DISSERTATION

CULTURE-EXPANDED ARTICULAR CHONDROCYTES: A POTENTIAL CELLULAR THERAPEUTIC FOR OSTEOARTHRITIS WITH MSC-LIKE PROPERTIES

Submitted by

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ABSTRACT

CULTURE-EXPANDED ARTICULAR CHONDROCYTES: A POTENTIAL CELLULAR THERAPEUTIC FOR OSTEOARTHRITIS WITH MSC-LIKE PROPERTIES

Osteoarthritis (OA) is a highly prevalent and debilitating joint disease in horses, dogs, and humans. OA affects more than 303 million people globally with an annual economic loss to Americans approaching $200 billion. It has a considerable impact on the patient, resulting in pain and disability and more than 1 million people undergo knee arthroscopy or joint replacement surgery each year due to end-stage OA in the United States. Therefore, OA therapies that produce lasting symptom- and disease-modifying effects are a medical priority.

Mesenchymal stromal cells (MSCs) are considered ‘medicinal signaling cells’ that have been postulated to treat OA by reducing inflammation and restoring joint function. However, IA injection of MSCs into diseased human or companion animal joints has demonstrated only a modest benefit to date, as symptom-modifying effects are often temporary, and evidence of disease-modification has been minimal. It has been reported that culture-expanded chondrocytes (CECs) can assume many of the hallmark properties of MSCs, such as immunomodulation and immunophenotype. However, unlike MSCs, chondrocytes are known to thrive in suspension, which is important as IA injections release cells into synovial fluid.

The goal of this research aims to characterize the growth, immunomodulatory properties, and gene expression of equine CECs as a function of expansion *in vitro* as well as CEC persistence in the joint after intra-articular injection using a validated model of OA in rats. Additional goals of this research are to 1) determine how CECs may (persistence) or may not (immunomodulation and molecular fingerprint) differ from bone marrow derived MSCs, and 2) compare cellular properties of CECs across age to determine an ideal donor for generating allogeneic therapies.

The results shown in chapters 2 and 3 indicate that chondrocytes retain a strong propensity for immunomodulation, that increases with expansion and dedifferentiation does not coincide with other temporal changes in gene expression. Further, these data do not indicate a benefit of neonatal donors. Future *in vitro* studies should further characterize the immunomodulatory, redifferentiation (chondrogenic) and angiogenic potential of CECs. The preliminary results described in chapter 4 indicate that CECs may have greater persistence than MSCs in the first 3 days post IA injection. Future *in vivo* studies should focus on determining the symptom- and disease-modifying effects following IA injection of CECs in relevant preclinical models, such as the rodent, horse, and dog.

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CHAPTER 1: INTRODUCTION

1.1 Importance of osteoarthritis

"…from Hippocrates down to the present age, we shall find, that an ulcerated cartilage is universally allowed to be a troublesome disease…and that, when destroyed, it is never recovered." - William Hunter, anatomist, 1743[1]

Osteoarthritis (OA) is a debilitating joint disease that is commonly diagnosed in dogs,[2] horses[3] and humans.[4] The disease is hallmarked by loss of articular cartilage, formation of osteophytes, synovial hyperplasia, inflammation and thickening of subchondral bone.[5,6] These structural changes often result in decreased range of motion, pain, and even disability for the affected individual, contributing to a decreased quality of life.[7] Age[6,8] and obesity[9] are the two largest risk factors associated with OA. However, other identified risk factors include trauma to the joint, female sex, African American race, and physically demanding occupations that wear on the joint over time causing mechanical breakdown ("wear and tear").[10–13]

OA is often categorized as primary or secondary. Primary OA, which is often referred to as spontaneous, naturally-occuring, or idiopathic OA, has no known predisposing cause or event that incites OA pathology, but is related to the risk factors listed above.[14] This is in contrast to secondary or post-traumatic OA (PTOA) where a single event, such as traumatic injury to the joint, initiates the onset of disease.[12,13] Despite the different etiologies that lead to the onset of disease, the chronic changes

observed within the joint are similar between primary and secondary OA[12], suggesting that therapeutics to promote healing may be independent of etiology.

Unfortunately, there are currently no therapeutic regimens that successfully restore the damaged cartilage to its normal phenotype or slow the progression of joint destruction.[15] Therefore, treatments are designed to address symptoms, such as pain and immobility.[16] Pain management with non-steroidal anti-inflammatory drugs (NSAIDs) is common, but can result in a host of adverse effects in numerous organ systems, with gastrointestinal ulcers being common, and may not provide satisfactory pain relief.[17] Consequently, more than 1 million people undergo knee arthroscopy or joint replacement surgery each year due to end-stage OA in the United States.[18] The total annual economic loss to Americans associated with OA, including both direct and indirect costs, is approaching $200 billion.[19] Thus, more research is required to develop therapeutic options that restore damaged cartilage and heal the joint or prevent cartilage degradation, synovitis and osteophytes before pathologic changes occur.

1.2 Current understanding of OA pathogenesis

The pathogenesis of OA is complex and has not been fully elucidated. However, it has been reported that local and systemic inflammation, cartilage, subchondral bone and synovium all participate in OA pathogenesis (Fig 1).[20–22] Regarding pathogenesis, it has been reported that mechanical abrasions within the knee leads to a progressive loss of type I and type II collagen (COL1, COL2) in the meniscus[22] as well as inflammation occuring as a response to injury within the joint.[20] The progression of OA activates the innate immune system.[23] Chondrocytes, osteoblasts, and synoviocytes release cytokines, such as interleukin-1 (IL-1), IL-4, IL-9, IL-13 and tissue necrosis

factor alpha (TNFa), matrix-degrading enzymes, such as disintegrin and metalloproteinase thrombospondin-like motifs 4 (ADAMTS4) and ADAMTS5, and collagenases/matrix metalloproteinases (MMPs), such as MMP1,

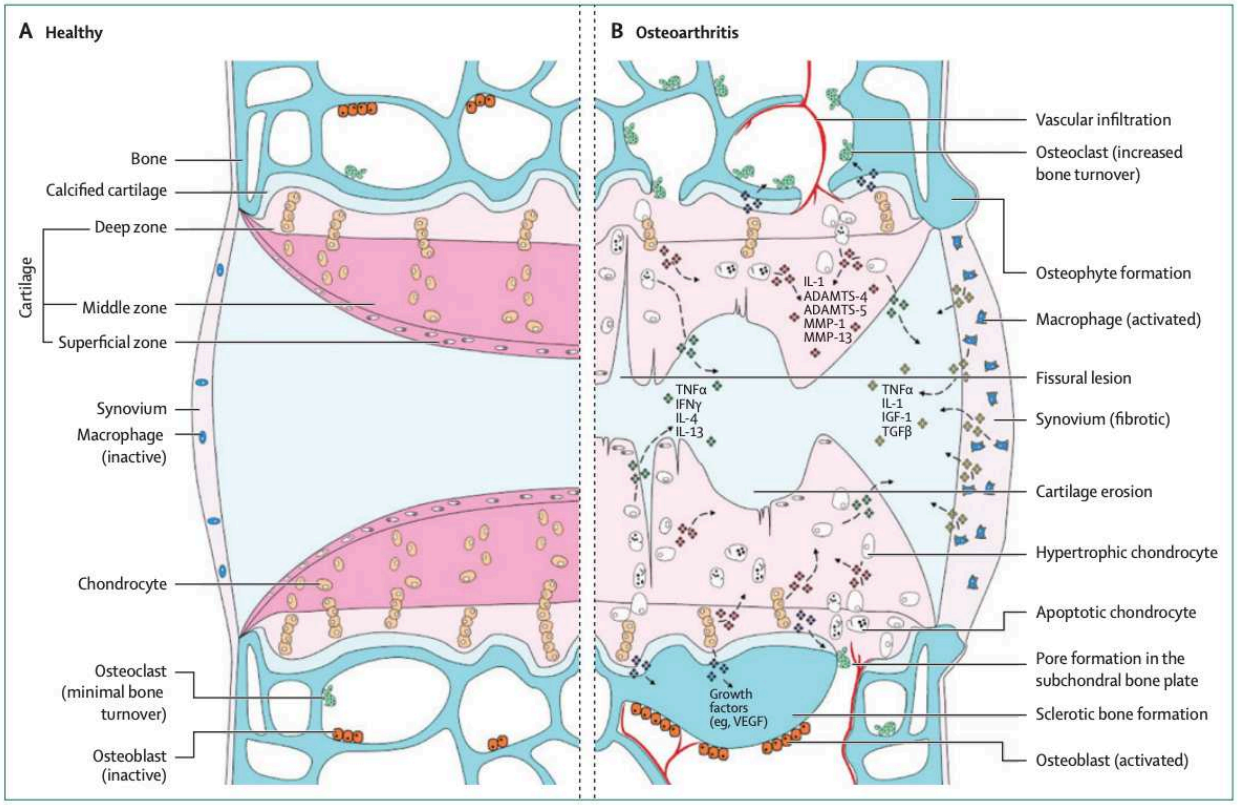

Figure 1.1. Signaling pathways and structural changes in the development of osteoarthritis. A) Healthy/normal joint. B) Osteoarthritic joint. ADAMTS: a disintegrin and metalloproteinase with thrombospondin-like motifs, IL: interleukin, MMP: matrix metalloproteinase, TNF: tumor necrosis factor, IFN: interferon, IGF: insulin-like growth factor, TGF: transforming growth factor, VEGF: vascular endothelial growth factor; taken from Glyn-Jones et al.[21]

MMP3 and MMP13,[21,22,24,25] into the local environment that degrades the joint tissues. While cartilage is experiencing collagen matrix degradation, chondrocytes also undergo hypertrophy and lose their ability to from new cartilage matrix.[24,26] Further, the release

of vascular endothelial growth factor (VEGF) by chondrocytes promotes angiogenesis of the synovium, contributing to more inflammation and chronic synovitis.[24,25,27,28] Additionally, the subchondral bone undergoes pathologic remodeling and invades past the interface between the bone and calcified cartilage, leading to subchondral cysts and osteophytes.[21] Due to the multifactorial nature of OA pathogenesis, a deeper understanding of the cross-talk between tissues within the microenvironment would aid in the progression of targeted therapeutic development.

1.3 Cellular therapies for treating OA

The need for new OA therapies is a medical priority, and there is great interest in the potential of cellular therapies to produce lasting symptom- and disease-modifying effects.[29] Mesenchymal stromal cells (MSCs) are thought to have a strong potential for treating OA as 'medicinal signaling cells'. In other words, MSCs do not rebuild damaged tissues themselves, but instead secrete factors that reduce inflammation and promote repair by endogenous cells.[30] In the context of OA, therapeutics with immunomodulatory properties would be expected to suppress local joint inflammation to slow the progression of disease.[20–22,31] Therefore, identifying cell sources that could modulate the local immune response is a priority and should be researched further.

In humans, interest in treating OA with IA injections of MSCs was first generated by Osiris Therapeutics when they reported disease-modifying effects in a goat partial meniscectomy model.[32] However, a systematic review by Ha et al., focusing on the treatment of OA with intra-articular (IA) injections with MSCs in 17 human studies concluded there was limited evidence for clinical outcomes, such as pain and function improvement, and cartilage repair.[15] In dogs, IA injections of MSCs have been reported

to be safe, with select studies indicating symptom-modifying effects, such as increased mobility, decreased pain scores, and increased daily activity.[33–37] However, improvement in joint function has been temporary[35–37] and regeneration of diseased joint tissues has not been reported.[38] Similarly, IA injection of equine MSCs have been reported to be safe; but, again, testing in experimental models of OA or cartilage defects have not shown strong evidence of efficacy.[39,40] Therefore, there is a critical need to improve upon the effectiveness of cell therapies for treating OA with IA injection.

1.4 Mesenchymal stromal cells (MSCs)

There are many types of stem cells, and they are often characterized by their ability to self-renew and differentiate along multiple lineages. Embryonic stem cells, which are derived from the inner cell mass of blastocysts, are considered pluripotent because they possess the ability to differentiate along the endodermal, mesodermal, and ectodermal lineages.[41] However, adult stem cells, such as mesenchymal stromal cells (MSCs) are only considered multipotent due to their trilineage differentiation potential[42,43] which consists of chondrogenesis, osteogenesis, and adipogenesis.

In the 1960's, A.J. Freidenstein discovered what we now call MSCs[44] where he isolated adherent, "fibroblast-like", clonogenic cells from bone marrow.[45] Since then, investigators have isolated MSCs from a variety of tissues with similar properties[45,46] and unfortunately, there are no universally accepted criteria to define MSCs. Therefore, the International Society for Cellular Therapy (ISCT) published a position statement in 2006 with the minimum criteria to define MSCs: 1) adherence to plastic in standard culture conditions, 2) >95% positive for CD105, CD73, and CD90 and <2% positive for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA class II or HLA-DR.[47]

Additionally, it's been hypothesized that all fibroblasts possess hallmark properties of MSCs[48–51] and this theory is supported by comparative studies in which culture-expanded cells from various connective tissues, such as cartilage, tendon, synovium and intervertebral disc, resemble MSCs. [49–52] Therefore, other cell types, such as chondrocytes, could possess unrecognized potential as 'medicinal signaling cells'.

1.5 Chondrocytes

Articular cartilage functions as a lubricating and load-bearing surface for joints.[53,54] Mature articular cartilage has a unique zonal architecture, with the different zones characterized by variations in matrix biochemical composition, gene expression, cell density, cell distribution, cell metabolism, and pericellular matrix (described in Fig 1.2).[1,53,54] Chondrocytes are the only cell type in articular cartilage, with type II collagen being the main structural protein, that works with a meshwork of other types of collagen and non-collagenous proteins, to provide cartilage with its compressive strength.[21] Chondrocytes strictly regulate the cartilage architecture and biochemical composition in response to changes in the chemical and biomechanical environment.[55]

1.6 Manufacturing allogeneic cellular therapies

Extensive culture expansion is critical for manufacturing allogeneic therapies because millions of doses, each consisting of millions of cells, can be produced from a single, ideal donor. Additionally, allogeneic therapies are appealing because they eliminate pain associated with autologous tissue harvest, simplify clinical logistics by creating an off-the-shelf therapy, create a commercial opportunity, and are more cost effective than autologous cells to embark on the expensive regulatory pathway.[56]

Zonal structure of mature articular cartilage

The superficial zone (tangential layer) consists of two to three layers of small, flattened chondrocytes arranged parallel to the surface that produce high quantities of a specific proteoglycan lubricant (proteoglycan 4, also known as lubricin). In the middle or transitional zone, chondrocytes are spherical; in the deep or radial zone, chondrocytes are enlarged and form columns perpendicular to the joint surface. In the middle and deep zones, the cartilage ECM is composed mainly of type II collagen, aggrecan and hyaluronan. Finally, in the calcified zone, chondrocytes are hypertrophic and embedded in a calcified ECM connected to the subchondral bone, separated from the uncalcified zone by a histologically distinct structure, the tidemark.

Abbreviation: ECM, extracellular matrix.

