concentration is used, the amount of protein in systemic circulation will vary proportionally. In making these predictions, we assumed that any protein that is cleared out by the joint capsule enters the systemic circulation and the proteins are not cleared out by the kidneys or liver. So these predictions represent approximate values for the maximum protein quantities that will be present in systemic circulation. If it is found out that there is a threshold beyond which the proteins can cause undesirable side effects, then the model predictions can be used to back-calculate the maximum dose that would be safe for intra-articular injection.

Chapter 5

Developing *In Vitro* Cartilage Synovium Co-Culture Models for Post-Traumatic Osteoarthritis (PTOA)

5.1 Introduction

Osteoarthritis is a multifactorial disease that primarily affects articular hyaline cartilage but also involves other joint tissues such as the subchondral bone and synovium [150][160][161]. The risk factors for developing OA include aging, being overweight, being female, abnormal joint alignment and having a history of traumatic joint injury [252]. The exact mechanisms that lead to the symptoms of OA within the different subtypes are not completely understood. But it is known that mechanical, structural and biological factors all play a role in disease progression.

On the biological side, chronic low grade inflammation with the involvement of the innate immune system plays a role in disease progression [172]. Cartilage, synoviocytes and macrophages in synovial tissue and subchondral bone all produce cytokines, chemokines, proteases and other inflammatory mediators [172][253]. These can directly contribute to disease progression by damaging tissues and also indirectly contribute by stimulating inflammatory pathways in other tissues. Even in the context of a disease like post-traumatic

osteoarthritis where the primary trigger is an acute traumatic mechanical injury, there is a significant increase in the levels of cytokines such as IL-6, IL-1 β and TNF- α in the days and weeks following injury [254][165]. These cytokines can then stimulate the upregulation of proteases (e.g., metalloproteinases and aggrecanases) that lead to cartilage degeneration.

While the crosstalk between joint tissues that is enabled by the innate immune system plays an important role in OA progression [255], this is not captured in traditional *in vitro* models that have mono-cultures of cartilage subjected to externally induced mechanical or biochemical injury. As a first step in capturing these interactions, human and bovine co-culture models with cartilage and synovium have been set up in the present study.

Some of the earliest cartilage-synovium co-culture studies were performed with porcine tissues [256][257]. It was found that in the presence of synovium, the rate of collagen and proteoglycan degradation in cartilage was elevated and the rate of aggrecan biosynthesis (as measured by ^{35}S radiolabel incorporation) was reduced. However when the synovium was removed from the co-culture after 8 days and the cartilage culture was continued for 4 more days, ^{35}S radiolabel incorporation recovered to levels similar to or higher than the levels seen in cartilage that had been in monoculture throughout [257].

In vitro cartilage-synovium co-cultures have also been studied with canine [258][259], equine [260][261] and bovine tissues [262][263][264][265]. Bovine synovial tissue was found to release ADAMTS-5 and increase proteoglycan degradation in bovine cartilage [262][263]. Co-culture with bovine synovium also led to a decrease in proteoglycan biosynthesis in cartilage, and this could not be rescued by IL-1 blockade or IL-1 + TNF- α blockade [265].

In experiments *in vitro* with human cartilage and synovium tissues harvested from end-stage OA joints (obtained from knee replacement surgeries), MMP activity was detected in co-cultures of cartilage with synovium [266]. Cartilage that was co-cultured with synovium also had lower glycosaminoglycan (GAG) content and biosynthesis [266]. Cartilage and synovium monocultures were also included in these studies and both tissue types produced different subsets of cytokines. The cytokines produced by the synovium were more similar to those seen in the synovial fluid of OA patients [266].

In the present study, we established an *in vitro* co-culture model starting with nor-

mal adult human cartilage and synovium (as opposed to end stage OA tissues). Immature bovine cartilage-synovium co-culture experiments were also set up, with the aim of comparing results with human tissue experiments and gaining insights into the effects of similarities or differences in tissue responses based on species and age.

We note that since the time these experiments were completed, extensive co-culture studies with human cartilage, synovium and subchondral bone have been completed by other lab members [267]. Since these experiments included subchondral bone (unlike the present study which only includes cartilage and synovium), they represent a more physiologically relevant *in vitro* OA model.

5.2 Materials and Methods

5.2.1 Harvest and culture of adult human cartilage and synovium

Two human knee joint distal femurs (36 year old male donor; Collins grade 0 and 75 year old; Collins grade 2) along with the joint capsule synovium were obtained from the Gift of Hope Organ and Tissue Donor Network (Itasca IL). All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT.

Full thickness cartilage explants were harvested with a 3mm biopsy punch, and synovium tissue was cut into approximately 5x5 mm explants. Cocultures with one cartilage explant and one synovium explant were set up in serum-free medium: low-glucose DMEM; 1g/L (Corning Cellgro, Manassas, VA) supplemented with 10mM HEPES buffer (Gibco, Grand Island, NY), 0.1mM nonessential amino acids (Sigma Aldrich, St. Louis, MO), 0.4mM proline (Sigma), 20µg/ml ascorbic acid (Sigma), 100units/ml penicillin G, 100µg/ml streptomycin, and 0.25µg/ml amphotericin B (Sigma) for 2-3 days (5% CO₂; 37°C). Monoculture cartilage and synovium control cultures were also set up (Figure 5.1).

Media changes with serum-free culture medium were performed every two to three days. The media collected during media changes was immediately frozen at -20°C and later thawed to perform multiplex ELISA assays. Cartilage biosynthesis rates were measured

during the last 24 to 48 hours of each experiment. One experiment was terminated at the 7-day time point and another was terminated at day 14. On the day of termination, some explants were used for the cell viability assay using FDA/PI. All other explants were washed, weighed and digested using proteinase K. The digested explants were frozen at -20°C and later used for downstream assays.

5.2.2 Harvest and culture of young bovine cartilage and synovium

Bovine knee joints from three young (1-2 week old) calves were procured on the day of slaughter (Research 87, Boylston, MA). The joints were carefully opened and the joint capsules were harvested first. Following this, cartilage explants were harvested from the femoropatellar groove with a 3mm biopsy punch and trimmed down to 1mm thickness. The joint capsule synovium was cut into explants with scissors, and one cartilage explant was incubated with one synovium explant for the coculture conditions (Figure 5.2). Mono-culture cartilage and synovium control cultures were also set up.

Culture medium consisted of low-glucose DMEM; 1g/L (Corning Cellgro, Manassas, VA) supplemented with 10mM HEPES buffer (Gibco, Grand Island, NY), 0.1mM nonessential amino acids (Sigma Aldrich, St. Louis, MO), 0.4mM proline (Sigma), 20µg/ml ascorbic acid (Sigma), 1% ITS, 100units/ml penicillin G, 100µg/ml streptomycin, and 0.25µg/ml amphotericin B (Sigma) for 2-3 days (5% CO₂; 37°C). Media changes were performed every two to three days, and experiments were terminated at the 1-week, 2-week and 3-week timepoints. Media was collected and frozen (-20°C) during media changes, and was later used for IL-6, IL-1 β and TNF- α ELISA assays. Cartilage biosynthesis rates were measured during the last 24 to 48 hours of the each experiment. On the experiment termination day, all tissues were washed, weighed and digested using proteinase K. Some explants were not digested and were used for the cell viability assay using FDA/PI. The digested explants were frozen at -20°C and later used for downstream assays.

5.2.3 Multiplex ELISA for human proinflammatory cytokines

Culture media samples were analyzed using the V-Plex Proinflammatory Panel 1 Human Kit from Meso Scale Discovery (MSD). This kit measures the levels of IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-1 β , TNF- α , IFN- γ , and IL-12p70. The protocols provided by the kit manufacturer were followed for performing the assay, reading the plates and analyzing the data.

5.2.4 Singleplex ELISA for bovine IL-6, IL-1 β , and TNF- α

Cytokine levels in bovine culture media samples were measured using a bovine IL-6 ELISA kit (Thermo Fisher), a bovine IL-1 β ELISA kit (Thermo Fisher) and a bovine TNF- α ELISA kit (R&D systems). The protocols provided by the kit manufacturer were followed for performing the assay, reading the plates and analyzing the data.

5.2.5 sGAG biosynthesis rate and explant sGAG content

To measure the chondrocyte biosynthesis rate of sulfated glycosaminoglycans (sGAGs), ^{35}S -sulfate radiolabel was added to the culture medium during the final 24 to 48 hours of each experiment, using 10 $\mu\text{Ci/mL}$ ^{35}S -sulfate for human tissues and 5 $\mu\text{Ci/mL}$ for bovine tissues. Explants were then washed with PBS with non-radioactive sulfate to wash out excess radiolabel, weighed and digested with proteinase K. A liquid scintillation counter (PerkinElmer) was used to measure the amount of radiolabel incorporated within the extracellular matrix of the tissues. Radiolabel incorporation data for cartilage were normalized by the duration of exposure to radiolabel and by the wet weight of the explants [186]. The sGAG content of the explants at the end of the experiment were measured using the dimethylene blue (DMMB) dye binding assay [217].

5.2.6 Alamar blue assay for explant metabolic rate measurement

Tissues in each well in the culture plate (cartilage alone for monoculture groups, or cartilage and synovium for coculture control and coculture treatment groups) were incubated for 2.5

hours in a 1X solution of Alamar Blue (ThermoFisher) dissolved in culture medium. Absorbance measurements at 570 nm and 600 nm were performed using a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek) and used to calculate the percentage reduction of Alamar Blue, which is directly proportional to the explant metabolic rate. These values were normalized by explant weights. These weight-normalized readings for the treatment groups were then normalized by the weight-normalized mean value for the untreated controls.

5.2.7 Cell viability

Cartilage explants were sectioned longitudinally into $\sim 100\ \mu\text{m}$ slices, incubated in the dark for 2 minutes in PBS containing FDA (fluorescein diacetate; $4\ \mu\text{g}/\text{ml}$) and PI (propidium iodide; $40\ \mu\text{g}/\text{ml}$). Explant slices were then washed by incubating in PBS for 2 minutes. This was followed by another quick wash with PBS and imaging using a Nikon fluorescence microscope at 4X magnification. The FDA stained (live) cells are green fluorescent and the PI stained (dead) cells are red fluorescent. In the microscopy images, green was replaced with blue and red was replaced with yellow to make them more accessible to color-blind readers.

5.2.8 Statistical Analysis

Statistical analysis was performed using the JMP Pro 14 software package. Bovine and human sGAG biosynthesis data and explant sGAG content data and bovine metabolic rate data, were analyzed using the linear mixed effects model with the donor (human or bovine) as a random factor. This was followed by the post-hoc Tukey's Honestly Significant Difference (Tukey's HSD) test, and p-values less than 0.05 were considered to be statistically significant.

5.3 Results

5.3.1 Proinflammatory cytokine release is upregulated in the presence of human synovium

Cartilage and synovium explants from a 36 year old male donor were cultured individually as monoculture and also together.

There was increased release of cytokines and chemokines to the medium of synovium monocultures and that of the cartilage-synovium co-culture groups (Figure 5.3 and 5.4). IL-6 and IL-8 levels in the media reached the ng/mL range throughout the culture duration, while the levels of all other cytokines (TNF- α , IL-1 β , IFN- γ , IL-2, IL-13, IL-4, IL-10 and IL-12p70) were in the pg/mL range.

Additionally, the levels of the proinflammatory cytokines IL-6, TNF- α , and IL-1 β as well as the anti-inflammatory cytokine IL-10 were demonstrated to be at levels comparable to those seen *in vivo* after a joint injury (Figure 5.3). IL-8 levels released *in vitro* were an order of magnitude higher than post-injury levels *in vivo*.

5.3.2 Bovine IL-6 release is upregulated in the presence of bovine synovium

Bovine co-culture experiments were performed with cartilage and synovium tissues obtained from four different animals. ELISA assays for IL-6, TNF- α , and IL-1 β were performed on culture media samples from three of these four experiments. In each case, the IL-6 levels in media from synovium mono-cultures and cartilage-synovium cocultures were in the ng/mL range (Figure 5.5). TNF- α , and IL-1 β were undetectable in monoculture and co-culture media samples. The lower limit of detection for the bovine IL-1 β and TNF- α assays were higher than the levels of these cytokines detected in human co-cultures. It may be possible that the bovine co-cultures also have these cytokines, but were not detected due to the limitations of the assay.

There was marked animal-to-animal variability in the IL-6 levels in the medium (Figure 5.5). While all the cartilage explants were nearly identical in size and weight, there

was variability in the weights of the synovium explants. In order to determine whether the animal-to-animal variability was mostly caused by different synovial explant sizes (and thereby the number of synoviocytes in the explants), the IL-6 concentrations were normalized by synovium explant wet weights (Figure 5.6). While the weight normalization reduced the magnitude of the variability, the normalized IL-6 levels from the three animals still differed markedly. For example, the normalized IL-6 level in the synovium monoculture in 5.5C was more approximately 3 times higher than the normalized IL-6 level in the synovium monoculture in 5.5A.

5.3.3 Effect of coculture with synovium on human cartilage sGAG content and biosynthesis rate

The sGAG biosynthesis rate of cartilage explants was measured during the final 24 hours of the 1-week and 2-week experiments. Even though pro-inflammatory cytokine levels were highly elevated in the co-culture groups, there were no differences between the biosynthesis rate of monoculture cartilage and co-culture cartilage (Figure 5.7A). Similarly, there were no significant differences between the final sGAG content for monoculture cartilage explants and co-culture cartilage explants at the 1-week and 2-week time points (Figure 5.7B).

5.3.4 Effect of coculture with synovium on bovine cartilage sGAG content, biosynthesis rates and metabolic rate

In experiments with bovine tissues, co-culture with synovium led to a significant decrease in the sGAG biosynthesis rate of cartilage at the 1-week, 2-week and 3-week time points (Figure 5.8A). The difference between the monoculture cartilage biosynthesis rate and co-culture cartilage biosynthesis rate is highest at the end of week 1, and the difference decreases with time. Similar to this, co-culture causes a significant decrease in the tissue metabolic rate (Figure 5.8B), and this effect decreases at later time points. Finally, co-culture with synovium causes a significant decrease in the sGAG content of cartilage explants at the 1-week, 2-week and 3-week time points. The difference between the monoculture and co-culture cartilage increases with time, and is highest at the end of 3 weeks.

