STEM CELL THERAPY COMBINED WITH CRISPR TO ENGINEER ANTI-INFLAMMATORY CHONDROCYTES TO IMPROVE NEUROINFLAMMATION IN RHEUMATOID ARTHRITIS

By

LAKESHIA R. GARY

A thesis submitted to the

Graduate School-Camden

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of Master of Science

Graduate Program in Biology

Written under the direction of

Dr. Nathan Fried

And approved by

_____

Dr. Nathan Fried, Thesis Director

_____

Dr. William Saidel, Committee Member

_____

Jamie Dunaev, Committee Member

_____

Kaitlin Laws, Committee Member

Camden, New Jersey

October 2020

THESIS ABSTRACT

Stem cell therapy combined with CRISPR to engineer anti-inflammatory chondrocytes to improve neuroinflammation component in Rheumatoid Arthritis

by LAKESHIA R. GARY

Thesis Director:
Dr. Nathan Fried

Regenerative medicine has the potential to improve several medical issues**,** such as tissue damage, diminished cell maintenance/repair and failed organ systems. CRISPR technology provides the opportunity to expand stem cell therapy to not only replace lost cells, but also genetically engineer them to treat certain conditions. Rheumatoid arthritis is one disorder where combining both therapies can improve the patient outcome by genetically engineering replacement chondrocytes to constitutively release anti-inflammatory cytokines. This unique strategy presents the opportunity to not only target the pain produced from joint deterioration, but also continuously replace the cartilage to increase the reduced mobility of Rheumatoid Arthritis diagnosed patients.

Preface

Maybe it's not supposed to be easy for you. Maybe you're one of the rare few who can handle tough times and still choose to be a loving person. Maybe it's going how it's going because you're built for it... Don't stress a thing. It's going to work out because you're not going to stop putting the work in. ~Robert Hill Sr.

…for such a time as this Ester 4:14b

Acknowledgement/ Dedication

Thank you to my thesis director, Doctor Fried for the countless hours that we spent discussing and editing this paper. As well as thank you to all my committee members for all the time that you dedicated to editing, motivating me and speaking positively. I appreciate how each of you were truthful and understanding that I could endure any and all critiques in order to become better. Lastly, I would like to dedicate this paper to my mother Letitia. Y Davis, she is the reason that this idea even happened.

Table of Contents

List of Illustrations

Introduction

Rheumatoid arthritis (RA) is often a life changing diagnosis since it can lead to debilitating symptoms. RA is very difficult to treat because the underlying etiology is unknown. Though the molecular origin is unclear, RA can attack all joints, treatment options are both necessary and limited, with several featuring challenging side effects (Fuggle et al., 2014). Cartilage deterioration is the major underlining physiological issue that begins the activation of joint pain associated with rheumatoid arthritis. New technological advances in gene editing and stem cell technology may offer an avenue not only to intermittently treating the disorder, but to provide continuous treatment that could be considered true RA remission. This could theoretically be done by targeting both the degeneration of the joint and the inflammation that induces chronic pain. Currently, most treatment options focus only on treating inflammation. CRISPR genome editing technology can allow physicians to genetically engineer stem cells that would be used to produce tissue-regenerative chondrocytes within a joint (Han et al., 2019). In doing so, the joint cartilage would be replaced by anti-inflammatory chondrocytes, restoring the joint while preventing future inflammatory-induced deterioration.

Rheumatoid Arthritis

Arthritis is a medical term that simply means swelling and inflammation of the joints. Arthritis is involved in more than 100 different conditions. These conditions include not only swelling of the joint(s) but swelling of the tissue around the joint. Joint pain and stiffness results from this deterioration and inflammation (Barbour et al., 2017). In 2015 the Center for Disease Control and Prevention (CDC) reported that one in six adults (54 million) were diagnosed with a form of arthritis. Two-thirds of these patients are women. Arthritis has a substantial effect on society, both medically and economically. Each diagnosis comes at a price. The yearly out-of-pocket cost for each patient is $2,117.00, totally $140 billion nation-wide. If including lost wages, this total is even higher at $164 billion (CDC, 2018). Although all forms of arthritis feature pain, RA is vastly more debilitating than other forms of arthritis because of rapid joint deterioration that leads to nearly 50% of patients becoming disabled 10 years after diagnosis (Heidari, 2011). This diagnosis not only limits mobility, but also produces several challenges with normal day-to-day patient behaviors. Given this, RA isn't just painful but also is associated with depression/anxiety.

RA is an autoimmune disease in which the body mistakenly identify the synovium cells as unknown pathogens. This misidentification results in joint deterioration and inflammation at a specific body region (hands, wrist, knees, spine) or throughout the entire body. Some symptoms include redness of skin, swollen tissue in a localized area, skin warm to the touch and pain (McInnes, 2011). RA is often not diagnosed in the early stages since there are limited symptoms or signs early on. Although the underlying etiology is unknown, RA appears to be multi-causal with a combination of genetic, environmental, and lifestyle

choices being correlated with the chance of developing the disorder. Since multiple risk factors can contribute to the progression of RA, each patient's symptoms may differ.

An influx of osteopontin (OPN), a multi-functional protein biomarker is an RA biomarker involved in the mineralization of bone, calcification inhibition and immune cell function regulation. OPN is expressed at high levels during the proinflammatory state in the synovial fluid in bones which lead to the bulk formation of proinflammatory cytokines. OPN can regulate the immune response by increasing degradation of extracellular enzymes and the expression of Th1 cytokines. These proinflammatory cytokines include Interleukin (IL)-17, IL-6 and tumor necrosis factor alpha (TNFa) which all interact with CD-44, a cell surface adhesion receptor largely expressed in diseases such as cancer (Roy et al., 2015). Widespread damage does not occur until later into disease progression which is when patients may complain of discomfort and pain. Most RA diagnosed patients do not see a specialist until they have experienced discomfort for a lengthy amount of time. Unfortunately, this level of discomfort is well passed the beginning of significant damage to the joints. To be diagnosed with RA, the patient must also feature high levels of RA-associated monoclonal and polyclonal antibodies within their blood (rheumatoid factor, RF and anti-citrullinated protein antibody ACPA). Coupled with these biomarkers, patients also undergo a physical assessment to identify symmetrically inflamed joints. There are currently no treatments to stop disease progression, but instead only ones that slow its progression (NIH, 2020).

The two primary problems with RA are 1) the degeneration of the joint and 2) the associated pain, both of which advance the breakdown of cartilage and production of excessive inflammation.

The current treatments for RA can range from mild to invasive. Some of these treatments are home remedies (compresses) and others are medications with differing side effects (like immunosuppression). These treatments have broad efficacy as some of these approaches treat symptoms and others attempt to treat causes. The forms of treatment that target inflammation are hot/cold compresses and disease-modifying antirheumatic drugs (DMARDs). A hot compress increases the blood flow and reduces muscle spasms. A cold compress restricts blood flow and reduces inflammation and swelling. DMARDs, such as Methotrexate and Leflunomide, decrease flare-ups and slow progression of the disease. Though they could drastically reduce the symptoms of RA, they greatly suppress the immune system, leaving patients open to an array of opportunistic infections (i.e., cold or flu). Patients receiving this biologic cannot take antibiotics during or following for two weeks because the antibiotics will not have therapeutic effects. When DMARDs are given regularly, they can have substantial autoimmune side effects that can lead to the development of lupus (hyperactive immune system attacks healthy tissue), vasculitis (blood vessel inflammation) and psoriasis (excessive skin cell growth) (Adkar, 2017). The forms of treatment that target cartilage deterioration are hyaluronic acid injections and surgery. Hyaluronic acid injections (Euflexxa, Synvisc, Monovisc, GelOne) lubricates eroded joints. Efficacy varies among patients and insurance providers only allow for these injections 2-3 times a year, which is not sufficient for some patients (Mohd et al., 2018). Surgery is a last option treatment involving the removal of the diseased joint and fusing the bone together to limit movement and muscle stiffness (Adkar, 2017).