Figure 1.2. Zonal structure of mature articular cartilage; From Jiang and Tuan.[1]

Due to extensive regulatory testing, it has been postulated that 20 population doublings (PDs) would serve as a general guideline for the minimum MSC expansion required to produce allogeneic therapies.[57] It has been widely reported that MSCs can be extensively expanded to 30-70 PDs,[57–60] so there is great enthusiasm for MSCs are a potential cell source for allogeneic therapies. However, reports for extensive chondrocyte expansion are extremely limited. Kolettas et al., reported adult human culture-expanded chondrocytes (CECs) had a lifespan of ~35 PDs,[61] but it has not been characterized in other species. Therefore, it is critical that chondrocytes from various species be studied for their ability to undergo extensive expansion if CECs are to be considered for allogeneic cell therapies in preclinical and clinical animal models.

1.7 Donor age

Age should be considered when determining the ideal donor to develop an allogeneic therapy as age-dependent differences have been reported for chondrocytes.

For example, Tran-Khanh et al., reported that fetal (last trimester) and young (1.5 years old but skeletally mature) primary bovine chondrocytes behaved similarly when cultured in agarose, while aged (5-7 years) chondrocytes had 2x less proliferation, 20% less proteoglycan accumulation per cell, and 55% less collagen accumulation per cell.[62,63] When comparing human articular chondrocytes (donor age range: 1-87 years), it was reported that senescence-associated beta-galactosidase activity increased with age while mitotic activity and telomere length decreased with age,[64] indicating a younger donor may be ideal. Further, PD time was significantly lower (~2 days) for chondrocytes derived from polydactyly children (aged 0.5-3.5 years) compared to chondrocytes isolated from adults undergoing knee arthroscopy (aged 42-62 years; PD time ~4-8 days).[65] However, it should be noted that these cells were not extensively expanded, with the polydactyl chondrocytes going through ~5 PDs. To compare immunomodulatory differences across age, the MSC literature must be review.[66] Numerous reports indicate that birth-associated tissue MSCs, such as umbilical cord, cord blood and placenta, are more immunomodulatory[67–70] than bone marrow derived MSCs, while other studies report no difference.[71–73] Therefore, the effect of age should be considered when determining the ideal cell donor for allogeneic CEC therapies.

1.8 Progenitor-like properties of expanded chondrocytes

While chondrocytes exist in a near-quiescent state in mature cartilage, isolated chondrocytes that have undergone *in vitro* expansion have been shown to quickly lose their ability to produce cartilaginous extracellular matrix, a phenomenon referred to as dedifferentiation.[74] However, at the same time, it has been shown that CECs gain

properties associated with progenitor cells, such as gene expression, trilineage differentiation, immunophenotype and immunosuppression.[75–82]

Gene expression is commonly used to show chondrocyte dedifferentiation as COL2 is a marker of differentiated chondrocytes while COL1 is highly expressed by MSCs.[83–86] Therefore, a decrease in COL2 expression while gaining COL1 expression is considered a marker of chondrocyte dedifferentiation. Barlic et al., reported a 5-fold decrease in COL2 while COL1 increased 300-fold over 10 days of chondrocyte expansion.[85] Additionally, CECs have been shown to undergo osteogenesis, chondrogenesis, and adipogenesis,[75–77,80,81] which are hallmarks of MSCs.

Cluster of differentiation (CD) antigens, which are commonly associated with MSCs, have also been shown to change with chondrocyte expansion.[76–80] Interestingly, many of these markers, such as CD44, CD49e, CD151 are expressed within 24 hrs, while others, such as CD90, CD105 and CD166 are expressed within 14 days of being seeded into monolayer culture.[79,80] However, some CD markers, such as CD146, show more gradual increases with serial passaging.[77] Jiang et al., reported that increased CD146 expression in expanded human chondrocytes coincided with a loss of COL2,[77] suggesting that progenitor properties are adopted with expansion.

MSCs are immunosuppressive[31] but lack immunogenicity[87] and it has been reported that primary chondrocytes and chondrocytes that had undergone limited expansion possess similar immunomodulatory properties.[88–90] For example, Lohan et al., reported chondrocytes that had undergone limited expansion (1.8 PDs) suppressed stimulated lymphocyte proliferation similarly to MSCs when co-cultured *in vitro*.[89] Further, CECs with limited expansion suppressed lymphocyte activation and cytotoxic

granzyme B expression as well as modulated pro-inflammatory macrophage activity by reducing MHC-II expression and TNFa secretion.[91] However, future studies are required to determine the immunomodulatory properties of chondrocytes after extensive expansion.

1.9 MSC and chondrocyte survival in suspension

Following IA injection in experimental models, the vast majority of MSCs persist in the joint for only a period of days, with few cells engrafting into joint tissues.[32,92–95] The limited joint residence time reported may be due to an inherent inability of MSCs to survive in suspension. Anoikis is a process in which cells undergo apoptosis when they are unable to bind to the appropriate extracellular matrix[96] or cell culture substrate *in vitro*. Therefore, anoikis has been reported as a reason for poor MSC survival following implantation[97] and laboratory studies using suspension culture support this concept.[98–101] For example, decreases in cell viability have been reported for bone marrow MSCs that were suspended in hydrogels.[98–100] Furthermore, MSCs had poor viability when suspended in plasma or serum, despite cold storage in an effort to maintain viability.[101] Conversely, it has been recognized for decades that chondrocytes possess a unique ability to thrive in suspension. For example, hydrogel suspension cultures have been used extensively to study the biology of chondrocytes.[102–104] Therefore, CECs are inherently more likely to persist in the joint, overcoming a limitation of MSC therapies for treating OA.

1.10 Global hypothesis and rationale

Characterizing the properties of CECs, and how those properties change over time, is critical to recognize their potential as an allogeneic therapy for OA. The overarching hypothesis for this work is CECs possess MSC-like properties, such as immunomodulation and molecular fingerprint, but survive significantly longer in suspension, overcoming a limitation of MSC therapies for treating OA. Therefore, the research described in this dissertation aims to characterize the growth, immunomodulatory properties, and gene expression of CECs as a function of expansion *in vitro* as well as CEC persistence in the joint after intra-articular injection *in vivo*. Additional goals of this research were to determine how CECs may or may not differ from bone marrow derived MSCs, the current cell of choice for IA injection. Lastly, we aimed to compare adult and neonatal CECs to investigate how age effects their cellular properties as an ideal donor for generating allogeneic therapies has yet to be determined.

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CHAPTER 2: SUPPRESSION OF STIMULATED LYMPHOCYTE PROLIFERATION BY ADULT EQUINE CHONDROCYTES INCREASES WITH CULTURE EXPANSION

2.1 Summary

Chondrocytes may hold promise for treating diseased joints as they resemble mesenchymal stromal cells (MSCs) in their propensity for immunomodulation. Culture-expansion is an important aspect of generating cell therapies, although the extent to which chondrocytes retain immunomodulatory properties with expansion has not been reported. The objective of this study was to evaluate immunomodulation by chondrocytes with extensive expansion via suppression of proliferation of stimulated lymphocytes in a co-culture laboratory model. Chondrocytes were evaluated for expression of cluster of differentiation (CD) 146, which has been postulated to be a marker of therapeutic potency for MSCs. Chondrocytes were obtained from adult or neonatal equine cartilage, while MSCs were isolated from adult equine bone marrow. For adult chondrocytes, suppression of stimulated lymphocyte proliferation significantly increased between 12 and 19 population doublings (PDs) and did not further change through 33 PDs. Neonatal chondrocytes also experience an increase in suppression of stimulated lymphocyte proliferation with expansion, while immunomodulation by MSCs was unaffected by expansion. When evaluated after ~19 PDs, suppression of stimulated lymphocyte proliferation was not significantly different between adult and neonatal chondrocytes. CD146 was at least partially expressed by each group, although CD146 expression correlated with suppression of stimulated lymphocytes for adult chondrocytes only. In conclusion, chondrocytes retained a strong propensity for

immunomodulation with expansion that is sufficient for current recommendations for manufacturing MSC therapies. These data do not indicate a benefit of immature donors, and generally do not support the concept that CD146 expression indicates high immunomodulatory potency.

2.2 Introduction

Mesenchymal stromal cells (MSCs) secrete therapeutic cytokines that are thought to hold promise for treating damaged or diseased joints.[1] While commonly sourced from bone marrow, cells that possess MSC properties have been derived from many connective tissues, including articular cartilage.[2–11]Culture-expanded chondrocytes (CECs) have been shown to possess many hallmark properties of MSCs, such as expression of CD markers associated with MSCs and multipotent differentiation.[2,3,6–11] Further, like MSCs from bone marrow and other connective tissues, laboratory studies have demonstrated that CECs possess potent immunomodulatory properties,[12–15] which are believed to be significant mechanisms by which MSCs can lower inflammation in diseased joints.[16] These data suggest that CECs may be an effective cell source for treating joints.

As for all MSCs therapies, culture expansion is expected to be a critical aspect of generating treatments for diseased joints. For example, allogeneic MSC treatments requires extensive expansion to produce multiple doses of millions of cells from a single donor.[17] Likewise, autologous therapies require expansion to produce millions of cells from small cartilage biopsies.[18] CECs are anticipated to possess a tolerance for extensive expansion as indicated by a study demonstrating stable growth through greater than 20 population doublings (PDs),[19] which has been proposed as sufficient

growth to support MSC therapies.[17] However, the extent to which CECs acquire and retain immunomodulatory properties with extensive expansion has not been reported. Therefore, the objective of this study was to evaluate immunomodulation by CECs as a function of growth in expansion culture.

It is well-recognized that chondrocytes undergo a temporal dedifferentiation with proliferation in expansion culture.[2,20–22] Also, it has been proposed that MSC properties are associated with a fibroblast phenotype, which is a concept that has been supported by similarities between adipose and bone marrow MSCs as well as multiple sources of fibroblasts.[4,23] Taken together, we hypothesize that immunomodulatory properties of chondrocytes increase with expansion of primary cells. To test this hypothesis, we evaluated chondrocytes and MSCs from adult horses, a large animal species that is used as a preclinical model for cartilage repair and osteoarthritis.[24] Also, neonatal chondrocytes were evaluated based on their purported improved healing potential relative to adult cells.[25,26] Chondrocytes and MSCs were expanded using a protocol that was previously effective in promoting extensive expansion of MSCs[27] and connective tissue cells.[5,8,11]. Changes in phenotype were monitored by the expression of CD146, a commonly-used marker of MSCs[28] that has been reported to indicate therapeutic potency.[29] Immune properties were primarily measured via co-culture of CECs/MSCs in monolayer with stimulated or unstimulated lymphocyte.[13–15] Additionally, a novel approach to co-culture in which CECs or MSCs were maintained in suspension with lymphocytes was validated and used to compare immunomodulatory properties among all cell types.

2.3 Materials and Methods

Isolation of chondrocytes and MSCs. *Chondrocytes*: All culture medium contained 10 mM HEPES and 0.5% antibiotics/antimycotics solution (Thermo Fisher Scientific, Waltham, MA). Tissues were collected from 3-4-year-old mixed breed mares (n=5) and 1-day to 3-month-old foals (n=5) that were euthanized for reasons unrelated to this study. Articular cartilage was harvested from the talus or stifles and digested in alphaMEM basal medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.4% w/v pronase (EMD Millipore, Burlington, MA) for 60 mins, followed by an overnight incubation in 0.1% w/v type II collagenase (Sigma-Aldrich, St. Louis, MO). Isolated chondrocytes were filtered through a 40 μm cell strainer and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) and 5% DMSO (Thermo Fisher Scientific, Waltham, MA). *MSCs*: Bone marrow was harvested from the ilium of 3-5-year-old mixed breed mares (n=5) that were euthanized for reasons unrelated to this study. Nucleated cells were isolated via centrifugation and then seeded into low glucose DMEM (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS at $25x10^4$ cells/cm^2, as previously described.[27] At 24 hours, the medium was changed to expansion medium (alphaMEM supplemented 10% FBS and 2 ng/ml fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ).[11] Colonies of MSCs were collected after 7-10 days and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% FBS and 5% DMSO.

Expansion of chondrocytes and MSCs. Chondrocytes and MSCs were recovered from cryopreservation, seeded 20-$40x10^3$ cells/cm^2 overnight, and then reseeded at 500 cells/cm^2 in expansion medium. Most cultures were passaged every 5

days, although chondrocytes were first passaged after 3 and then 4 days due to areas that were locally confluent, as described in the results. Population doublings (PDs) were calculated after each passage.

Flow cytometry for analysis of CD146 expression. Cells were washed in FACS buffer (phosphate buffered saline with 2% FBS and 0.1% sodium azide), blocked for 20 mins at 4C with 5% normal equine serum in FACS buffers, and immunostained with the fluorochrome-conjugated antibody CD146:AF647 (clone OJ79c; Bio-Rad, Hercules, CA) for 30 mins. Additional cell aliquots were stained with an isotype-matched antibody, mouse IgG1 kappa isotype control:AF647 (Invitrogen, Carlsbad, CA) to serve as a control. Flow cytometry was performed using an Attune NxT (Invitrogen, Carlsbad, CA), and data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Lymphocyte proliferation in co-culture with CECs or MSCs. PMBCs were collected from horses that were not donors for cartilage or bone marrow. Peripheral blood was drawn into CPDA-anticoagulated whole blood bags (Jorgensen, Loveland, CO). PBMCs were isolated by separation over a Ficoll gradient (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions and cryopreserved at $15x10^6$ cells/ml in 95% fetal bovine serum and 5% DMSO. *Monolayer* - PBMCs were recovered for 2 hours in alphaMEM supplemented with 10% FBS. 0.2×10^6 PBMCs were seeded into wells of 96 well plates that contained near-confluent monolayer cultures of MSCs or CECs, at ratios of 1:20 or 1:40. Cultures were maintained in the presence or absence of 5 ug/ml Concanavalin A (ConA; Sigma-Aldrich, St. Louis, MO) for 96 hours. After 72 hours, 3uM of 5-ethynyl-2'-deoxyuridine (EdU) was added to label proliferating cells. At 96 hours, PBMCs were collected and stained with LIVE/DEAD

Fixable Violet (Thermo Fisher Scientific, Waltham, MA) and then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), permeabilized with saponin-based reagent, and stained for equine CD3:FITC (clone CD3-12; Bio-Rad, Hercules, CA) and Alexa Fluor 647 to detect incorporated EdU. T cell proliferation was measured as the percent AF647-positive within the live, CD3+ population of cells. The T cell proliferation index was calculated as the proliferation percentage normalized to the value of stimulated PBMCs alone, which was assigned a value of 1.[30] *Suspension* - Surfaces of a 96-well plate were coated with 80ul of 50mg/ml polyHEME (Sigma-Aldrich, St. Louis, MO) to inhibit cell adhesion. One percent methylcellulose was added to co-culture medium to increase the viscosity and reduce large cell clustering in suspension.[31] PBMCs were mixed with CECs or MSCs at a ratio of 1:20. Cultures were maintained and analyzed as described above.

Statistical analysis. Statistical tests were performed using R software (version 4.0.5). CD146 expression and lymphocyte proliferation data were analyzed using a linear mixed effects model with time in culture as the fixed effect and donor as the random effect with a Tukey adjustment for multiple pairwise comparisons. When comparing two groups, t-tests were performed. To determine the association of CD146 expression with lymphocyte proliferation, a linear mixed model was fitted with lymphocyte proliferation as the response variable, CD146 expression and cell type as fixed effects, with their interaction included. Additionally, donor was utilized as a nested random effect to account for homogeneity of donors. A Kenward-Roger degrees-of-freedom method was used. Packages lme4, nlme, lmerTest, and emmeans were used to complete these analyses. P-values and confidence intervals at a significance level of

0.05 were considered statistically significant. Data are presented as mean ± standard error.