5.3.5 Effect of coculture with synovium on human and bovine cartilage viability

The viability of monoculture and co-culture cartilage was qualitatively characterized using FDA/PI staining followed by fluorescence microscopy. The green fluorescence of FDA (live cells) was replaced with yellow and the red fluorescence of PI (dead cells) was replaced by blue during post-processing in Fiji (Fiji Is Just ImageJ). The results for human cartilage are shown in Figure 5.9, and the bovine cartilage images are shown in Figure 5.10.

There were no differences between monoculture cartilage viability and co-culture cartilage viability at the 1 week time point. At the 2 week time point, co-cultured human cartilage had a higher fraction of dead cells compared to monoculture cartilage. In the experiments with bovine tissues, co-cultured cartilage had a higher fraction of dead cells compared to monoculture cartilage at the 1 week, 2 week and 3 week time points.

5.4 Discussion

Post-traumatic osteoarthritis (PTOA) is a disease of the entire joint. Cartilage, bone and joint capsule synovium are all affected in PTOA, and each tissue contributes to PTOA progression both individually and through crosstalk with other tissues.

Culturing cartilage and synovium together can capture some of the interactions that occur *in vivo*, leading to a more physiologically relevant *in vitro* model. Previous co-culture models are based on porcine, bovine, canine, equine tissues or on end-stage human OA tissue obtained from total knee replacement surgeries.

In the present study, a co-culture system starting with normal (non-OA) human cartilage and synovium was developed, with the hypothesis that it would be more relevant for simulating the initial evolution of PTOA. A co-culture model with immature bovine tissues was also set up to investigate the effect of species and age, and compare results with human co-culture results.

In the experiments with human tissues, cytokine and chemokine levels were highly elevated in the culture medium of groups containing synovium tissue (synovium monoculture

and cartilage-synovium co-culture) at early time points. Furthermore, the levels of several cytokines in these *in vitro* cultures were comparable to the corresponding levels in synovial fluid after ACL rupture in humans. The cytokine levels in the synovium monoculture and cartilage-synovium co-culture groups decrease with time, and trend is also similar to what happens *in vivo* during the days/weeks following ACL rupture.

In the bovine tissue experiments, cytokine levels had to be measured using individual ELISA assays as a multiplex ELISA kit was not commercially available. The bovine IL-1 β and TNF- α ELISA kits were not as sensitive as the multiplex human ELISA kit, and this could have contributed to the finding that IL-1 β and TNF- α were not detected in any of the media samples. However, IL-6 levels were very high in synovium monoculture medium and cartilage-synovium co-culture medium, and these concentrations were similar to those seen in human co-cultures. There was also marked animal-to-animal variability in the quantitative values of IL-6 concentrations, and these differences were not completely eliminated even after normalizing concentrations with explant wet weights.

The elevated cytokine levels in human cartilage-synovium cocultures did not cause changes in the sGAG biosynthesis rate or the sGAG content of cartilage explants even after two weeks of culture. There was also no effect on cartilage cell viability at the 1-week time point. However, the co-cultured cartilage had lower cell viability compared to monoculture cartilage at the end of 2 weeks.

In the bovine system, co-culture with synovium caused significant decreases in the metabolic rate, sGAG content and biosynthesis rate of cartilage. While the total sGAG content of monoculture cartilage increased with time, a similar trend was not observed for co-culture cartilage. The decreases in sGAG biosynthesis rate and metabolic rate of co-cultured cartilage were highest at the 1 week time point, and the difference between monoculture and co-culture cartilage decreased at later time points. These results are consistent with the decrease in cytokine levels with time.

Immature (1-2 week old) bovine cartilage has more than 5 times higher cell density compared to adult human cartilage, and the immature bovine chondrocytes are also much more metabolically active than adult human chondrocytes. Together, these factors could make the bovine cartilage more sensitive to high cytokine levels compared to human car-

tilage, and contribute to the rapid PTOA-like changes (reduced sGAG biosynthesis rate, lower GAG content and viability) seen in the co-cultured tissue. While this means it may not be feasible to use the bovine co-culture system as a substitute for the adult human co-culture system, the rapid PTOA-like changes may make it a good system for initial screening of potential disease modifying OA drugs. In this work, experiments were performed with knees from only two donors due to a lack of availability of normal or near-normal human knee cartilage and associated synovium. Future work could include performing experiments with tissues obtained from donors belonging to different age-groups, sexes, body weights and other relevant factors to investigate the effects of these factors on tissue responses.

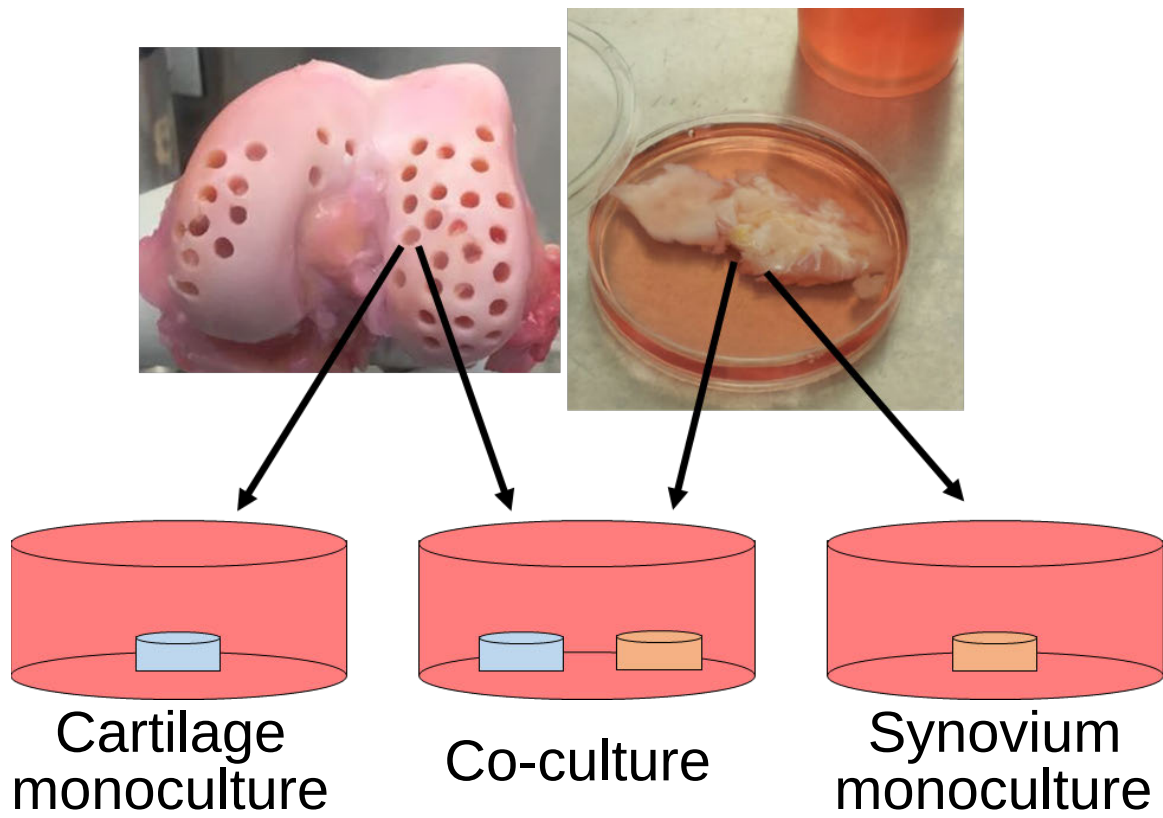


Figure 5.1: Experimental setup for human cartilage-synovium co-culture experiments.

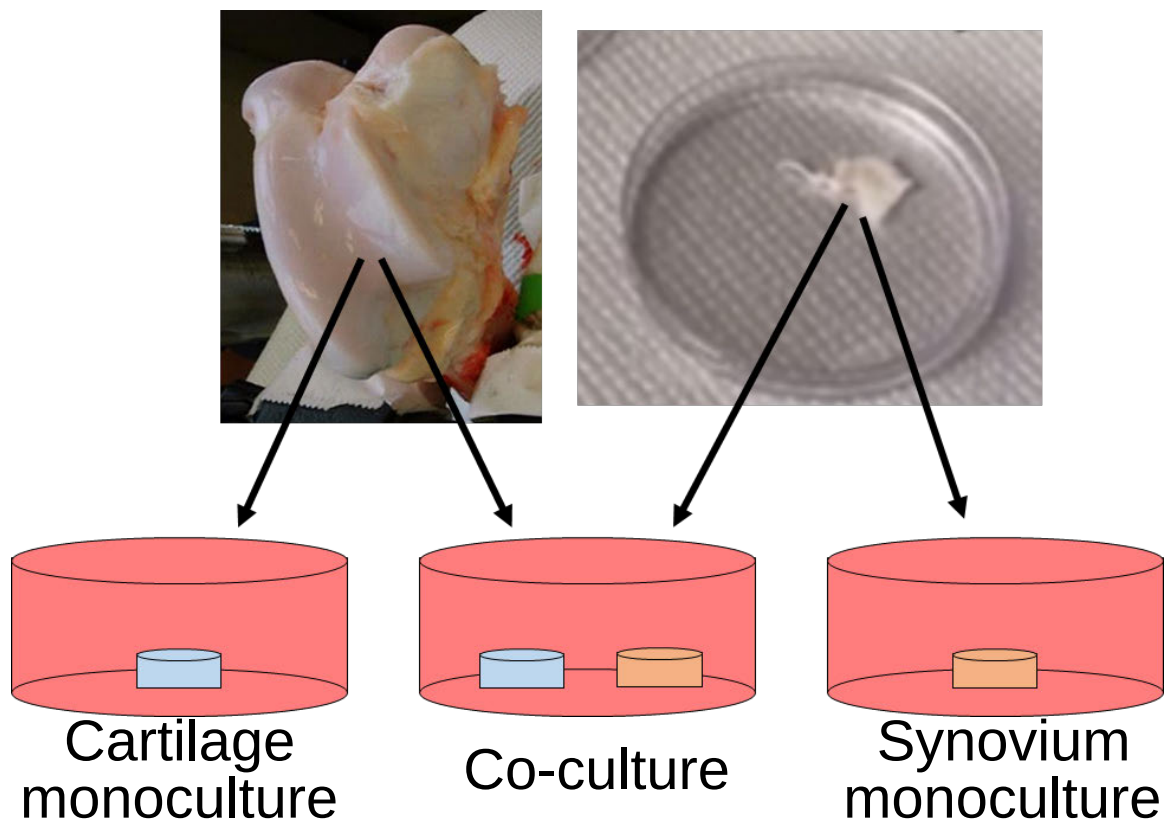


Figure 5.2: Experimental setup for bovine cartilage-synovium co-culture experiments.

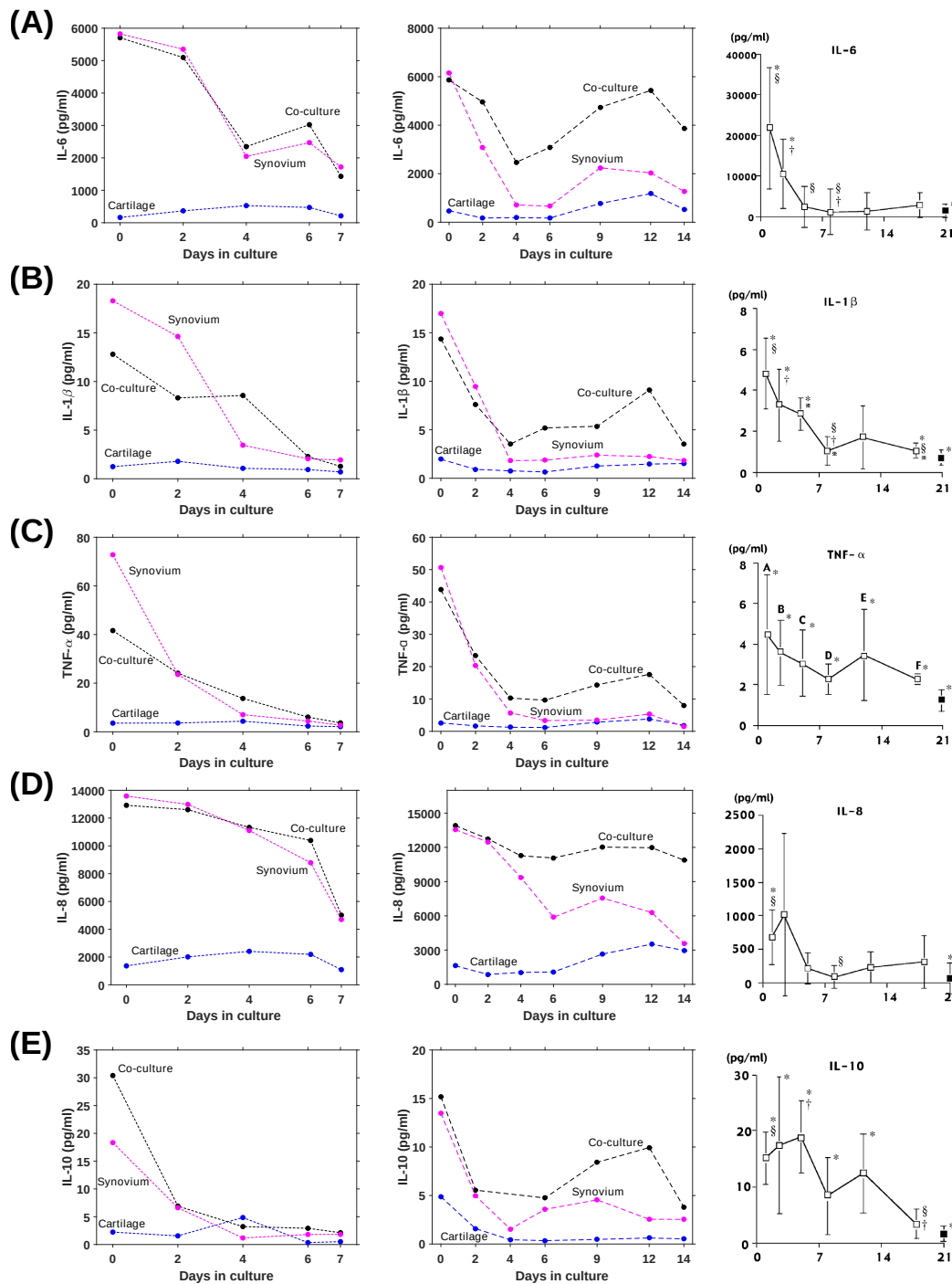


Figure 5.3: Culture medium levels of IL-6, TNF- α , IL-1 β , IL-8 and IL-10 levels in co-culture experiments with human cartilage and synovium tissues (36 year old male donor). The first and second columns show the results for experiments terminated at 1 week and 2 week time points respectively. Blue dots: Cartilage monoculture data; magenta dots: synovium monoculture data; black dots: cartilage-synovium co-culture data. The third column shows *in vivo* cytokine levels in human knees following ACL injury ([254])