The forms of treatment that target pain are non-steroidal inflammatory drugs (NSAIDs) and opioids. NSAIDs reduce pain and glucocorticoids that decrease the production of

proinflammatory cytokines. They cannot be used for long-term treatment due to the risk of cardiovascular, kidney or gastrointestinal damage/toxicity. Opioids reduce pain but due to their risk of abuse, are not ideal options for long-term treatment (Adkar, 2017). DMARDs are thus the standard of care for RA but are used with other forms of therapeutics due to their inability to address the patient's cartilage deterioration.

Cartilage is connective tissue that is both firm and flexible. It provides structural support and form to various parts of the body, for instance, the ears, larynx and intervertebral discs. Cartilage limits the stress that constant motion causes on joints as well as the friction of bones by covering the ends of bones. Healthy cartilage permits bones to glide over each other, allowing the body to exhibit a full range of motion (MedlinePlus 2015). There are three types of cartilage present in the human body: hyaline, elastic, and fibrocartilage. RA largely affects the fibrocartilage of inflamed joints. Fibrocartilage is a layered matrix of compact collagenous bundles reinforced by thin fibers with an open spongy structure with gaps between the lacunae containing a chondrocyte (Benjamin & Evans 1990). The fibrocartilage poses great tensile strength; it can absorb shock very well and is the best to encompass the knee area. The knee endures a lot of stress and wear due to human bipedalism. Walking upright puts a constant pressure on the cartilaginous joints of the long bones and the spine/knee joints are the load bearing areas of the body that endure the most stress. Even simple activities such as standing wears on the intervertebral disc. With many years of just normal day-to-day activity, the intervertebral disc begins to naturally deteriorate and degrade; in RA patients, this process is exacerbated. Over time, the intervertebral disc wear induces the fibrocartilage to decrease in density (Tendulkar et al., 2019).

Targeting Degeneration

Regenerative medicine is the ability of a body to recreate what was once there, may that be a cell, tissue, organ or even a limb. Stem cells play a major role since they are used to regenerate the cells of damaged tissue to a fully functional state. The body requires a stem cell population with pluripotent capabilities in various parts of the body to repair, replace and maintain cells as needed (Ivankovic et al., 2019).

Reduction in cartilage density leads to symptoms of pain and limited movement, which can lead to joint damage and deformities. Common effects from obesity, repetitive trauma, age and genes is cartilage deterioration, can lead to life changing debilitating diagnosis such as degenerative disc disease (DJD), bone spurs, herniated discs and ankylosing spondylitis. DJD occurs naturally with age due to years of lifting and straining. Most individuals will experience some form of degeneration in their spine by the age of 40. As a result of this deterioration, the usual form and capabilities will change indefinitely. The most consequential effect of DJD is chronic pain (Donnally III, 2019). Ankylosing spondylitis is similar to RA as it is also a rheumatic autoimmune disease coupled with chronic inflammation with a lack of identifiable causes for flare-ups. Flare-ups often become identifiable based on each individual patient's triggers. Patient triggers unfortunately vary significantly, some resulting in immobility, disfigured digits, irregular posture, and pain in various areas of the body (McVeigh, 2006). Given the importance of healthy cartilage for maintaining joint mobility and avoiding debilitating chronic issues caused by RA, new studies are needed to protect and regenerate cartilage. One way of doing this is through focusing on chondrocyte regeneration using stem cell therapy.

Chondrocytes initially derive from mesenchymal stem cells (MSCs) that are located in the bone marrow and first become chondroblasts, an active cell that produces the extracellular matrix that will eventually house itself in a lacunae. After the chondroblast is entrapped, it begins to secrete chondroitin. Chondroitin is a sulfated glycosaminoglycan that produces the matrix that actively builds and repairs the cartilage. Once lacunae are full of the chondroitin, the chondroblast transforms into the chondrocyte. The number of chondrocytes present in collagenous bundles of thin fibers determines how flexible the cartilage will be. To ensure that fibrocartilage is generated appropriately, the chondrocyte must be placed in a specific pattern (van der Kraan et al. 2002; Craft et al. 2013).

There are three distinctive characteristics of stem cells: unspecialized, long-term self-renewal and ability to generate in to at least one specialized cell. Stem cells are undifferentiated cells that are classified by their origin and potency. Origin includes embryonic, biopsied tissues, or already specialized cells. The stem cells origin will determine their potency, which could be totipotency, pluripotency, multipotency, or unipotency, limitations vary. These specialized cells are derived from the process of differentiation that occurs in a specific embryonic signaling environment. There are four distinctive phases, specification (which is reversible), determination (which is when it becomes irreversible), proliferation (the cell numbers increase) and differentiation (in which a cell is morphologically, chemically and functionally specialized). Conrad Waddington describes these four stages as cells hitting a series of check points as it develops into a specific specialized state. This process is coined as Waddington's Epigenetic Landscape (Daley, 2015).

Embryonic stem cells are derived from the inner mass of a blastocyst, which is a fertilized egg on day 6 of embryonically cleaving. The inner mass is removed, placed in a petri dish, then periodically separated as the cells continue to divide. These cells generate primitive germ layers from which all tissues can develop. Induced pluripotent stem cells (iPSCs) are derived from somatic tissue and once treated then put in an environment, will replicate that type of specialized cell. Adult stem cells derived from an already specialized cell can only develop into tissue specific cells. Gastrulation is the phase of embryonic development where three germ layers are formed. These germ layers are ectoderm (form outer tissues of body), mesoderm (forms tracts and organs), endoderm (forms epithelial linings of organs and tracts). The development of these three germ layers will determine if a cell is pluripotent. Pluripotent classification can only come from testing an individual cell to verify the development of these three germ layers (Mummery et al., 2014).

Stem cells have the ability to regenerate damaged or degenerative tissue via self-reproduction or self-renewal and plasticity of differentiation. Self-renewal is the cells' ability to divide asymmetrically either on an individual or a population level, this is really important for tissue homeostasis. The plasticity of differentiation refers to the ability to differentiate into various types of cells, which is sometimes instructed by the surrounding tissue environment. Pluripotent embryonic stem cells and iPSCs are excellent in their potency ability to reproduce and differentiate into various tissues. While multipotent adult stem cells can have a limited ability of reproduction or differentiation compared to embryonic stem cells or iPSCs. Stem cells can be used as a means to provide a therapeutic application. A possible means of therapy is the replacement of chondrocytes in cartilage

due to neural inflammation caused by chronic pain because of rheumatoid arthritis in the lower spine (Mummery et al., 2014).