2.4 Results

Expansion in monolayer cultures. *Chondrocyte growth patterns and morphology*: Primary cultures of adult and neonatal chondrocytes proliferated in close proximity to form locally confluent colonies by day 3 that did not disperse across the growth surface (Fig 2.1A).

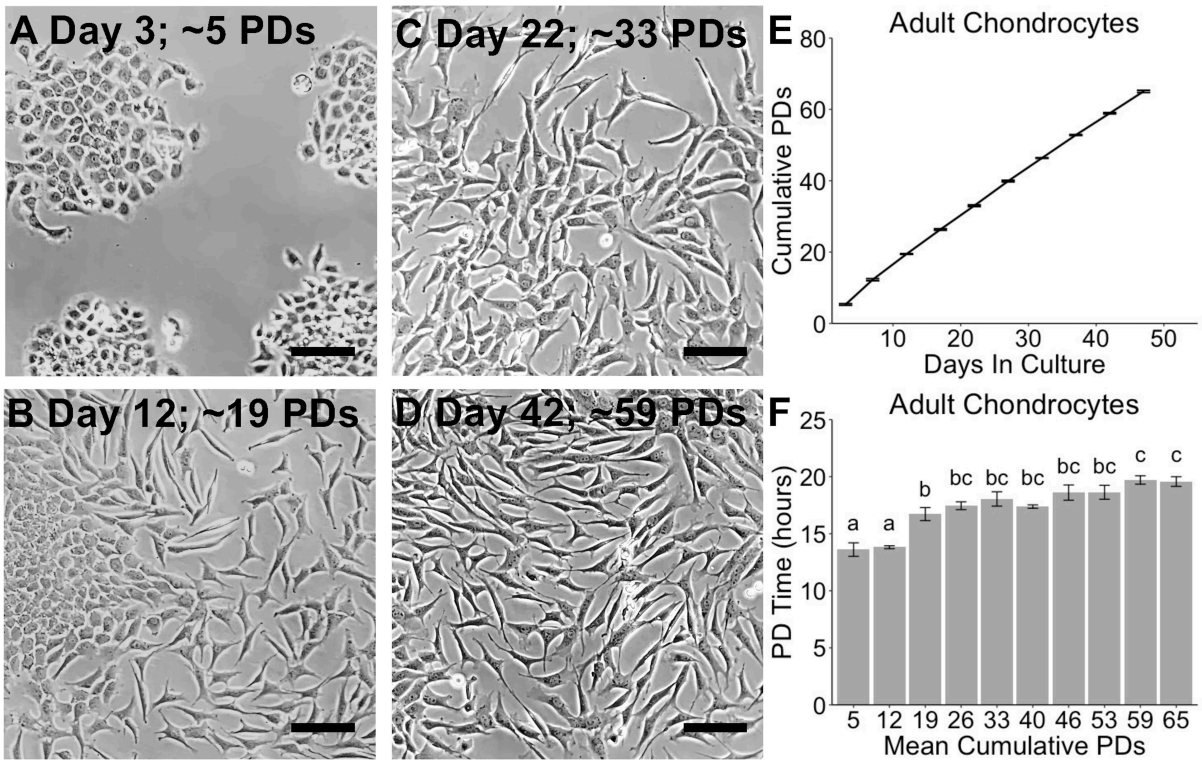

Figure 2.1. Expansion timecourse for adult equine chondrocytes. A-D) Representative images of adult equine chondrocytes over 47 days. Bar = 100 μm. E) Cumulative population doublings. F) Population doubling time with cumulative growth. N=5 donors for days 0-22, and n=3 donors for days 22-47. Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denotes statistically significant differences (P<0.05).

Therefore, cultures were passaged at this point. A similar pattern of clustered growth was observed for the first subculture, and the cells were passaged after 4 days (day 7). In subsequent subcultures, the propensity of chondrocytes to proliferate in clusters decreased, concomitant with a shift in cell morphology to a fibroblastic, spindle-shape that remained until the end of the time course (Fig 2.1B-D). At this point, cultures were passaged after 5 days (day 12), and subsequently every 5th day.

Growth kinetics: *Adult chondrocytes* – Primary chondrocytes (n=5 donors) were expanded for 22 days, which resulted in mean cumulative growth of 33.0 PDs (Fig 2.1E). Over the first 12 PDs, corresponding to the first two passages, mean population doubling times (PDTs) were 13.6-13.8 hrs (Fig 2.1F). With subsequent expansion, the mean PDT significantly increased to 16.7 hrs at 19 PDs ($P<0.05$; Fig 2.1F), and did not further change through 33 PDs. Cells from 3 donors were maintained in expansion culture for 47 days, resulting in a mean cumulative PD total of 65.1 (Fig 2.1E). The mean PDT did not significantly change between 33 and 65 PDs ($P<0.05$; Fig 2.1F). *Neonatal chondrocytes* – Primary chondrocytes (n=5 donors) were expanded for 22 days, resulting in mean cumulative growth of 32.7 +/- 0.4 PDs and PDT of 16.1 +/- 1.1 hours (data not shown). *Adult MSCs* – MSCs isolated from colony-forming cultures (n=5 donors) were expanded for 25 days, and were passaged every 5th day. Over this period the mean cumulative growth was 31.0 +/- 0.7 PDs and PDT of 19.5 +/- 0.8 hrs (data not shown).

CD146 expression. *Adult chondrocytes* – Adult chondrocytes were evaluated for CD146 expression at each passage in Fig. 2.2A (n=5 donors for 0-33 PDs, n=3 donors for 33-65 PDs).

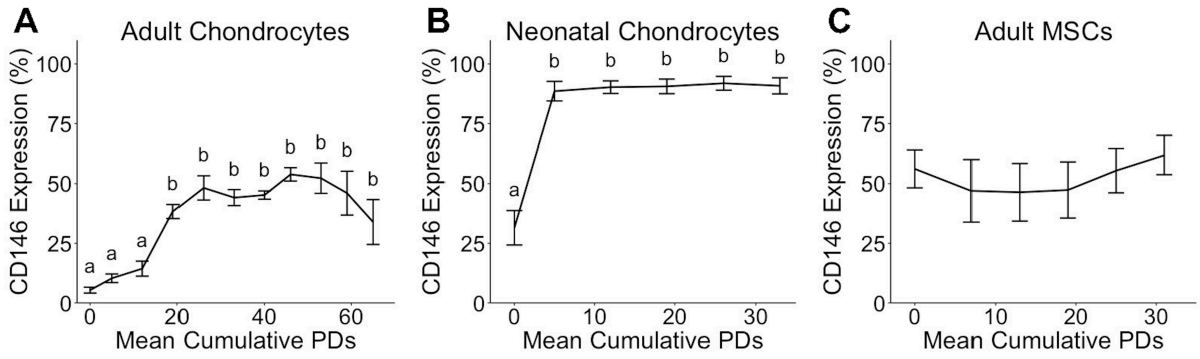

Figure 2.2. CD146 expression of adult chondrocytes (A, n=5 donors for days 0-22, and n=3 donors for days 22-47), neonatal chondrocytes (B, n=5 donors) and adult MSCs (C, n=5 donors) with cumulative growth. Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denotes statistically significant differences (P<0.05).

Mean CD146 expression for primary (P0) cells was 5%, and did not significantly change after 5 (10%, P=0.99) or 12 (14%, P=0.71) PDs. By 19 PDs, CD146 expression significantly increased to 38% (P<0.05). There were no significant changes in CD146 expression for the remainder of the timecourse (P=0.14-1.00) with mean values ranging between 34-54%. *Neonatal* chondrocytes - Neonatal chondrocytes (n=5 donors) were evaluated at each passage over the 22 day expansion timecourse. Primary chondrocytes were 32% CD146 positive, with expression significantly increasing to 89% by 5 PDs (P<0.05) and did not significantly change through the remainder of the timecourse (P=0.99-1 (Fig 2.2B). *Adult bone marrow MSCs* - MSCs (n=5) donors were evaluated at each passage of the 25 day expansion timecourse, corresponding to ~31 PDs . Mean CD146 expression ranged between 46-62% and did not significantly change among expansion timepoints (P=0.29-1.00; Fig 2.2C). Compared to CECs, variability in CD146 among donors was relatively high for MSCs. For examples, the

difference between maximum and minimum values was 66% (12-75%) at the first passage, while the largest difference between maximum and minimum values for CECs at a given timepoint was 25%.

Lymphocyte proliferation. <u>*Stimulated monolayer culture*</u>: *Adult chondrocytes* - CECs (n=5 donors) were evaluated over five passages corresponding to 5-33 PDs. Experiments were repeated for 3 PBMC donors at 1:20 CEC:PBMC. CECs significantly reduced lymphocyte proliferation by at least 50% at all timepoints ($P<0.05$). Lymphocyte proliferation was highest at 5 PDs and 12 PDs, averaging 46% and 39% of PBMC-only controls, respectively (Fig 2.3A). With subsequent expansion, lymphocyte proliferation significantly decreased to 19-23% of controls ($P<0.05$) and was not significantly different between 19-33 PDs ($P=0.95$-1.00; Fig 2.3A). (*Neonatal chondrocytes* - neCECs (n=3 donors) were evaluated over three passages corresponding to 5, 12, and 19 PDs.

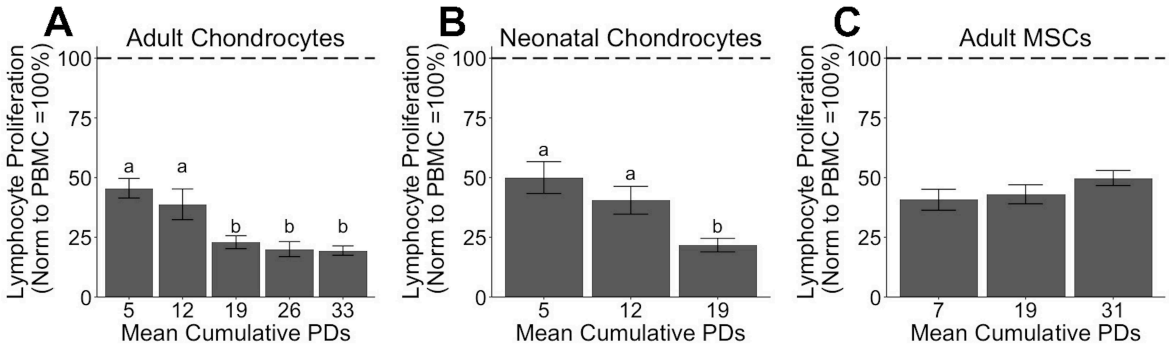

Figure 2.3. Proliferation of stimulated lymphocytes in co-culture with chondrocytes or MSC in monolayer. Each cell type was evaluated as a function of growth in expansion culture. A) Adult chondrocytes (n=5 donors). B) Neonatal chondrocytes (n=3 donors). C) Adult MSCs (n=5 donors). Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denotes statistically significant differences ($P<0.05$).

Experiments were repeated for 2 PBMC donors at 1:20 neCEC:PBMCs. Stimulated lymphocyte proliferation was not significantly different after 5 PDs and 12 PDs (50% and 41% of controls, respectively, P=0.08, Fig 2.3B). With subsequent expansion, lymphocyte proliferation significantly decreased to 22% of controls at 19 PDs ($P<0.05$; Fig 2.3B). *Adult MSCs* – MSCs (n= 5 donors) were evaluated over three passages corresponding to 7, 19, and 31 PDs. Experiments were repeated for 2 PBMC donors. In preliminary studies seeding of MSCs at the same concentration as CECs (10,000/well) resulted in contraction of the cell layer with 1-3 days, which is problematic as lymphocyte contact is an important aspect of immunomodulation.[32,33] To address this issue, the seeding density of MSCs was reduced 2-fold, resulting in a MSC:PBMC ratio of 1:40. MSCs suppressed stimulated lymphocyte proliferation by 41-50% of controls, and did not significantly change as a function of cumulative growth (P=0.18-0.89, Fig 2.3C).

Unstimulated monolayer culture: For adult chondrocytes (5-33 PDs), lymphocyte proliferation ranged from 0.55% +/- 0.21% to 1.65% +/- 0.51%, was not significantly different than PBMC-only controls (1.95% +/- 0.90%) for all expansion timepoints (P = 0.25-1), and did not change as a function of cumulative growth (P = 0.16-1; data not shown). For neCECs, lymphocyte proliferation ranged from 0.94% +/- 0.16% to 1.27% +/- 0.23% and did not significantly change with cumulative growth (P = 0.65-1, data not shown). For MSCs, lymphocyte proliferation ranged from 1.73% +/- 0.35% to 2.91% +/- 0.59% and did not significantly change with cumulative growth (P = 0.051-1, data not shown).

Comparison of cell types: – Monolayer: Adult and neonatal chondrocytes (n=3 donors/cell type) that that had been expanded through 19 PDs were evaluated in parallel using 3 PBMC donors. Lymphocyte proliferation was not significantly different between adult and neonatal chondrocytes (P=0.10; Fig 2.4A). Suspension: The three cell types were compared in suspension given that stable monolayer cultures could

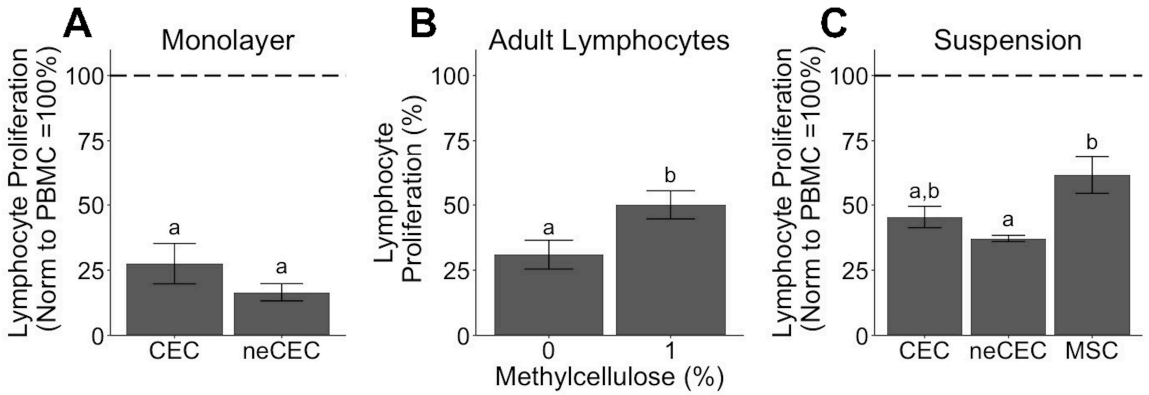

Figure 2.4. Comparison of cell types in stimulated lymphocyte co-culture. A) Monolayer cultures of adult and neonatal chondrocytes. Chondrocytes were evaluated after expansion through ~19 PDs. N=3 donors for each cell type. Suspension cultures – (B) Stimulated lymphocyte proliferation in the absence or presence of 1% methylcellulose using 3 PBMC donors, (C) Comparison of adult and neonatal chondrocytes and MSCs in suspension culture containing 1% methylcellulose. MSCs were evaluated after expansion through 12 PDs. Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denotes statistically significant differences (P<0.05).

not be established with the same cell number, as described above for MSCs. *Validation of control cultures*: Using unstimulated and stimulated PBMCs only (n=3 donors), suspension cultures were compared to monolayer. Unstimulated lymphocyte proliferation in suspension (4.2 +/- 0.6%) was higher than proliferation in monolayer (1.9 +/- 0.4%, P<0.05, data not shown). Stimulated lymphocyte proliferation in suspension

(50% of the total population) was moderately higher than proliferation in monolayer conditions (31% $P<0.05$; Fig 2.4B). These data demonstrated that suspension culture did not greatly alter proliferation of lymphocytes. *Stimulated co-culture*: Experiments were conducted using 3 donors per cell type and 3 PBMC donors. CECs were evaluated after ~19 PDs to capture their maximum immunomodulatory potency. CECs were compared to MSCs that had been expanded through 12 PDs, which approximately replicates the total growth of CECs given that MSCs undergo proliferation as part of primary colony-forming cultures. Over 4 days, chondrocytes and MSCs formed small clusters that appeared grossly similar in size, while lymphocytes appeared to associate with the cell clusters (data not shown). CECs suppressed lymphocyte proliferation by 45%, which was not significantly different than MSCs (62%; $P=0.09$) or neCECs (37%; $P=0.43$; Fig 2.4C). Suppression of lymphocyte proliferation was significantly greater for neCECs compared to MSCs ($P<0.05$).