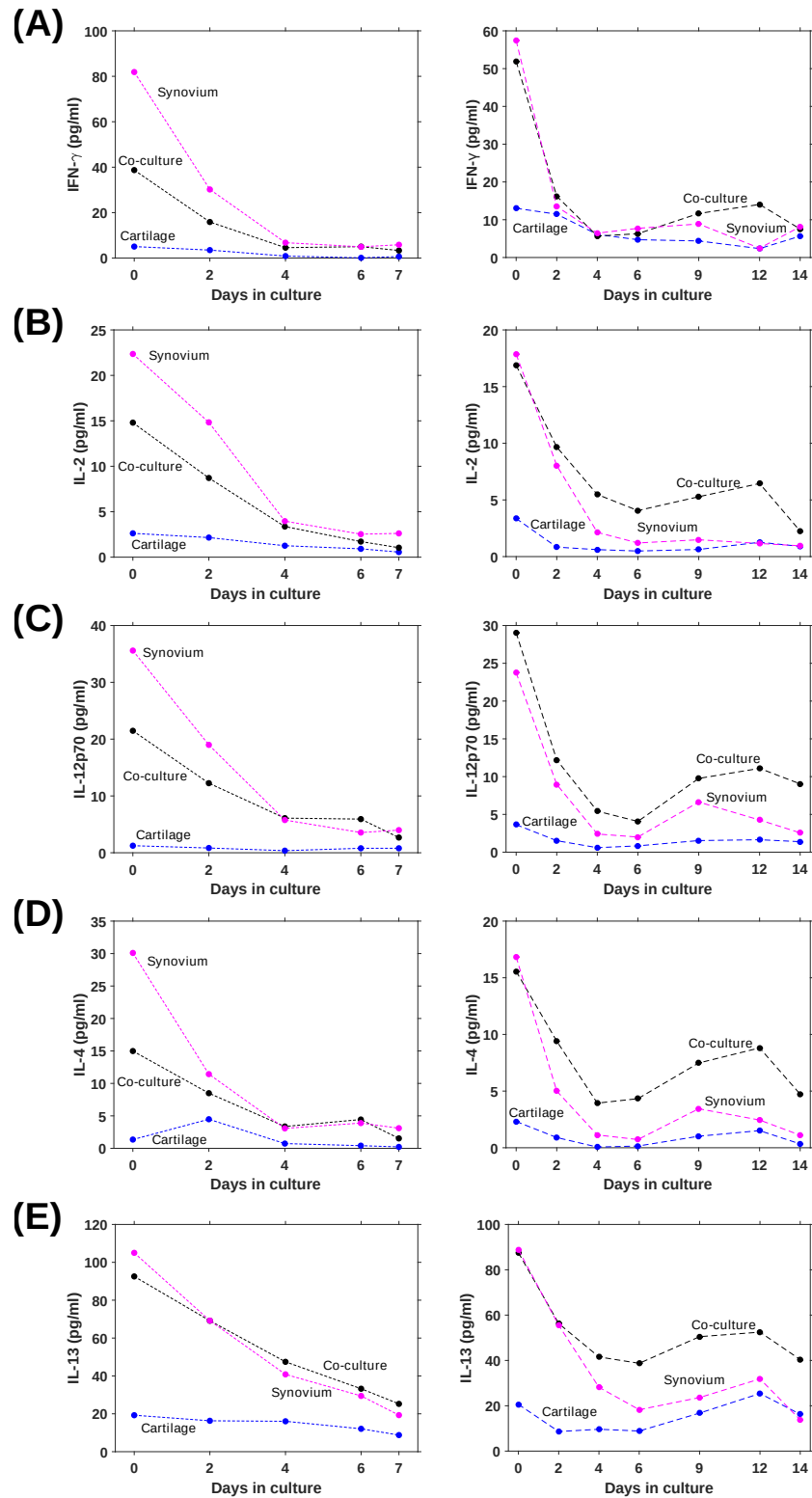


Figure 5.4: Culture medium levels of IFN- γ , IL-2, IL-13, IL-4 and IL-12p70 levels in 1 week (column 1) and 2 week (column 2) co-culture experiments with human cartilage and synovium tissues (36 year old male donor). The blue dots are data from cartilage monocultures, magenta dots are data from synovium monocultures and black dots are the cartilage-synovium co-culture data.

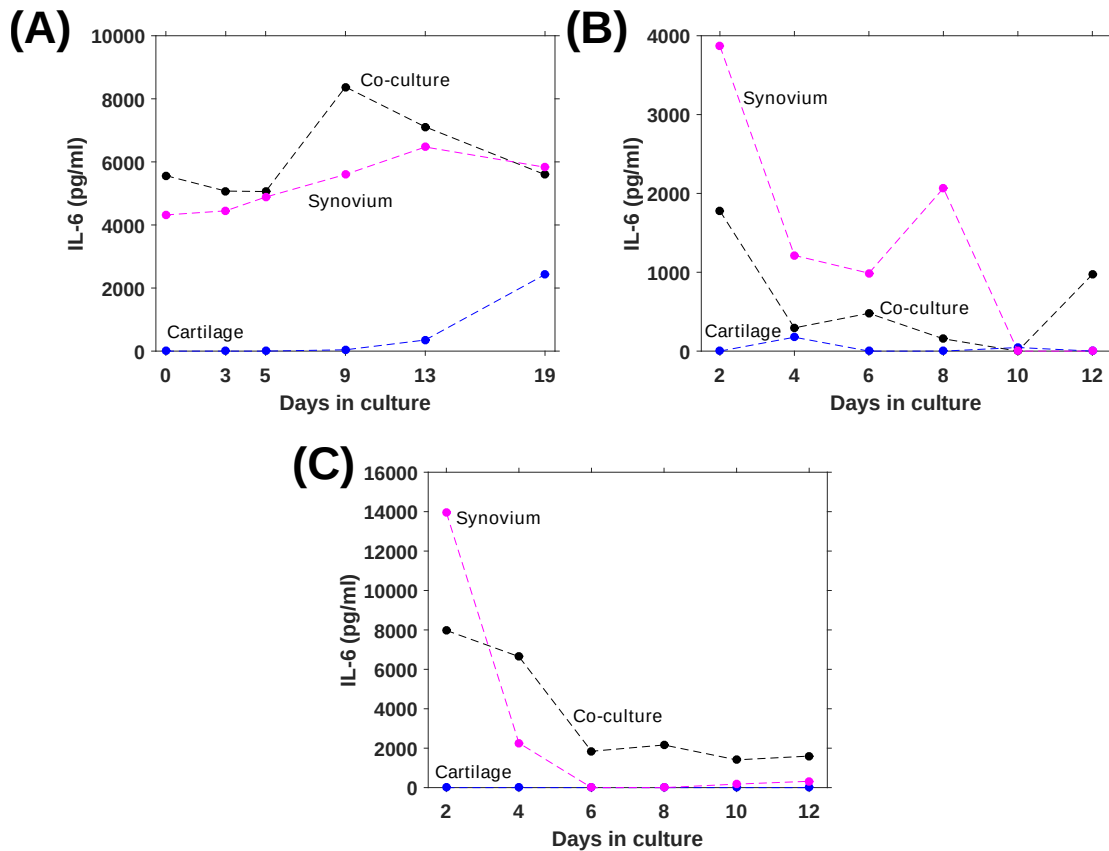


Figure 5.5: In *in vitro* cultures with tissues from 3 different animals, bovine synovial explants release IL-6 at levels comparable to those seen *in vitro* cultures with human synovium explants as seen in Figure 5.3A.

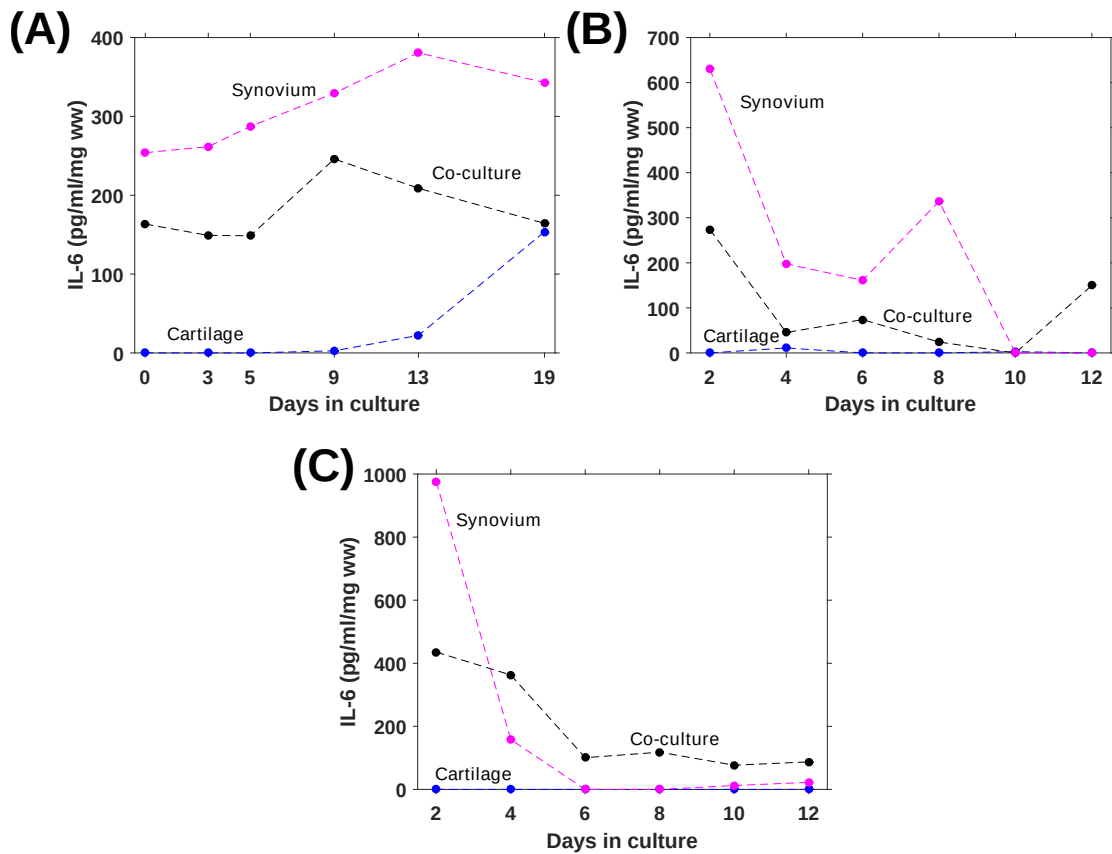


Figure 5.6: Bovine monoculture and coculture IL-6 concentrations normalized to explant wet weights.

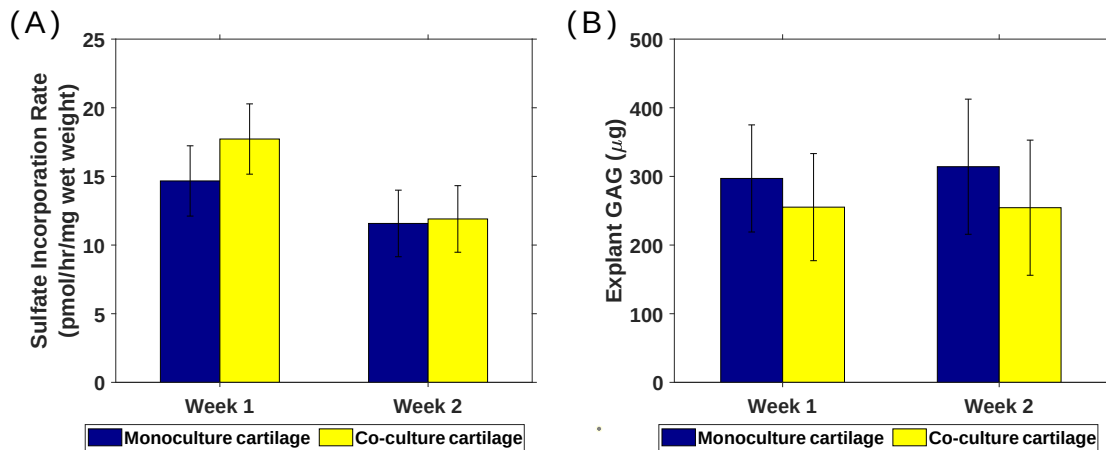


Figure 5.7: (A) sGAG biosynthesis rate for human monoculture cartilage and co-culture cartilage measured at the 1 week and 2 week time points. (B) Monoculture and co-culture cartilage sGAG content at the end of 1 week and 2 weeks time points (same explants shown in (A)). Bars: Mean $\pm$ SE; N = 2 donors with 3 to 5 explants/condition/donor.