Researchers are finding ways to push a developing stem cell into specific types of cells, i.e. fibroblasts, neurons, kidney cells, etc. Specifically, a couple of researchers from the University of Cambridge have identified a way to nudge stem cells to become both pancreatic and hepatic cells. They were able to achieve this by using a fluorescence ubiquitination cell cycle indicator (FUCCI), this is genetically encoded florescence that follows the cell division within a cell population. Using this technology, they were able to determine that the early G1 phase of mitosis could develop into both endoderm cells as well as mesoderm cells, while the late G1 stage would develop into neuroectodermal cells. Cyclin-D is a protein that regulates cell cycle progression. Low expression of Cyclin-D during early G1 stage allows entry of the effector signaling cell into the nucleus, which results in either endoderm or mesoderm cell generation. High expression of Cyclin-D during the late G1 stage blocks the entry of the signaling effector cell into the nucleus by CDK4 binding causing phosphorylation, this would result in neuroectoderm cell formation. In this study, they were also able to decrease differentiation of the formed stem cells pluripotency by increasing c-peptide positive cells and the expression of endocrine markers specifically for pancreatic cells and hepatic cells were able to develop (Pauklin S & Vallier L., 2014). The same technology can be used to replace chondrocytes – the cells that are necessary to regenerate the lost cartilage that RA patients have.

Targeting Inflammation

Inflammation is crucial to the immune system being able to respond to injury and/or infection. Inflammation acts as a signal to trigger the immune system to defend against foreign pathogens that are invading the body. When inflammation arises, the immune system releases Major histocompatibility complex (MHC) Class 2 cells and macrophages to fight the invading pathogens. These cells are released into the bloodstream or the affected tissue area. The immune system fends off viruses and bacteria, while also repairing and healing the damaged tissue. Loss of function, swelling, pain, redness and warmth to the injured area are all signs of inflammation. When inflammation is present, individuals will experience varying levels of pain, discomfort and stiffness. The degree of pain differs with each RA patient, with descriptions varying from pulsating, throbbing, steady, stabbing or constant (InformedHealth.org, 2018).

Neuroinflammation is initiated in the brain or spinal cord due to an inflammatory response. Neuroinflammation is the primary reason for severe unrelenting chronic pain, this is due to microglia activation. Microglia are innate immune cells of the central nervous system (CNS) that are responsible for immune surveillance primarily and microphage like behaviors such as production of chemokines and cytokines (DiSabato et.al, 2016). When there is an hyper production of synovial fluid it accumulates in one area, forming a pannus. A pannus is an increased amount of fibrovascular or granulation tissue that forms over a normal body structure, in this case joint, cartilage (Towns & Bathon, 2010).

The falsely identified synovial cells targeted by the MHC Class 2 cells that are responding to an increased production of cytokines. Cytokines are signaling proteins that are produced by cells that regulate and mediate immunity, hematopoiesis, and inflammation. They have

various functions throughout the body, which can include both growth and differentiation factors (tissue maintenance and repair), colony-stimulating factors (production of blood cells), immunoregulatory and proinflammatory (functions of the immune system). These cytokines are categorized by the cell that releases them: interleukins released by leukocytes, monokines released by monocytes, lymphokines released by lymphocytes, and chemokines released by innate immune cells. Cytokines can exert both proinflammatory and anti-inflammatory responses. Proinflammatory cytokine production causes activation of macrophages the initiation of the inflammatory response. Certain proinflammatory cytokines (IL-1 beta, IL-6 and TNF- alpha) are distinctively involved in the development of chronic pain. Chronic pain transpires when the pain persists even after the following damage or injury has healed. TNF acts on numerous distinctive signaling pathways via two cell surface receptors (TNFR1 and TNFR2) to control apoptotic (programmed cell death) pathways. NF-kB activation of inflammation initiates stress-activated protein kinases.

Proinflammatory cytokines can facilitate a sensory neuron mechanism that produces excess Substance P (neuropeptide acting as neurotransmitter/modulator). Substance P stimulates microglia to produce proinflammatory cytokines resulting in pain and neuroinflammation. Substance P and proinflammatory cytokines will decrease the threshold that is required for the pain neurons to fire. Essentially making the pain neurons more sensitive to mechanical, thermal and chemical stimulation resulting in chronic pain (Kim et.al, 2018). The production of proinflammatory cytokines causes inflammation that attacks synovial joint tissues causing increased joint fluid, joint swelling, muscle loss and bone/cartilage damage. Anti-inflammatory cytokines are immunoregulatory molecules that control the proinflammatory cytokine response. The anti-inflammatory cytokines work alongside

specific soluble cytokine receptors and cytokine inhibitors to control the immune response. A major anti-inflammatory cytokine is IL-10, as it is very potent in reducing the expression of proinflammatory cytokines (IL-6, TNF- alpha, IL -1) (Chakravarthy et al., 2017; Zhang, JM, 2007).

The IL-10 gene is located on chromosome 1q32.1 of the human genome (Uniprot.org, 2020). IL-10 binds to heterotetramic receptors, IL-10RA and IL-10RB which initiates the activation of Janus kinase (JAK)-1 (protein-coding gene essential for signaling cytokines) and signal transducer and activators of transcription (STAT)-2 (transcription activator). The triggering of this pathway allows STAT3 to translocate into the nucleus, activating transcription to drive the expression of anti-inflammatory mediators. The release of these anti-inflammatory mediators reduces inflammation by inhibiting proinflammatory cytokines. They also block antigen-presenting cells (macrophages and monocytes) and reduce MHC Class 2 costimulatory molecules (Iyer, 2012). IL-10 injections have been used in various studies to reduce pain. In 2001 Plunkett et al, used an adeno-associated viral (serotype 2; AAV2) vector that encodes IL-10 (AAV2-r-IL-10) where they intrathecally injected mice with chronic inflammatory pain from a sciatic nerve lesion. AAV2-r-IL-10 inhibited mechanical allodynia for eight days after the initial administration, as assessed with von Frey hairs. The von Frey test uses reflex based assays with paw/tail, by touching the animal in order to see if it will move its paw in response to painful stimuli (Milligan et al., 2005). Another research study demonstrated that injections of quisqualic acid (QUIS) into the spinal cord of rats would induce a spinal cord injury that caused a surge in IL-1 beta mRNA, tumor necrosis factor related apoptosis-inducing ligand (TRAIL), inducible nitric oxide synthase (iNOS) and CD95 ligand, all indicating

proinflammatory events. Rats receiving regular intrathecal injections (every 30 minutes) of IL-10, however, exhibited a substantial decrease in these molecular markers of inflammation and pain-related behaviors (Plunkett et al., 2001).