Correlation of CD146 expression and immunomodulation. The main effects of CD146 expression and cell type were not statistically significant. However, interactions between CD146 expression and cell type had a significant effect on lymphocyte proliferation. To interpret the association of CD146 expression with lymphocyte proliferation, the model output for each individual cell type was considered. For adult CECs, the model estimated a statistically significant negative effect of CD146 expression on lymphocyte proliferation (C.I. = -0.54 +/- 0.13; Fig 2.5). Conversely, MSCs showed a nonsignificant negative effect of CD146 expression on lymphocyte proliferation (C.I. = -0.10 +/- 0.12) while neCECs showed a nonsignificant positive effect (C.I. = 0.28 +/- 0.48; Fig 2.5).

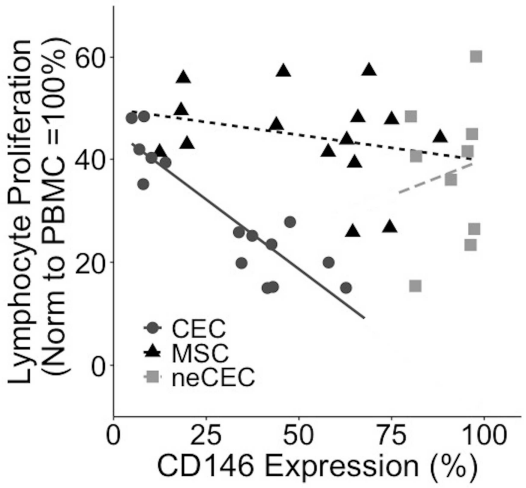

Figure 2.5. Correlation of CD146 expression and stimulated lymphocyte proliferation. Only adult chondrocytes had a statistically significant estimate for the slope of CD146 expression. Points indicate raw data from Figs. 1 and 2 while lines indicate prediction.

2.5 Discussion

The primary objective of this study was to characterize immunomodulation by CECs with expansion that extends beyond existing studies[13,14] and surpasses growth that has been proposed as necessary for MSC therapies.[17] As a first step, we evaluated the ability of adult equine chondrocytes to proliferate for nearly 50 days in expansion culture. Using a culture strategy that has proven effective for expansion of bone marrow MSCs[5] as well as cells from different connective tissues,[5,11] adult equine chondrocytes proliferated through 65 PDs with only minor decreases in population doubling time. Neonatal chondrocytes resembled adult as proliferation was robust through 33 PDs. This degree of expansion greatly exceeds many studies that have analyzed expansion of chondrocytes, which have been largely motivated by more modest expansion objectives (<10 PDs) for autologous cartilage tissue engineering.[18] However, our data is

consistent with Kolletas et. al., who reported expansion of human articular chondrocytes up to 37 PDs.[19] Further, tolerance for extensive expansion is consistent with MSCs.[17,34] Therefore, our data supports the potential to expand large numbers of CECs that are necessary for MSC therapies.

For the use of CD expression as a marker of the MSCs phenotype, it is important to note that the panel recommended by the International Society for Cellular Therapy[35] was not evaluated. This decision was based on previous studies indicating that recommended MSC markers, such as CD105 and CD44, can be expressed by primary chondrocytes within 24 hours of isolation and expansion,[2] when very little dedifferentiation has occurred. Conversely, Jiang et. al. reported that chondrocyte dedifferentiation coincided with increasing CD146 expression for human cells.[8] Further, conventional markers have not correlated with therapeutic potency as has been proposed for CD146.[29] Here, adult chondrocytes acquired partial CD146 expression with expansion, which has been previously reported for human chondrocytes,[8,36,37] and is generally consistent with equine MSCs in this study as well as previous reports for horse[38] and human[39] cells. Relatively high and consistent levels of CD146 expression by neonatal chondrocytes is generally consistent with fetal bone marrow MSCs, placental stromal cells, liver fibroblasts,[40,41] which suggests that aging may be associated with a decreased propensity for CD146 expression.

Historically, unstimulated lymphocytes have been reported to undergo modest proliferation *in vitro,* and studies involving co-culture with MSCs[42] or chondrocytes[13] report no to modest lymphocyte proliferation. These findings are consistent with the current study where proliferation of unstimulated lymphocytes was low and not

significantly different from co-cultures. For stimulated cultures, suppression of lymphocyte proliferation of at least 50% in co-cultures here is consistent with previous reports evaluating adult and neonatal chondrocytes from multiple species.[12–15,43] Additionally, suppression of lymphocyte proliferation by MSCs was consistent with previous reports for human[42] and equine[44,45] MSCs. Therefore, the effects of co-culture here is generally consistent with existing data across different species and cell types.

To characterize lymphocyte proliferation when co-cultured with adult CECs, we limited our analysis to 33 PDs (corresponding to 5 passages) based on the assumption that a ~10-billion-fold increase in cell numbers would be conservatively sufficient to meet the needs for both autologous and allogeneic therapies. With expansion, the statistically significant and substantial decrease in lymphocyte proliferation (>50%) indicated that growth through at least 19 PDs maximizes the immunomodulatory properties of adult chondrocytes. Also, the improved propensity for immunomodulation appears to be independent of age as neCECs demonstrated a similar reduction in stimulated lymphocyte proliferation at 19 PDs. While extensive expansion was favorable for immunomodulation, it should be noted that CECs with minimal expansion (5 PDs) were still capable of suppressing stimulated lymphocyte proliferation. These data are consistent with previous studies in which rat or human (1.8 PDs) CECs demonstrated robust immunomodulation with limited expansion.[13,14]

To compare lymphocyte proliferation between cell types, it is important that the ratio of MSCs or CECs to lymphocytes is the same as it has been shown that immunomodulation is strongly influenced by this parameter[12,14,15,32,43,44] Given the difficulty in establishing comparable monolayer cultures for CECs and MSCs, we

validated the use of a suspension culture system The propensity of CECs and MSCs to form small clusters is consistent with previous studies using MSCs,[31] and lymphocytes appeared to associate with the cell clusters in a manner that resembles MSCs in monolayer.[46] For adult CECs and MSCs, the lack of significant difference in lymphocyte proliferation is consistent with numerous monolayer studies that have reported comparable immunomodulatory properties between these cell types.[12–14] For CECs, stimulated lymphocyte proliferation was not significantly different between adult and neonatal donors, which is consistent with monolayer cultures (Fig 2.4A). Further, these results are consistent with comparative studies using MSCs from adult and birth-associated tissues that do not show a clear pattern of differential immunomodulatory potency with aging.[47] The significant difference in stimulated lymphocyte proliferation between neonatal CECs and adult MSCs suggest the potential for a differential propensity for immunomodulation with cell type and age, although additional data are needed to better elucidate the effect of each factor.

Given the hypothesis that CD146 expression is a marker for therapeutic potency,[29] we investigated this relationship by comparing CD146 expression with modulation of stimulated lymphocyte proliferation in three cell types over time. A linear mixed model indicated that this association was significant for adult CECs because the statistically significant increase in CD146 expression at 19 PDs coincided with a marked decrease in stimulated lymphocyte proliferation. However, associations were not statistically significant for adult MSCs or neonatal CECs. It should be noted for MSCs that while neither CD146 expression nor suppression of stimulated lymphocyte proliferation changed with expansion as a group, there was a particularly high degree of

variability in CD146 expression among donors, ranging between 12.4-75.0% at each timepoint. However, as seen in Fig 2.5, these differences in CD146 expression between individual donors did not correlate to differences in stimulated lymphocyte proliferation during co-culture, resulting in a weakly negative association that lacked statistical significance. Additionally, neonatal CECs were at least 88.6% CD146 positive at all timepoints, but still experienced a significant decrease in stimulated lymphocyte proliferation at 19 PDs, resulting in a weakly positive association that lacked statistical significance. Taken together, these data do not broadly support the concept that CD146 is indicative of immunomodulatory potency, although age and cell type may be important variables for this association.

From these data we conclude that adult and neonatal chondrocytes possess a strong tolerance for extensive expansion, and that a moderate degree of growth enhances immunomodulatory potency. Further, because adult and neonatal chondrocytes have similar suppressive effects on lymphocyte proliferation, both age groups can be considered as sources for MSC therapies. For bone marrow MSCs, robust suppression of lymphocyte proliferation up to 31 PDs suggests that the recommendation of expansion to 20 PDs for generating cell therapies[17] may be conservative for applications in which immunomodulation is prioritized. There are several limitations to this study. While lymphocyte co-culture has been commonly used to characterize immunomodulation, additional assays such as macrophage polarization[48,49] and analysis of secretome[50] can provide a more complete understanding of immunomodulatory potency. Also, it's possible that with these additional assays, a more robust association of CD146 and immunomodulation may be

identified. Cells from horses were evaluated based on their relevance for preclinical models[24] and availability of neonatal tissues. However, comparative studies are needed to determine how well horse cells reflect the behaviors of human chondrocytes and MSCs. Finally, while comparative studies often result in similar MSC properties among tissue sources, a greater understanding of how CECs may differ from MSCs is needed to identify whether CECs may or may not be favorable for different applications.

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CHAPTER 3: CHARACTERIZATION OF miRNA and mRNA OF CULTURE-EXPANDED CHONDROCYTES OVER TIME WITH COMPARISON TO MESENCHYMAL STROMAL CELLS

3.1 Summary

Chondrocytes, the only cell type in cartilage, exist in a near-quiescent state *in vivo*. However, it has been shown that isolated chondrocytes that have undergone *in vitro* expansion quickly lose their ability to produce cartilaginous extracellular matrix, a phenomenon referred to as dedifferentiation. However, at the same time, it has been shown that culture-expanded chondrocytes (CECs) gain properties associated with mesenchymal stromal cells (MSCs), such immunophenotype, immunosuppression, and trilineage differentiation. Therefore, it is believed that CECs hold promise as a cellular therapy for regenerative medicine. However, when CECs transition from a chondrocyte to an MSC-like phenotype has not been fully characterized and gene expression analysis serves as a starting off point to understand this temporal change. CECs would most likely be used as an allogeneic therapy, which requires extensive expansion to create millions of treatments from a single donor. This is important to maintain uniformity between lots as well as minimize costs and the ideal donor has yet to be determined. However, neonatal chondrocytes are considered more metabolically active and thought to possess superior healing potential. Therefore, we hypothesize that neonatal CECs will have greater changes in gene expression over time compared to adults. The objective of this study was to compare equine adult to neonatal CECs for

changes in gene expression over time and to determine if these changes were significantly different from MSCs.

3.2 Introduction

While chondrocytes exist in a near-quiescent state in mature cartilage, isolated chondrocytes that have undergone *in vitro* expansion have been shown to quickly lose their ability to produce cartilaginous extracellular matrix, a phenomenon referred to as dedifferentiation.[1] However, at the same time, it has been shown that CECs gain properties associated with progenitor cells, such as gene expression, trilineage differentiation, immunophenotype and immunosuppression.[2–9] For example, MSCs are immunosuppressive[10] but lack immunogenicity[11] and it has been reported that chondrocytes that had undergone limited expansion possess similar immunomodulatory properties.[12–14] Lohan et al., reported chondrocytes that had undergone ~2 population doublings (PDs) suppressed stimulated lymphocyte proliferation similarly to MSCs when co-cultured *in vitro*.[13] Data presented in Ch 2 agree with these findings. Additionally, cluster of differentiation (CD) antigens, which are commonly associated with MSCs, have also been shown to change with chondrocyte expansion.[3–7] For example, it has been reported that CD146 expression resulted in a gradual increases with serial passaging.[Ch1,4] Jiang et al., reported that increased CD146 expression in expanded human chondrocytes coincided with a loss of COL2,[4] suggesting that the adoption of progenitor properties is associated with dedifferentiation.

Gene expression is commonly used to show chondrocyte dedifferentiation as type II collagen (COL2) is a marker of differentiated chondrocytes while type I collagen (COL1) is expressed by MSCs.[15–18] Therefore, a decrease in COL2 expression while

gaining COL1 expression is considered a marker of chondrocyte dedifferentiation. Additional growth factors associated with chondrocytes and MSCs will be analyzed to observe if a change in expression coincides with dedifferentiation. For example, SRY-box transcription factor 9 (SOX9) has been reported as the master regulator of chondrogenesis. Simental-Mendia et. al, reported that transgenic overexpression of SOX9 in articular chondrocytes resulted in increased levels of COL2 expression, decreased COL1 expression and contributed to cartilage deposition.[19] Similarly, the transforming growth factor beta (TGFB) family of growth factors play an important role in cartilage formation and maintenance,[20] and are more highly expressed in chondrocytes than MSCs, however these mechanisms are poorly understood. Lastly, vascular endothelial growth factor (VEGF) is known to be secreted by MSCs, but is detrimental in articular cartilage.[21,22] These growth factors could be analyzed as biomarkers to delineate the dedifferentiation of chondrocytes, differentiate between MSCs and chondrocytes, and may also serve a role in tissue regeneration for chondrogenesis.

MicroRNAs are short (17-24 nucleotide), single stranded, non-coding RNAs that regulate gene expression through mRNA degradation and post-transcriptional silencing. This genetic regulation has an important role in many biological processes, including but not limited to: cell differentiation, cell development, cell growth, and cell survival.[23,24] The miRNA expression profile of cells have been shown to regulate their phenotype and changes in expression can lead to changes in function.[25,26]

Among miRNAs regulating chondrogenesis, miR-140 is one of the more highly studied miRNAs. miR-140 maintains chondrocyte proliferation[27] and has been shown to have an effect on expression of Ras-related protein Ral-A (RALA).[28,29] Levels of miR-

140 are upregulated in chondrogenesis and chondrogenic differentiation of MSCs.[29] Alternatively, miRNAs can also negatively affect chondrogenesis. It has been reported that miR-29a, which is highly expressed in MSCs, decreased chondrogenesis by suppressing the expression of SOX9, aggrecan (ACAN), and COL2.[29] As an initial screening tool, miRNAs that were reported to be highly expressed by MSCs or chondrocytes were analyzed.

