Abstract

Title of Document: DEVELOPING AN EXTRACELLULAR VESICLE BASED TREATMENT FOR OSTEOARTHRITIS

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Osteoarthritis (OA) is a disease characterized by the degradation of articular cartilage. Extracellular vesicles (EVs) are cargo-filled bodies that mediate intercellular communication and are influential in OA pathogenesis. This study utilized parallel methodologies to investigate whether EV signaling can be manipulated to combat OA. The first approach aimed to identify cells lines that produce EVs with therapeutic activity against OA, while the second introduced miRNA in EVs to induce cartilage regeneration. EVs derived from synovial fibroblasts (SFBs) induced further inflammation. Moreover, miRNA did not impact MMP-13 production. While SFB-EVs were pro-inflammatory, increasing the amount of MMP-13 present, human bone marrow-derived mesenchymal stem cell (BM-hMSC) EVs did not stimulate a change in MMP-13 production. Future studies should further characterize these results to maximize therapeutic impact.

DEVELOPING AN EXTRACELLULAR VESICLE BASED TREATMENT FOR OSTEOARTHRITIS

By

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List of Abbreviations

ACAN: Aggrecan

ADAMTS5: Aggrecanase

ACI: Autologous chondrocyte implantation

BCA: Bicinchoninic acid

BM-hMSC: Human bone marrow-derived mesenchymal stem cell

BMP: Bone morphogenetic protein

CDC: Cardiosphere-derived cell

COL2A1: Collagen type 2

ECM: Extracellular matrix

EV: Extracellular vesicles

GAG: Glycosaminoglycan

hC: Human chondrocyte

HFLS: Human fibroblast-like synoviocytes

hOA-C: Osteoarthritic human chondrocyte

IGF-I: Insulin-like growth factor

IL-1β: Interleukin-1β

miRNA: microRNA

MMP: Matrix metalloproteinases

mRNA: Messenger RNA

NSAID: Nonsteroidal anti-inflammatory drugs

OA: Osteoarthritis

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RT-PCR: Real time PCR

SFB: Human Synovial Fibroblast

TGF-β: Transforming growth factor

TKA: Total knee arthroplasty

TNF-α: Tumor necrosis factor

Chapter 1: Introduction

Individuals in the United States are living longer, healthier lives because of improvements made in healthcare and nutrition over the last 100 years. Associated with living longer, however, is an increased risk of developing degenerative diseases including osteoarthritis (OA). This, compounded with other factors such as obesity, diabetes, among others can contribute to the development of OA symptoms. OA results from the degradation of articular cartilage, which, in turn, leads to an increase in friction between bones and causes pain and joint immobility. More than thirty million adults in the United States currently suffer from OA, and the number of cases is projected to grow significantly as the population ages (Center for Disease Control and Prevention, 2018). Additionally, women are more likely to develop OA and often present with more severe forms of the disease due to a wide array of individual factors such as knee anatomy, kinematics, and hormonal influences, among others. (Hame & Alexander, 2013). There is currently no cure for OA, and the most common OA treatments address the symptoms of the disease instead of targeting the underlying causes. These treatments provide temporary pain relief, but do not halt the progression of the disease and force patients to settle for a brief respite from chronic pain (National Collaborating Centre for Chronic Conditions - Great Britain, 2008). As such, it is of vital importance to find a treatment that will prevent further deterioration and ultimately repair damage done by OA so that patients can return to living pain-free lives.

A promising new OA treatment involves using extracellular vesicles (EVs) as delivery vehicles for targeted therapies. EVs are vital components of intercellular communication within the body. They can transport a wide array of molecules, including

microRNA (miRNA) that can prevent the translation of specific **messenger RNA** (mRNA) sequences and downregulate gene expression. EVs also possess membrane proteins that allow them to target and deliver cargo to specific cells. An example of a cell that receives this cargo is a **chondrocyte**, which is a cell type that helps maintain articular cartilage (György, Hung, Breakefield, & Leonard, 2015). **Extracellular matrix** (ECM) is the main constituent of articular cartilage, and ECM maintenance is closely related to articular cartilage health. (Fox, Bedi, & Rodeo, 2009). In joints affected by OA, ECM is degraded, negatively affecting joint function and health. Promoting the expression of healthy ECM proteins by controlling the cargo delivered by EVs to chondrocytes could provide for articular cartilage regeneration.

According to Kato et al. (2014), **human synovial fibroblasts** (SFBs) and bone marrow-derived **mesenchymal stem cells** (BM-hMSCs) could be treated to release EVs containing cargo that would impact gene expression in chondrocytes and induce OA. Alternatively, isolating and loading EVs with therapeutic miRNA molecules via **sonication**, an established EV loading technique, prior to introducing them to chondrocytes could upregulate the production of ECM proteins or downregulate the production of degradative enzymes (Alvarez-Erviti et al., 2011). miRNA molecules downregulate these degradative proteins by interacting with specific mRNA sequences to hinder the translation of the target sequence (Ha & Kim, 2014). Ultimately, the goal of the study is to determine how EVs can be manipulated to promote the generation of ECM proteins and inhibit the production of degradative enzymes that break down articular cartilage, thus promoting cartilage tissue regeneration for the treatment of OA.

Research Questions and Hypotheses

Based upon the literature review, it is hypothesized that both cell source and miRNA cargo will play integral roles in determining the therapeutic potential of EVs. The study aims to develop an EV-based OA treatment that utilizes a practical and effective cell source as well as engineered miRNA cargo. Specifically, the proposed research questions and hypotheses are as follows:

Research Questions

1. How does cell source affect the therapeutic potential of EVs for OA treatment?
2. Can EVs be loaded with therapeutic miRNAs that amplify their therapeutic activity?

Hypotheses

1. Hypothesis: The cell source and miRNA content of EVs dictate their therapeutic potential, which can be measured by observing ECM protein generation and the progression of OA pathology in chondrocytes.
    a. SFBs and BM-hMSCs are potential cell sources that release therapeutic EVs because of their ability to influence chondrocyte bioactivity;
    b. miRNA-140 and miRNA-125b are known to play a role in cartilage regeneration, so they have potential as therapeutic cargo.
2. Null Hypothesis: EV cell source and miRNA cargo have no significant effect on OA pathology.

Additional Guiding Thoughts and Questions

1. To test the hypotheses, it must be possible to first induce the release of modified EVs from producer cells. How can we treat various cell types to release EVs that downregulate OA-associated proteins and upregulate normal ECM protein synthesis? Can we apply that treatment to cartilage tissue?
2. After the necessary release of EVs is accomplished, loading EVs with cargo that targets OA sequences is the next step. Regulation of gene expression through miRNA could target specific mRNA, allowing for a more consistent treatment approach. How does the direct loading of certain miRNA into EVs impact the downregulation of OA-associated proteins and ECM development?

Chapter 2: Literature Review

Osteoarthritis

Overview. OA induces articular cartilage degradation and results in increased friction between bones (Figure 1) (Lawrence et al., 2008). This friction gives rise to swelling and stiffness that may result in a loss of joint strength and induce chronic pain over time. Mature human articular cartilage cannot regenerate, and current treatments for OA are typically focused on minimizing pain instead of providing long-term solutions (Yanke & Chubinskaya, 2015).

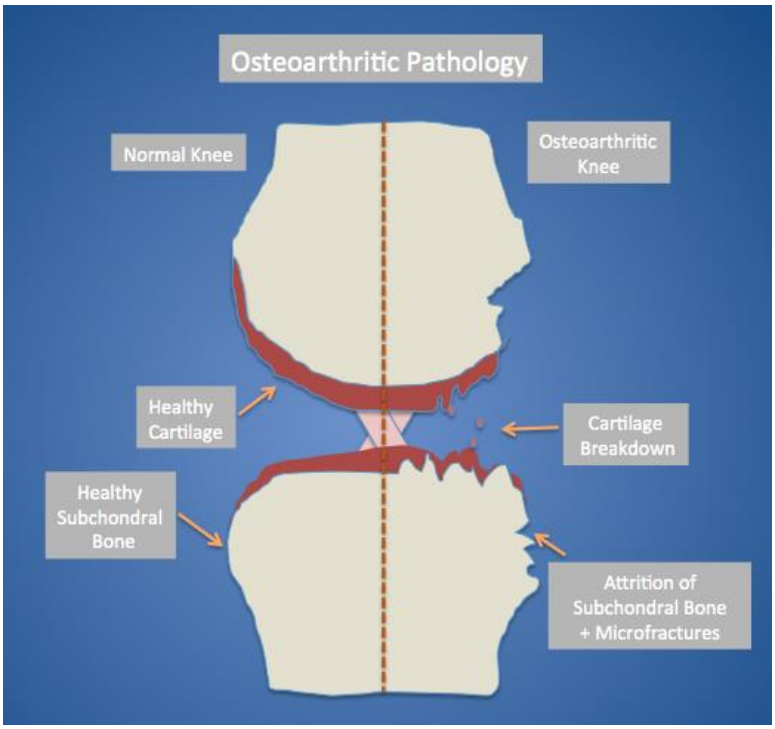

Figure 1: Osteoarthritic Pathology. Note the cartilage breakdown and accompanying mechanical stress visible in the OA knee on the right (*Adapted from Roman-Blas & Herrero Beaumont, 2013*)

Cell Biology. Articular cartilage gets its characteristic form and function from unique cells called chondrocytes, and there are many differences between normal chondrocytes and chondrocytes that have developed symptoms of OA (Lin, Willers, Xu,

& Zheng, 2006). Under normal circumstances, articular chondrocytes display little activity; the **cytokines** and **growth factors** that stimulate division during development are expressed at very low levels (Sandell & Aigner, 2001). These factors—transforming growth factor (TGF-β), bone morphogenetic protein (BMP), and insulin-like growth factor (IGF-I)—also promote the synthesis of ECM proteins such as **collagen** and **aggrecan** (Grunder et al., 2004). Likewise, cytokines that promote the expression of matrix metalloproteinases (MMPs), which degrade ECM proteins, are also expressed at low levels (Sandell & Aigner, 2001; Vu & Werb, 2000). These cytokines include interleukins (IL-1,17,18) and tumor necrosis factor (TNF-α).

However, in **osteoarthritic human chondrocytes** (hOA-Cs), both the anabolic factors that stimulate growth and the catabolic factors that trigger degradation are expressed at high levels. Thus, in the early stage of the disease, the biosynthesis phase, the degradative effects of OA are offset by the increase in ECM synthesis (Sandell & Aigner, 2001). Nonetheless, this dynamic situation is unsustainable; as the disease progresses, the chondrocytes' regenerative efforts are overwhelmed by the catabolic activity of the MMPs. Consequently, the degradative phase of OA is brought on by the abundance of fragmented matrix proteins present in the joint.

Certain types of matrix proteins upregulate the expression of MMPs when degraded (Jayasuriya et al., 2016). This positive feedback loop ensures that degradative enzyme production will always outpace anabolic activity in hOA-Cs (Loeser, Goldring, Scanzello, & Goldring, 2012). Stopping and reversing these destructive mechanisms at the molecular level are key components of current OA research, but researchers have struggled to find means by which to target and stimulate OA cells.

Current Therapies. Clinical treatments of OA are currently limited, and no cure exists (National Collaborating Centre for Chronic Conditions - Great Britain, 2008). The three major categories of treatment—lifestyle changes, medication, and surgery—fail to adequately address the biological causes of OA. Another form of treatment, cell therapy, seeks to treat OA on a cellular level, but studies have thus far reported limited effectiveness at treating OA and repairing joint damage.

***Lifestyle Changes*.** Lifestyle changes recommended by clinicians to patients with OA can include some or all of the following: weight loss, exercise, rest for the affected joint, and physical and occupational therapy (Clark, 2014). These measures can reduce a patient's pain and improve joint function but cannot cure the disease (Roddy & Doherty, 2006).