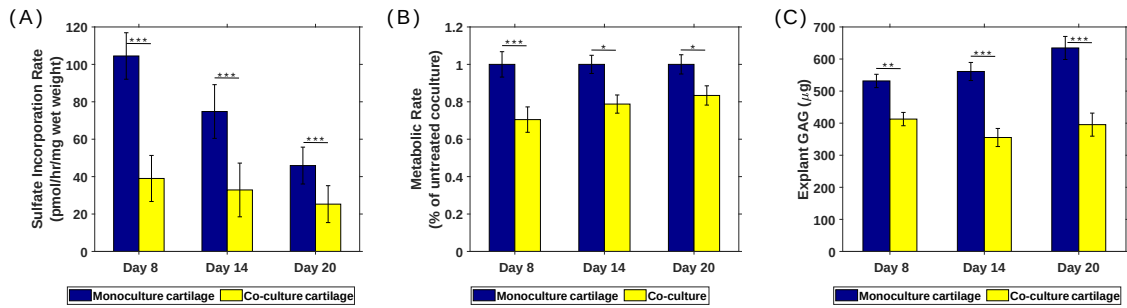


Figure 5.8: (A) sGAG biosynthesis rate for bovine monoculture cartilage and co-culture cartilage measured at the 1 week, 2 week and 3 week time points. (B) Tissue metabolic rates (measured by the Alamar Blue assay) for the explants shown in (A). (C) Monoculture and co-culture cartilage sGAG content at the end of 1 week, 2 weeks or 3 weeks in culture (same explants shown in (A)). Bars: Mean $\pm$ SE; N = 4 animals with 5 explants/condition/donor; *:p<0.05, **:p<0.01; ***:p<0.001.

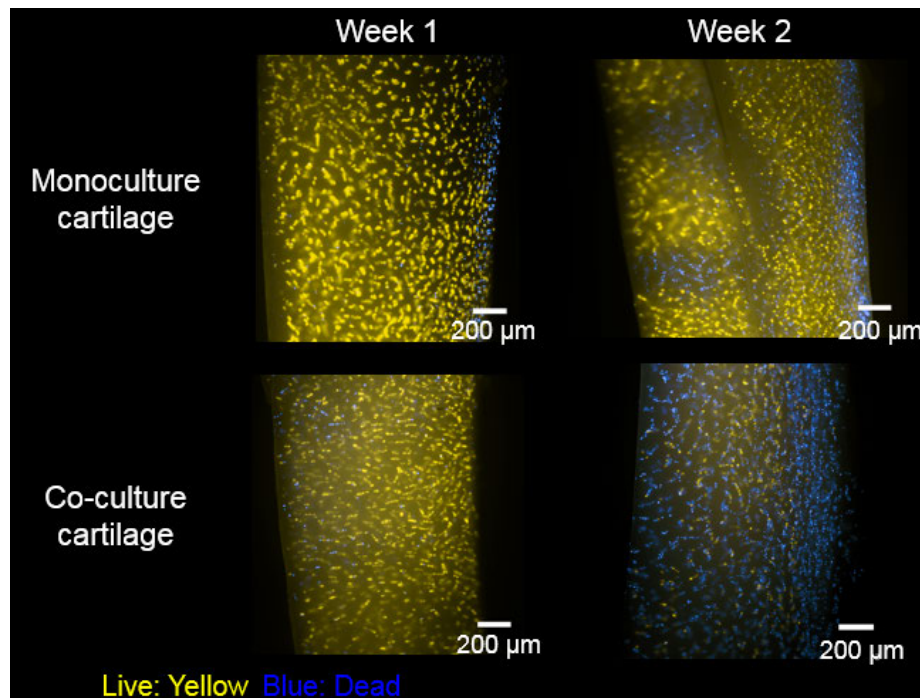


Figure 5.9: Viability of human mono-culture (top row) and co-culture (bottom row) cartilage at 1-week and 2-week time points. (Yellow: live cells, Blue: dead cells; Scale bar: 200 μm; Superficial zone (SZ) faces right for all explants.)

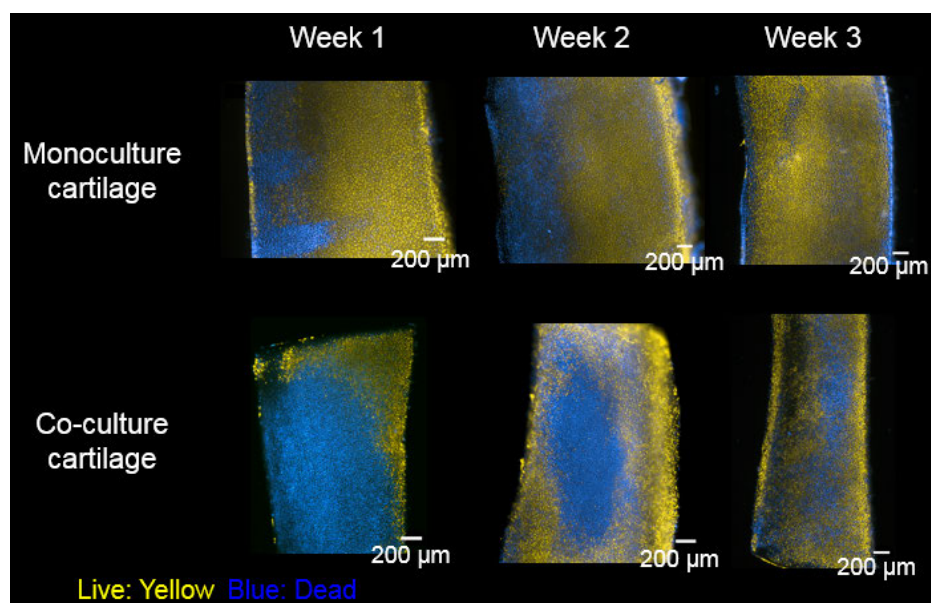


Figure 5.10: Viability of bovine mono-culture (top row) and co-culture (bottom row) cartilage at 1-week and 2-week time points. (Yellow: live cells, Blue: dead cells; Scale bar: 200 μm ; Superficial zone (SZ) faces right for all explants.)

Chapter 6

Effects of Kinase and Toll-Like Receptor (TLR) Inhibitors in a co-culture PTOA model

6.1 Introduction

Post-traumatic osteoarthritis (PTOA), a subtype of OA that accounts for approximately 12% of all cases, is triggered by acute traumatic joint injuries such as an ACL tear. While PTOA is triggered by mechanical injury to the joint, there is an inflammatory response that can contribute to disease progression. There is a significant increase in the levels of cytokines (IL-6, IL-1 β , TNF- α etc.) and chemokines (IL-8 etc.), in the days and weeks following injury [254][165]. These cytokines can then stimulate the upregulation of proteases and aggrecanases that lead to cartilage degeneration.

One of the mechanisms by which the inflammatory responses are triggered after injury involves the activation of the innate immune system through pattern recognition receptors (PRRs). Injury to joint tissues leads to the release of cartilage extracellular matrix fragments, intracellular alarmins, plasma proteins and other damage associated molecular patterns (DAMPs) into the joint space[167][168]. These DAMPs are recognized by several types of receptors, collectively known as PRRs, that are found on cell membranes, endo-

somes, and cytoplasm. While PRRs are most commonly found in immune cells, within joints they are also expressed by chondrocytes, synoviocytes as well as osteoblasts and osteoclasts in the subchondral bone [171].

When these cells are activated by DAMPs through the PRRs, they produce multiple inflammatory mediators such as cytokines, prostaglandins, leukotrienes and pro-catabolic enzymes such as matrix metalloproteases (MMPs) and aggrecanases [167][171][172]. These inflammatory mediators can act on their own tissue targets and lead to further inflammation, cartilage degradation and bone remodeling. The tissue degenerative processes can release new DAMPs, which will start the vicious cycle all over again [268]. DAMPs in the joint can also activate the complement system (which is a part of the innate immune system that is mediated by proteins), which can contribute to cartilage degradation by killing chondrocytes or through sublytic activation of inflammatory pathways [167][172].

Previous studies have shown that glucocorticoid drugs such as Dexamethasone have anti-inflammatory and anti-catabolic effects in models of OA both *in vitro* and *in vivo*. Dexamethasone is currently injected intra-articularly to achieve short-term symptomatic relief in OA patients, but repeated use is avoided due to concerns about the safety of the drug [269]. Drug delivery methods are currently under development to locally deliver low doses of Dexamethasone in a sustained manner so that the beneficial effects (such as preventing matrix degradation and rescuing cell viability) are achieved while avoiding harmful side effects [206]. Recent studies have also shown that combining a pro-anabolic drug such as Insulin-like growth factor 1 (IGF-1) with Dexamethasone is more effective than using either drug alone [186].

While IGF-1 and Dexamethasone may be able to protect cartilage from damage in the presence of cytokines, it would potentially be beneficial to also intervene at a more upstream level in the process. Since DAMPs induce the production of cytokines and other inflammatory mediators through the activation of PRRs, using drugs that inhibit this process could directly reduce the levels of these mediators and further help in preventing the progression of PTOA. Toll-like receptors 2 and 4 (TLR2 and TLR4) are two of the major PRRs involved in recognizing OA-specific DAMPs [167], and TLR4 by itself recognizes more DAMPs than any other known PRR [171].

Synovial fibroblasts express high levels of TLR4 and they produce proinflammatory cytokines such as IL-1 β and TNF when they recognize DAMPs through TLR4 [171]. The extent of bone-erosion in the presence of high levels of IL-1 β was found to be dependent on TLR4 [270], which indicates that targeting TLR4 may help to slow down the bone changes seen in OA.

TLR2 and TLR4 expression has been found to be upregulated in lesional areas of human OA cartilage [271][171]. In 2 to 3 week cultures of human cartilage explants with peptidoglycan and LPS (which stimulate TLR2 and TLR4 respectively), there was a significant increase in sGAG loss and CTX II levels in the culture medium [271]. Peptidoglycan and LPS also caused significant increases in the production of MMPs, NO, and PGE₂ in cultures of human monolayer chondrocytes and human cartilage explants [271]. These effects were inhibited when anti-TLR2 and anti-TLR4 antibodies were used, confirming that the effects seen with peptidoglycan and LPS were due to TLR signaling.

These effects were also independently inhibited by MAPK inhibitors (JNK inhibitor, p38 inhibitor and MEK-1/2 inhibitor) [271]. Additionally, JNK, p38 and ERK were all phosphorylated in chondrocytes treated with peptidoglycan or LPS [271]. These findings indicate that there is interaction between TLR signaling pathways and MAPK signaling pathways [271].

MAPK pathways are also stimulated by IL-1 β , one of the major cytokines in OA [272][273]. MAP kinase inhibitors had anti-catabolic effects in an *in vitro* cytokine challenge model with normal or near normal human cartilage, and both anti-catabolic and pro-anabolic effects in a model with bovine tissues [272].

In the present study, OxPAPC, a dual inhibitor of TLR2 and TLR4, was tested in the *in vitro* cartilage synovium co-culture model described in Chapter 5. We hypothesized that the injury caused by cutting the synovium explants released DAMPs into the culture medium, which in turn stimulated the release of the high levels of cytokines and chemokines. We further hypothesized that OxPAPC would inhibit TLR signaling by these DAMPs and thereby reduce the levels of cytokines and chemokines. Since TLR signaling pathways are potentially interacting with MAPK pathways, and kinase inhibitors previously had beneficial effects in an *in vitro* cytokine challenge model, we further tested the effects of a JNK in-

hibitor (SP600125), an ERK5 inhibitor (XMD17-109) and a p38 inhibitor (SB203580) in the co-culture model. CP690550, a JAK3 inhibitor, was also included in the present study based on previous work showing beneficial effects in an *in vitro* cytokine challenge model [272].

6.2 Materials and Methods

6.2.1 Harvest of adult human cartilage and synovium

One human distal femur (69 year old female donor; Collins grade 1) and one ankle joint (63 year old male donor; Collins grade 1) were obtained from the Gift of Hope Organ and Tissue Donor Network (Itasca IL). Knee joint capsule synovium tissues were also obtained from both donors. All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT. Full thickness cartilage explants were harvested with a 3mm biopsy punch and equilibrated in serum-free medium: low-glucose DMEM; 1g/L (Corning Cellgro, Manassas, VA) supplemented with 10mM HEPES buffer (Gibco, Grand Island, NY), 0.1mM nonessential amino acids (Sigma Aldrich, St. Louis, MO), 0.4mM proline (Sigma), 20µg/ml ascorbic acid (Sigma), 100units/ml penicillin G, 100µg/ml streptomycin, and 0.25µg/ml amphotericin B (Sigma) for 2-3 days (5% CO₂; 37°C). While the cartilage explants were equilibrating, the complete joint capsule tissue was also incubated in serum-free medium. At the end of the equilibration period, synovium explants were cut using scissors and cocultures containing one cartilage explant and one synovium explant were immediately set up. Monoculture controls with cartilage alone were also included in the experiments using tissues from the 63 year old male donor.

6.2.2 Effects of kinase inhibitors in the cartilage-synovium coculture model

We tested the effects of OxPAPC (InvivoGen), which inhibits TLR2 and TLR4, as well as several kinase inhibitors, specifically SP600125 (JNK1/2 inhibitor, Sigma), XMD17-

109 (ERK5 inhibitor), SB203580 (p38 α/β inhibitor, CST) and CP-690550 (JAK3 inhibitor, Sigma), in the cartilage-synovium co-culture model. The doses for the kinase inhibitors were chosen based on previous studies [272], and doses ranging from 100 nM to 100 μ M were tested for the TLR2 + TLR4 inhibitor. The major outcome measure was the quantitative level of levels of cytokines and chemokines released by the tissues. Other outcome measures included the tissue metabolic rate, cartilage sGAG biosynthesis, and cartilage sGAG content.

6.2.3 Multiplex ELISA for proinflammatory cytokines and chemokines

Culture media samples from the experiment with 69 year old female donor tissues were analyzed using the V-Plex Proinflammatory Panel 1 Human Kit and the V-PLEX Chemokine Panel 1 Human Kit from Meso Scale Discovery (MSD). Media samples from the 63 year old male donor tissues were analyzed using a custom MSD U-Plex kit with IL-6, IL-1 β , TNF- α , IL-8, IL-4, IL-10, MCP-1, MIP-1 α , MIP-1 β , and Eotaxin chosen as the analytes. The protocols provided by the kit manufacturer were followed for performing the assay, reading the plates and analyzing the data.

6.2.4 sGAG biosynthesis rate and sGAG content of cartilage explants

To measure the chondrocyte biosynthesis rate of sulfated glycosaminoglycans (sGAGs), 10 μ Ci/mL ³⁵S-sulfate radiolabel was added to the culture medium during the final two days of each experiment. Explants were then washed with PBS with non-radioactive sulfate to wash out excess radiolabel, weighed and digested with proteinase K. A liquid scintillation counter (PerkinElmer) was used to measure the amount of radiolabel incorporated within the extracellular matrix of the tissues. Radiolabel incorporation data for cartilage were normalized by the duration of exposure to radiolabel and by the wet weight of the explants [186]. The sGAG contents of the cartilage explants were measured using the dimethylene blue (DMMB) dye binding assay [217] and normalized to the explant wet weights.