If CECs are to be considered as an allogeneic cellular therapy, extensive expansion is required to create numerous treatments from a single donor. Ch 2 established that equine CECs are capable of extensive expansion, which is important to maintain uniformity as well as minimize costs. Using an allogeneic approach means any donor could be considered. Neonatal chondrocytes are considered more metabolically active and thought to possess superior healing potential.[30,31] Before large preclinical trials are initiated, the ideal donor must be determined. Therefore, we hypothesized that neonatal CECs will have greater changes in gene expression over time compared to adult CECs. Additionally, we hypothesized that dedifferentiation would coincide with changes in miRNA and growth factor gene expression. The objective of this study was to compare equine adult CECs to neonatal CECs for changes in gene expression over time and to determine if these changes were significantly different from MSCs.

3.3 Materials and Methods

Isolation of chondrocytes and MSCs. *Chondrocytes*: All culture medium contained 10 mM HEPES and 0.5% antibiotics/antimycotics solution; all from Thermo Fisher Scientific, Waltham, MA). Tissues were collected from 3-4-year-old mixed breed

mares (n=5) and 1-day to 3-month-old foals (n=5) that were euthanized for reasons unrelated to this study. Articular cartilage was harvested from the talus and stifles and digested in alphaMEM culture medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.4% w/v pronase (EMD Millipore, Burlington, MA) for 60 mins, followed by an overnight incubation in 0.1% w/v type II collagenase (Sigma-Aldrich, St. Louis, MO). Isolated chondrocytes were filtered through a 40 μm cell strainer and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) and 5% DMSO (Thermo Fisher Scientific, Waltham, MA). *MSCs*: Bone marrow was harvested from the ilium of 3-5-year-old mixed breed mares (n=5) that were euthanized for reasons unrelated to this study. Nucleated cells were isolated via centrifugation and then seeded into low glucose DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% FBS at $25x10^4$ cells/cm^2, as previously described.[32] At 24 hours, the medium was changed to expansion medium (alphaMEM supplemented 10% FBS and 2 ng/ml fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ).[9] Colonies of MSCs were collected after 7-10 days and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% FBS and 5% DMSO.[33]

Expansion of chondrocytes and MSCs. Chondrocytes and MSCs were recovered from cryopreservation, seeded into expansion medium at $20\text{-}40x10^3$ cells/cm^2 overnight, and then reseeded at 500 cells/cm^2 in expansion medium. Most cultures were passaged every 5 days, although chondrocytes were first passaged after 3 and then 4 days due to areas that were locally confluent, as previously described (Ch 1). CEC cultures we maintained for 22 days, corresponding to 5 passages, while MSC cultures were maintained for 15 days, corresponding to 3 passages. At each passage,

$5x10^5$ CECs (n=4/cell type) were washed with PBS, pelleted via centrifugation at 200 xg for 5 min, snap frozen in liquid nitrogen and stored at -80C until RNA extraction.

RNA extraction and quantification. Frozen cell pellets were homogenized in 700ul Qiazol Lysis Reagent (Qiagen, Germantown, MD) and allowed to sit at room temperature for 5 mins. Chloroform (140ul) was added to the homogenate, shaken vigorously for 15 sec, and left at room temperature for 3 min. Samples were centrifuged at 12,000 xg for 15 min at 4°C, separating the sample into 3 distinct layers (RNA phase, DNA phase, and protein phase). The top aqueous phase containing RNA was transferred to a new 1.7-mL tube. miRNA and mRNA were isolated using the miRNeasy Micro Kit (Qiagen, Germantown, MD) according to the manufacturer's recommendations. RNA purity and quantification were assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Samples were used for PCR if they had 260:280 nm and 260:230 nm values above 1.7. Total RNA was processed for reverse transcription using the miScript II RT Kit (Qiagen, Germantown, MD) for miRNA or iScript cDNA Synthesis (Bio-Rad, Hercules, CA) for mRNA. One microgram of total RNA was added to each reverse transcription reaction and performed following the manufacturer's specifications. Briefly, reverse transcription for miRNA used 60 min at 37°C, 5 min at 95°C, while mRNA used 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and then holding at 4°C until cDNA was stored at -20C for future use in semi-quantitative real time PCR (qRT-PCR).

Primer design. Equine transcript sequences were acquired from Ensembl (https://uswest.ensembl.org/index.html). Equine specific forward primers were used for

miRNA (Table 3.1). For mRNA, validated equine specific forward and reverse primers were purchased (Table 3.2, Qiagen, Germantown, MD).

Table 3.1. Primer sequences and melt temperature (Tm) for each mircoRNA.

microRNA	Forward	Tm
RNU43	CGACTGCATAATTTGTGGTAGTGG	63
U6 snRNA	CTTATTGACGGGCGGACAGAAAC	65
miR-125b	TCCCTGAGACCCTAACTTGTGA	63
miR-138	AGCTGGTGTTGTGAATCAGGCCG	66
miR-140-3	TACCACAGGGTAGAACCACGG	65
miR-140-5	CAGTGGTTTTACCCTATGGTAG	61
miR-143	TGAGATGAAGCACTGTAGCTC	65
miR-145	GTCCAGTTTTCCCAGGAATCCCT	65
miR-146a	TGAGAACTGAATTCCATGGGTT	59
miR-146b	TGAGAACTGAATTCCATAGGCT	59
miR-16	TAGCAGCACGTAAATATTGGCG	61
miR-21	TAGCTTATCAGACTGATGTTGA	57
miR-22	AAGCTGCCAGTTGAAGAACTGT	61
miR-221	AGCTACATTGTCTGCTGGGTTTC	63
miR-222	AGCTACATCTGGCTACTGGGT	63
miR-27a	TTCACAGTGGCTAAGTTCCGC	63
miR-29a	TAGCACCATCTGAAATCGGTTA	59
miR-let-7a	TGAGGTAGTAGGTTGTATAGTT	57
miR-let-7f	TGAGGTAGTAGATTGTATAGTT	55

Table 3.2. Primer NCBI reference and Qiagen ID for each gene.

Gene	NCBI Reference Sequence	Qiagen ID
ACTB	NM_001081838	PPE00105A
GAPDH	NM_001081838	PPE00120A
COL1A1	XM_005597481	PPE00104A
COL2A1	NM_001081764	PPE00009A
CD146	XM_005611639	PPE06566A
VEGF	NM_001081821	PPE00074A
TGFB1	NM_001081849	PPE00123A
SOX9	XM_005597898	PPE00130A
TGFB3	XM_001492687	PPE01196A
RALA	XM_001494123	PPE07850A
DUSP6	XM_001491999	PPE01128A
FOXO1	XM_005601179	PPE00716A
HDAC7	XM_005611111	PPE07573A

Real-Time PCR. *miRNA:* The miScript SYBR Green PCR Kit (Qiagen, Germantown, MD) was used according to manufacturer's instructions, with cDNA at a concentration of 5ng/ul and a final reaction volume of 10ul. qRT-PCR cycle conditions were per the manufacturer's protocol: 15 min at 95°C and 40 cycles of denaturing at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 70°C for 30 sec. Relative expression was normalized using U6 snRNA and RNU43 and then normalized to the mean relative expression of MSCs. Each reaction was conducted in duplicate. The comparative cycle threshold (Ct) method was used to determine relative expression levels.[34] *mRNA:* For each mRNA qRT-PCR reaction, 5 µL of LightCycler 480 SYBR Green I Master (Roche, Indianapolis, IN) was added to 2 µL of nuclease-free water, 1 µL of cDNA at a concentration of 5 ng/µL, and 2 µL of primer mix at a concentration of 10 µM to reach a final volume of 10 µL. Samples were loaded into 384 well plates and analyzed in duplicate using a LightCycler 480 PCR System (Roche, Indianapolis, IN). Reactions were incubated at 95°C for 5 mins before undergoing 45 cycles of amplification (10 s at 95°C, 10 s at 60°C, 10 s at 72°C) and melt curve analysis with 5 acquisitions per °C from 65°C to 95°C. Relative expression was normalized using GAPDH and ACTB and then normalized to the mean relative expression of MSCs. Each reaction was conducted in duplicate. The comparative Ct method was used to determine relative expression levels.[34]

Statistical analysis. Statistical tests were performed using R software (version 4.0.5). Relative miRNA and mRNA expression data were analyzed using a linear mixed effects model with the log (base 10) transformed relative expression as the response variable, time in culture and miRNA/mRNA as fixed effects, with their interaction

included, and donor as the random effect. Post-hoc pairwise comparisons with a Tukey adjustment were used to compare levels of time in culture. Packages lme4, nlme, and emmeans were used to complete these analyses. P-values and confidence intervals were used to assess the statistical significance at a significance level of 0.05. Data were back transformed and are presented as mean $\pm$ confidence level limits.

3.4 Results and Discussion

Markers of dedifferentiation. Changes in the COL2/COL1 ratio have been reported to determine chondrocyte dedifferentiation.[15–18] Barlic et al., reported a 5-fold decrease in COL2 while COL1 increased 300-fold over 10 days of chondrocyte expansion[17] and similar trends in expression were observed in this study. For COL1, primary chondrocytes were 1,869-fold lower than MSCs (CI = -3.66 – -2.88) and significantly decreased with expansion to 3-fold lower than MSCs (CI = -0.89 – -0.11) by 19 PDs ($P<0.05$; Fig 3.1A), resulting in a >1,800-fold change in 12 days of expansion. There were no significant differences in expression beyond 19 PDs (P=0.10-0.84). Conversely, primary chondrocytes were 29,800-fold higher than MSCs (CI = 4.08 – 4.86) for COL2 and significantly decreased with expansion to 42-fold higher than MSCs (CI = 1.24 – 2.02) by 19 PDs ($P<0.05$; Fig 3.1B), resulting in a >29,000-fold change in 12 days of expansion. COL2 expression continued to significantly decrease and was 9-fold higher than MSCs (CI = 0.56 – 1.34) by 33 PDs ($P<0.05$).

miRNA. Although many miRNAs resulted in statistically significant differences between individual PDs and/or between cell types, they had only modest trends and fold-changes were small (<10-fold). These modest trends complicated analyses so

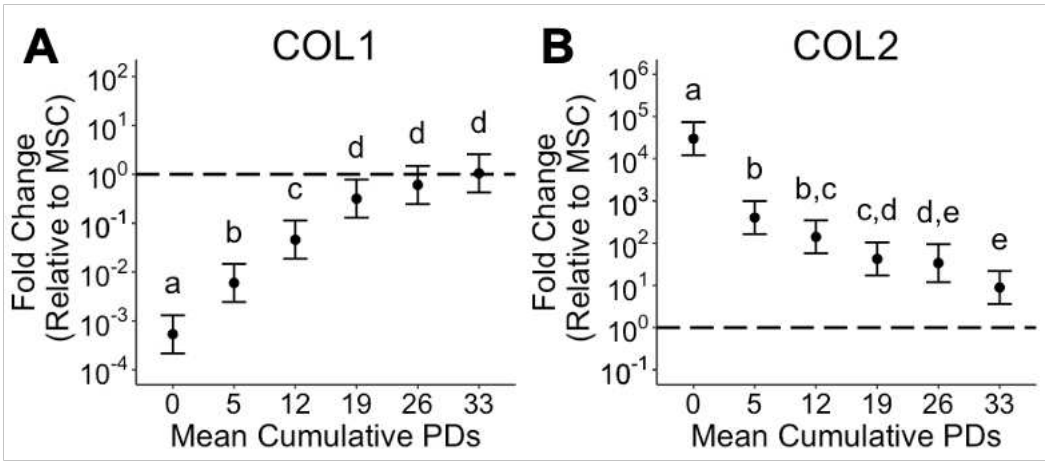

Figure 3.1. Adult CEC mRNA fold changes relative to MSCs for markers of cartilage dedifferentiation. A) COL1, B) COL2. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time (P<0.05). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

further investigation is needed to understand which changes are biologically meaningful. However, we anticipate that the miRNAs resulting in clear positive or negative trends overall provide the best starting point for investigation. Therefore, the following data has been divided into miRNAs that resulted in modest trends (Figs 3.2 and 3.4) and those that resulted in clear trends (Figs 3.3 and 3.5).

Adults: Of the 17 miRNAs tested, there were four miRNAs that showed clear positive (miR-143 and miR-145) or negative (miR-140-3 and miR140-5) trends in CECs over time (Fig 3.3). Additionally, all four of these miRNAs in CECs converged towards MSCs at later timepoints (Fig 3.3).

For miR-140-3, primary chondrocytes were 523-fold higher than MSCs (CI = 2.24 – 3.17) and significantly decreased with expansion to 4-fold lower than MSCs (CI = -1.00 – -0.19) by 26 PDs (P<0.05; Fig 3.3A). There were no significant differences

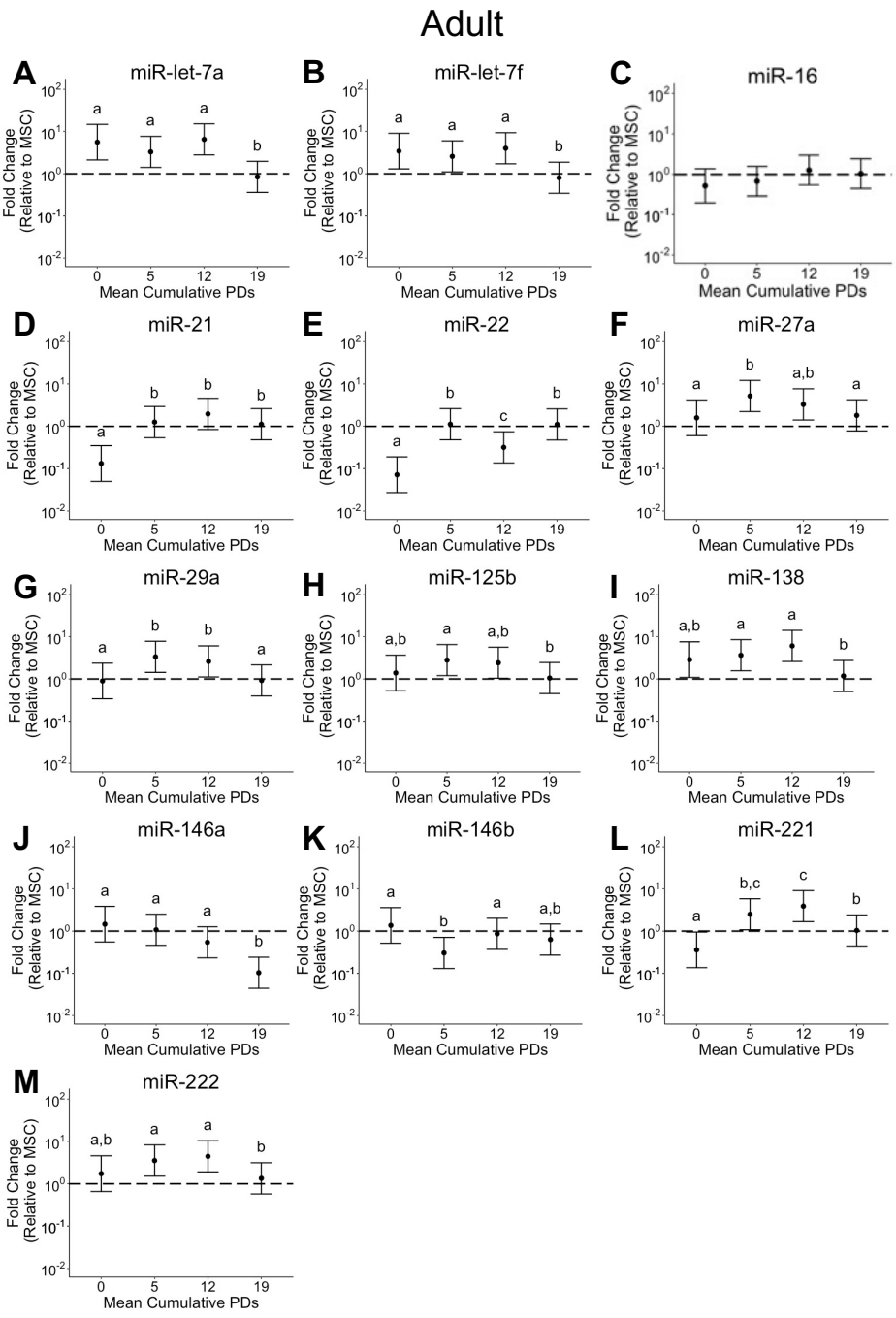

Figure 3.2. Adult CEC miRNA fold changes relative to MSCs with modest trends. A) miR-let-7a, B) miR-let-7f, C) miR-16, D) miR-21, E) miR-22, F) miR-27a, G) miR-29a, H) miR-125b, I) miR-138, J) miR-146a, K) miR-146b, L) miR-221, M) miR-222. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time ($P<0.05$). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