Medication. Medications for OA patients typically target two symptoms of the disease: pain and inflammation. Acetaminophen, a commonly used medication, is an effective painkiller but has no impact on the progression of OA or on inflammation (Towheed et. al., 2006). Nonsteroidal anti-inflammatory drugs (NSAIDs) can alleviate a patient's pain and reduce joint inflammation but do not treat OA's underlying causes (National Collaborating Centre for Chronic Conditions - Great Britain, 2008). Common examples of drugs in this category are ibuprofen and naproxen. Stronger versions of these drugs are available by prescription and can reduce pain and inflammation more effectively than over-the-counter options, but these drugs can cause a variety of side effects, including cardiovascular problems as well as liver and kidney damage (NHS, 2014).

Direct Injections. In more serious cases of OA, patients may receive more invasive treatments. Cortisone shots injected directly into the affected joint are one such option. However, these treatments provide only temporary pain relief and increased joint mobility (Kruse, 2008). If shots are administered too frequently, possible side effects can include osteonecrosis, nerve damage, and deterioration of the joint cartilage. For this reason, doctors typically limit patients to no more than four injections per year (National Collaborating Centre for Chronic Conditions - Great Britain, 2008). Another treatment option is the injection of hyaluronic acid, which mimics natural joint fluid (National Collaborating Centre for Chronic Conditions - Great Britain, 2008). While some studies have reported this method to be a safe and effective pain relief option, others have found that these shots produce marginal benefits when compared to a placebo treatment (Migliore & Granata, 2008; Lo, LaValley, McAlindon, & Felson, 2003). Regardless of pain relief effectiveness, hyaluronic acid-based treatments have no effect on the biological causes of OA.

Surgery. In many extreme cases of OA, surgical treatments may be recommended after noninvasive methods have been exhausted (Mayo Clinic, 2014). These treatment options are invasive and are accompanied by the risks associated with any major surgery. Furthermore, patients who undergo surgical treatments often require significant physical therapy and undergo lengthy rehabilitation periods. The most common surgical procedure for knee OA is total knee arthroplasty (TKA). TKA is a costly procedure that involves inserting metal alloys into the knee joint (Ivirico et al., 2017). Although innovations in TKA have led to the development of a minimally invasive procedure, the alloy replacement has the potential to fail, which would require patients to undergo follow-up

procedures to fix it (Escobar Ivirico et al., 2017; King et al., 2007). Patients can also expect a painful recovery following TKA, even if no replacement surgeries are necessary.

A more recently-developed surgical treatment known as microfracture surgery is an attempt to induce cartilage synthesis without any joint replacement (Yanke & Chubinskaya, 2015). During the microfracture procedure, surgeons drill small holes in the bone adjacent to damaged cartilage tissue. The blood and bone marrow released from these fractures create conditions conducive to cartilage synthesis (Ma, 2014). Compared to TKA, this treatment method is inexpensive and the operation simple. However, the number of long-term benefits associated with this procedure is limited; in most cases, it produces no new cartilage, and patients report little to no relief from pain (Yanke & Chubinskaya, 2015).

Another surgical method of cartilage regeneration, autologous chondrocyte implantation (ACI), involves two separate procedures (Yanke & Chubinskaya, 2015). The first procedure is a cartilage biopsy in which a patient's cartilage cells are sampled for a 3-5 week expansion in culture. In the second procedure, the cultured cells are implanted into the targeted joint and covered with a patch made of tissue from the periosteum, the thick tissue that covers the shin bone, to hold the implanted cartilage in place (Brigham and Women's Hospital, 2011). In previous studies, 75% of patients reported improvement at the 10-year mark. While this is a promising treatment for cartilage defects that could lead to OA, ACI will not reverse existing OA. Additionally, prior microfracture procedures may negatively affect the outcome of ACI (Minas, Von Keudell, Bryant, & Gomoll, 2014). While TKA, microfracture, and ACI are fairly effective, they are invasive, taxing on the patient, and expensive.

Cell Therapy. Another approach that has been examined to treat OA is cell therapy. Cell therapy is the process of injecting live cells into a damaged area of tissue to reverse and repair the damage. Cell therapy can be used in conjunction with surgical treatment or alone. Several studies have sought to treat OA via cell therapy alone. These studies have resulted in mixed outcomes. Most commonly, bone marrow-derived mesenchymal stem cells (BM-hMSCs) are injected into the knees of OA patients. After cell injections, OA progression is generally slowed but not halted (Murphy, Fink, Hunziker, & Barry, 2003). BM-hMSC injections have been shown to reduce cartilage and bone destruction, and some studies have reported regeneration of subchondral bone defects caused by OA (Murphy et al., 2003; Wakitani et al., 1994). Additionally, cartilage-like tissue growth has been observed at the site of cell injections, strengthening the damaged joint. However, this tissue lacks the stiffness and mechanical strength of natural, healthy cartilage (Wakitani et al., 1994; Wakitani et al., 2002; Murphy et al., 2003).

Additional studies performed with similar methods of BM-hMSC cell therapy have examined the different effects. Rather than focusing on internal results such as cartilage degradation and tissue regeneration, these studies used clinical measurements of knee health such as joint mobility and stiffness, patient pain, and ambulatory ability to quantify treatment success. Though BM-hMSC injections into the knees of OA patients have yet to cause large improvements in patient outcomes, the results seem promising and future development might allow this technology to become a more effective treatment method (Davatchi, Abdollahi, Mohyeddin, Shahram, & Nikbin, 2011; Koh & Choi, 2012).

However, OA presents a host of challenges that cell therapy treatments fail to address. The first challenge is the fact that OA usually presents in patients of advanced age and/or patients who are obese; these factors have been shown to influence autologous stem cell quality and viability (Diekman & Guilak, 2013). Cells extracted from these patients may not be healthy enough to implant or may exhibit a reduced capacity for differentiation and would thus do little to combat OA pathology (Diekman & Guilak, 2013). Using cells from younger, healthier donors may require patients to take immunosuppressive drugs, which are associated with other health complications (Diekman & Guilak, 2013).

The second obstacle preventing cell therapy from becoming a viable treatment method is the fact that OA develops in an inflammatory environment (Diekman & Guilak, 2013). Injecting cells that can modify their behavior in accordance with environmental cues could serve to make the problem worse if the cells become stimulated by inflammatory EVs (Diekman & Guilak, 2013). These stimulated cells could further complicate a patient's condition by augmenting the production of degradative enzymes.

Extracellular Vesicles

Overview. EVs are cell-derived lipid vesicles crucial to the transfer of information between cells (Lamichhane et al., 2014). Studies have shown that EVs can store and transport a wide array of molecules and interact with many different types of tissues in the body (van der Pol, Böing, Harrison, Sturk, & Nieuwland, 2012). EVs are formed when specific regions of the plasma membrane encapsulate target molecules and detach from the parent cell (Raposo & Stoorvogel, 2013). The compounds that they contain are known to include proteins, nucleic acids, other organic molecules, and

cellular waste, which all have the potential to affect activity in recipient cells (van der Pol et al., 2012). The biochemical properties of EVs allow them to identify target cells (via clathrin mediated endocytosis, macropinocytosis, etc.), enter them, and deliver chemical payloads that modulate intracellular machinery (György et al., 2015; Mulcahey et al., 2014).

When delivering its contents, an EV first must attach to receptors on the plasma membrane of a target cell. It is then taken up via endocytosis, a process in which the plasma membrane fuses with the EV and draws it into the cytoplasm (Figure 2). After the EV is internalized, the membrane breaks down, and the materials inside are released and transferred to different sites in the cell. These materials have the ability to influence a wide range of intracellular biochemical pathways (Raposo & Stoorvogel, 2013).

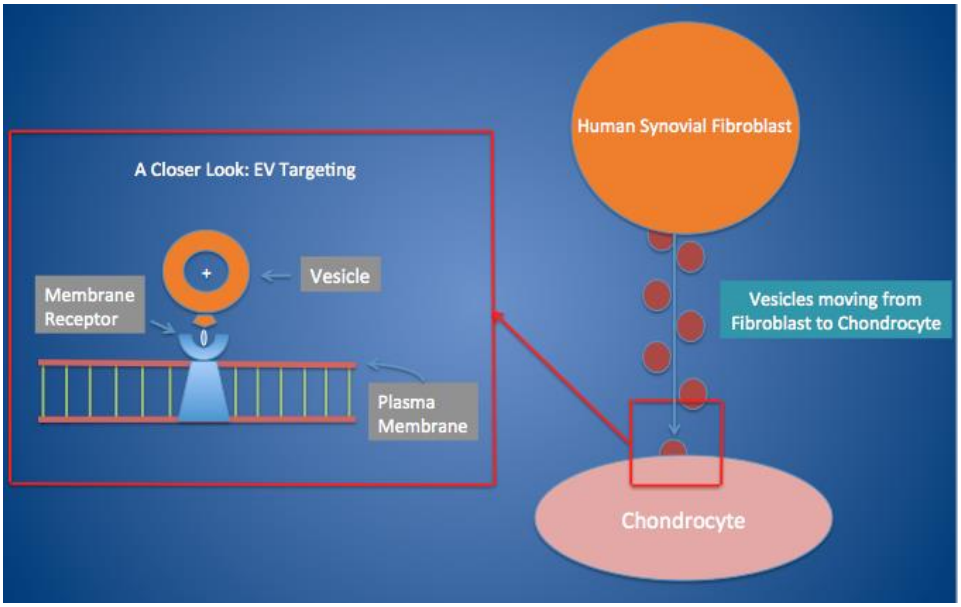

Figure 2: The mechanism of endocytosis. The EV membrane interacts with receptors on surface of the cell, fuses with the cell membrane, and moves into the cytoplasm.

Functionality and Potential Applications. The targeting specificity and carrying capacity of EVs make them a promising subject for medical research; however, scientists

have encountered several problems when trying to classify, isolate, and understand how these EVs form and act. In terms of classification, EVs have been grouped into four categories: exosomes, microvesicles, microparticles, and apoptotic vesicles (van der Pol et al., 2012). Classification efforts have been hampered by overlaps in the sizes of these EVs, making isolation and analysis difficult (van der Pol et al., 2012). Cell culture conditions and isolation methods can alter the contents, purity, and activity of the EVs (Andaloussi, Mäger, Breakefield, & Wood, 2013). Isolation methods can also damage EVs, affecting their structures and functions. Fortunately, researchers are finding ways to surmount these barriers and create new knowledge. In fact, researchers have been able to isolate, investigate, and shed light on the activity of many specific EVs by using differential centrifugation, electron microscopy, and tests on cultured cells.

EVs are widely utilized by the body; they have been found to play roles in immunosuppression, antigen presentation, inflammation, tumor growth, **angiogenesis**, metastasis, genetics, waste management, and cell adhesion (van der Pol et al., 2012). When obtained from a patient's own cells, EVs have the potential to be employed in personalized medicine to limit unfavorable immune responses associated with allogenic transplants (Lamichhane et al., 2014). This diverse range of bioactivity makes EVs ideal vessels for potential use in targeted therapeutics like improved OA treatments. In addition, the stability of the EVs in the body make it an ideal therapeutic delivery mechanism (Burke et. al., 2016). EVs have the capability to provide an alternative to current pain alleviation methods and, with further research, could be used for novel OA treatments with increased efficacy and fewer side effects than conventional methods.