6.2.5 Alamar blue assay to measure explant metabolic rate

The metabolic rate of cartilage explants was measured using the Alamar Blue assay (ThermoFisher). Tissues in each well in the culture plate (cartilage alone for monoculture, or cartilage and synovium for coculture control and treatment groups) were incubated for 2.5 hours in a 1X solution of Alamar Blue dissolved in culture medium. At the end of the incubation period, the fluorescence (530nm ex, 590nm em) of the medium at was measured using a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek). These readings were corrected for the fluorescence of the blank controls (1X Alamar Blue incubated by itself without any tissues). The blank corrected readings were normalized by individual explant weights. These weight-normalized readings for the treatment groups were then normalized by the weight-normalized mean value for the untreated controls.

6.2.6 Statistical Analysis

Statistical analysis was performed using MATLAB. Metabolic rate data, sGAG biosynthesis data and explant sGAG content data were analyzed using a one-way ANOVA, followed by the post-hoc Tukey's Honestly Significant Difference (Tukey's HSD) test, and p-values less than 0.05 were considered to be statistically significant.

6.3 Results

6.3.1 Effect of TLR inhibitors and kinase inhibitors on cartilage explant biosynthesis rate

Co-culture experiments were set up with tissues obtained from two donors: a 69 year old female and a 63 year old male donor. For the 69 year old donor, knee cartilage (Collins grade 1) and knee synovium explants were harvested. Co-cultures of 1 cartilage explant cultured together with 1 synovium explant were set up, and the effects of a JNK inhibitor (SP600125), an ERK5 inhibitor (XMD17-109) and a TLR2 + TLR4 inhibitor (OxPAPC) were tested. An untreated co-culture group was included as a control. The following

doses were used for the different inhibitors: 25 μ M SP600125, 5 μ M XMD17-109, and four different doses (100 nM, 1 μ M, 10 μ M and 100 μ M) of OxPAPC. While SP600125 and XMD17-109 were dosed continuously, OxPAPC was added only on Day 0 and Day 3 of the culture. The duration of the experiment was 17 days, and the normalized sulfate incorporation rate of cartilage was measured between days 14 and 17 of the experiment.

For the 63 year old donor, ankle cartilage (Collins grade 1) and knee synovium explants were harvested. Co-cultures of 1 cartilage explant cultured together with 1 synovium explant were set up, and the effects of a TLR2 + TLR4 inhibitor (OxPAPC), a JNK inhibitor (SP600125), a combination of a JNK inhibitor and IGF-1 (SP600125 + IGF-1), an ERK5 inhibitor (XMD17-109), an ERK5 inhibitor and IGF-1 (XMD17-109 + IGF-1), a JAK3 inhibitor (CP690550), a JAK3 inhibitor and IGF-1 (CP690550 + IGF-1), a p38 inhibitor (SB203580) and a p38 inhibitor with IGF-1 (SB203580 + IGF-1) were tested. An untreated monoculture cartilage group and an untreated co-culture group were included as controls. The following doses were used for the different inhibitors: 25 μ M SP600125, 5 μ M XMD17-109, 10 μ M SB203580, 10 μ M CP690550, 300 ng/ml IGF-1, and four different doses (100 nM, 1 μ M, 10 μ M and 100 μ M) of OxPAPC. While SP600125, XMD17-109, SB203580, CP690550 and IGF-1 were all dosed continuously, OxPAPC was added only on Day 0 and Day 3 of the culture. The duration of the experiment was 9 days, and the normalized sulfate incorporation rate of cartilage was measured between days 7 and 9 of the experiment.

A cartilage monoculture control was not included in experiments with the 69 year old female tissues. In the experiments with the 63 year old male donor tissues, a monoculture cartilage control was included. There was no statistically significant difference between sGAG biosynthesis rate of monoculture cartilage and coculture cartilage (Figure 6.1), which is consistent with results in the previous thesis chapter.

In both sets of experiments (one with the 69 year old female donor tissues and the other with the 63 year old male tissues), SP600125 and OxPAPC did not cause any changes in sulfate incorporation rates, but treatment with XMD17-109 led to significantly lower biosynthesis rate (Figure 6.1). Combining IGF-1 with XMD17-109 rescued the biosynthesis loss caused by XMD17-109 alone: there was no statistically significant difference

between the normalized sulfate incorporation rate of the XMD17-109 + IGF-1 group and the monoculture control group or the co-culture control group in Figure 6.1D.

The p38 (SB203580) and JAK3 (CP690550) inhibitors were not tested with 69 year old female donor tissues. But in cocultures with 63 year old male tissues, they did not cause any changes in the sGAG biosynthesis rate compared to the untreated controls (Figure 6.2). Combining IGF-1 with SP600125, SB203580 or CP690550 led to significantly higher biosynthesis rate compared to the untreated controls.

6.3.2 Effect of TLR inhibitors and kinase inhibitors on metabolic rate of tissues in coculture

In experiments with 69 year old female donor tissues, treatment with SP600125 and XMD17-109 caused significant decreases in tissue metabolic rate (as measured by the Alamar Blue assay) compared to untreated control (Figure 6.3A). In experiments with 63 year old male donor tissues, the SP600125 and XMD17109 treatment groups had a trend of lower metabolic rate compared to the untreated controls, but the differences were not statistically significant. There were also no statistically significant differences between any of the other treatment groups (SP600125 + IGF-1, XMD17-109 + IGF-1, SB203580, SB203580 + IGF-1, CP690550, CP690550 + IGF-1 and OxPAPC) and the untreated control groups (Figure 6.3 and 6.4).

6.3.3 Effect of TLR inhibitors and kinase inhibitors on cartilage explant GAG content

In experiments with 69 year old female donor tissues, the normalized explant GAG content of the XMD17-109 treatment group was significantly lower than that of the SP600125 treatment group (Figure 6.5A). There were no other differences in the sGAG contents (Figure 6.5 and 6.6).

6.3.4 Effect of JAK inhibitor on cytokine and chemokine levels

The JAK inhibitor (CP690550) was not tested with the 69 year old female tissues. It was tested individually and in combination with IGF-1 in co-cultures with 63 year old male tissues. The CP690550 treatment group had lower levels of IL-6, IL-1 β , TNF- α , IL-8 and MCP-1 compared to the untreated co-culture control group (Figure 6.7). However the combination therapy with CP690550 + IGF-1 resulted in higher cytokine and chemokine levels compared to CP690550 alone. In some cases such as IL-6, the concentrations in the CP690550 + IGF-1 group were in between the CP690550 group and the untreated co-culture control group. In other cases such as IL-8 or TNF- α , the concentrations in the CP690550 + IGF-1 group were similar to or higher than the corresponding concentrations in the untreated co-culture control group.

Similar trends were found in MIP-1 α and MIP-1 β concentrations where CP690550 treatment led to a decrease in the chemokine concentrations, and the combination of CP690550 + IGF-1 brought them back up but not as high as the untreated co-culture control (Figure 6.8A and B). With regards to Eotaxin concentrations, the cartilage monoculture group had levels comparable to the co-culture control and the treatment groups (Figure 6.8C). This was unlike any of the other cytokines or chemokines where the monoculture levels were negligible compared to the co-culture levels.

The readings for IL-4 and IL-10 concentrations were either below the lowest standard or undetectable and are not reported here.

6.3.5 Effect of p38 inhibitor on cytokine and chemokine levels

The p38 inhibitor (SB203580) was not tested with the 69 year old female tissues. It was tested individually and in combination with IGF-1 in co-cultures with 63 year old male tissues. Both the SB203580 treatment group and the SB203580 + IGF-1 groups had lower levels of IL-6, IL-1 β , IL-8 and MCP-1 compared to the untreated co-culture control group (Figure 6.9). At the day 3 and day 5 timepoints, the TNF- α levels in the SB203580 group were lower than the untreated control, whereas the TNF- α levels in the SB203580 + IGF-1 group were similar to the untreated control (Figure 6.9C). At the day 7 and day 9 timepoints,

TNF- α was undetectable in the media from the SB203580 and SB203580 + IGF-1 groups, due to which these data points are not shown in Figure 6.9C.

The readings for IL-4 and IL-10 concentrations were either below the lowest standard or undetectable and are not reported here. The results for MIP-1 α , MIP-1 β and Eotaxin are shown in Supplementary Figure 6.10.

6.3.6 Effect of JNK and ERK5 inhibitors on cytokine and chemokine levels

In experiments with both the 69 year old female donor and the 63 year old male donor, co-culture groups treated with SP600125 (a JNK inhibitor) and XMD17-109 (an ERK5 inhibitor) had lower concentrations of MCP-1, IL-8, and IL-6 compared to the untreated co-culture control groups (Figure 6.11). At the day 3 time point of the 69 year old female donor experiment, there were no differences in the IL-1 β levels between the groups; but the SP600125 group and the XMD17-109 group had lower IL-1 β levels compared to the untreated control at all other time points (Figure 6.11D; first column). In the experiments with 63 year old male donor tissues, the SP600125 and XMD17-109 treatment groups had lower IL-1 β concentrations compared to the untreated coculture control at all time points (Figure 6.11D; second and third columns).

Additionally, the MCP-1, IL-8, IL-6 and IL-1 β levels in the SP600125 + IGF-1 combination treatment co-culture group were lower than the untreated co-culture control, and were very similar to the levels seen in the SP600125 treatment group (Figure 6.11; second column). However, unlike the XMD17-109 group, the XMD17-109 + IGF-1 combination treatment condition had IL-8 and IL-6 levels similar to the untreated co-culture control (Figure 6.11; third column). The MCP-1 levels for the XMD17-109 + IGF-1 group were in between the concentrations for the XMD17-109 group and the untreated co-culture control group. The IL-1 β concentrations in the XMD17-109 + IGF-1 group and the XMD17-109 group were similar to each other.

There were no clear trends in the TNF- α concentrations (Figure 6.11E). For the 69 year old female donor, there were no differences between the TNF- α concentrations of

the untreated control group and the SP600125 or XMD17-109 groups. For the 63 year old male, the SP600125 and SP600125 + IGF-1 groups had lower TNF- α levels than the untreated controls while the XMD17-109 and XMD17-109 + IGF-1 groups had lower TNF- α levels than the untreated controls.

6.3.7 Effect of TLR inhibitors on cytokine and chemokine levels

In experiments with 69 year old female donor tissues, co-culture groups treated with 10 μ M and 100 μ M doses of OxPAPC had lower levels of IL-8, IL-6, TNF- α , MCP-1 and IL-1 β compared to the untreated coculture control at all time points (Figure 6.12A-C and Figure 6.13A-B).

The trends were less clear in experiments with the 63 year old male donor tissues (Figure 6.12D-F and Figure 6.13C-D). In these experiments, the IL-8 and IL-6 levels in the OxPAPC treated groups were less than those of the untreated control group at the 3 day time point (Figure 6.12D and E). Additionally, the 100 μ M OxPAPC group had a lower MCP-1 level at day 3 compared to the untreated control (Figure 6.13C). However the IL-1 β and TNF- α levels in the OxPAPC treated groups are either very similar to or somewhat higher than the corresponding cytokine levels in the untreated control group (Figure 6.12F and Figure 6.13D).

The readings for IL-4 and IL-10 concentrations were either below the lowest standard or undetectable for the experiment with 63 year old male donor tissues. However, in the experiment with 69 year old female donor tissues, the co-culture group treated with co-cultures with 100 M OxPAPC had a lower IL-4 concentration compared to the untreated control group (Figure 6.14A). Additionally, both the 10 M OxPAPC group and the 100 M OxPAPC group had lower levels of IL-10 compared to the untreated control group (Figure 6.14B).

6.4 Discussion

Innate immune responses and inflammatory interactions between joint tissues play an important role in OA progression. In the context of PTOA, traumatic joint injury can release

cartilage extracellular matrix fragments, plasma proteins and damage associated molecular patterns (DAMPs) into the joint space. These DAMPs are recognized by pattern recognition receptors (PRRs) which then trigger inflammation [268].

PRRs are usually found in innate immune cells, but within the joint, they are also expressed by cartilage, synoviocytes and the bone [271][171]. The inflammatory mediators that are released when PRRs are activated act on targets on multiple joint tissues, thereby causing more inflammation, inducing further tissue damage and releasing more DAMPs.

We established a cartilage-synovium co-culture model and observed that cytokine and chemokine levels in the culture medium were highly elevated in the presence of joint capsule synovium. The levels were the highest at the beginning of the experiments and went down with time, and the trends were similar to those observed *in vivo* after a traumatic joint injury. We hypothesized that the damage inflicted on the synovium when it was cut into explants at the start of the experiment released DAMPs. These DAMPs could have then interacted with PRRs and triggered the immune response. The cytokines and chemokines released in this initial response can act on their tissue targets and trigger further inflammation.

In the context of PTOA, Toll-like receptors 2 and 4 (TLR2 and TLR4) are two of the major PRRs involved [167]. TLR signaling activates NF- κ b and MAPK signaling [271][274]. MAPK signaling is also stimulated by inflammatory cytokines, growth factors, mechanical stimuli and matrix proteins [268][275]. We tested the effects of MAPK pathway inhibitors (SP600125: a JNK inhibitor; XMD17-109: an ERK5 inhibitor; and SB203580: a p38 inhibitor), a dual TLR2 and TLR4 inhibitor (OxPAPC), and a JAK3 inhibitor (CP690550; the JAK-STAT pathway is involved in cytokine signaling). In addition to measuring the effects of these inhibitors on cytokine and chemokine levels in the culture medium, we also measured their effects on cartilage.

OxPAPC, CP690550 and SB203580 did not affect the metabolic rate, cartilage biosynthesis rate or cartilage explant GAG content. SP600125 did not affect cartilage biosynthesis rate or cartilage explant GAG content, but there was a decrease in metabolic rate in one donor. XMD17-109 suppressed cartilage biosynthesis in both donors and metabolic rate in one donor. But combining IGF-1 with XMD17-109 rescued the loss of biosynthe-

sis.