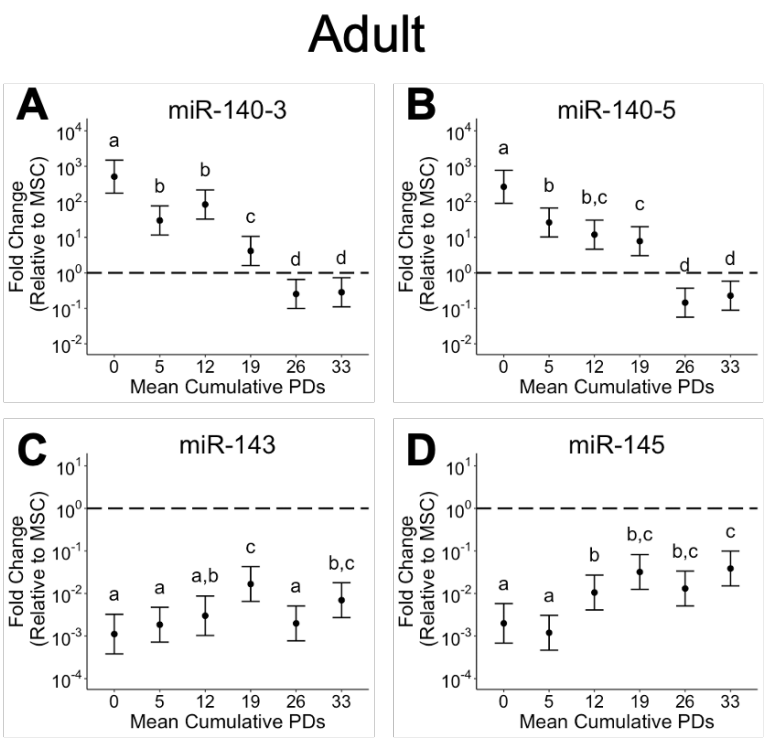

Figure 3.3. Adult CEC miRNA fold changes relative to MSCs with positive or negative trends. A) miR-140-3, B) miR-140-5, C) miR-143, D) miR-145. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time (P<0.05). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

beyond 26 PDs (P=1.00). miR-140-5 was similar to miR-140-3 where primary chondrocytes were 264-fold higher than MSCs (CI = 1.96 – 2.89) and significantly decreased with expansion to 6-fold lower than MSCs (CI = -1.25 – -0.43) by 26 PDs (P<0.05; Fig 3.3B). Again, there were no significant differences beyond 26 PDs (P=0.84). miR140-3 and miR140-5 have been published as pro-chondrogenic, so it is not surprising that the strong negative trends herein correlated with the strong negative trend in COL2 expression.

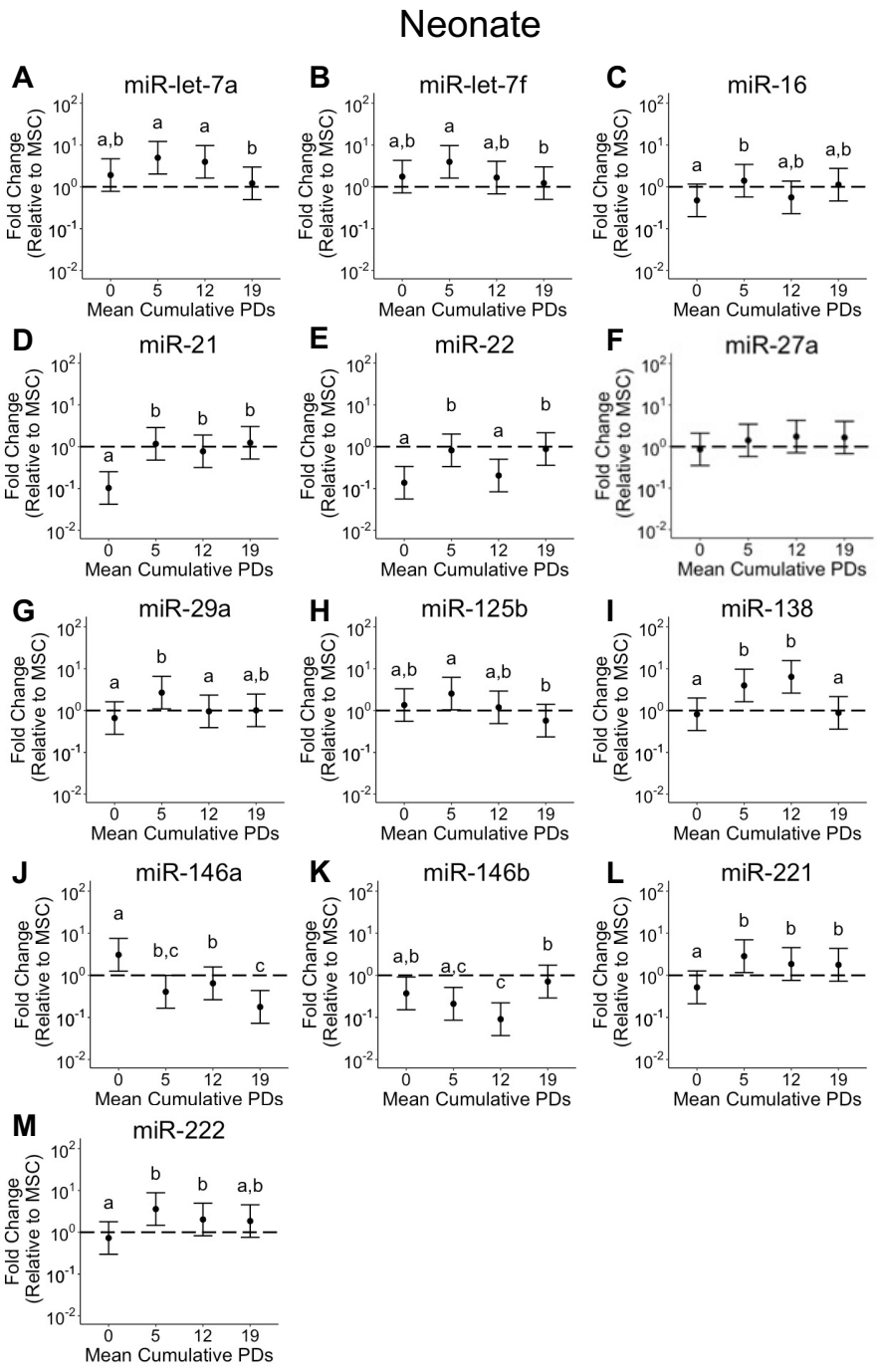

Figure 3.4. Neonatal CEC miRNA fold changes relative to MSCs with modest trends. A) miR-let-7a, B) miR-let-7f, C) miR-16, D) miR-21, E) miR-22, F) miR-27a, G) miR-29a, H) miR-125b, I) miR-138, J) miR-146a, K) miR-146b, L) miR-221, M) miR-222. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time ($P<0.05$). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

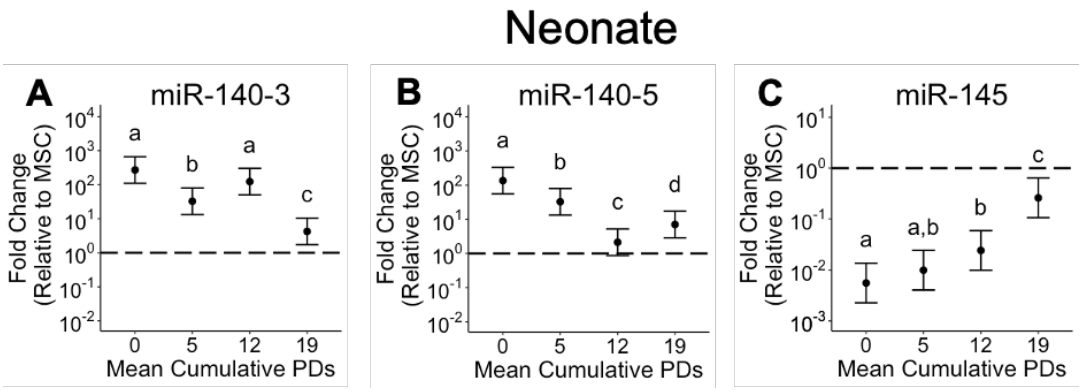

Figure 3.5. Neonatal CEC miRNA fold changes relative to MSCs with positive or negative trends. A) miR-140-3, B) miR-140-5, C) miR-145. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time (P<0.05). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

In contrast, miR-143 and miR-145 showed positive trends over time. For miR-143, primary chondrocytes were 799-fold lower than MSCs (CI = -3.42 – -2.49) and significantly increased with expansion to 137-fold lower than MSCs (CI = -2.57 – -1.75) by 33 PDs (P<0.05; Fig 3.3C). Again, miR-145 was similar to miR-143 where primary chondrocytes were 410-fold lower than MSCs (CI = -3.16 – -2.24) and significantly increased with expansion to 25-fold lower than MSCs (CI = -1.82 – -1.00) by 33 PDs (P<0.05; Fig 3.3D).

Neonates: Of the 16 miRNAs tested, there were three miRNAs that showed clear positive (miR-145) or negative (miR-140-3 and miR140-5) trends in CECs over time (Fig 3.5). It's notable that these three miRNAs showed the same trend as adult. Additionally, all three of these miRNAs in CECs converged towards MSCs at later timepoints (Fig 3.5). For miR-140-3, primary chondrocytes were 276-fold higher than MSCs (CI = 2.04 – 2.82) and significantly decreased with expansion to 4-fold higher than MSCs (CI = 0.24

– 1.02) by 19 PDs (P<0.05; Fig 3.5A). miR-140-5 was similar to miR-140-3 where primary chondrocytes were 137-fold higher than MSCs (CI = 1.74 – 2.52) and significantly decreased with expansion to 7-fold higher than MSCs (CI = 0.46 – 1.24) by 19 PDs (P<0.05; Fig 3.5B). Again, due to miR-140-3 and miR-140-5 being associated with chondrogenesis, it is not surprising that the strong negative trends herein correlated with the strong negative trend in COL2 expression.

Similar to adult, neonatal CECs showed a negative trend for miR-145 over time. Primary chondrocytes were 181-fold lower than MSCs (CI = -2.65 – -1.88) and significantly increased with expansion to 4-fold lower than MSCs (CI = -0.97 – -0.19) by 19 PDs (P<0.05; Fig 3.5C).

Donor age comparison: The three miRNAs with the largest change over time (miR-140-3, miR-140-5, and miR-145) were used to compare differences between age. The relative fold change for primary chondrocytes were used for each miRNA comparison as they resulted in the greatest difference from MSCs. For miR-140-3 and miR-145, the relative expression for adult CECs (523-fold and 0.0024-fold, respectively) was not statistically different from neonatal CECs (276-fold and 0.0129-fold, respectively; P=0.09 and P=0.41; Fig 3.6A,C) The relative expression for adult CECs (264-fold) was significantly greater than neonatal CECs (137-fold) for miR-140-5 (P<0.05; Fig 3.6B). However, even though some comparisons lacked statistical significance, the average fold change relative to MSCs for adult CECs was greater than neonatal CECs for all three miRNAs. Therefore, the variable of age was removed from subsequent studies and only adult CECs were analyzed.

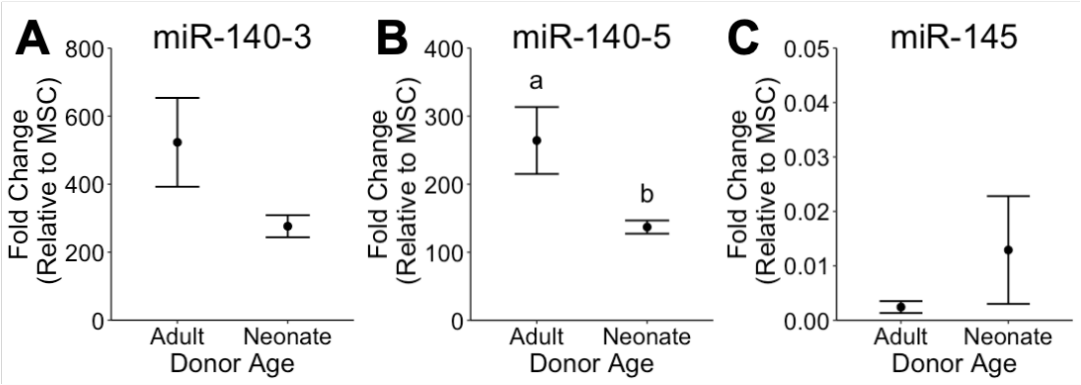

Figure 3.6. Adult versus neonatal CEC miRNA fold changes relative to MSCs. A) miR-140-3, B) miR-140-5, C) miR-145. Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denotes statistically significant differences over time ($P<0.05$).

mRNA. Following the initial miRNA screen, targeted mRNAs were selected to study relative gene expression in adult CECs over time (0-33 PDs) and compared to adult MSCs (19 PDs). mRNAs fall into the following categories: gene targets of specific miRNAs that resulted in large changes over time (DUSP6, FOXO1, HDAC7, and RALA), genes associated with cartilage and/or MSCs (SOX9,[35] TGFB1 and TGFB3),[36] angiogenesis (VEGFA)[37] and CD marker that may be associated with therapeutic potency (CD146).[38,39]

Gene targets of miRNAs: miRNAs affect expression of specific mRNAs through the RNA-induced silencing complex. In cartilage, miR-140-5 has been shown to down regulate RALA,[28] miR-143 targets HDAC7[40] and miR-145 represses DUSP6[35] and FOXO1.[41] Despite large expression changes over time for miR-140-3, miR-140-5, miR-143 and miR-145 in adult CECs, there were only modest changes in expression of their target genes (Fig 3.7). Again, some mRNAs resulted in statistically significant

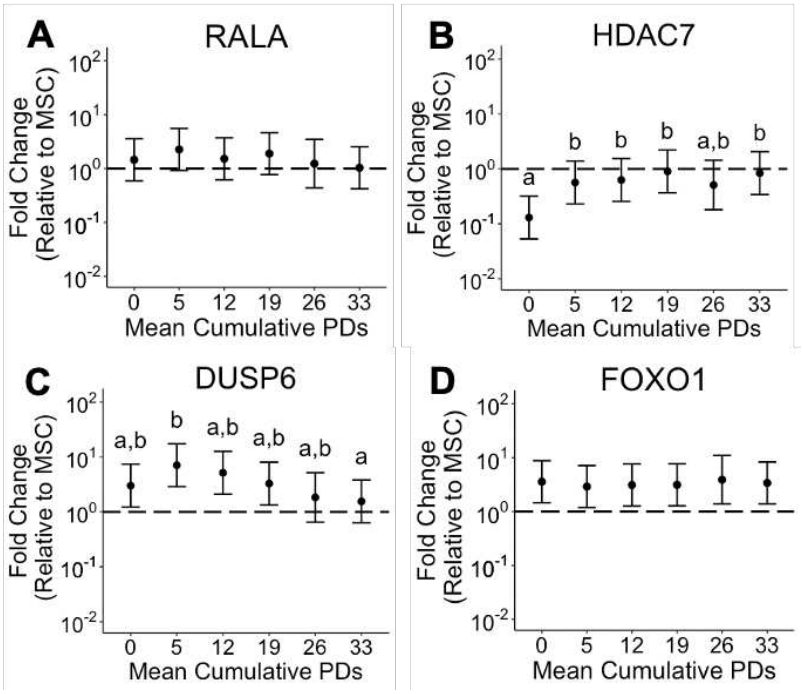

Figure 3.7. Adult CEC mRNA fold changes relative to MSCs for genes that are targets of specific miRNAs. A) RALA, B) HDAC7, C) DUSP6, D) FOXO1. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time (P<0.05). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

differences between individual PDs and/or between cell types, they had only modest trends and fold-changes were small (<10-fold). These modest trends complicated analyses so further investigation is needed to understand which changes are biologically meaningful. For example, miR-140-5 resulted in >250-fold change from 0 PDs to 26 PDs but there were no significant differences over time nor significant differences between CECs and MSCs for RALA (P=0.53-1.00; Fig 3.7A), a target of miR-140-5. It is surprising that although some miRNAs had large changes in expression (>250), their target mRNAs did not have similar changes in expression. Previous reports

have shown that target genes have modest changes in response to miRNA expression.[28,35,40,41] Therefore, mRNA expression appears to be a difficult way to confirm miRNA activity. Protein would be a more robust analysis and should be considered for future studies.