Assessing EV Bioactivity. Several studies have shown different methods to quantify EV activity. A study by Ibrahim, Cheng, and Marbán in 2014 showed that vesicles derived from cardiosphere-derived cells (CDCs) encouraged formation of new blood vessels, through the process known as angiogenesis, and regeneration of damaged heart tissue. Angiogenesis is extremely important to the development of healthy tissue because the formation of new blood vessels allows nutrients and signaling molecules from all over the body to interact with, promote, and nourish new cells (Li & Li, 2003). In the study, CDC-EVs were isolated and plated on an angiogenesis assay to demonstrate an increase in angiogenesis in response to the addition of these EVs. Overall, this study demonstrates an example of utilizing EVs as an effective treatment for damaged tissue (Ibrahim et al., 2014). A 2014 study from Bian et al. showed that BM-hMSC-derived EVs on can promote proliferation and angiogenesis in human umbilical vein endothelial cells. Shabbir, Cox, Rodriguez-Menocal, Salgado, and Badiavas (2015) found that EV activity in damaged cell populations promotes fibroblast migration and angiogenesis. Fibroblast cells play an integral role in the synthesis and maintenance of the ECM that gives tissues their characteristic structure, strength, and functionality (Provenzano, Alejandro-Osorio, Valhmu, Jensen, & Vanderby, 2015). Since EV activity directly influences these physiological processes, it is possible that an EV-based therapy could be developed to reverse tissue damage and degradation.

Effect of EV Cargo. In order to boost the therapeutic activity of EVs, specific cargo can be loaded into the EV membrane. Proteins, nucleic acids, lipids and miRNA can all be added as cargo into EVs. However, miRNA in particular is appealing due to its ability to target specific RNA sequences. miRNA is also capable of inhibiting entire

biological pathways (Sutaria et al., 2017). In the study by Ibrahim, Cheng, and Marbán (2014), miRNA-146a, which is naturally present in cardiosphere-derived cell (CDC)-EVs, was found to increase the thickness of cardiac tissue. In order to solidify this conclusion, miR-146a rich CDC-EVs were compared to miRNA-146a depleted CDC-EVs (Ibrahim, Cheng, & Martin, 2014). miRNA-146a enriched CDC-EVs had higher levels of angiogenesis than miR-146a depleted EVs (Ibrahim, Cheng, & Martin, 2014). In another study by Fang et al., miRNA targeted the phosphoinositide-3-kinase/Akt pathway, which is known to trigger hepatocellular carcinoma tumor formation. It was determined that miRNA-7 downregulated this pathway, as tumor volume and proliferation of hepatocellular carcinoma cells were significantly decreased post-treatment (Fang et al., 2012).

There are several relevant miRNAs that have been linked to cartilage homeostasis. Upregulation of miRNA-125b has been linked to the downregulation of several different degradative enzymes, like aggrecanase (ADAMTS5), collagenase (MMP-13), and synoviolin (SYVN1) (Matsukawa et al., 2013; Xu et al., 2012; Ge, Li, & Yin, 2017). miRNA-140 has also been found to downregulate ADAMTS5 and MMP-13, as well as upregulate aggrecan (ACAN) and collagen type 2 (COL2A1) (Miyaki et al., 2009; Liang et al., 2012; Yang et al., 2011). It is expected that miRNA-125b and miRNA-140 would slow or even prevent the destruction of the ECM, and miRNA-140 would also increase the production of new, healthy ECM proteins. EVs loaded with miRNA-125b and miRNA-140 could have regenerative effects on joints affected by OA.

Regenerative Medicine. The therapeutic application of EVs is a relatively new idea with potential avenues including treatment of disease and the healing of tissue

injuries (György et al., 2015). Because of EVs' integral role in intercellular communication and their ability to interact with a wide variety of cell types, EVs offer a way to combat the cause of diseases at a cellular level. EVs are multi-functional, as they have the capacity to serve different purposes depending on the condition of the patient. EVs can carry ribonucleic acid (RNA), proteins, or drugs, all of which can manipulate recipient cells (György et al., 2015).

In a 2014 study, Kato et al. concluded that exosomes play a role in the degradation of cartilage caused by OA. In the study, SFBs from articular cartilage were first isolated and treated with Interleukin-1β (IL-1β); the results showed that this treatment promoted the degradation of collagen matrix and led cells to exhibit OA behavior. Kato et al. (2014) analyzed gene expression with real-time PCR (RT-PCR) and monitored effects on the cartilage with a **glycosaminoglycan** (GAG) release assay, and further analytical testing revealed the upregulation of proteins that participate in degradation of cartilage and the downregulation of proteins responsible for building cartilage. A major takeaway from the Kato et al. (2014) study is that extracellular vesicles from inflamed human SFB cells can modulate chondrocyte phenotype (in this case negatively). This suggests that healthy EVs may be able to elicit the opposite effect and induce chondrocyte production of ECM proteins and suppress the production of degradative enzymes.

This *in vitro* study is a logical step for scientists studying EVs, as recent developments in EV research have led to applications of EVs in regenerative medicine. As Kato et al. (2014) demonstrated, EVs can be applied to different tissues in the body to treat a range of diseases. A 2015 study showed that MSC-derived EVs improve post-

stroke recovery by promoting brain tissue regeneration and reducing inflammation through angiogenesis (Doeppner et al., 2015). As another example, a study focused on communication among cartilage tissue cell receptors showed that targeted delivery of insulin growth factors to OA cartilage tissue, independent of EVs, promoted chondrocyte response and ECM cartilage synthesis (Tokunou et al., 2008). Though delivery of growth factors can be used to promote responses in cartilage tissue, EVs have the potential to induce a wider array of cellular responses, which could lead to a more lasting, effective change.

In a separate study, researchers investigated an EV-based therapeutic approach for osteochondral defects in the femurs of rats. Isolated EVs from human embryonic mesenchymal stem cell culture media were injected weekly into the damaged joint area. After 12 weeks, the researchers observed complete healing of the originally damaged area (Zhang, et. al., 2016). The methods covered in these studies suggest EVs could be used to combat degenerative diseases like OA. Furthermore, applying previously established techniques to a study of articular cartilage regeneration will be beneficial to the millions who lack sufficient treatment for their OA. To conduct further research on EVs and OA, similar techniques to those used to isolate EVs and analyze gene expression will be necessary.

Conclusion

OA affects approximately 30 million adults in the United States population, and as that population continues to age, the disease will become more prevalent ("Osteoarthritis Fact Sheet", 2017). Lacking effective treatment options, patients and clinicians are forced to manage chronic pain through invasive procedures. Ultimately

treating the underlying pathology of OA in a noninvasive manner would be more effective than current symptom-focused procedures. Currently, cell therapy is the only known method that seeks to treat OA pathology on a microbiological level, but as discussed, this method is complicated by the inflammatory environment of an OA joint. The application of EV technology has the potential to address these concerns because it harnesses the benefits of cell therapy while allowing researchers to conduct all producer cell manipulations in strictly controlled *in vitro* environments. The static nature of EVs preclude the possibility of cell behavior being hijacked by the inflammatory signals present within the OA joint and allow for precise control over the delivered treatment dosage. Therefore, EV technology could potentially be used to create a therapy for OA that would stop the mechanisms that lead to the degradation of ECM and repair tissue without forcing the patient to undergo expensive, taxing procedures.

Chapter 3: Methodology

The goal of this research is to investigate the relationship between EV mediated intercellular communication and development or amelioration of an osteoarthritic phenotype in articular chondrocytes. OA phenotype is characterized by increased production and excretion of degradative enzymes relative to healthy chondrocytes, which are largely quiescent (Sandell & Aigner, 2001). An optimal treatment would not only prevent cartilage degradation through halting the increased production of degradative enzymes, but would also result in regenerative behavior through stimulating production of ECM proteins. The uptake of EVs from SFBs by human chondrocytes (hCs) has been shown to stimulate OA phenotype in the target cells (Kato et al., 2014). SFBs communicate with hCs within the physiological environment of joints, therefore this methodology utilizes that natural mechanism. BM-hMSCs were also used to produce EVs for hC treatment as they have been shown to have regenerative capabilities in several applications (Murphy et al., 2003; Wakitani et al., 1994). This has resulted in a hypothesized link between this EV intercellular communication and OA pathogenesis. If hCs receive signals from EVs produced by healthy cells, it is possible that the inverse process could occur to a certain extent, and degradative activity could be reduced. Stemming from this line of thinking, a second hypothesis was developed, positing that this process can further be manipulated by the loading of isolated EVs with miRNA to elicit or amplify therapeutic effects upon EV treatment. Alternatively, EV uptake by hCs could be part of an inflammatory pathway regardless of fibroblast conditioning. In this case, blocking this communication could have therapeutic effects. A series of experiments was designed to begin investigating these hypotheses.

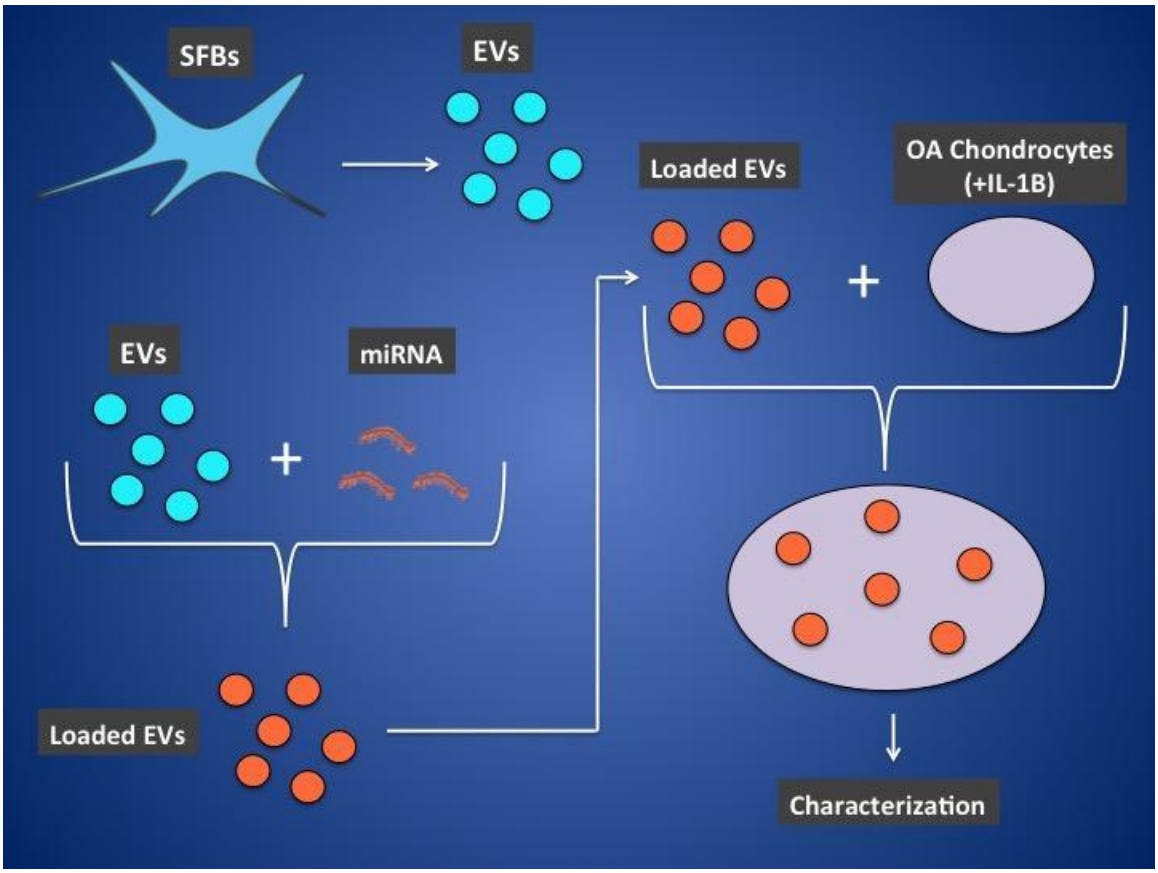

Figure 3. Overview of methodology, showing progression from producer cell culturing and EV isolation steps, through miRNA loading, and finishing with chondrocyte treatment and characterization through ELISA.