CRISPR Technology

Clustered regularly interspaced short palindromic repeats (CRISPR) Type 2 system is a bacterial immune system that has been modified for genome engineering. CRISPR consists of two components: a guide RNA (gRNA) and a nonspecific CRISPR associated endonuclease (Cas9). The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas9 binding in a user defined ~20 nucleotides spacer or targeting sequence, which defines the genetic target to be modified. The genomic target of Cas9 can be altered by simply changing the targeting sequence present in the gRNA. CRISPR uses a particular mechanism that makes cuts at specific locations based on the sequence of gRNA to allow for gene editing. The DNA may be transcribed into RNA and integrated into Cas9 protein. Cas9 is a endonuclease that uses the short bacteriophage sequences of RNA as a template to recognize and destroy all of the preferred bacteriophage DNA that it encounters in the cell through double strand breaks. After the desired bacteriophage DNA sequences have been removed, then gRNA forms a complex at each particular site inducing methylation to inhibit gene expression. Once gene expression is inhibited then a designed RNA sequence can be inserted in that area producing a desired effect via location specific histone acetylation to increase that specific gene expression. CRISPR Cas9, the nuclease can identify and cut DNA of random sequence with high specificity and efficiency (Jinek et al., 2012 & Doudna et al. 2014). Jennifer Doudna is the biochemist of UC-Berkley that established this bacterial system could be co-opted and modified not only for gene editing purposes, but for correcting mutations, development of gene-based medication, human disease modeling and other types of modifications that might increase/decrease gene expression. CRISPR was realized for its part in bacterial inherent immunity. Humans and

some other species take up segments of the bacteriophage DNA and integrate them into their own genome. The incorporation of the bacteriophage and virus DNA into the body's' genome provides a resistance to reinfection.

CRISPR editing technology has been used before to engineer cells into releasing anti-inflammatory cytokines. In 2017 Farhang et al used CRISPR Cas9 to control inflammatory signaling by fusing to Krüppel Associated Box (KRAB) domain while coexpressed with single gRNA to suppress TNFR1 and IL1R1 (cytokines/receptors). In this study, the researchers used human adipose derived stem cells (hADSCs) in a 3- dimensional cell culture *in vitro*. hADSCs are cells used for musculoskeletal disease treatment and the behavioral responses in 3D cultured cell environment is more contemplative of an *in vivo* cellular response. The researchers were able to successfully demonstrate that epigenome editing of hADSCs effectively defend the musculoskeletal tissue from inflammatory signaling, while in culture when experiencing inflammatory difficulties. In doing so, the hADSCs defensive response confirmed that epigenome edits not only foster self-survival, but extracellular membrane deposition. The epigenome edits were also able to sustain and promote cell protective immunomodulation and stem cell differentiation, all while enduring inflammatory challenges. In using CRISPR epigenome editing the hADSCs are expected to be able to protect endogenous cells from apoptotic interactions and age induce deterioration. The hADSCs are also expected to decrease proliferation of proinflammatory signaling due to the engineering of the therapeutic hADSCs cells implantation into inflammatory environments (Farhang et al., 2017).

In another study, researchers tested the efficacy of using engineered cytokine activation to control feedback expression as a form of biologic therapy. They used murine (mice)

pluripotent stem cells for this experimentation. The researchers were able to engineer articular cartilage stem cells. The cells had the capacity to induce transient anti-inflammatory responses to fight off IL1Ra (proinflammatory cytokine) and/ or TNF (inflammation cytokine signaling protein) catabolic effects. CRISPR made it possible for them to add gene IL1Ra or the soluble TNF receptor closer to the transcription factor of the cDNA. Closer proximity to the promoter for CCL2 biomarker was crucial in order to engineer induced pluripotent stem cells (iPSCs). CCL2 is a macrophage chemoattractant protein-1 gene that regulates the operating of T-cells, basophils and monocytes/macrophages, which are antagonist to IL1Ra and TNF activity. The iPSCs were able to create a negative feedback loop when stimulated by proinflammatory cytokines. In doing so, iPSCs were able to differentiate into chondrocyte like cells, as a response to the proinflammatory stimulation. As the stimulation continued, there was a reduced expression of catabolic enzymes as well as a decreased maintenance of glycosaminoglycan (rich cartilage matrix, used as bodies lubricate/ shock absorber) (Adkar, 2017 & Brunger et al., 2017). CRISPR epigenome editing technology makes room for constant release of anti-inflammatory cytokines, allowing for distinctive personalized therapeutic treatments that could relieve the issues of inflammation in rheumatoid arthritis patients.

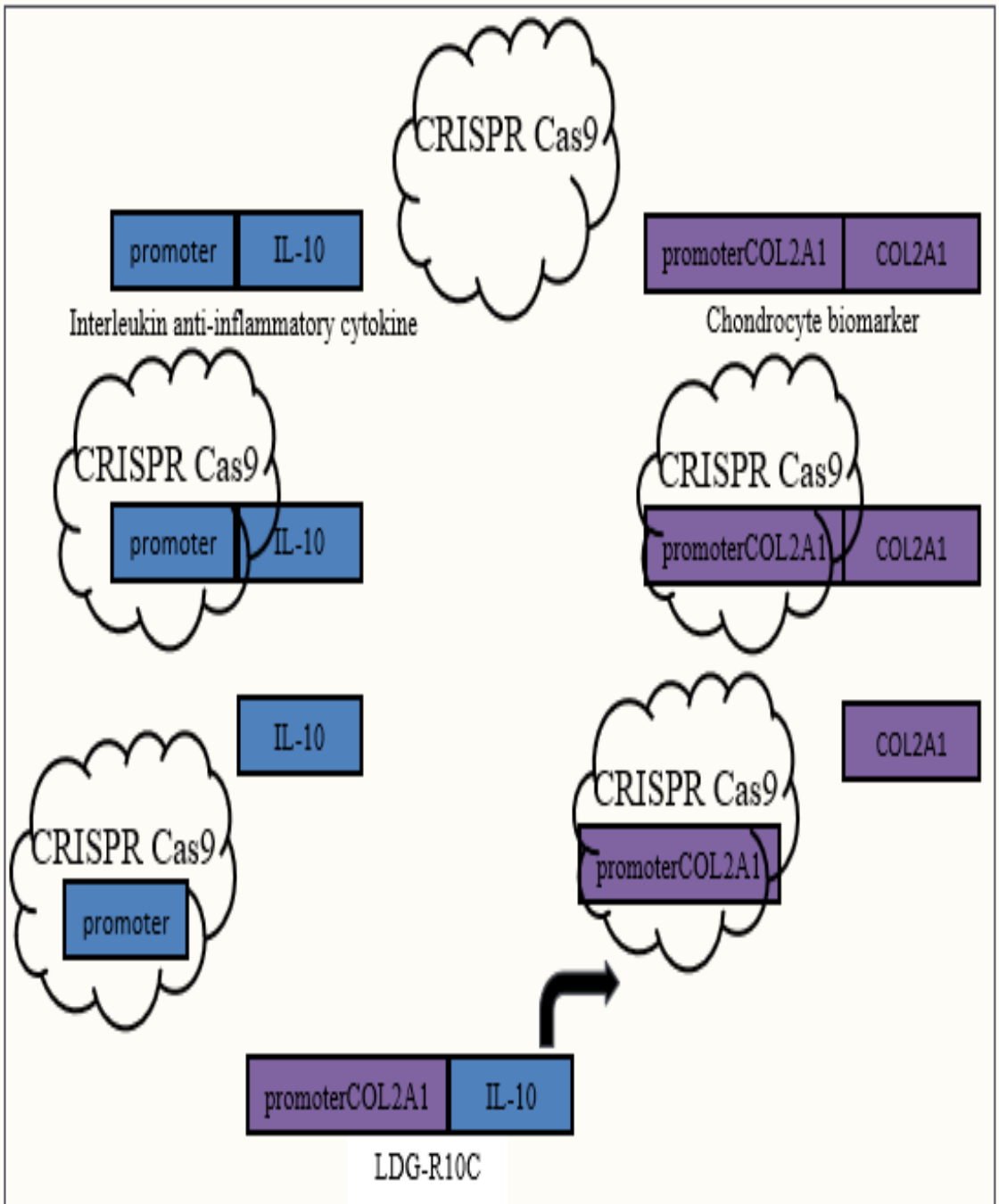

Figure 1: LDG-R10C Formation – Using CRISPR Cas9 to remove the promoter of IL-10 and then replacing it with the promoter sequence of the C0L2A1 chondrocyte molecular biomarker. This will in turn create chondrocytes (LDG-R10C) that will naturally produce anti-inflammatory IL-10 every time a chondrocyte is formed.