Genes associated with cartilage, angiogenesis and therapeutic potency: The transcription factor SOX9 has been demonstrated to play an essential role in chondrocyte differentiation, and has been termed a master regulator of chondrogenesis.[42] For SOX9, primary chondrocytes were 66-fold higher than MSCs (CI = 1.43 – 2.21) and significantly decreased to 15-fold higher than MSCs (CI = 0.79 – 1.57) by 5 PDs ($P<0.05$; Fig 3.8C). There were no significant differences in expression beyond 5 PDs ($P=0.47$-1.00), but CECs had significantly higher expression than MSCs at all time points ($P<0.05$; Fig 3.8A). Even though SOX9 expression decreased between 0 and 5 PDs, SOX9 expression from CECs remained consistently higher (~15-fold) than MSCs from 5-33 PDs. Further studies are needed to understand if this is biologically meaningful. The modest change in SOX9 expression over time does not appear to coincide with dedifferentiation.

Members of the TGFB family play essential roles in numerous aspects of cartilage formation and homeostasis. In postnatal cartilage maintenance, TGFBs inhibit terminal hypertrophy in chondrocytes. TGFB1 was shown to inhibit chondrocyte hypertrophy at an early stage in bovine synovial explants[43] while TGFB3 was shown to inhibit terminal hypertrophy in chondrogenic mesenchymal stem cells.[44]

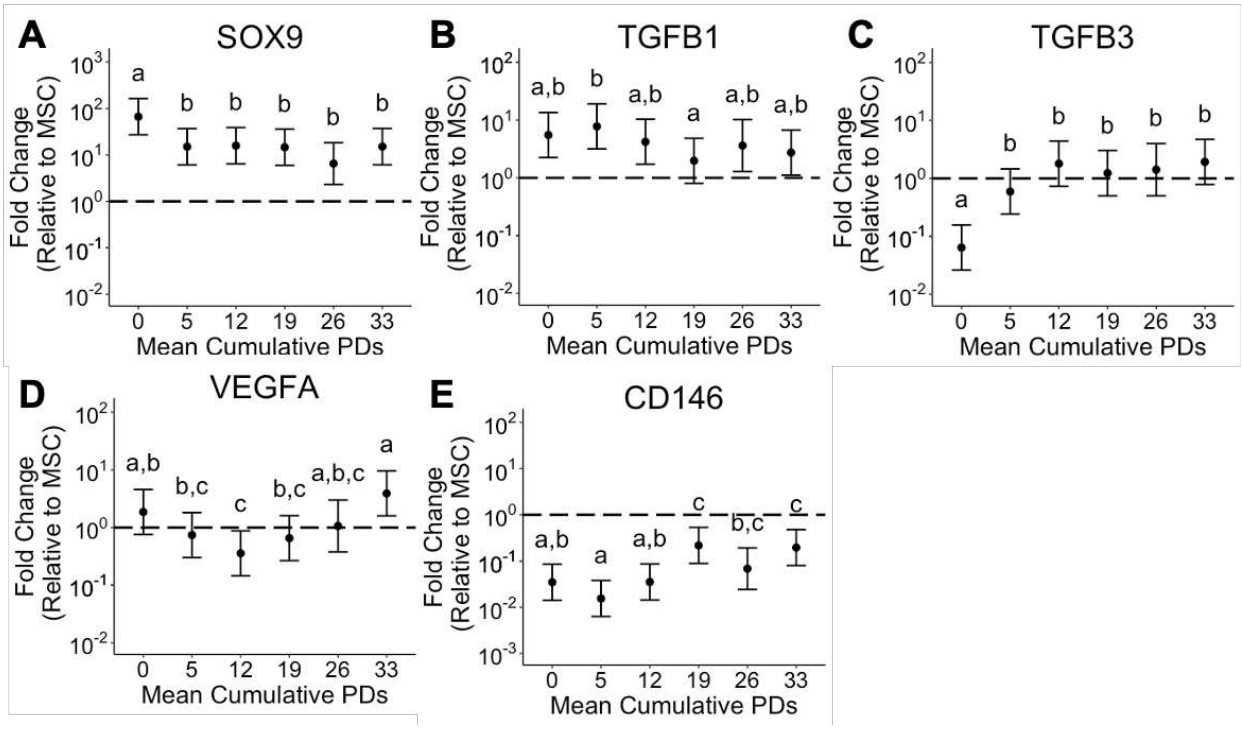

Figure 3.8. Adult CEC mRNA fold changes relative to MSCs for genes that are associated with cartilage and/or MSCs. A) SOX9, B) TGFB1, C) TGFB3, D) VEGFA, E) CD146. Data are mean +/- confidence level limits. For each figure, data that are labeled with different letters denotes statistically significant differences over time ($P<0.05$). CECs are significantly different from MSCs if the confidence interval bars does not intersect with the dashed line.

TGFB1 resulted in statistically significant differences over time and between cell types ($P<0.05$; Fig 3.8B), but overall changes were small (<10-fold). TGFB1 expression from CECs was ~5-fold higher than MSCs for most time points ($P<0.05$; Fig 3.8B) However, further work is necessary to elucidate if these relatively small changes in TGFB1 between cell types are biologically meaningful. TGFB3 expression for primary chondrocytes was 16-fold lower than MSCs (CI = 1.43 – 2.21) but significantly decreased to 2-fold lower than MSCs (CI = 0.79 – 1.57) by 5 PDs ($P<0.05$; Fig 3.8C). Expression stabilized beyond 5 PDs and there were no significant differences in

expression over time for CECs or between CECs and MSCs (P=0.15-1.00). These results do not indicate there is an association of TGFB expression changing with dedifferentiation.

VEGF is directly involved in pathologies of OA, including cartilage degeneration, synovitis, pain, and subchondral bone cysts and sclerosis.[45,46] Increased levels of VEGF are associated with OA progression while inhibiting VEGF has been shown to slow the progression.[22] VEGFA expression resulted in statistically significant differences over time and between cell types (P<0.05; Fig 3.8D), but overall changes were small (<10-fold). VEGFA expression in primary chondrocytes was not significantly different from MSCs (CI = -0.12 – 0.66). As expansion progressed to 12 PDs, VEGFA expression significantly decreased to 3-fold lower than MSCs (CI = -0.84 – -0.06). Interestingly, continued expansion increased VEGFA expression to 1-4-fold higher than MSCs (P=0.24-0.92). These changes were relatively small overall, and it does not appear that these changes coincide with dedifferentiation. Additional studies are required to determine the biological significance.

Jiang et al., reported that CECs acquired CD146 expression with serial passaging,[4] which was similar to the adult CEC CD146 expression results reported in this dissertation (Ch 1). The relative expression of CD146 ranged from 28 – 65-fold lower than MSCs from 0-12 PDs (P=0.46-1.00) and then significantly decreased to 5-fold lower than MSCs at 19 PDs (P<0.05; Fig 3.7G). The relative expression of CD146 ranged from 5 – 15-fold lower than MSCs from 19-33 PDs (P=0.18-0.99). The relative expression of CD146 for CECs was significantly lower than MSCs at all time points (P<0.05; Fig 3.8E). The changes in relative CD146 gene expression are similar to the

phenotypic changes seen in Ch 1, where CD146 expression significantly increased at 19 PDs. However, then CD146 gene expression decreased at 26 PDs, which was not reflected in CD146 expression seen in Ch 1. Again, these results do not indicate there is an association of CD146 expression changing with dedifferentiation.

3.5 Conclusions

As indicated by the robust changes in COL1 and COL2 over time, the CECs transition to a vastly different state with time in expansion culture. Unfortunately, this did not reveal patterns of differential expression for the majority of the miRNA and mRNA tested herein. For example, it has been reported that miRNA can target downstream of transcription. Therefore, it's possible that the mRNA targets for this study were affected downstream. For example, mRNAs are influenced through multiple pathways. It has been reported that RALA can be activated by Ras,[47] Arf6,[48] and insulin.[49] Additionally, it's possible that other miRNAs could target upstream regulators leading to an increased expression of the targeted mRNA. Additional protein expression studies are required to characterize the temporal changes of CECs.

3.6 References

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CHAPTER 4: EVALUATION OF CHONDROCYTE AND MESENCHYMAL STROMAL CELL PERSISTENCE AFTER INTRA-ARTICULAR INJECTION IN A RAT MODEL

4.1 Summary

Osteoarthritis (OA) is a highly prevalent and debilitating joint disease that affects more than 303 million people globally in 2017. Further, OA has a considerable impact on the patient, resulting in pain and disability. Consequently, it is not surprising that more than 1 million people undergo knee arthroscopy or joint replacement surgery each year due to end-stage OA in the United States, with the annual economic loss to Americans approaching $200 billion. Therefore, OA therapies that produce lasting effects are a medical priority. Mesenchymal stromal cells (MSCs) are considered 'medicinal signaling cells' that have been postulated to treat OA by reducing inflammation and restoring joint function. Therefore, providing MSCs into the joint space via intra-articular (IA) injection was hypothesized to treat OA. However, IA injection of MSCs into diseased human or companion animal joints has demonstrated only a modest benefit to date, as symptom-modifying effects are often temporary and have minimal evidence of disease-modification. This may be due to MSCs having limited persistence within the joint and very few cells engrafting into the host tissue. We propose to improve upon the use of MSCs from conventional tissue sources by using chondrocytes from articular cartilage. Of importance, connective tissue cells become MSC-like with isolation and expansion. It has been reported that culture-expanded chondrocytes (CECs) can assume many of the hallmark properties of MSCs, such as

immunomodulation, propensity for extensive expansion, and a similar immunophenotype. Further, chondrocytes are known to thrive in suspension, which is important as IA injections release cells into synovial fluid. Due to their durability, we hypothesize that CECs persist longer than MSCs in the joint space after IA injection. This chapter describes the results of n=4 rats, initial interpretations, and future cohorts to complete the study.

4.2 Introduction

Osteoarthritis (OA) is a debilitating joint disease that is commonly diagnosed in dogs,[1] horses[2] and humans[3]. As a leading cause of pain and disability, OA is thus a major contributor to a decreased quality of life.[4] Consequently, it is not surprising that more than 1 million people undergo knee arthroscopy or joint replacement surgery each year due to end-stage OA in the United States,[5] with the annual economic loss to Americans approaching $200 billion.[6] Unfortunately, there are currently no therapeutic regimens that successfully restore damaged cartilage to its normal phenotype or slow the progression of joint destruction,[7] reflecting a void in the current understanding of OA pathophysiology.

The need for new OA therapies is a medical priority, and there is great interest in the potential of cell therapies to produce lasting symptom- and disease-modifying effects.[8] Mesenchymal stromal cells (MSCs) are thought to possess strong potential to treat OA as ‘medicinal signaling cells’ that do not rebuild damaged tissues themselves, but instead secrete factors that reduce inflammation and promote repair by endogenous cells. For example, MSCs are immunomodulatory, which can suppress the joint inflammation that drives the progression of OA.[9] For humans, interest in treating OA

with IA injections of MSCs was first generated by Osiris Therapeutics when they published a compelling paper demonstrating disease-modifying effects in a goat partial meniscectomy model.[10] However, a recent review summarizing the results of 17 human studies focused on treatment of established OA concluded only limited benefits of IA MSC injections.[11] In dogs, intra-articular (IA) injections of MSCs have been reported to be safe, with select studies indicating a symptom-modifying effect.[12–16] However, improvement in joint function has been temporary[14–16] and regeneration of diseased joint tissues has not been reported.[17] Similarly, IA injection of equine MSCs are safe; but, again, testing in experimental models of OA or cartilage defects has not shown strong evidence of efficacy.[18,19] Therefore, there is a critical need to improve upon the effectiveness of cell therapies for treating OA with IA injection.

Chondrocytes may be an alternative to MSCs for cell therapies. While chondrocytes exist in a near-quiescent state in mature cartilage, chondrocytes that have been isolated from their host matrix and propagated *in vitro* adopt properties that are associated with MSCs.[20–27] Chondrocytes adopting MSC-like properties may be explained by the well-documented shift from a chondrocyte phenotype to a fibroblast phenotype, as it has been postulated that hallmark properties of MSCs are possessed by all fibroblasts.[28–31] This theory is supported by comparative studies in which culture-expanded cells from various connective tissues resemble MSCs phenotypically.[29–32] Importantly, we and others have reported that CECs resemble MSCs in their propensity for immunomodulation *in vitro.*[Ch 1,33–35] Similar findings have been reported for culture-expanded cells from different connective tissues, which further supports the expectation that fibroblastic cells are capable of immunomodulation.[9,33–37]

Given that the therapeutic potential of CECs resembles MSCs (Ch 1), CECs may address a critical limitation of MSCs. Specifically, after IA injection in experimental models, the vast majority of MSCs persist in the joint for only a period of days, with few cells engrafting into joint tissues.[10,38–41] We postulate that the limited joint residence time is due to an inability of MSCs to survive when suspended in synovial fluid, based on the phenomena known as anoikis, a process in which cells undergo apoptosis when they are unable to bind to the appropriate extracellular matrix[42] or cell culture substrate *in vitro*. Indeed, anoikis has been noted as a reason for poor MSC survival following implantation[43] and laboratory studies support the possibility that MSCs are inherently limited by anoikis. For example, decreases in viability have been reported for bone marrow MSCs that are suspended in hydrogels[44–46] or suspension in plasma or serum despite cold storage in an effort to maintain viability.[47]

Conversely, it has been recognized for decades that chondrocytes possess a unique ability to thrive in suspension. For example, hydrogel suspension cultures have been used extensively to study the biology of chondrocytes.[48,49] Therefore, CECs are expected to overcome the inability of MSCs to persist in the joint. The objective of this study was to compare the joint residence time of CECs and MSCs in injured rat stifles. We hypothesized that CECs persist in the joint space after IA injection longer than MSCs due to chondrocytes inherent ability to thrive in suspension. This hypothesis will be tested using fluorescently labeled CECs or MSCs and imaging using the IVIS Spectrum.