Producer Cell EV Isolation and Characterization

Research Design. The effects of EVs isolated from SFBs and BM-hMSCs on OA phenotype in HCs were tested *in vitro*. SFBs were chosen as an EV producer cell because EVs isolated from SFBs have been implicated in OA pathogenesis and naturally communicate with hCs in the natural environment of the joint (Kato et al., 2014). To gauge the impact of producer cell type on treatment efficacy, the impact of BM-hMSC-derived EV treatment was also studied. Recent research suggests that exosomes derived from BM-hMSCs have regenerative capacity, which may come from their integral role in growth and development (Murphy et al., 2003; Wakitani et al., 1994). We aimed to test

the baseline effects of isolated EVs on hCs that had been stimulated to express osteoarthritic behavior, to evaluate their suitability for use as a vehicle to deliver therapeutic miRNA cargo and to determine whether unmodified EVs have some regenerative capacity on their own. After treating loaded EVs with therapeutic miRNA and treating osteoarthritic hCs with both regular and loaded EVs, we expected to see a decrease in degradative enzyme production on osteoarthritic hCs treated with regular EVs, and an even greater decrease in those treated with loaded EVs.

All cell types were cultured in monolayer in tissue culture treated polystyrene flasks (TCPS). SFBs used in this methodology were from an immortalized human cell line SW-982. BM-hMSCs used in this methodology were passage 2 of culturing after sample was taken from a human patient. All protocols used in this study, including cell culture procedures, can be found in the Appendices. SFB culture procedures are described in Appendix A, BM-hMSC culture procedure is described in Appendix B, hC monolayer culture procedures are described in Appendix C. EVs were isolated using differential centrifugation, which allows for the separation of biological components based on size and density. Differential centrifugation techniques for isolating EVs are described in Appendix D. To ensure consistency in subsequent experiments, the protein concentrations of solutions containing isolated EVs was measured using the bicinchoninic acid (BCA) assay, which colorimetrically detects the reduction of Cu^{2+} to Cu^{1+} caused by proteins in an alkaline solution (Pierce BCA Protein Assay Kit). BCA assays were performed using the Pierce BCA Protein Assay Kit from Thermo Fisher Scientific™; procedures used for this assay can be found in Appendix E. EV samples were also analyzed with a Malvern NanoSight LM10 instrument to determine size

distribution, phenotype, and quantity of EVs within each solution, as found in Appendix F. To produce an *in vitro* OA model, hC cells were primed to exhibit the OA phenotype via contact with the pro-inflammatory cytokine IL-1β (Kato et. al., 2013). IL-1β has been shown to induce the production of MMPs, other proteinases, and other proinflammatory cytokines that promote OA behavior in hCs (Goldring & Otero, 2011). hCs were treated with IL-1β for 48 hours before EV treatment and characterization of cell output was done after an additional 96 hours. Full hC treatment procedures are described in Appendix G. Procedures for preparing IL-1β supplemented media are described in Appendix H. Two samples of hCs were not treated with IL-1β to be used to gauge baseline MMP-13 production (prior to OA induction with IL-1β). IL-1β stimulated hCs were then treated with both unloaded EVs and EVs loaded with miRNA-140 and miRNA-125b. Samples of miRNA-140 and miRNA-125b, which have been shown to downregulate the aggrecanase signalling pathway, were obtained from Dharmacon, Inc. The corresponding sequences and their polarity are described along with the sonication-dependent EV loading protocol in Appendix I. Sonication disrupts the integrity of the lipid bilayer of the EVs and allows miRNA to enter the intra-vesicular space via simple diffusion. The membranes spontaneously reassemble after the EVs are removed from the sonicator (Lamichhane 2016). Treatment groups are specified in Figure 4.

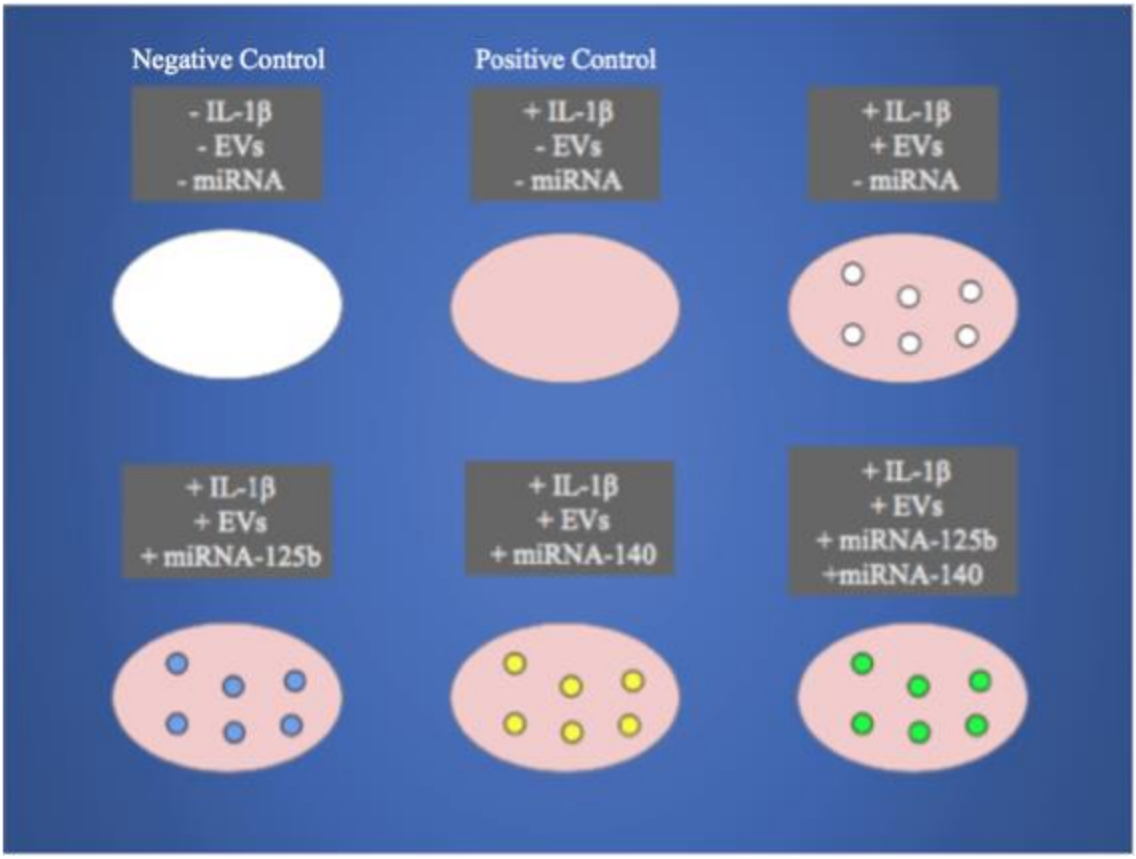

Figure 4. Treatment groups. The six experimental groups: untreated hCs, hCs treated with only IL-1β, hCs treated with IL-1β and unmodified EVs, hCs treated with IL-1β and EVs loaded with miR125b, hCs treated with IL-1β and EVs loaded with miR140, and hCs treated with IL-1β and EVs loaded with both miR125b and miR140.

The protocol for the treatment of hCs with EVs can be found in Appendix J. Cell culture media samples were taken 48 hours after treating EVs with IL-1β to measure secreted MMP-13 for the quantification of inflammatory phenotype in chondrocytes via ELISA. Loaded and unloaded EVs were added after these media samples were collected and media sample were collected 48 hours later (96 hours from OA induction) to gauge the effects of the treatment on the progression of the OA phenotype via measuring expression of proteins characteristic of OA.

Data Collection. The cell culture media samples taken at either 48 hours or 96 hours post induction were analyzed via ELISA to quantify MMP-13 content. MMP-13 content was measured with the Human MMP-13 ELISA kit purchased from Thermo

Fisher Scientific. The kit uses HRP conjugated secondary antibodies to detect concentrations of the target protein bound by a primary antibody. The protocol used to analyze the MMP-13 content of the samples is provided in Protocol 11. Absorbance values were quantified (450 nm) with a SpectraMax M Series UV/Vis Microplate reader. A standard curve was used to calculate protein concentrations from the A450 data. A single factor ANOVA was used to determine whether the mean MMP-13 content differed between samples. A p-value cutoff of 0.01 was used, although all statistically significant results reported here corresponded to p values that were at least five orders of magnitude below this threshold.

Chapter 4: Results

In an effort to analyze the effect of miRNA-140 and miR-125b with SFB EVs, a Western blot was performed with treated hC lysates and analyzed for the presence of aggrecanase and collagenase expression. However, the immunoblot did not demonstrate any bands. The likely reason for the lack of visible expression of the degradative enzymes was that the protein concentration of the lysate was generally low. This may be due to the lysing procedure, size of the treatment wells, or a combination of both. Therefore, an ELISA was a more suitable approach due to the ability to detect protein expression at a lower concentration.

Characterization of EVs

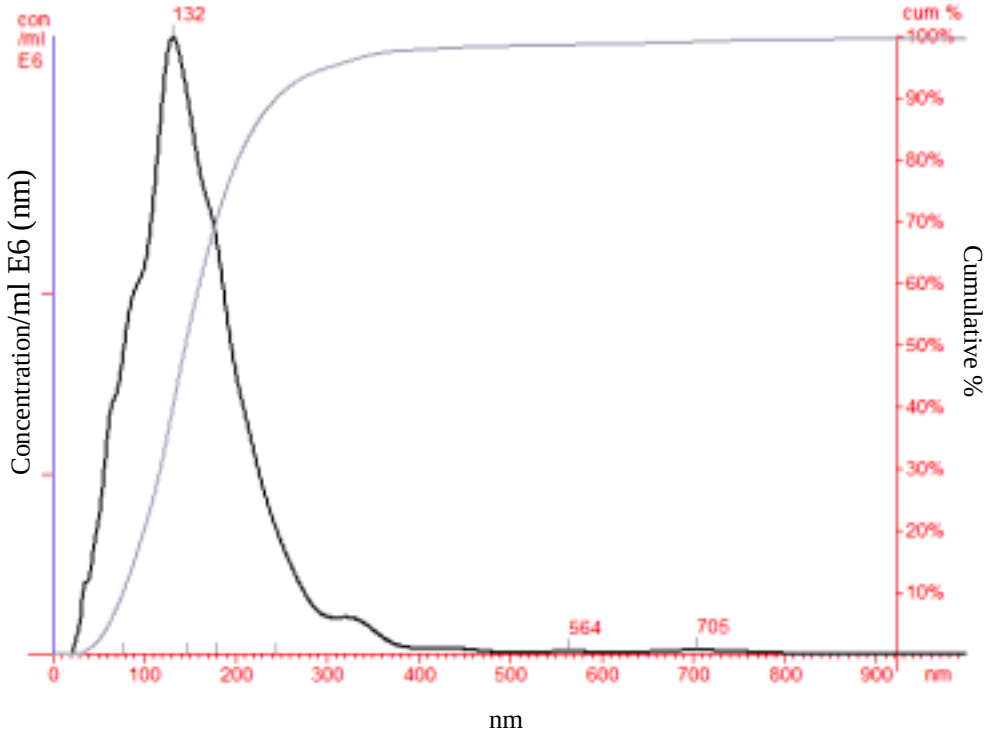

Figure 5. Sample histogram of SFB EV size (nm) and concentration. Data from graphs like the one above were used to characterize EV samples. The peak of the trace of the black line represents the mode of the size distribution.