The following proinflammatory cytokines, chemokines and anti-inflammatory cytokines were measured in all samples based on their relevance to OA: IL-6, IL-1 β , TNF- α , IL-8, MCP-1, MIP-1 α , MIP-1 β , Eotaxin, IL-4 and IL-10 [276][277][278][279][280][281].

Treatment with CP690550, SB203580 and the combination of SB203580 + IGF-1 reduced the levels of most cytokines and chemokines compared to untreated co-culture controls. While these are promising results, these drugs were tested with tissues from only one donor (63 year old male), and there is a need for further investigation with multiple donors.

Treatment with SP600125 and XMD17-109 reduced cytokine and chemokine levels compared to the untreated co-culture control in both donors. The treatment group receiving SP600125 + IGF-1 had lowered cytokine and chemokine levels that were similar to SP600125 treatment alone. However, when IGF-1 was combined with XMD17-109, the cytokine and chemokine levels were higher than XMD17-109 alone.

Since treatment with XMD17-109 alone also suppressed cartilage biosynthesis rate and metabolic rate (both of which were rescued upon combination treatment with IGF-1), these results indicate that the lower cytokine and chemokine levels with XMD17-109 are potentially due to cytotoxic and/or catabolic effects, and not due to anti-inflammatory effects. On the other hand, the combination of SP600125 + IGF-1 had pro-anabolic effects on cartilage but lowered the cytokine and chemokine levels similar to SP600125 alone, which indicates that this may primarily be due to anti-inflammatory effects. While the consistency in results between both donors is promising, there is a need for further experiments with multiple donors.

The 10 μ M and 100 μ M doses of OxPAPC caused decreases in IL-6, IL-1 β , TNF- α , IL-8, IL-10 and MCP-1 compared to the untreated co-culture control in the experiment with 69 year old female donor tissues. The 100 μ M dose of OxPAPC also caused a decrease in IL-4 concentration in the 69 year old female donor experiment. These effects were seen even up to day 11 of the experiment, even though OxPAPC was added only on day 0 and day 3 of the experiment. These preliminary results indicate that early suppression of TLR signaling may be effective in arresting the inflammatory cascades.

In the experiment with 63 year old male donor tissues, the effects of OxPAPC on cy-

tokine and chemokine levels were either much more modest (for example, in the case of IL-8 or TNF- α) compared to those seen in the 69 year old female donor experiment, or non-existent (for e.g., IL-1 β). Further experiments with multiple donors will be necessary to account for donor-to-donor variability and to make reliable conclusions about the effects of this drug.

A limitation of the present study was that we could run the experiments with tissues obtained only from two human donors. One of them was a 69 year old female donor, and co-cultures were set up with knee cartilage and knee synovium. The second donor was a 63 year old male, and co-cultures were set up with ankle cartilage and knee synovium. While the trends in cytokine and chemokine levels released in untreated co-culture groups were consistent with the results in the previous chapter, there were large differences in the absolute values of the concentrations between the two donors.

In the case of IL-8, the difference between the two donors is potentially due to a limitation with the assay. The IL-8 concentrations for the 69 year old female patient were measured with a V-Plex MSD assay in which the highest standard was 66,700 pg/ml and the samples were diluted by a factor of 4. This means that IL-8 concentrations as high as 266,800 pg/ml could be measured accurately for the 69 year old female donor samples. On the other hand, the samples for the 63 year old male donor were analyzed using a U-Plex plate in which the highest IL-8 concentration that could be measured accurately was only 8,320 pg/ml. Some of the measurements for the 63 year old male donor were at or slightly above this level; in these cases, the assay could have reached saturation and the true concentrations may have been far higher than the measured value.

Despite this limitation, most of the other concentration measurements were within the range of the assays and there was still a large difference between the two donors. These differences are most likely due to high donor-to-donor variability in the human population. The experiments need to be repeated with many more donors before conclusions can be made about the effects of the drug. If future experiments lead to the conclusion that one or more of these drugs has the potential to be used as a DMOAD, conjugating them to appropriately charged GFP carriers would enable the delivery of low doses in a targeted and sustained manner to joint tissues. Furthermore, under conditions in which addition of

free IGF-1 was found to be of additional benefit above, a combination therapy could utilize the newly developed GFP-IGF-1 fusion proteins (described in detail in Chapter 3 of this thesis).

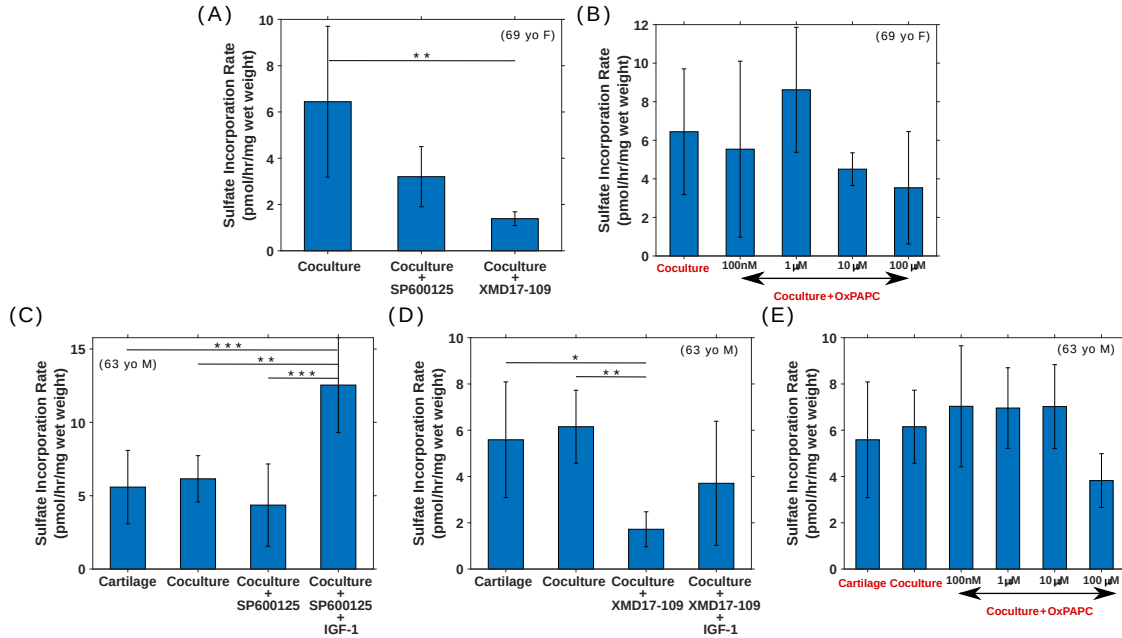


Figure 6.1: Normalized sulfate incorporation rate for human co-culture explants treated with TLR inhibitors or kinase inhibitors. Human cartilage-synovium cocultures were treated with 25 μ M SP600125 (JNK inhibitor), 5 μ M XMD17-109 (ERK5 inhibitor) or different doses (100 nM to 100 μ M) of OxPAPC (TLR2 + TLR4 inhibitor). Treatment groups with combinations of SP600125 + IGF-1 and XMD17-109 + IGF-1 were also included in experiments with 63 year old male donor tissues (C,D,E). SP600125 and XMD17-109 were dosed continuously, while OxPAPC was added only on Day 0 and Day 3 of culture. An untreated co-culture control group was included in experiments in (A,B), while experiments in (C,D,E) included an untreated cartilage monoculture control and an untreated co-culture control. Bars: Mean $\pm$ SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

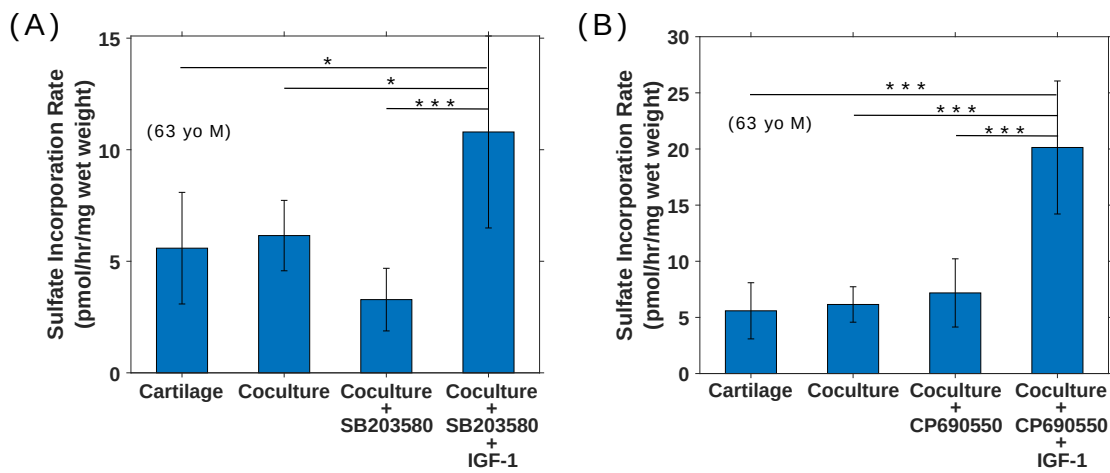


Figure 6.2: **Normalized sulfate incorporation rate for human co-culture explants treated with kinase inhibitors.** Human cartilage-synovium cocultures were treated continuously with 10 μ M SB203580 (p38 inhibitor) (A) or 10 μ M CP690550 (ERK5 inhibitor) (B) individually or in combination with 300 ng/ml IGF-1. An untreated cartilage monoculture control and an untreated co-culture control group were included. Bars: Mean $\pm$ SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

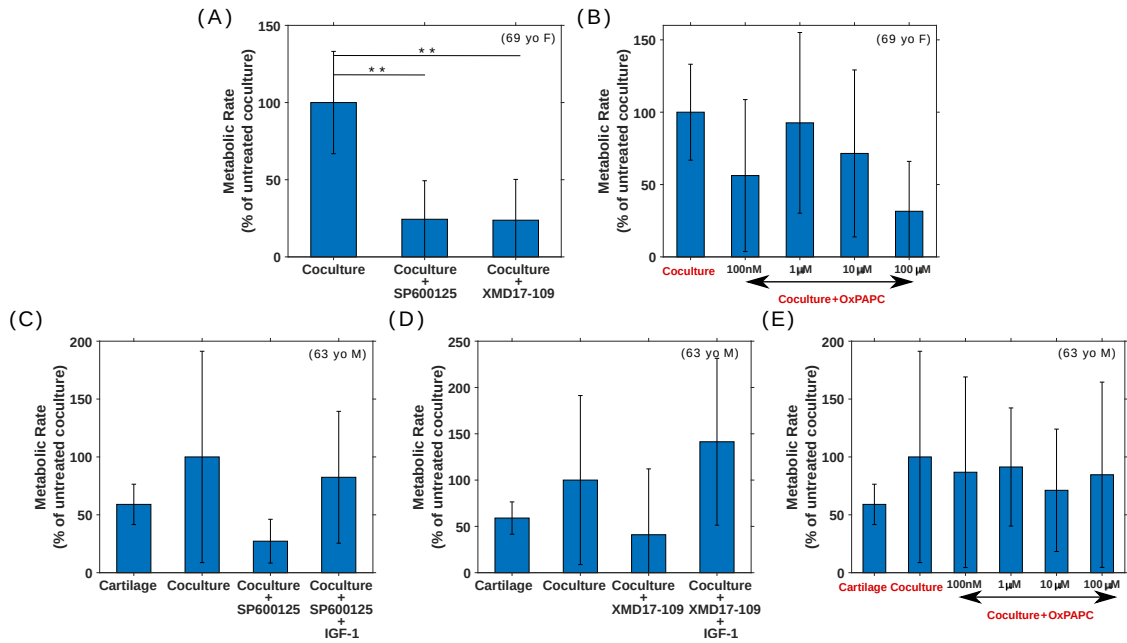


Figure 6.3: Normalized metabolic rate for human co-culture explants treated with TLR inhibitors or kinase inhibitors. Human cartilage-synovium cocultures were treated with 25 μ M SP600125 (JNK inhibitor), 5 μ M XMD17-109 (ERK5 inhibitor) or different doses (100 nM to 100 μ M) of OxPAPC (TLR2 + TLR4 inhibitor). Treatment groups with combinations of SP600125 + IGF-1 and XMD17-109 + IGF-1 were also included in experiments with 63 year old male donor tissues (C,D,E). SP600125 and XMD17-109 were dosed continuously, while OxPAPC was added only on Day 0 and Day 3 of culture. An untreated co-culture control group was included in experiments in (A,B), while experiments in (C,D,E) included an untreated cartilage monoculture control and an untreated co-culture control. Bars: Mean $\pm$ SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

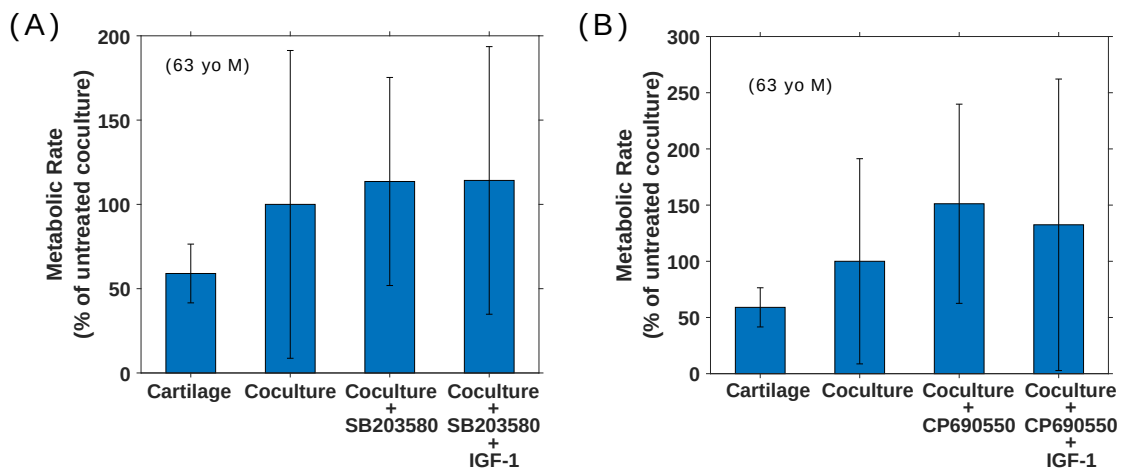


Figure 6.4: Normalized metabolic rate for human co-culture explants treated with kinase inhibitors. Human cartilage-synovium cocultures were treated continuously with 10 μ M SB203580 (p38 inhibitor) (A) or 10 μ M CP690550 (ERK5 inhibitor) (B) individually or in combination with 300 ng/ml IGF-1. An untreated cartilage monoculture control and an untreated co-culture control group were included. Bars: Mean $\pm$ SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