Stem Cell Therapy & CRISPR as RA Treatment

In combining stem cell therapy with CRISPR, there are new possibilities for medical treatments. Stem cell capabilities of self-renewal, proliferating, differentiating combined with gene therapy expression leads to a more efficient way for the body to heal itself. Gene expression is how a cell regulates which of the many genes from your genome are actually expressed. Gene expression can be displayed in different patterns of various cells, they have different sets of proteins, which make each cell type distinctive because they all specialize in a different job. A familiar example of gene expression is the control of insulin. Gene expression actually gives the signal for blood glucose regulation. The ability to increase or decrease the signal for insulin could be very helpful to people suffering from glycemic level issues. Gene therapy expression can help the cells better respond to their changing external environment, giving that ability to better control when and how much proteins are expressed (Goverdhana et al., 2005). By manipulating which genes are properly expressed we can use this therapy to overcome the greatest two issues associated with rheumatoid arthritis, autoimmune induced inflammation and cartilage degeneration.

The body does not naturally repair cartilage deterioration as chondrocytes are not able to cellularly divide and this causes cartilage to stop regenerating in adults. The cartilage in adults are usually inhabited with quiescent chondrocytes that maintain their matrix in a minimal turnover state, in lieu of this using stem cell therapy to target degenerating cartilage is imperative (Goldring, 2012). By initiating chondrogenesis, the development of cartilage, physicians can begin to address the joint pain associated with RA. Chondrogenesis starts with the transcription factor Pax 9 (chondrocyte progenitor), that starts the induction of chondrogenesis. Pax 9 leads to the second stage of activating of NCAM (2nd stage chondrocyte progenitor) this reaction last for a very short moment before

the process of differentiation begins. NCAM then activates NKX 3.2, which is the chondrogenic transcription regulator. The key to cartilage engineering is centered around Sox 9, the chondrogenic transcription regulator whose binding efficiency is increased in the presence of Sox 5 & 6. Their presence allows Sox 9 to bind to chondrocyte phenotypic gene COL2A1. COL2A1 acts as a molecular biomarker for chondrocytes, protocol for collagen production, provides strength and structure to the connective tissues that sustains the body's joints, skin, muscles and organs support wise. When COL2A1 is expressed in the presence of Hyaluronan (polymer glycosaminoglycan), it biochemically allows for cartilage generation to occur (Suchorska et al., 2017).

During the production of chondrocytes from stem cells, it'll also be possible to harness CRISPR to genetically engineer chondrocytes to express IL-10 constituently. In order for IL-10 to automatically be expressed by chondrocytes this can be accomplished by removing the promoter of IL-10 and then replacing it with the promoter sequence of the COL2A1 molecular biomarker (Hissnauer et al., 2010). By switching the promoters this will in turn create chondrocytes (LDG-R10C) that will naturally produce anti-inflammatory IL-10 every time a chondrocyte is formed. The IL-10 gene sequence will then be under the control of the chondrocyte-specific gene. Therefore, creating an environment that will not only target the issue of cartilage degeneration by producing the anti-inflammatory IL-10. LDG-R10C production will also be able to combat the production of proinflammatory cytokines that are produced by the patient's autoimmune system. The LDG-R10C has the capabilities to provide solutions to the current one sided treatment of RA focused on the reduction of inflammation. Also, the issue of replacing the degenerated cartilage will be addressed, in turn that will alleviate the aspects of chronic pain. As

reviewed above, the present treatments for rheumatoid arthritis only addresses either the cartilage degeneration issue or the proinflammatory cytokines issue, the LDG-R10C could do both and in doing so could effectively increase the percentage of patients with rheumatoid arthritis ability to go into remission.

Proposal

To test this idea, we will use a rat model with induced RA and measure inflammation, pain, and regeneration in four groups of animals (those without RA induced, those with RA induced but not treated, those with RA and treated with stem cell therapy and those with RA and treated with combinatorial stem cell/CRISPR technology.

RA model of pain: Rats and mice are the most commonly used animal models for inflammatory arthritic experimentation. Two types of rodent models are strains of mice that spontaneously develop arthritis and other strains are manipulated via arthritogenic stimuli to induce them into being models. These models are able to simulate features of disease progression, disease onset and duration, which contributes to their use in pain studies as well. Two strains of rodents that spontaneously develop arthritis R/KBxN models (joint erosion similar to human RA joints) and TNF transgenic mouse models (expresses human proinflammatory TNF gene). These mice models are heavily focused on because they show full penetrance and replicable disease progression (Fischer et al., 2017). Inducible arthritis models can be manipulated in various ways in order to initiate disease onset. Some are injected intradermally with an immune stimulating antigen, the mice reaches maximal severity within one week, then goes into remission. Immunizing mice with type 2 collagen triggers severe polyarthritis that will persist into a chronic state within two weeks also, producing autoimmune capabilities. Serum injections from other models cause a lacking antibodies triggering acute disease. A single dose of pristane can be injected subcutaneously causing acute severe inflammation resulting in chronic relapsing in the mice (Asquith et al., 2009).

Measuring pain in Rats: Pain will be measured using the von Frey hair test, where the animal's tail or hind paw is stimulated with a series of calibrated monofilaments that correlate to different levels of force. If an animal is experiencing pain, they will respond to a lower amount of force. The rats micromovements in response to pain will be captured using high speed videography and measured with statistic modeling that can determine the level of pain a particular rat is in (Abdus-Saboor et al., 2019).

Measuring inflammation in RA Rats: Inflammation will be measured using immunohistochemistry and blood samples. Immunohistochemistry is the detection of antigens in tissue sections/ slices of sacrificed models to initiate a reaction between monoclonal and polyclonal antibodies in order to measure for the presence of increased inflammation (NIH, 2020). Immunohistochemical analysis of synovial tissue specimens from the models will analyzed. Blood samples will be taken from the models in order to test for rheumatoid factor (RF) (antibody for RA), c-reactive protein (CRP) (joint inflammation) and anti–citrullinated protein antibody (ACPA) (joint deterioration) these test are primarily used to determine a diagnosis for rheumatoid arthritis. In order to be considered a positive diagnosis for RA the RF would have to be 14 IU/mL or above, CRP 10 mg/L or above and >60 EU/mL a strong positive ACPA (Mouterde et al., 2019).

Measuring regeneration in RA Rats: The proliferation of chondrocytes and/ or generated cartilage will be measured using bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU), which is a thymidine analog that can be integrated into DNA. Integration occurs during S phase of DNA replication to identify cells *in vivo* (in live animal) and *in vitro* (cell lines/ cultures) in order to specifically mark the cells as they replicate (Abcam.com, 2020).