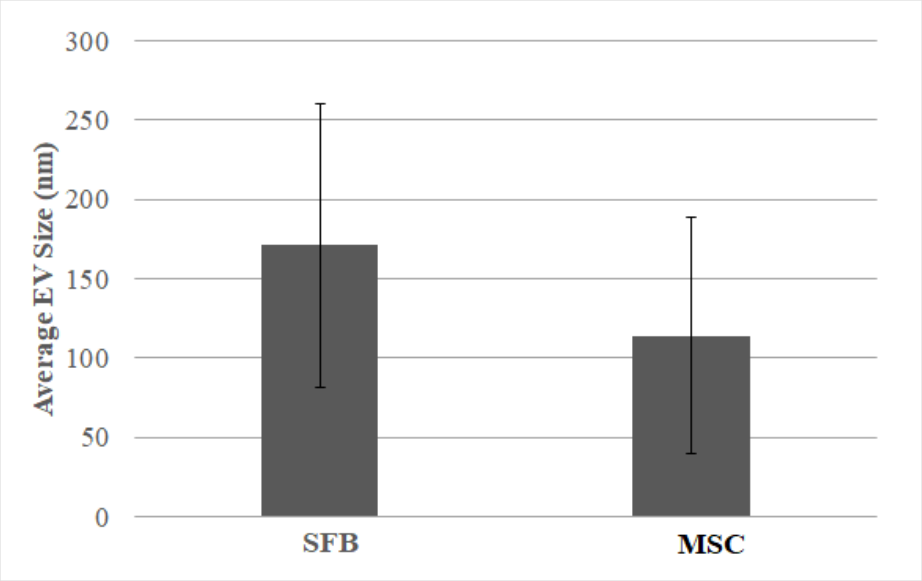

Figure 6. Averaged EV size distribution of SFB and BM-hMSC EV samples. The size distributions were determined via NanoSight. Error bars represent standard deviation between the trials.

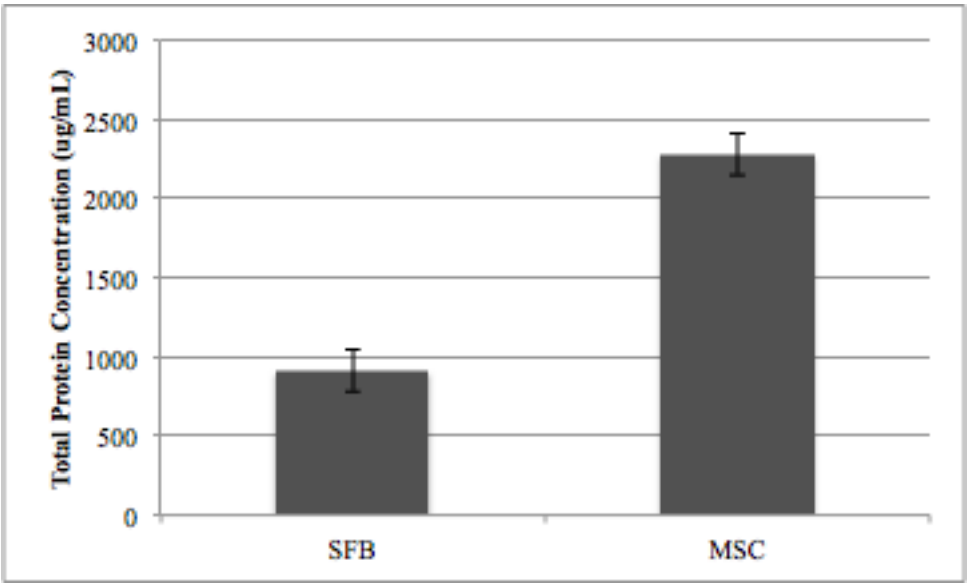

Figure 7. Averaged total protein concentrations of SFB and BM-hMSC EV samples. The total protein present in EV samples from SFB and BM-hMSC cell cultures was determined via BCA assay. Error bars represent standard deviation between the trials.

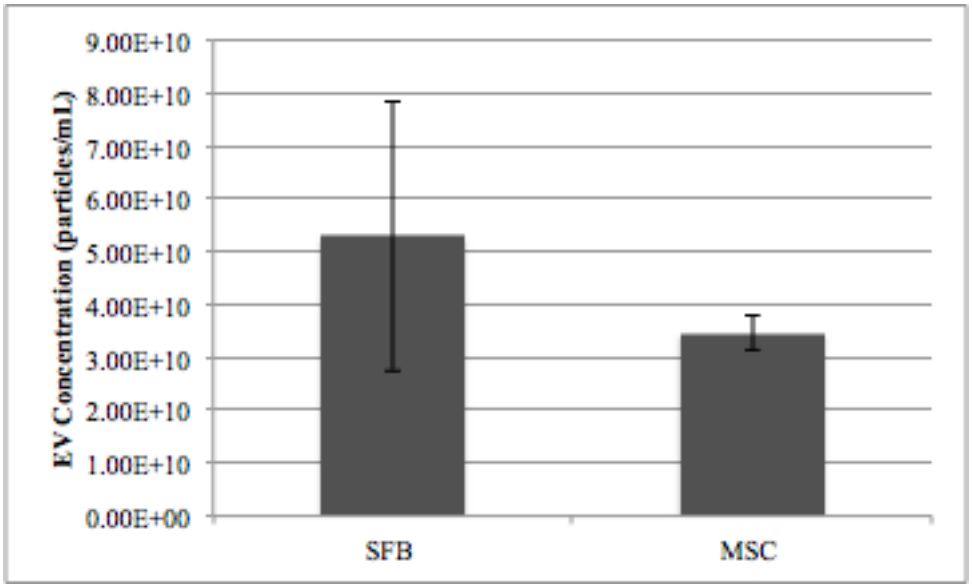

Figure 8. Average particle density of SFB and BM-hMSC EV samples. The concentration of EVs in samples from SFB and BM-hMSC cell cultures was determined via NanoSight analysis. Error bars represent standard deviation between the multiple measurements taken.

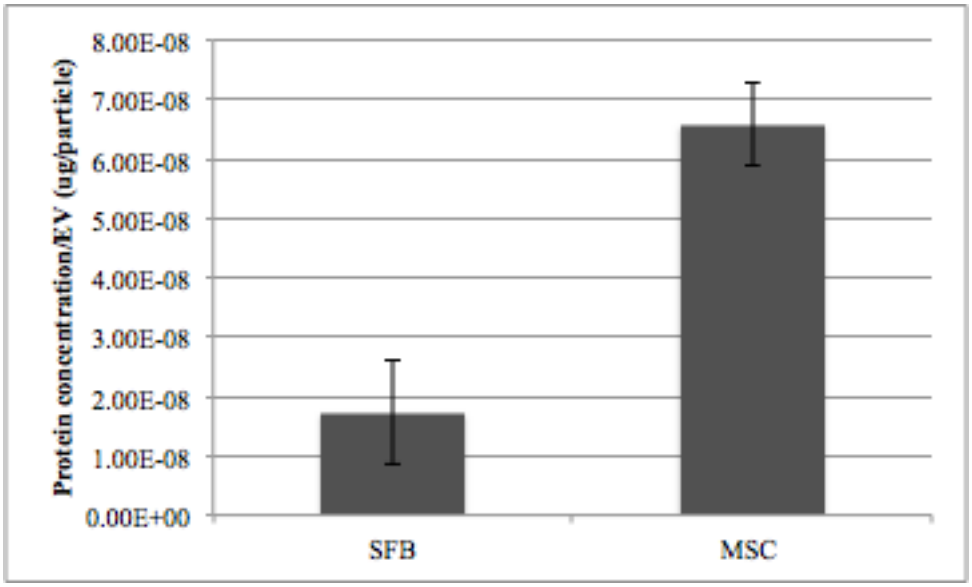

Figure 9. Average protein concentration per SFB and BM-hMSC EV particle. The average concentration of protein in each EV was determined by dividing the average total protein concentration by the EV concentration for the SFB and BM-hMSC EV samples, respectively. Error bars represent the propagated error from the measurements used in the calculation.

EV samples were run through NanoSight analysis to determine the size and concentration, and data were reported in histograms as in Figure 5. Based on NanoSight, average size of the SFB EV sample was determined to be 171 nm and the average size of

the BM-hMSC sample was determined to be 114 nm (Figure 6). Based on results from BCA and NanoSight, respectively, it was determined that the SFB EV sample used had an average protein concentration of 914 ug/mL (Figure 7) and an average EV concentration of 5.29×10^{10} particles/mL (Figure 8). By dividing the average protein concentration by the average EV concentration, it was found that the average protein concentration per EV was 1.73×10^{-8} ug/particle (Figure 9). Using the same methods, the BM-hMSC EV sample had an average protein concentration of 2280 ug/mL (Figure 7), an average EV concentration of 3.46×10^{10} particles/mL (Figure 8), and an average protein concentration per EV of 6.58×10^{-8} ug/particle (Figure 9).

Experimentation

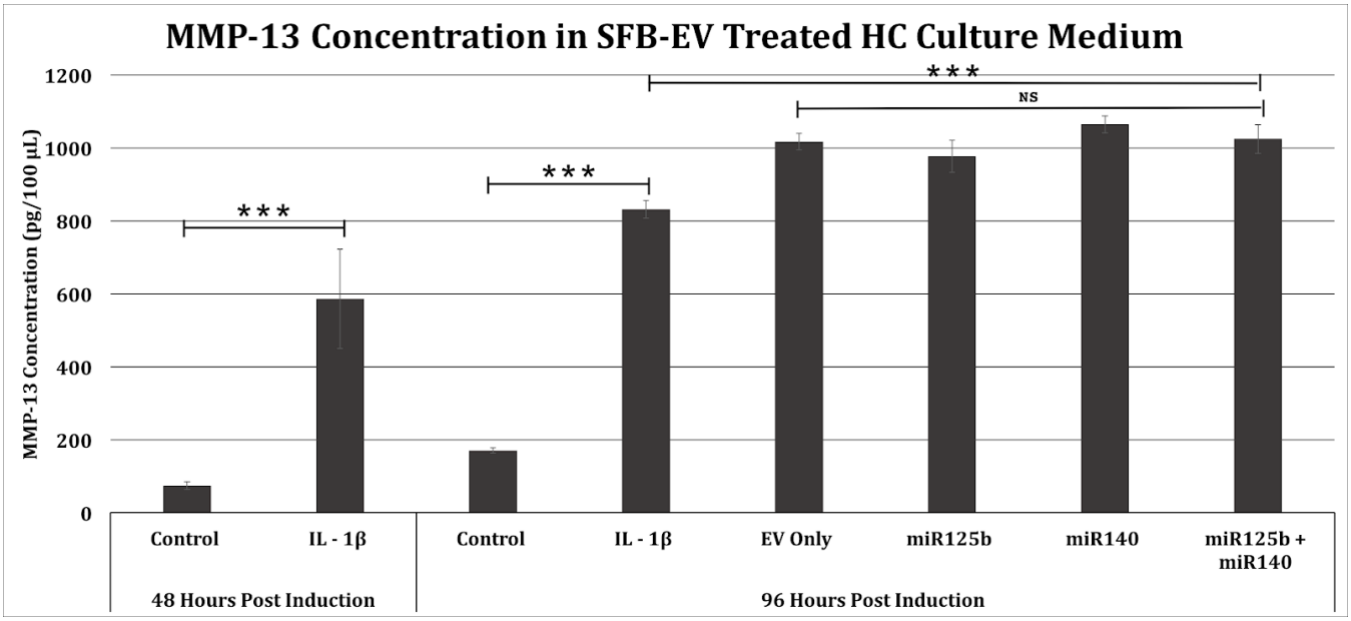

Figure 10. ELISA results showing MMP-13 concentration in IL-1β treated OA-HC samples subsequently treated with SFB EVs (*** indicates $p < 0.001$). Note significant increase in MMP-13 concentration between IL-1β and EV treated samples.