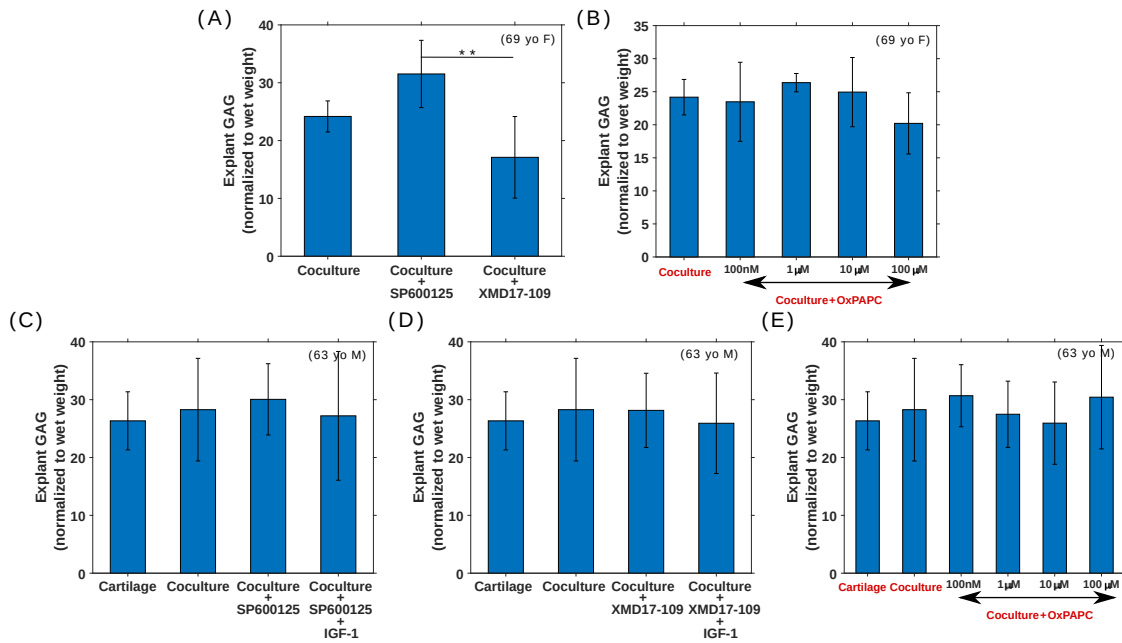


Figure 6.5: Explant sGAG content (normalized to wet weight) for human co-culture explants treated with TLR inhibitors or kinase inhibitors. Human cartilage-synovium cocultures were treated with 25 µM SP600125 (JNK inhibitor), 5 µM XMD17-109 (ERK5 inhibitor) or different doses (100 nM to 100 µM) of OxPAPC (TLR2 + TLR4 inhibitor). Treatment groups with combinations of SP600125 + IGF-1 and XMD17-109 + IGF-1 were also included in experiments with 63 year old male donor tissues (C,D,E). SP600125 and XMD17-109 were dosed continuously, while OxPAPC was added only on Day 0 and Day 3 of culture. An untreated co-culture control group was included in experiments in (A,B), while experiments in (C,D,E) included an untreated cartilage monoculture control and an untreated co-culture control. Bars: Mean ± SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

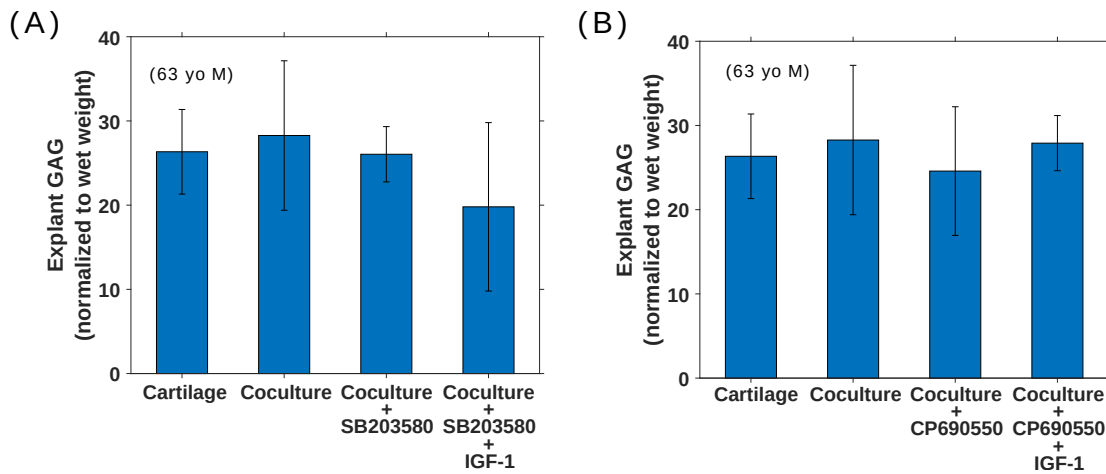


Figure 6.6: **Explant sGAG content (normalized to wet weight) for human co-culture explants treated with kinase inhibitors.** Human cartilage-synovium cocultures were treated continuously with 10 μ M SB203580 (p38 inhibitor) (A) or 10 μ M CP690550 (ERK5 inhibitor) (B) individually or in combination with 300 ng/ml IGF-1. An untreated cartilage monoculture control and an untreated co-culture control group were included. Bars: Mean $\pm$ SE; N = 5 to 6 explants/condition; *:p<0.05, **:p<0.01; ***:p<0.001.

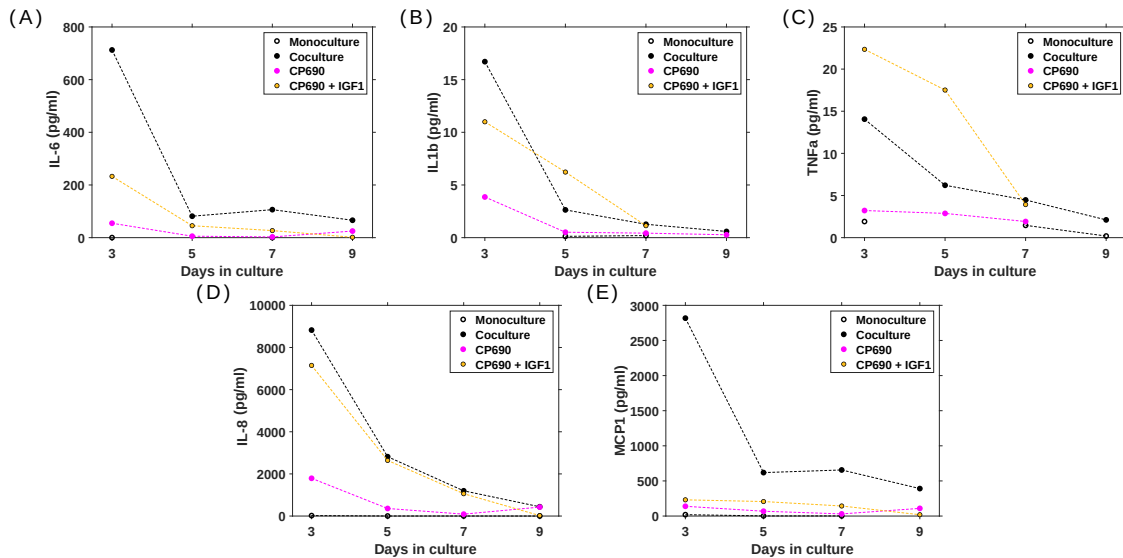


Figure 6.7: Culture medium levels of IL-6, IL-1 β , TNF- α , IL-8 and MCP-1 in co-culture groups (63 year old male donor) treated continuously with a JAK3 inhibitor (CP690550) individually or in combination with 300 ng/ml IGF-1. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; magenta dots: Coculture with 10 μ M CP690550; gold dots: Coculture with 10 μ M CP690550 + 300 ng/ml IGF-1.

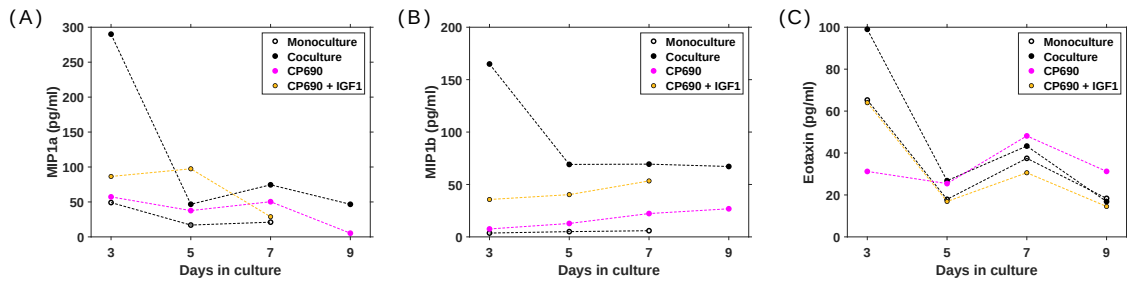


Figure 6.8: Culture medium levels of MIP-1 α , MIP-1 β , and Eotaxin in co-culture groups (63 year old male donor) treated continuously with a JAK3 inhibitor (CP690550) individually or in combination with 300 ng/ml IGF-1. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; magenta dots: Coculture with 10 μ M CP690550; gold dots: Coculture with 10 μ M CP690550 + 300 ng/ml IGF-1.

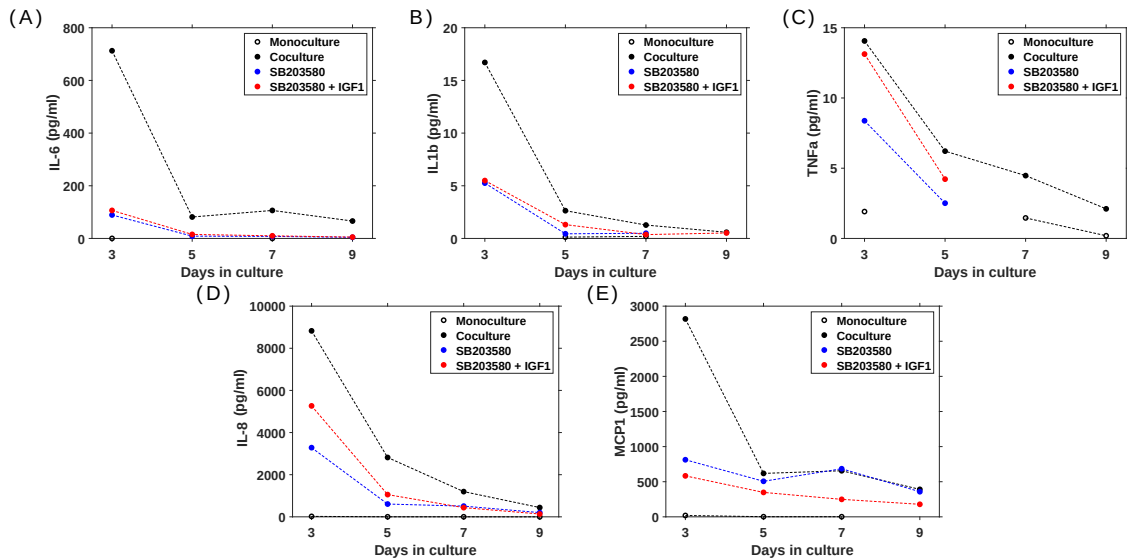


Figure 6.9: Culture medium levels of IL-6, IL-1 β , TNF- α , IL-8 and MCP-1 in co-culture groups (63 year old male donor) treated continuously with a p38 inhibitor (SB203580) individually or in combination with 300 ng/ml IGF-1. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 μ M SB203580; red dots: Coculture with 10 μ M SB203580 + 300 ng/ml IGF-1.

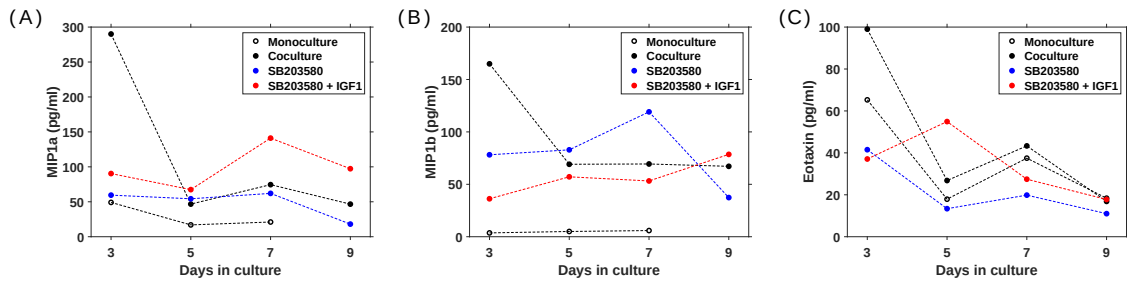


Figure 6.10: Culture medium levels of MIP-1 α , MIP-1 β , and Eotaxin in co-culture groups (63 year old male donor) treated continuously with a p38 inhibitor (SB203580) individually or in combination with 300 ng/ml IGF-1. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 μ M SB203580; red dots: Coculture with 10 μ M SB203580 + 300 ng/ml IGF-1.