Cartilage thickness will also be measured using MRI imaging to visually compare the thickness of cartilage before and after treatments (Koo et al., 2005).

Experimental Groups: Using a power analysis to determine the amount for each group, there will be a total of 160 rats that will be equally divided into four groups of 40. Group 1: induce RA treat with stem cells without CRISPR technology, Group 2: no RA and no treatment, Group 3: induce RA and inject with saline cells and Group 4: induce RA and inject with stem cells with CRISPR engineered cells (LDG-R10C).

Anticipated results: I anticipate that Group 2 will show the least amount of joint inflammation and deterioration, a negative rheumatoid factor, C-reactive protein and ACPA levels, as well as a no change in miotic index percentage and the greatest amount of cartilage thickness as it is the control. When in reference to the other groups I anticipate that Group 4 the group treated with stem cells and CRISPR technology will display the greatest amount of improvement especially in increased numbers when looking at the cartilage thickness and mitotic index after treatment. I expect the mitotic index will show an increased rate of proliferation proving that the chondrocytes were able to effectively replicate and in doing so the cartilage was able to increase in thickness. I expect the joint inflammation and deterioration as well as the CRP, ACPA and rheumatoid factor antibodies present in the blood to have significantly decreased improving the state of the actual rheumatoid arthritis diagnosis implicating that Group 4 is going into remission. Lastly, I am anticipating that the amount of force needed via the von Frey hair test would increase due to the reduced sensitivity to pain implicating minimum reflex paw withdrawal, which will also correlate with rheumatoid arthritis going into remission. Furthermore, I expect Group 1 to show minimal improvement, if any being as though this group has been

induced with RA and is being treated with stem cells. There is a possibility that the stem cells may actually be able to replicate in the environment they are placed in without CRISPR technology, but the likelihood of that is minimal. I expect Group 3 will display the highest level of joint inflammation, joint deterioration, CRP level, ACPA level and rheumatoid factor antibodies in the blood. Group 3 will also have a higher sensitivity to the least amount of force needed in the von Frey hair test further exemplifying increased pain. There will not be an increase to the mitotic index percentage as no chondrocytes are being injected, this will also be exemplified in the thickness of cartilage continuing to decrease. These results are conclusive as Group 3 is not receiving any form of treatment it is only showing how rheumatoid arthritis will progress as time goes on acting as another control.

ANTICIPATED OUTCOME

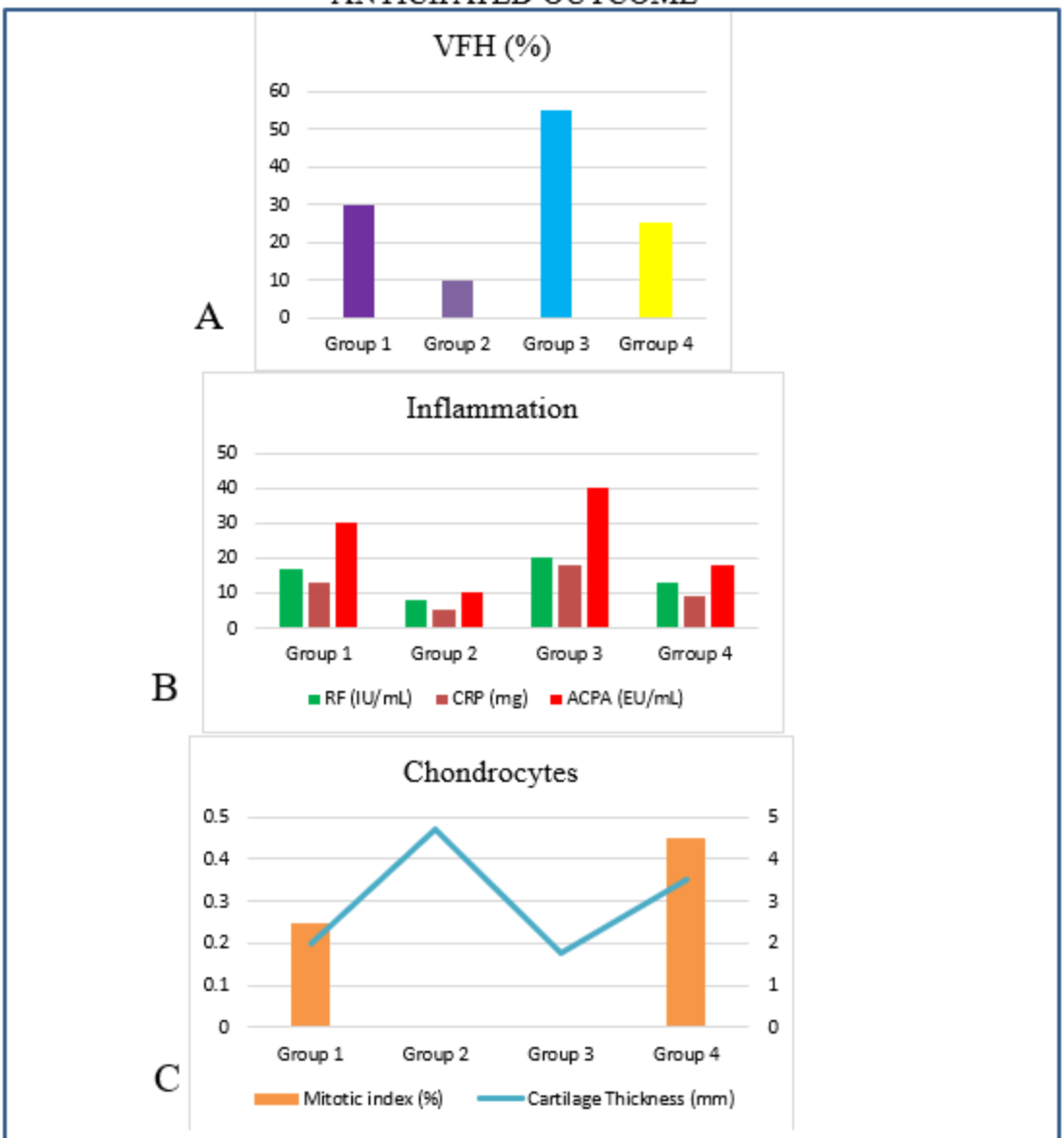

Figure 2: **A.** Group 3 displays the highest percentage of reaction in the VFH test as there is an increased sensitivity of pain & it is the negative control. Group 2 has the least % of movement, it is a positive control. Group 4 has a reduced % amount a reaction, RA is in remission and due to receiving treatment. Group 1 is being treated with stem cell as the RA is still able to progress. **B.** Group 3 shows the highest numbers of inflammation the RF is above 14, CRP is above 10 and ACPA is 60, which implicates severe RA. Group 4 shows a noticeable improvement when compared to Group 3 as it is exemplifying a patient in remission as a result of treatment. Group 2 is a positive control, so the inflammation is nonexistent. Group 1 is being treated with stem cell as the RA is still able to progress. **C.** Group 2 has no miotic index percentage and the largest amount of cartilage thickness as it is the positive control. Group 4 displays a miotic index percentage that is significantly high correlating with an increased cartilage thickness when compared to Group 2. Group 3 is a negative control, no mitotic index percentage, significant decreased cartilage thickness. Group 1 is being treated with stem cell as the RA is still able to progress.