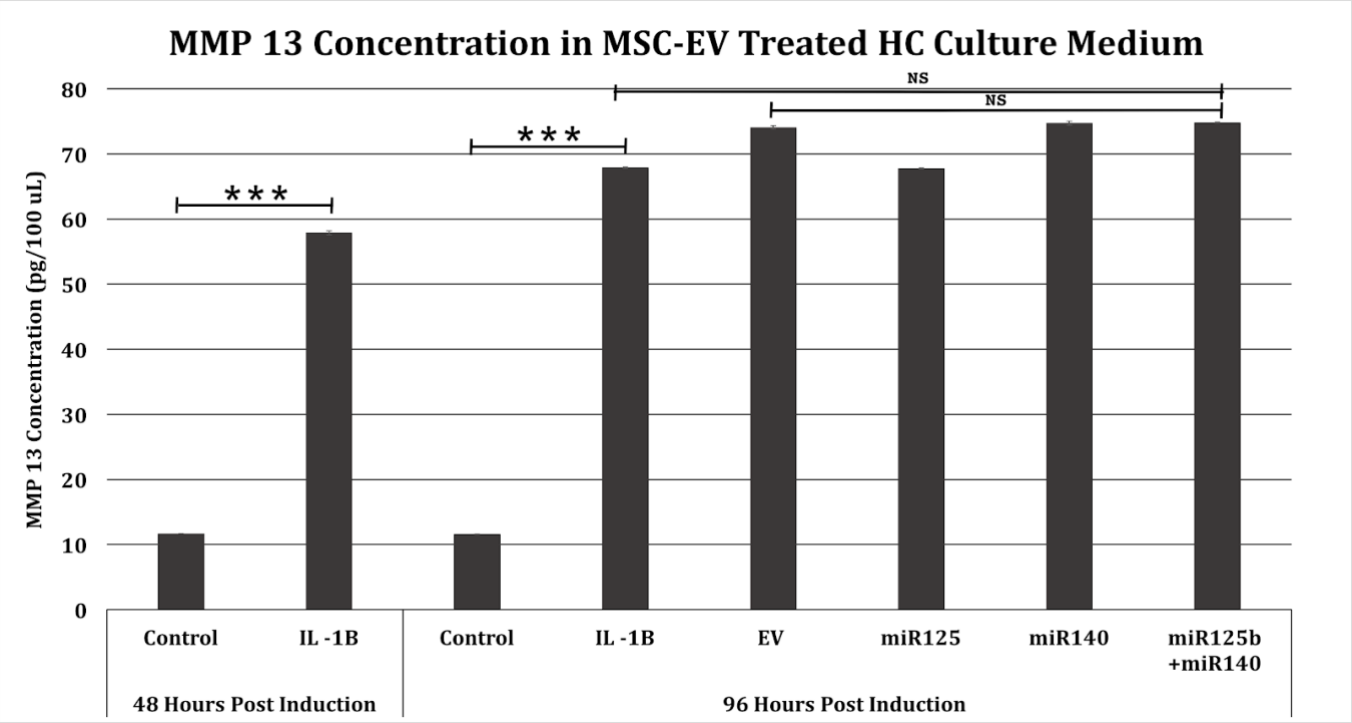

Figure 11. ELISA results showing MMP-13 concentration in IL-1β treated OA-HC samples subsequently treated with BM-hMSC EVs (*** indicates $p < 0.001$). No change in MMP-13 concentration was detected between IL-1β and EV treated samples.

ELISA results indicated that there was a statistically significant increase ($p <<< 0.001$) in MMP-13 concentration after 48 hours post IL-1β mediated OA induction, indicating a successful induction protocol. The samples collected post-SFB EV treatment showed a statistically significant ($p <<< 0.001$) increase in MMP-13 production relative to the IL-1β control and the non-OA control. The BM-hMSC-EV treated samples did not show a statistically significant increase in MMP-13 concentration relative to the IL-1β control. No statistically significant differences were seen between the different EV/miRNA treatment groups in either of the experiments. The concentration of MMP-13 in the EVs themselves was not quantified.

Chapter 5: Discussion

It was anticipated that IL-1β would induce OA associated genes in hCs and EV treatment would reduce OA phenotype. The ELISA showed that hCs treated with IL-1β significantly increased MMP-13 production with respect to the control both 48 and 96 hours after treatment, suggesting OA associated genes were successfully induced in the hCs due to the expression of the inflammatory cytokine. However, contrary to the hypothesis, SFB EV treatment did not yield promising results; the ELISA showed that SFB EV treatment, regardless of cargo, significantly increased MMP-13 expression compared to inflamed hCs receiving no SFB EV treatment. As such, it could be inferred that SFB EVs induce an inflammatory response in hCs; however, an additional experimental group consisting of non-OA hCs treated with SFB EVs is needed to solidify this claim. The SFBs used in these experiments were relatively high passage (> P20), and this high passage number may have made the cells behave significantly differently from primary SFB cells. This disparity may have induced the inflammation observed. An increase in MMP-13 production in this additional group would have solidified the claim that SFB EVs increase inflammation in hCs. However, due to mycoplasma contamination that greatly hindered hC cell growth, the amount of experimental groups and time available for further experimentation was severely limited. All contaminated cell lines, and the products derived from those cell lines, were disposed of and new materials were ordered to prepare all of the data presented here.

A recent study has suggested that EVs derived from BM-hMSCs have therapeutic potential for treating OA. For example, Cosenza et al. showed via PCR that treatment of BM-hMSC EVs could promote gene expression of structural proteins while inhibiting the

production of degradative enzymes in OA-like hCs. This study failed to replicate these results. While an OA associated gene was successfully induced as in the previous experiment, the results show that BM-hMSC EVs, regardless of cargo, induced no significant change in MMP-13 expression, suggesting that cartilage synthesis and OA phenotype are not significantly affected. The discrepancy between these results and the literature could potentially be explained by inadequate loading of miRNA. Since BM-hMSC EVs did not have observable adverse effects on the hCs, relative to the samples treated with SFB EVs, there is still potential that with proper loading of miRNA, genes encoding degradative enzymes could be effectively blocked. While a nanosight experiment was conducted to ensure the EVs were intact after sonication, a test to quantify the loaded miRNA would provide insight into the dosage needed for BM-hMSC EVs to be used as a treatment for OA. For example, a colorimetric assay could be done on EVs loaded with fluorescently labeled miRNA due to the fact that the detection threshold for the fluorophore used was several orders of magnitude above the concentration used to load the EVs. A possibly remedy for this issue would be using a different fluorophore with a lower detection threshold, or using polymerase chain reaction (PCR) to quantify the levels of the target miRNA present in loaded EVs washed in PBS to remove excess miRNA. Additionally, quantifying the amount of MMP-13 present in the EVs themselves would be a crucial step in future experiments, as it was not clear whether the EVs themselves carried the MMP-13 detected by the ELISA.

More comprehensive results could be achieved with additional time and experimentation. For example, the ELISA used in this study was only able to detect one substance, MMP-13. Subsequent ELISA studies could quantify other degradative

enzymes, such as ADAMTS5, or ECM proteins indicative of cartilage synthesis, such as ACAN or COL2A1. Downregulation of degradative enzymes and upregulation of ECM proteins post-treatment would show that the treatment was effective. Other experiments, such as Western Blots and qRT-PCR, could also verify the results obtained from the ELISA.

Chapter 6: Conclusion

The incidence of OA has risen in correlation with the increasing average life expectancy in the United States. Current treatments for OA, such as surgery, simply address symptoms of the pain. Thus, targeting the underlying cellular mechanism of OA provides a promising approach to curing the disease. This study focused on the intercellular communication between cell types involved in the pathology of OA. This was specifically addressed by manipulating the EV cargo derived from SFBs and BM-hMSCs and investigating their impact on hC gene expression.

While a significant difference in the expression of MMP-13 was observed between the hC control and hCs treated with IL-1β, an even greater expression of MMP-13 was observed in hCs treated with SFB EVs, regardless of manipulation. This implies that SFB EVs actually promote the induction of one of the inflammatory pathways implicated in OA pathogenesis. The same experiment was repeated with BM-hMSC-derived EVs to confirm this inference. There was no significant change in MMP-13 expression between the inflamed hCs and the BM-hMSC-EV-treated inflamed hCs, indicating that BM-hMSC EVs neither worsened nor reduced inflammation relative to the IL-1B only controls.

Overall, a future investigation focused on manipulating the cargo of BM-hMSC-EVs for therapeutic results would be appropriate. This study confirmed that the manipulated EVs were intact after sonication. In the future, experiments are needed to confirm that the manipulated EVs are successfully loaded with the miRNA cargo. Likewise, further experimentation with higher doses of BM-hMSC EVs, confirmation of

successfully loaded BM-hMSC EVs, and a wider variety of miRNA targeted at the MMP-13 gene would provide greater insight into the viability of EVs as a medium for OA treatment. If future work replicated the results of this study, EVs would likely not be a promising route to revolutionizing the way OA is treated.

Appendices

Appendix A: General Instructions for Culturing Human Fibroblast-Like Synoviocytes (HFLS)

(Cell Applications, Inc., 2005)

1. Storage
    a. Cryopreserved Vials (408-05)
        i. Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.
    b. Proliferating Flasks (409-25, -75)
        i. Examine under a microscope to check if all the cells are attached to the bottom of the flask. If not, notify CAI or your distributor immediately.
        ii. Decontaminate the exterior of the flask with 70% alcohol.
        iii. Place the sealed flask in a 37°C, 5% CO_2 humidified incubator for 2 hours as shipped.
        iv. In a sterile Biological Safety Cabinet, open the cap of the flask very slowly and carefully.
        v. Remove the Transport Medium by aspiration. Add fresh Growth Medium: 5 ml for a T-25 flask and 15 ml for a T-75 flask.
        vi. Place the flask in a 37°C, 5% CO_2 humidified incubator with loosened cap to allow gas exchange.
        vii. Change medium every other day.
    c. Growth Medium (415-500)
        i. Store the Growth Medium at 4°C in the dark immediately upon arrival.
    d. Subculture Reagent Kit (090K)

i. Store at -20°C immediately upon arrival.

ii. Store at 4°C after thawing.

2. Preparation for Culturing

    a. Make sure the Class II Biological Safety Cabinet, with HEPA filtered laminar airflow, is in proper working condition.

    b. Clean the Biological Safety Cabinet with 70% alcohol to ensure it is sterile.

    c. Turn the Biological Safety Cabinet blower on for 10 min. before cell culture work.

    d. Make sure all serological pipettes, pipette tips and reagent solutions are sterile.

    e. Follow the standard sterilization technique and safety rules:

        i. Do not pipette with mouth.

        ii. Always wear gloves and safety glasses when working with human cells even though all the strains have been tested negative for HIV, Hepatitis B and Hepatitis C.

        iii. Handle all cell culture work in a sterile hood.