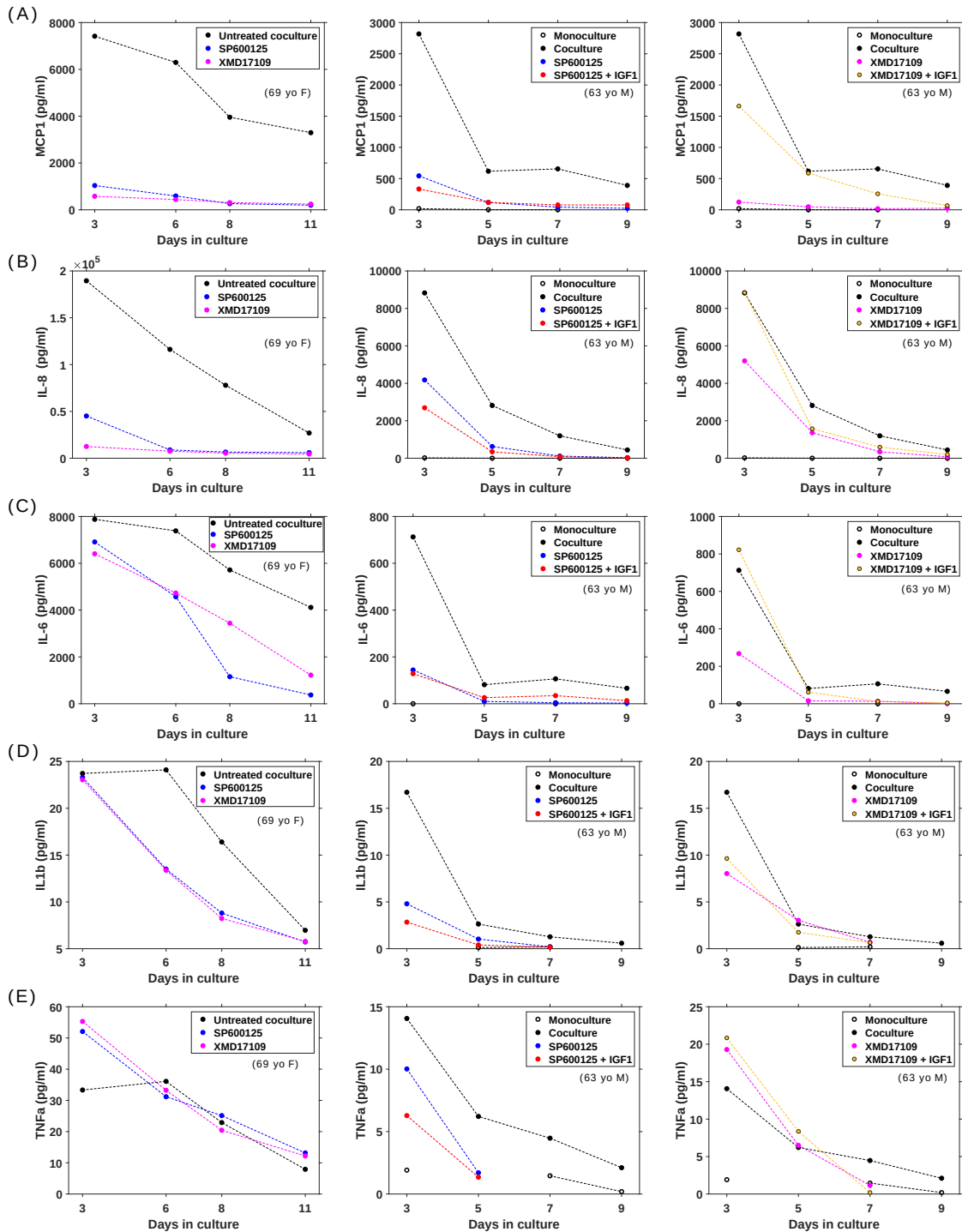


Figure 6.11: Culture medium levels of MCP-1, IL-8, IL-6, IL-1 β and TNF- α in co-culture groups treated with JNK inhibitor (SP600125), ERK5 inhibitor (XMD17-109), JNK inhibitor + IGF-1, or ERK5 inhibitor + IGF-1. First column: Data from experiments with 69 year old female donor tissues. Second and third columns: Data from experiments with 63 year old male donor tissues. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 25 μ M SP600125; magenta dots: Coculture with 5 μ M XMD17-109; red dots: Coculture with 25 μ M SP600125 + 300 ng/ml IGF-1; gold dots: Coculture with 5 μ M XMD17-109 + 300 ng/ml IGF-1.

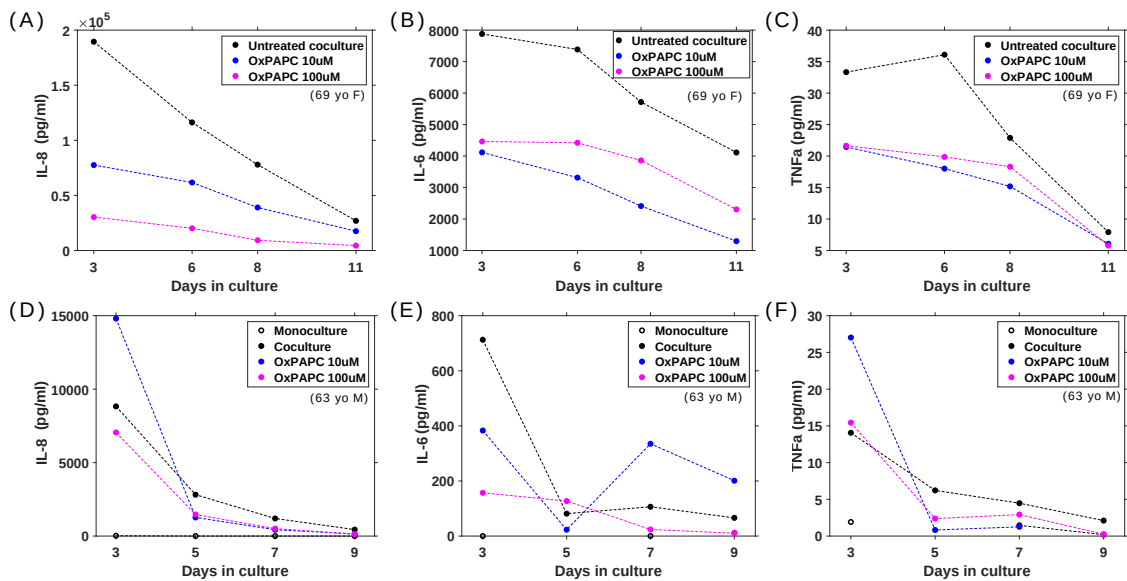


Figure 6.12: Culture medium levels of IL-8, IL-6 and TNF- α in co-culture groups treated with OxPAPC. (A, B, C): Data from experiments with 69 year old female donor tissues. (D, E, F): Data from experiments with 63 year old male donor tissues. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 μ M OxPAPC; magenta dots: Coculture with 100 μ M OxPAPC.

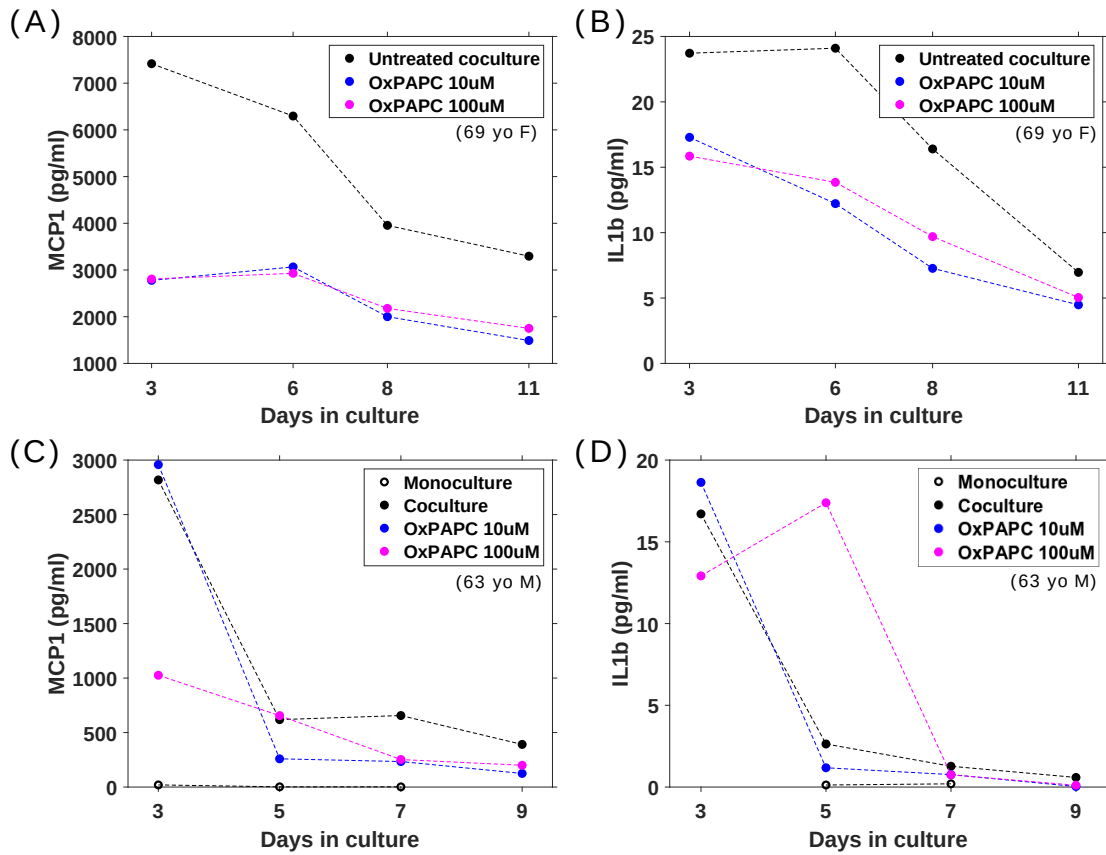


Figure 6.13: Culture medium levels of MCP-1 and IL-1 β in co-culture groups treated with OxPAPC. (A, B, C): Data from experiments with 69 year old female donor tissues. (D, E, F): Data from experiments with 63 year old male donor tissues. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 μ M OxPAPC; magenta dots: Coculture with 100 μ M OxPAPC.

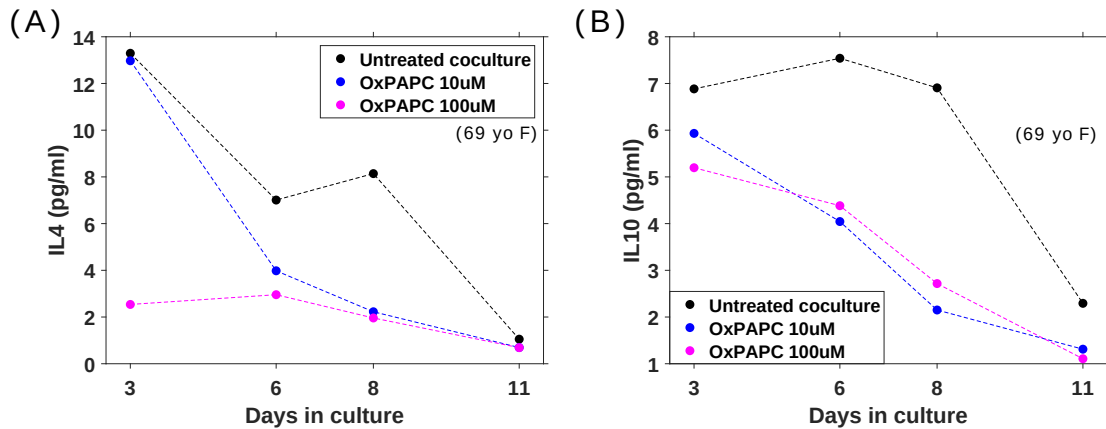


Figure 6.14: Culture medium levels of IL-4 and IL-10 in co-culture groups (69 year old female donor) treated with OxPAPC. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 µM OxPAPC; magenta dots: Coculture with 100 µM OxPAPC.

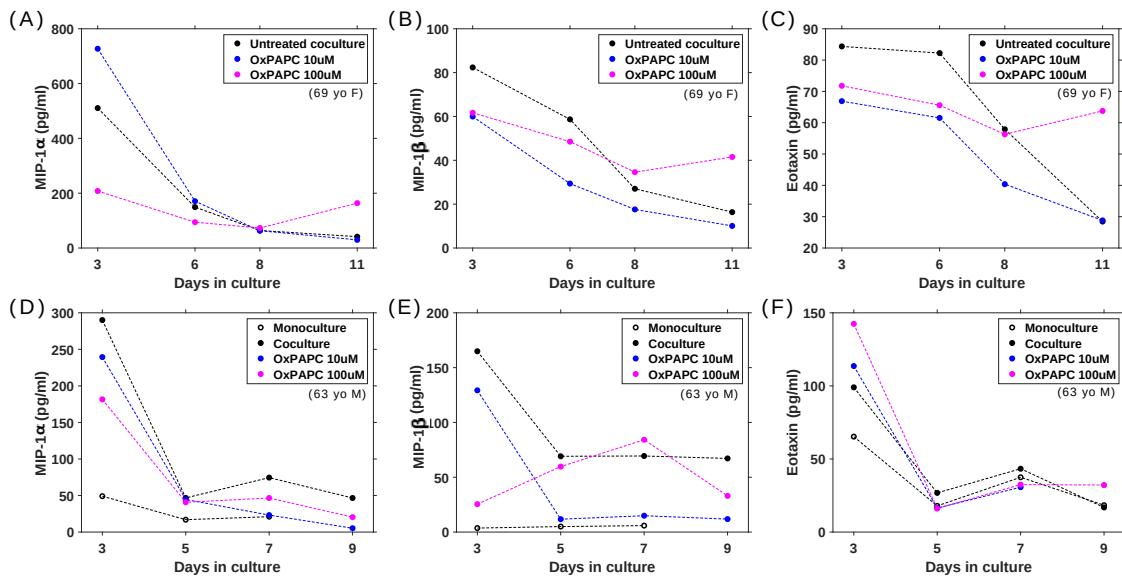


Figure 6.15: Culture medium levels of MIP-1α, MIP-1β and Eotaxin in co-culture groups treated with OxPAPC. (A, B, C): Data from experiments with 69 year old female donor tissues. (D, E, F): Data from experiments with 63 year old male donor tissues. **Legend:** Black open dots: Untreated monoculture cartilage control; black filled dots: Untreated coculture control; blue dots: Coculture with 10 µM OxPAPC; magenta dots: Coculture with 100 µM OxPAPC.

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