Conclusion

Engineering COL2a1 gene to be expressed in the pattern of Interleukin10 gene by giving it COL2a1 gene's promoter at the endogenous locus, is effectively genetically changing the sequence of the DNA. By changing the sequence of DNA this could lead to great advancements in treatment not only in Rheumatoid arthritis, but could be an effective outline for approaches to other autoimmune diseases such as lupus, multiple sclerosis, diabetes, etc. At this point in time treatment for RA is greatly focused on joint inflammation and as previously reviewed above these can have detrimental side effects that result in other crucial diseases being able to form in the body. By approaching treatment from a holistic approach and targeting both joint inflammation and cartilage deterioration it leaves room for the physician not to have to choose. The patient will have a chance at forming a new normal that is not debilitating to their psyche or their way of life. The patient may not be as agile or fast as they used to be, but they won't be in the current broken depleted state they are today. As previously stated above 40% of the patients become disabled ten years after receiving the RA diagnosis. The molecular experimentation I am proposing gives an opportunity of hope for the patient to be able to push forward in an improve state. When RA is detected in an earlier stage the CRISPR engineered cells (LDG-R10C) can prevent the experience that is possible with this diagnosis.

Opioid addiction has led to a public health crisis. Abuse of these prescriptions have led to heavy addiction that has not been seen in years. By reducing joint inflammation, this in turn will reduce pain sensitivity as it refers to chronic pain that is caused by the neuroinflammation itself. By relieving one issue, the positive offset is that it reduces another issue.

Currently the physician's approach to cartilage deterioration is Hyaluronic acid injections and surgery. The Hyaluronic acid injections lubricate eroded joints, efficacy varies, and insurance providers allow them 2-3 times a year. I believe the patient will have access to this treatment, because it is in an improved form of injection that has an anticipated higher efficacy with minimal variability. I believe that this can be covered by insurance though at what level in which the insurance will cover it can't be determined at this point in time. There are foundations, Grant funding and clinical trials that provide patients that cannot afford treatment with the opportunity. Each of these variables gives the patient a chance to receive full treatment without paying the cost.

This experiment is not just limited to male models, as it includes female models as well. Past experiments have proven to show that the body's ability to improve or respond to treatments may differ when it refers to the different sexes. This treatment could have multiple positive benefits for society, such as alleviation of the opioid epidemic, improvement in the quality of life for hundreds of thousands of Americans and savings from reduced healthcare costs. As this is a theoretical proposal/ experiment and I'm providing anticipated results there will be limitations. Such limitations are the possibility that there can be too much IL10 present in the joint, there will not be a significant amount of proliferation of cells in order to increase cartilage thickness, etc. The only way to determine if the experimentation proposed here will make any significant advancement in the field of rheumatology and other autoimmune disease fields are to perform the experiment. Although there are some limitations the future directions could prove to be insurmountable. My proposal can effectively change treatments across many different

specialties in medicine, if not it will at least push the ideas in the possibilities in way of treating in the field forward.

References

Abcam.com [Internet]. (2020). BrdU Staining protocol and BrdU assay protocol. *Abcam plc*. https://www.abcam.com/protocols/brdu-staining-protocol

Abdus-Saboor, I., Fried, N. T., Lay, M., Burdge, J., Swanson, K., Fischer, R., Jones, J., Dong, P., Cai, W., Guo, X., Tao, Y. X., Bethea, J., Ma, M., Dong, X., Ding, L., & Luo, W. (2019). Development of a Mouse Pain Scale Using Sub-second Behavioral Mapping and Statistical Modeling. *Cell reports*, Vol. 28(6): 1623–1634.e4. DOI: https://doi.org/10.1016/j.celrep.2019.07.017

Adkar, S. S., Brunger, J. M., Willard, V. P., Wu, C. L., Gersbach, C. A., & Guilak, F. (2017). Genome Engineering for Personalized Arthritis Therapeutics. *Trends In Molecular Medicine*, Vol. 23(10): 917–931. DOI: https://doi.org/10.1016/j.molmed.2017.08.002

Asquith, D. L., Miller, A. M., McInnes, I. B., & Liew, F. Y. (2009). Animal models of rheumatoid arthritis. *European journal of immunology*, Vol. 39(8): 2040–2044. https://doi.org/10.1002/eji.200939578

Barbour KE, Helmick CG, Boring MA, Brady TJ. (2017). Vital signs: prevalence of doctor-diagnosed arthritis and arthritis-attributable activity limitation - United States, 2013—2015. *Morb Mortal Weekly Rep*. Vol. 66: 246-253. DOI: http://dx.doi.org/10.15585/mmwr.mm6609e1

Benjamin M, Evans EJ. (1990). Fibrocartilage. *PubMed Central: Research Review* Vol. 171: 1-15

Brunger, J. M., Zutshi, A., Willard, V. P., Gersbach, C. A., & Guilak, F. (2017). Genome Engineering of Stem Cells for Autonomously Regulated, Closed-Loop Delivery of Biologic Drugs. *Stem cell reports*, Vol. 8(5): 1202–1213. DOI: https://doi.org/10.1016/j.stemcr.2017.03.022

Center for Disease Control and Prevention (CDC). (2018). Arthritis- Related Statistics. *Center for Disease Control and Prevention*. https://www.cdc.gov/arthritis/data_statistics/arthritis-related-stats.htm Access on April 29th, 2020.

Chakravarthy, K, et al. (2017). Stem Cell Therapy for Chronic Pain Management: Review of Uses, Advances, and Adverse Effects. *Pain Physician.* Vol. 20: 293-305

Craft AM, Ahmed N, Rockel JS, Baht GS, Alman BA, Kandel RA, Grigoriadis AE, Keller GM. (2013). Specification of chondrocytes and cartilage tissues from embryonic stem cells. *National Center for Biotechnology Information: PubMed*. Vol. 140(12): 2597-2610

Daley GQ. (2015). Stem cells and the evolving notion of cellular identity. *Philosophical Transactions of Royalty Society London B Biological Science*. Vol. 370(1680):20140376. DOI:10.1098/rstb.2014.0376

DiSabato, D. J., Quan, N., & Godbout, J. P. (2016). Neuroinflammation: the devil is in the details. *Journal of neurochemistry*, Vol. 139 Suppl 2(Suppl 2): 136–153. DOI: https://doi.org/10.1111/jnc.13607

Donnally III CJ, Hanna A, Varacallo M. Lumbar Degenerative Disk Disease. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; April 13, 2020 Available from: https://www.ncbi.nlm.nih.gov/books/NBK448134/

Doudna, JA et al., (2014). The new frontier of genome engineering with CRISPR-Cas9. Science. Vol. 346: 6213. DOI: 10.1126/science.1258096

Farhang, N., Brunger, J. M., Stover, J. D., Thakore, P. I., Lawrence, B., Guilak, F., Gersbach, C. A., Setton, L. A., & Bowles, R. D. (2017). CRISPR-Based Epigenome Editing of Cytokine Receptors for the Promotion of Cell Survival and Tissue Deposition in Inflammatory Environments. *Tissue engineering. Part A*. Vol. 23(15-16): 738–749. DOI: https://doi.org/10.1089/ten.TEA.2016.0441