3. Culturing HFLS

    a. Preparing Cell Culture Flasks for Culturing HFLS

        i. Take the Synoviocyte Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.

        ii. Pipette 15 ml of Synoviocyte Growth Medium* to a T- 75 flask. * Keep the medium to surface area ratio at 1ml per 5 cm^2. For example,

5 ml for a T-25 flask or a 60 mm tissue culture dish. 15 ml for a T-75 flask or a 100 mm tissue culture dish.

b. Thawing and Plating HFLS

i. Remove the cryopreserved vial of HFLS from the liquid nitrogen storage tank using proper protection for your eyes and hands.

ii. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.

iii. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath and watch the vial closely during the thawing process.

iv. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.

v. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.

vi. Remove the vial cap carefully. Do not touch the rim of the cap or the vial.

vii. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette. Be careful not to pipette too vigorously as to cause foaming.

viii. Pipette the cell suspension (1ml) from the vial into the T-75 flask containing 15 ml of Synoviocyte Growth Medium.

ix. Cap the flask and rock gently to evenly distribute the cells.

x. Place the T-75 flask in a 37°C, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.

xi. Change to fresh Synoviocyte Growth Medium after 24 hours or overnight to remove all traces of DMSO.

xii. Change Synoviocyte Growth Medium every other day until the cells reach 60% confluent.

xiii. Double the Synoviocyte Growth Medium volume when the culture is >60% confluent or for weekend feedings.

xiv. Subculture the cells when the HFLS culture reaches 80% confluent.

4. Subculturing HFLS

   a. Preparing Subculture Reagents

      i. Remove the Subculture Reagent Kit from the -20°C freezer and thaw overnight in a refrigerator.

      ii. Make sure all the subculture reagents are thawed. Swirl each bottle gently several times to form homogeneous solutions.

      iii. Store all the subculture reagents at 4°C for future use. The activity of Trypsin/EDTA Solution will be stable for 2 weeks when stored at 4°C.

      iv. Aliquot Trypsin/EDTA solution and store the unused portion at -20°C if only portion of the Trypsin/EDTA is needed.

   b. Preparing Subculture Flask

      i. Take the Synoviocyte Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.

ii. Pipette 30 ml of Synoviocyte Growth Medium to a T-175 flask (to be used in Section IV C Step 15).

c. Subculturing HFLS

i. Trypsinize cells at room temperature. Do not warm any reagents to 37°C.

ii. Remove the medium from culture flasks by aspiration.

iii. Wash the monolayer of cells with HBSS and remove the solution by aspiration.

iv. Pipette 10 ml of Trypsin/EDTA Solution into the T-75 flask. Rock the flask gently to ensure the solution covers all the cells.

v. Remove 9 ml of the solution immediately.

vi. Re-cap the flask tightly and monitor the trypsinization progress at room temperature under an inverted microscope. It usually takes about 2 to 5 minutes for the cells to become rounded. The cells may not become completely round during the trypsinization and some cells may maintain some processes even though they are loosened from the culture surface.

vii. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.

viii. Pipette 5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.

ix. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.

x. Rinse the flask with an additional 5 ml of Trypsin Neutralizing Solution and transfer the solution into the same conical tube.

xi. Examine the T-75 flask under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9.

xii. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells.

xiii. Aspirate the supernatant from the tube without disturbing the cell pellet.

xiv. Flick the tip of the conical tube with your finger to loosen the cell pellet.

xv. Resuspend the cells in 5 ml of Synoviocyte Growth Medium by gently pipetting the cells to break up the clumps.

xvi. Count the cells with a hemocytometer or cell counter. Inoculate at 10,000 cells per cm^2 for rapid growth, or at 7,000 cells per cm^2 for regular subculturing.

Appendix B: Culturing Human Mesenchymal Stem Cells (hMSC) in MSC NutriStem® XF

(Biological Industries, 2012)

1. Complete Ready-To-Use Medium Preparation
    a. The frozen MSC NutriStem® XF Supplement Mix should be thawed at room temperature or at 2-8°C. Avoid repeated freeze-thaw cycles (up to two times).
    b. For a complete medium, aseptically add 0.6ml of MSC NutriStem® XF Supplement Mix to 100ml of MSC NutriStem® XF Basal Medium.
    c. (Alternatively, add 3ml of MSC NutriStem® XF Supplement Mix to 500ml of MSC NutriStem® XF Basal Medium).
    d. MSC NutriStem® XF Basal Medium contains L-glutmine. Store at 2-8°C. Protect from light.
    e. The complete MSC NutriStem® XF Medium is stable at 2-8°C for up to 30 days.
2. Preparation of Pre-Coated Culture Dishes with MSC Attachment Solution (Cat. No. 05-752-1)
    a. Dilute MSC Attachment Solution 1:100 using sterile DPBS (without Ca++ and Mg++, Catalog No. 02-023-1) and gently mix using a pipette.
    b. Add the diluted MSC Attachment Solution to the cultureware. Gently agitate the coated cultureware and verify complete covering of the surface. Use Table 1 for recommended volumes.
    c. Incubate the coated cultureware for at least 30 minutes in a humidified CO_2 incubator (37°C).
    d. Following 30 minutes incubation:

i. For immediate use:
    1. Gently wash the cultureware with DPBS (For T-25 use at least 5ml).
    2. Seed cells immediately.
    3. It is critical that the coating does not dry out.

ii. For later use:
    1. Wrap the coated cultureware with Parafilm® and incubate at 2-8°C. Coated cultureware stored under sterile conditions at +2-8°C are stable for 1 week.
    2. Gently wash the cultureware with DPBS.
    3. Seed cells immediately.
    4. It is critical that the coating does not dry out.

3. Culturing of hMSC in the complete MSC NutriStem® XF Medium

a. Recovery of Cryopreserved hMSC

i. Pre warm 5-10 ml of complete MSC NutriStem® XF Medium in a 50 ml conical tube.

ii. Rapidly thaw frozen vial of hMSC in a 37°C water bath, with agitation untill a small amount of ice remains.

iii. Slowly add (drop by drop while gently swirling) the cells into the pre-warmed complete MSC NutriStem® XF Medium.

iv. Centrifuge cells at 300-400xg for 4-5 minutes at room temperature.

v. Remove supernatant and re-suspend cell pellet in 0.5-1 ml of complete MSC NutriStem® XF Medium.

vi. Perform a viable cell count (e.g., using Trypan Blue Exclusion Assay)

vii. Add the desired volume of complete MSC NutriStem® XF Medium.

viii. Transfer the cells into MSC Attachment Solution pre-coated culture dish (see above). Seeding densities should be calculated (see table 2).

ix. Incubate in a humidified CO_2 incubator (37°C).

1. Note: It is possible to avoid the centrifugation step after thawing. In this case skip steps 1.4-1.5 and transfer the thawed cells (from Step 1.3) directly into the pre-coated culture flask (using MSC Attachment Solution, Cat. No. 05-752-1) with the required volume of the complete MSC NutriStem® XF Medium, at a ratio of at least 1:10 (for the dilution of the DMSO).

b. Subculturing hMSC: MSC NutriStem® XF Medium was developed for optimal proliferation of hMSCs from a variety of sources (BM-hMSC, AT-hMSC, UCT-hMSC). The variety sources and the variability of donors may influence hMSC proliferation rate. For optimal proliferation of hMSC in MSC NutriStem® XF Medium, it is recommended to seed hMSCs at a concentration of 5000-6000 cell/cm^2 (Table 2), re-feed cells with fresh warmed complete MSC NutriStem® XF Medium every 2-3 days and subculture when the cells reach up to 80% confluence.

i. Subculturing Protocol

1. Pre-warm Recombinant Trypsin Solution (with or without EDTA, cat. no. 03-078-1, 03-079-1) to room temperature before use.
2. Remove culture medium and gently wash once with DPBS w/o Ca, Mg (Cat. No. 02-023-1).
3. For T25 culture flask add 1-3ml of Recombinant Trypsin Solution. (For any other culture dish, the appropriate volume should be adjusted). Note: The more the culture is confluence and/or highly passaged, the slower the detachment will be and the higher volume is recommended.
4. Incubate for 2-10 minutes at room temperature and verify cell detachment using inverted microscope. (Incubation at 37°C will not accelerate detachment). Usually, within 2-5 minutes (at R.T.) the cells will dissociate by gently tapping the flask.
5. Following detachment, add 5-10 ml of DPBS or pre-warmed MSC NutriStem® XF Medium. Collect cell suspension into sterile tube and re-wash the culture dish as necessary to collect the entire cells.
6. Centrifuge cells for 4-5 minutes at 300-400xg at room temperature. Carefully discard the supernatant.
7. Re-suspend cell pellet in minimal volume of pre-warmed complete MSC NutriStem® XF Medium. Take sample volume to perform a viable cell count

8. Re-seed cells into pre-coated culture dish (see above). Seeding densities and the required volume of complete MSC NutriStem® XF Medium to be added should be calculated (see Table 2).
9. Incubate in a humidified CO_2 incubator (37°C).
10. Re-feed cells with fresh warmed complete MSC NutriStem® XF Medium every 2-3 days.

c. Cryopreservation of hMSC

i. Rapidly re-suspend hMSC pellet with cold MSC Freezing Solution (Cat. No. 05-712-1) (recommended between 0.5-1x106 cells/ml, 1ml/vial).

ii. Immediately place the cryovials in appropriate freezing container (e.g., "Mr. Frosty") and place at -80°C for overnight.

iii. Transfer the cryovials into liquid nitrogen.

Appendix C: Human Chondrocytes Osteoarthritis (hOA-C) Culture Protocol

(Cell Applications, Inc., 2005)

1. Storage
    a. Cryopreserved Vials (402OA-05a)
        i. Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.
            1. Be sure to wear face protection mask and gloves when retrieving cryovials from the liquid nitrogen storage tank. The dramatic temperature change from the tank to the room could cause any trapped liquid nitrogen in the cryovials to burst and cause injury.
2. Preparation for Culturing
    a. Ensure the Class II Biological Safety Cabinet, with HEPA filtered laminar airflow, is in proper working condition.
    b. Sterilize the Biological Safety Cabinet with 70% alcohol.
    c. Turn the Biological Safety Cabinet blower on for 10 minutes before beginning cell culture work.
    d. Make sure all serological pipettes, pipette tips and reagent solutions are sterile.
    e. Follow the standard sterilization technique and safety rules:
        i. Do not pipette by mouth.

ii. Always wear gloves and safety glasses when working with human cells even though all the strains have been tested negative for HIV, Hepatitis B and Hepatitis C.

iii. Handle all cell culture work in a sterile hood.

3. Culturing HC-OA

a. Preparing Cell Culture Flasks for Culturing HC-OA

i. Take the Chondrocyte Growth Medium (411-500) from the refrigerator. Decontaminate the bottle with 70% alcohol before placing in a sterile hood.

ii. Pipette 15 ml of Chondrocyte Growth Medium (411-500)* to a T-75 flask (SIAL0641).

1. Keep the medium to surface area ratio at 1ml per 5 cm^2. For example:

a. 5 ml for a T-25 flask (SIAL0639) or a 60 mm tissue culture dish (SIAL0166).

b. 15 ml for a T-75 flask (SIAL0641) or a 100 mm tissue culture dish (SIAL0167).

b. Thawing and Plating HC-OA

i. Remove the cryopreserved vial of HC-OA from the liquid nitrogen storage tank using proper protection for your eyes and hands.

ii. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.

iii. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath and watch the vial closely during the thawing process.

iv. Remove the vial from the water bath when only a small amount of ice is left in the vial. *Do not let cells thaw completely.*

v. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.

vi. Remove the vial cap carefully. Do not touch the rim of the cap or the vial with your hands to avoid contamination.

vii. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette. Be careful not to pipette too vigorously as to cause foaming.

viii. Pipette the cell suspension (1ml) from the vial into the T-75 flask (SIAL0641) containing 15 ml of Chondrocyte Growth Medium (411-500).

ix. Cap the flask and rock gently to evenly distribute the cells.

x. Place the T-75 flask (SIAL0641) in a 37°C, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.

xi. Change to fresh Chondrocyte Growth Medium (411-500) after 24 hours or overnight to remove all traces of DMSO.

xii. Change Chondrocyte Growth Medium (411-500) every other day until the cells reach 60% confluency.

xiii. Double the Chondrocyte Growth Medium (411-500) volume when the culture is >60% confluent or for weekend feedings.

xiv. Subculture the cells when the HC-OA culture reaches 80% confluency.

4. Subculturing HC-OA

    a. Preparing Subculture Reagents

        i. Remove the Trypsin-EDTA solution (T3924) and Trypsin Inhibitor (T6414) from the -20°C freezer and thaw overnight in a refrigerator.

        ii. Make sure all the subculture reagents are thawed. Swirl each bottle gently several times to form homogeneous solutions.

        iii. Store all the subculture reagents at 4°C for future use.

        iv. Aliquot Trypsin/EDTA solution (T3924) and store the unused portion at -20°C if only a portion of the Trypsin/EDTA (T3924) is needed.

    b. Preparing Culture Flask

        i. Take the Chondrocyte Growth Medium (411-500) from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.

        ii. Pipette 30 ml of Chondrocyte Growth Medium (411-500) to a T-175 flask (SIAL1080) (to be used in Section IV C Step 15.)

    c. Subculturing HC-OA

        i. Trypsinize Cells at Room Temperature. Do Not Warm Any Reagents to 37°C.

        ii. Remove the medium from culture flasks by aspiration.

iii. Wash the monolayer of cells with HBSS (H6648) and remove the solution by aspiration.

iv. Pipette 10 ml of Trypsin/EDTA Solution (T3924) into the T-75 flask (SIAL0641). Rock the flask gently to ensure the solution covers all the cells.

v. Remove 9 ml of the solution immediately.

vi. Re-cap the flask tightly and monitor the trypsinization progress at room temperature under an inverted microscope. It usually takes about 2 to 5 minutes for the cells to become rounded. The cells may not become completely round during trypsinization and some cells may maintain some processes even though they are loosened from the culture surface.

vii. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.

viii. Pipette 5 ml of Trypsin Inhibitor Solution (T6414) to the flask to inhibit further tryptic activity.

ix. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.

x. Rinse the flask with an additional 5 ml of Trypsin Inhibitor Solution (T6414) and transfer the solution into the same conical tube.

xi. Examine the T-75 flask (SIAL0641) under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9.

xii. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells.

xiii. Aspirate the supernatant from the tube without disturbing the cell pellet.

xiv. Flick the tip of the conical tube with your finger to loosen the cell pellet.

xv. Resuspend the cells in 5 ml of Chondrocyte Growth Medium (411-500) by gently pipetting the cells to break up the clumps.

xvi. Count the cells with a hemocytometer or cell counter. Inoculate at 18,000 cells per cm^2 for rapid growth, or at 8,000 cells per cm^2 for regular subculturing.

Appendix D: Differential Centrifugation for EV Isolation

1. Isolation
    a. Centrifuge cell culture supernatant at 1000 x g at 4℃ for 10 minutes in a SX4750 Swinging-Bucket Rotor.
    b. Collect supernatant and centrifuge at 2000 x g at 4℃ for 20 minutes in a SX4750 Swinging-Bucket Rotor.
    c. Collect supernatant and centrifuge at 10,000 x g at 4℃ for 30 minutes in a FX6100 Fixed Angle Rotor.
    d. Collect supernatant and centrifuge at 40,000 rpm at 4℃ for 120 minutes in a Type 70 Ti Fixed Angle Rotor.
    e. Discard supernatant and resuspend pellet in PBS.
2. Washing
    a. Fill Pall Nanosep® centrifugal device with Omega membrane (MWCO 300 kDa) with EV suspension and centrifuge at 6000 g for 15 minutes.
    b. Discard supernatant and place the tube on ice.
    c. Repeat until entire suspension is filtered.
    d. Centrifuge with PBS 2x after to ensure all media is filtered out.
3. Storage
    a. Suspend pellet in PBS and store in freezer.

Appendix E: BCA Protein Assay

(Adapted from Thermo Fisher Scientific, 2015c; Lamichhane et al., 2015)

1. Create mixed working reagents using steps provided in the kit manual.
2. Place 1 mL of EV sample solution with 1 mL of mixed reagent solution.
3. Pipette up and down, thoroughly mixing.
4. Incubate at 60 degrees Celsius for 60 mins. Cool to room temp.
5. Run analysis in spectrophotometer. Read and note the absorbance at 562 nm.

Appendix F: NanoSight

(NanoSight NTA 2.1 Analytical Software, 2010)

1. Preparing Samples
    a. Make a dilution from the original sample of 400 uL of 5ug/mL of EVs in nonsterile PBS in a microcentrifuge tube.
    b. Vortex the sample.
    c. Withdraw 400 uL with a 1 cc syringe and slowly inject into the reader, taking care not to form bubbles.
2. Solution Analysis
    a. Using the NTA software, adjust the camera level until EVs are visible and large aggregates are not shown on the program screen.
    b. Assuming optimal concentration and high polydispersity, capture and record 60 seconds of footage.
    c. After recording footage, adjust the camera sensitivity (screen gain) until the smallest EVs present in the sample are made visible and are capable of being tracked and analyzed. Do not overexpose the image.
    d. Process the video sequence and record the concentration of particles/mL.

Appendix G: Treatment of monolayer cultured chondrocytes in T25 flasks with IL-1β

Note: Chondrocytes should be cultured in T25 flasks with 4 mL of media.

1. Preparation of IL-1β dilution
    a. Dissolve 5 μg of solid IL-1β into 1.22 mL of hC media. Label this 'Solution A'
    b. Combine 100 μL of solution A with 900 μL of media. Label this new solution 'Solution B'
    c. Combine 100 μL of solution B with 900 μL of media. Label this new solution 'Solution C'
    d. Sterile filter solution C.
    e. Add 100 μL solution C to each treatment group. Be sure each flask contains 4000 μL of media prior to addition of solution C.

Appendix H: Treatment of monolayer cultured chondrocytes in T25 flask with EVs

1. Obtain filters for 100cc syringes (equal to number of samples being treated) and place them into a sterile cell culture hood. If the treatment sample volumes are using are < 200 µL you may need to use needle tips to extract the full volume from the tube.
2. Remove the IL-1β treated HCs from cell culture incubator and place them in sterile cell culture hood.
3. Load one of the EV samples into a syringe and replace tip with filter unit. Open the cell culture flask set for treatment and push the EV solution through the filter unit to dispense sterile loaded-EVs into the cell culture medium.
4. Repeat for all samples and place cells back in cell culture incubator when finished.
5. Dispose of sharps in containers specially designated for disposal of sharps.

Appendix I: Sonication

1. Loading
    a. In a thin-walled 100 µL centrifuge tube, add 100 µg EVs.
    b. To the above solution, add 2 pmol of desired miRNA.
    c. To the above solution, add non-sterile PBS such that total volume of solution is 100 µL.
    d. Place tubes ice for 30 minutes
2. Sonication
    a. Place tubes in sonicator for 15 seconds.
    b. Place tubes on ice for 1 minute.
    c. Place tubes in sonicator for 15 seconds.
    d. Place tubes on ice for 1 minute.
3. Washing
    a. Transfer solution to Pall Nanosep® centrifugal device with Omega membrane (MWCO 300 kDa)
    b. Centrifuge at 6000 g for 5 minutes.
    c. Discard supernatant and resuspend with 1x PBS
    d. Repeat steps B&C 2 times

Appendix J: ELISA

1. Procedure
    a. Prepare all reagents, samples and standards as instructed.
    b. Add 100µL standard and sample to wells. Cover plate & incubate at RT for 2.5 hours.
    c. Wash plate four times
    d. Add 100µL Biotinylated Antibody to wells. Cover plate & incubate at RT for 1 hour.
    e. Wash plate four times
    f. Add 100µL of Streptavidin- HRP Reagent to each well.
    g. Cover & incubate plate at RT for 45 minutes.
    h. Wash plate four times
    i. Add 100µL TMB Substrate to each well.
    j. Develop plate at room temperature in the dark for 30 minutes.
    k. Add 50µL of Stop Solution to each well.
    l. Measure absorbance and calculate results
2. Precautions
    a. All specimens and reagents must be at room temperature (20-25°C) before use in the assay.
    b. Review all instructions carefully and verify components against the Kit Contents list (page 1) before beginning the assay.
    c. Do not use a 37°C water bath to thaw samples. Thaw samples at room temperature.

d. If using a multichannel pipettor, always use a new disposable reagent reservoir for the addition of each reagent. Use new disposable pipette tips for each transfer to avoid cross-contamination.
e. Use a new adhesive plate cover for each incubation step.
f. Avoid microbial contamination of reagents.
g. Avoid exposing reagents to excessive heat or light during storage and incubation.
h. Do not mix reagents from different kit lots. Discard unused ELISA components after completing the assay.
i. Do not use glass pipettes to measure the TMB Substrate Solution. Take care not to contaminate the solution. If the solution is blue before use, DO NOT USE IT.
j. Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
k. Some components of this kit contain sodium azide. Please dispose of reagents according to local regulations.

3. Sample Preparation
    a. Serum, plasma, and cell culture media sample types may be tested in this assay; 100µL per well of diluted sample is required. See reagent preparation step 3 for sample dilution recommendations.

b. Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
c. Samples and standards must be assayed in duplicate each time the assay is performed.
d. Equilibrate samples gradually to room temperature before beginning the assay. Do not use a heated water bath to thaw or warm samples.
e. Mix samples by gently inverting the tubes.
f. If samples are clotted, grossly hemolyzed, lipemic or contaminated, make a note on the template and interpret results with caution.

Glossary

Aggrecan (ACAN): Protein encoded by the ACAN gene, component of the extracellular matrix in articular cartilage, enables the joint to withstand compression

Aggrecanase (ADAMTS5): Enzyme that induces cleavage of aggrecan, causing extracellular matrix degradation

Angiogenesis: Process by which new blood vessels form from preexisting vessels

Articular Cartilage: Smooth tissue that caps the ends of bones, prevents friction between bones

Chondrocytes: Cells found in articular cartilage, crucial for cartilage form and function

Collagen: Main structural protein in the body, found in connective tissue, provides structure and support

Collagenase (MMP-13): Enzyme that induces cleavage of collagen, causing extracellular matrix degradation

Cytokines: Proteins secreted by cells, important in cell signaling, can enhance or inhibit other cytokines, can affect cell growth, maturation, and functioning

Electroporation: Process of running an electrical current through a cell, causes the cell membrane to become permeable and allow foreign objects to pass through

Extracellular Matrix (ECM): Collection of extracellular molecules secreted by cells, provides structural and biochemical support to surrounding cells

Extracellular Vesicle (EV): Lipid membrane structure, stores and transports cellular products

Fibroblast: Cell in connective tissue, produces extracellular matrix, collagen, and other fibers

Glycosaminoglycan (GAG): Long polysaccharide chains, polar and hydrophilic, implicated in cellular proliferation

Growth Factor: Protein or steroid hormone, signaling molecule, regulates a variety of cellular processes

Homogenate: Suspension of cells and cell fragments, obtained by homogenization, lacks cell structure

Human synovial fibroblast (SFB): Found in the **synovial fluid** (see Fibroblast above and synovial fluid below)

Mesenchymal stem cell (BM-hMSC): Stromal cells, possesses the ability to differentiate into osteoblasts, chondrocytes, myocytes, fibroblasts, stromal cells, and adipocytes

microRNA (miRNA): Noncoding RNA that target specific mRNA for degradation or repression, regulate gene expression

Messenger RNA (mRNA): Carry genetic information to ribosomes for translation

Northern blotting: Technique used to detect RNA, used in study of gene expression

OA human chondrocyte (hOA-C): Human chondrocytes affected by osteoarthritis

Polysaccharides: Lengthy chains of monosaccharides, carbohydrates

Synovial fluid: Viscous and dilatant fluid, found in synovial joints, reduces friction and provides cushion

Western blotting: Used to detect specific proteins in homogenate sample

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