Fischer, B. D., Adeyemo, A., O'Leary, M. E., & Bottaro, A. (2017). Animal models of rheumatoid pain: experimental systems and insights. *Arthritis research & therapy*. Vol. 19(1): 146. DOI: https://doi.org/10.1186/s13075-017-1361-6

Fuggle NR, Howe FA, Allen RL and Sofat N. (2014). New insights into the impact of neuro-inflammation in rheumatoid arthritis. *Front. Neurosci*. Vol. 8:357. DOI: 10.3389/fnins.2014.00357

Goldring M. B. (2012). Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Therapeutic advances in musculoskeletal disease*, Vol. 4(4): 269–285. https://doi.org/10.1177/1759720X12448454

Goverdhana, S., Puntel, M., Xiong, W., Zirger, J. M., Barcia, C., Curtin, J. F., Soffer, E. B., Mondkar, S., King, G. D., Hu, J., Sciascia, S. A., Candolfi, M., Greengold, D. S., Lowenstein, P. R., & Castro, M. G. (2005). Regulatable gene expression systems for gene therapy applications: progress and future challenges. *Molecular therapy : the journal of the American Society of Gene Therapy*, Vol. 12(2): 189–211. DOI: https://doi.org/10.1016/j.ymthe.2005.03.022

Han, Y. H., Kim, K. H., Abdi, S., & Kim, T. K. (2019). Stem cell therapy in pain medicine. *The Korean journal of pain*, Vol. 32(4): 245–255. DOI: https://doi.org/10.3344/kjp.2019.32.4.245

Heidari B. (2011). Rheumatoid Arthritis: Early diagnosis and treatment outcomes. *Caspian journal of internal medicine*, Vol. 2(1): 161–170.

Hissnauer, TN et al. (2010). Identification of molecular markers for articular cartilage. *Osteoarthritis and Cartilage*. Vol. 18(12): 1630-1638.

InformedHealth.org [Internet].(2018). What is an inflammation?. *Cologne, Germany: Institute for Quality and Efficiency in Health Care (IQWiG)*. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279298/

Ivankovic, M et al. (2019). Model systems for regeneration: planarians. *The Company of Biologists Ltd| Development*. Vol. 146. DOI: 10.1242/dev.167684

Iyer, S. S., & Cheng, G. (2012). Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Critical reviews in immunology*, Vol. 32(1): 23–63. DOI: https://doi.org/10.1615/critrevimmunol.v32.i1.30

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. Vol. 337(6096): 816–821. DOI: https://doi.org/10.1126/science.1225829

Kim, J. H., Park, J. S., & Park, D. (2018). Anti-allodynic effect of interleukin 10 in a mouse model of complex regional pain syndrome through reduction of NK1 receptor expression of microglia in the spinal cord. *Journal of pain research*, Vol. 11: 1729–1741. https://doi.org/10.2147/JPR.S166624

Koo, S., Gold, G. E., & Andriacchi, T. P. (2005). Considerations in measuring cartilage thickness using MRI: factors influencing reproducibility and accuracy. *Osteoarthritis and cartilage*, Vol. 13(9): 782–789. DOI: https://doi.org/10.1016/j.joca.2005.04.013

McInnes, I. B., & Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *New England Journal of Medicine*. Vol. 365(23): 2205-2219.

McVeigh, C. M., & Cairns, A. P. (2006). Diagnosis and management of ankylosing spondylitis. *BMJ (Clinical research ed.)*. Vol. 333(7568): 581–585. DOI: https://doi.org/10.1136/bmj.38954.689583.DE

MedlinePlus[Internet]. (2015). Cartilage Disorders. National Institute of Health: US National Library of Medicine. Available from: https://www.nlm.nih.gov/medlineplus/cartilagedisorders.html

Milligan, E. D., Sloane, E. M., Langer, S. J., Cruz, P. E., Chacur, M., Spataro, L., Wieseler-Frank, J., Hammack, S. E., Maier, S. F., Flotte, T. R., Forsayeth, J. R., Leinwand, L. A., Chavez, R., & Watkins, L. R. (2005). Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. *Molecular pain*. Vol. 1: 9. https://doi.org/10.1186/1744-8069-1-9

Mohd, I et al. (2018). Implantation of hyaluronic acid hydrogel prevents the pain phenotype in a rat model of intervertebral disc injury. *Science Advances.* Vol. 4(4). DOI: 10.1126/sciadv.aaq0597

Mouterde, G., Rincheval, N., Lukas, C., Daien, C., Saraux, A., Dieudé, P., Morel, J., & Combe, B. (2019). Outcome of patients with early arthritis without rheumatoid factor and ACPA and predictors of rheumatoid arthritis in the ESPOIR cohort. *Arthritis research & therapy*, Vol. 21(1): 140. DOI: https://doi.org/10.1186/s13075-019-1909-8

Mummery, C et al. (2014). Stem Cells: Scientific Facts and Fiction. *Academic Press*. 2 edition 54-100

National Institutes of Health (NIH): National Cancer Institute. (2020). Immunohistochemistry. *National Institutes of Health*. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/immunohistochemistry Access on April 30, 2020.

Pauklin S, Vallier L. (2014). The cell-cycle state of stem cells determines cell fate propensity. *Cell*. Vol. 155(1): 135-147. DOI:10.1016/j.cell.2013.08.031

Plunkett, JA et al. (2001). Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat. *Experimental Neurology*. Vol. 168:1 144-154

Roy K, Kanwar R, Kanwar J. (2015). Molecular targets in arthritis and recent trends in nanotherapy. *Int J Nanomedicine*. Vol.10(1):5407-5420 DOI: https://doi.org/10.2147/IJN.S89156

Schrepf, A., Kaplan, C.M., Ichesco, E. et al. (2018). A multi-modal MRI study of the central response to inflammation in rheumatoid arthritis. *Nature Communication*. Vol. 9: 2243. DOI: https://doi.org/10.1038/s41467-018-04648-0

Suchorska, W. M., Augustyniak, E., Richter, M., & Trzeciak, T. (2017). Gene expression profile in human induced pluripotent stem cells: Chondrogenic differentiation in vitro, part A. *Molecular medicine reports*, Vol. 15(5): 2387–2401. https://doi.org/10.3892/mmr.2017.6334

Tendulkar, G., Chen, T., Ehnert, S., Kaps, H., & Nüssler, A.,K. (2019). Intervertebral disc nucleus repair: Hype or hope?. *International Journal of Molecular Sciences*. Vol. 20(15). DOI: http://dx.doi.org.proxy.libraries.rutgers.edu/10.3390/ijms20153622

Towns, M & Bathon MD, J (2010). Inhibition of Inteleukin-1 as a Strategy for the treatment of rheumatoid arthritis. *John Hopkins Medicine: Rheumatology*. https://www.hopkinsarthritis.org/arthritis-info/rheumatoid-arthritis/ra-treatment/interleukin-1-inhibition/

Uniprot.org [Internet]. UniProtKB – P22301 (IL 10_HUMAN). *UniProt*. Available from: https://www.uniprot.org/uniprot/P22301

Van der Kraan PM, Buma P, van Kuppevelt T, van Den Berg WB. (2002). Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. *Osteoarthritis and Cartilage*. Vol. 10: 631-637

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International anesthesiology clinics*. Vol. 45(2): 27–37. DOI: https://doi.org/10.1097/AIA.0b013e318034194e