4.3 Materials and Methods

Isolation of chondrocytes and bone marrow. All culture medium contained 10 mM HEPES and 0.5% antibiotics/antimycotics solution (all from Thermo Fisher Scientific, Waltham, MA). Tissues were collected from 6-week-old male Sprague Dawley rats (n=5). *Chondrocytes*: Articular cartilage was harvested from stifles and digested in alphaMEM culture medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.4% w/v pronase (EMD Millipore, Burlington, MA) for 60 mins, followed by an overnight rocking incubation in 0.1% w/v type II collagenase (Sigma-Aldrich, St. Louis, MO) at 37ºC. Isolated chondrocytes were filtered through a 40 µm cell strainer and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) and 5% DMSO (Thermo Fisher Scientific, Waltham, MA). *MSCs*: Bone marrow was flushed from the diaphysis of the femur. Nucleated cells were isolated via centrifugation and then seeded into low glucose DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% FBS at $25x10^4$ cells/cm^2, as previously described.[50] At 24 hours, the medium was changed to expansion medium (alphaMEM supplemented 10% FBS and 2 ng/ml fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ).[27] Colonies of MSCs were collected after 7-10 days and cryopreserved at a concentration of $10x10^6$ cells/ml in 95% FBS and 5% DMSO.[44]

Expansion of chondrocytes and MSCs. Chondrocytes and MSCs were recovered from cryopreservation, seeded into expansion medium at 20-$40x10^3$ cells/cm^2 overnight, and then reseeded at 500 cells/cm^2 in expansion medium. Cultures were passaged every 5 days. When cells had expanded to the target cell number required to produce allogeneic treatments for the entire study, donors were pooled by cell type and

cryopreserved at a concentration of $10x10^6$ cells/ml in 95% FBS and 5% DMSO until needed for implantation.

Animals. All animal studies were performed with approval of the Colorado State University Institutional Animal Care and Use Committee (#2967) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Eight-week-old male rats (n=4), approximately 200-250g, were purchased from Charles River and acclimated to the vivarium for 14 days. Males were used to avoid confounding variables due to estrous. However, studies using females will be conducted in the future. Rats were dual-housed in solid bottom cages and maintained on a 12/12-hour light/dark cycle. Animals were allowed ad libitum water and AIN-93M rodent chow, which is chlorophyll free and does not interfere with imaging studies. Rats (n = 2/group) were randomized into either the CEC or MSC treatment group. Animal wellbeing was assessed daily. At the conclusion of the study, a cardiac stick was performed, followed by euthanasia via thoracotomy with confirmation via cervical dislocation.

Induction of OA in rat knees (DMM model). Prior to surgery, the hind-limbs of each animal were shaved and prepped using an alternating combination of 70% isopropyl alcohol and betadine. Rats were anesthetized by initial induction of 4% isoflurane and maintained at 1-3%. The right knee of all animals was injured. Briefly, after medial parapatellar arthrotomy, the infrapatellar fat pad (IFP) was temporarily repositioned laterally, allowing access to the anterior medial meniscotibial ligament, which was severed using a #15 blade. The IFP was repositioned, and the incision was closed. This model has been shown to produce mild-to-moderate OA at 4 weeks with

lesions primarily on the weight-bearing region of the medial tibial plateau and medial femoral condyles.[51] The left knee remained completely naïve.

Labeling of CECs and MSCs. Chondrocytes and MSCs were recovered from cryopreservation, seeded into expansion medium at 20-40x10^3 cells/cm^2 overnight. Immediately prior to implantation, CECs and MSCs were collected from monolayer culture using 0.25% trypsin (Gibco, Thermo Fisher Scientific, Waltham, MA) and labeled with VivoTrack 680 (PerkinElmer, Waltham, MA), a fluorescent dye that integrate into the cell membranes. Cells were resuspended in phosphate buffered saline (PBS) containing VivoTrack 680 or XenoLight DiR, incubated for 15-30 minutes at room temperature in the dark and then washed three times, according to manufacturer's instructions. The cells were resuspended to 10x10^3 cells/ul PBS just prior to implantation into the rat knees.

IA cell injections. For IA cell injections on day 7, the hind-limbs were prepped using an alternating combination of 70% isopropyl alcohol and betadine. VivoTrack 680 labeled cells (25x10^3 cells) were injected into the injured joint intra-articularly using a 25G needle.

Cell fate tracking analysis. Cells were tracked in vivo using a fluorescent imaging on the IVIS Spectrum system (PerkinElmer, Waltham, MA). Images were acquired on the day of treatment (day 0, immediately before IA injection, immediately after IA injection and 0.25 days post IA injection), and on days 1, 2, 3, 7, 10, and 14 post IA injection (Fig 4.1). Rats were anesthetized under 3% inhaled isoflurane and immediately imaged to record the presence and intensity of the fluorescent signal. VivoTrack 680 dye has a fluorescence emission of 696 nm and absorbance of 676 nm.

The 675 excitation and 720 emission filters were used. Blocking was utilized to isolate the right limb for imaging.

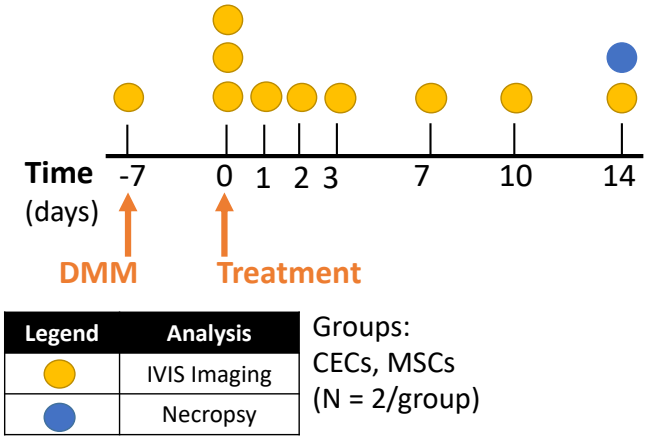

Figure 4.1. Experimental timeline to compare the joint residence time of allogeneic CECs and MSCs in a rat OA model.

IVIS imaging analysis. Using the IVIS software (Living Image 4.7.4), the fluorescent range (auto generated minimum and maximum) was determined for each rat based on the image taken immediately post IA injection and the fluorescent range was adjusted for all following images. Then the region of interest (ROI) was determined, and the max, average, and total radiant efficiency was recorded. The radiant efficiency was compared between treatment groups over time.

Statistical analysis of cell proliferation. Statistical tests were performed using R software (version 4.0.5). Growth kinetics data were analyzed using a linear mixed effects model with time in culture as the fixed effect and donor as the random effect with a Tukey adjustment for multiple pairwise comparisons. P-values and at a significance level of 0.05 were considered statistically significant. Data are presented as mean $\pm$ standard error.

Qualitative analysis of cell fate tracking. Mean radiant efficiency values were compared between treatment groups over time using GraphPad Prism 9 (version 9.3.1, San Diego, California).

4.4 Results and Discussion

Expansion in monolayer cultures. <u>*Growth kinetics:*</u> To understand the lifespan of the individual cell types, cells were expanded until an increase in the population doubling time was observed. Then, to produce the allogeneic treatments used for the study, additional cells were expanded and harvested before the decrease in expansion rate occurred. *Chondrocytes* - Primary chondrocytes (n=5 donors) were expanded for 14 days, which resulted in mean cumulative growth of 14.6 PDs. Over the first 11.6 PDs, corresponding to the first two passages, mean population doubling times (PDT) were 18.2-19.4 hrs (Fig 4.2A). With subsequent expansion, the mean PDT significantly increased to 39.3 hrs at 14.7 PDs ($P<0.05$; Fig 4.2A). Therefore, to produce the allogeneic CEC pool, primary chondrocytes (n=5 donors) were expanded for 8 days,

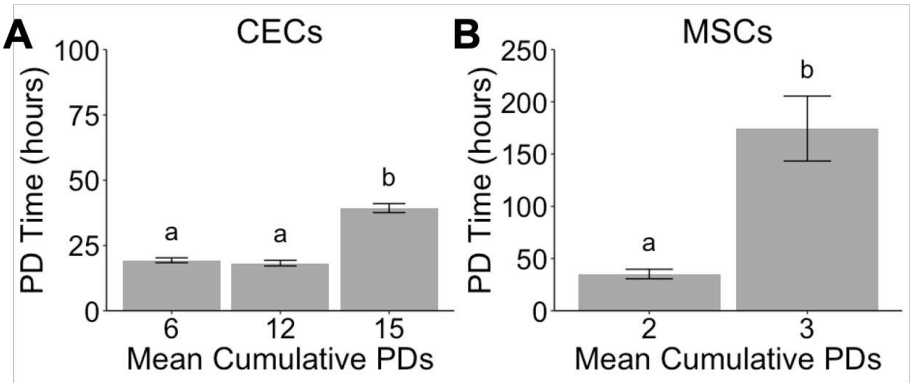

Figure 4.2. Growth kinetics of A) chondrocytes and B) MSCs. Data are mean +/- S.E.M. For each figure, data that are labeled with different letters denote statistically significant differences ($P<0.05$).

which resulted in mean cumulative growth of 9.3 PDs with mean PDT of 21.9 hrs (data not shown). *MSCs* – MSCs isolated from colony-forming cultures (n=5 donors) were expanded for 8 days, resulting in mean cumulative growth of 2.9 PDs. At the first passage (2.1 PDs), the average PDT was 35.2 hrs, which significantly increased to 174.5 hrs at the next passage (2.9 PDs; $P<0.05$; Fig 4.2B). Therefore, to produce the allogeneic MSC pool, MSCs (n=5 donors) were expanded for 7 days, resulting in mean cumulative growth of 1.9 PDs with mean PDT of 87.4 hrs (data not shown). It should be noted that although MSCs undergo limited proliferation to establish colony formation, PDs were not calculated until after cryopreservation and initiation of the study.

IVIS imaging analysis. The maximum, total and average radiant efficiencies were calculated based off the ROI's (representative images seen in Fig 4.3). The

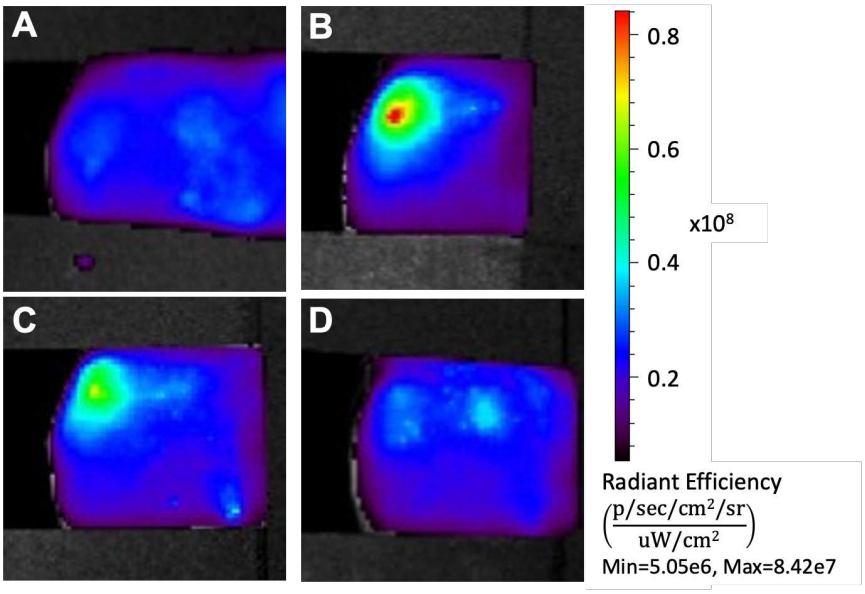

Figure 4.3. Representative images of ROI maximum radiant efficiencies over time. A) immediately prior to IA injection B) 0.25 days post-IA injection C) 3 days post-IA injection D) 7 days post-IA injection. Radiant efficiency scale is specific to individual donor.

maximum, total and average radiant efficiencies of CECs and MSCs were the same immediately post IA injection (0 days, Fig 4.4A-C). Then, the fluorescence of CECs increased compared to MSCs by 0.25 days post-IA injection, and remained higher

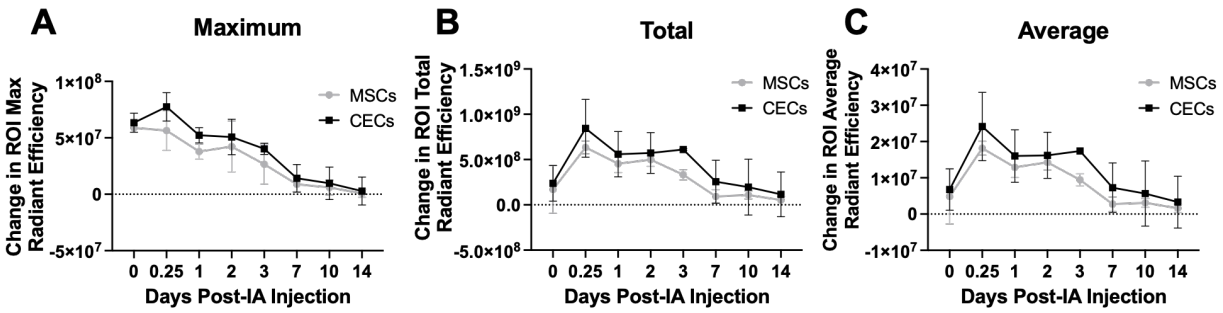

Figure 4.4 ROI radiant efficiency over time. A) maximum B) total C) average. Data are mean +/- S.E.M.

through 3 days (maximum; Fig 4.4A) and 7 days (total and average; Fig 4.4B-C) post-IA injection. Radiant efficiencies between treatment groups were the same for the remainder of the timecourse. Although there is only n=2/treatment, the increased fluorescence by CECs early in the timecourse could suggest greater persistence. This difference needs to be explored with a larger sample size to determine if the increased fluorescence is real and if it results in greater efficacy.

In three out of four animals, the maximum, total and average radiant efficiency increased between immediately post-IA injection and 0.25 days post-IA injection. This may be due to cellular dispersion throughout the joint space over time. For example, it's possible that cells are concentrated locally immediately post IA injection due to the viscosity of synovial fluid. However, after 0.25 days post IA injection, the cells may have had time to diffuse throughout the joint space, thus increasing the radiant efficiency. The

change in radiant efficiency in the first 0.25 days post IA injection needs to be explored with a larger sample size to determine if the increased fluorescence is significant.

As discussed in Ch 1, CECs are smaller than MSCs. Due to this, CECs emitted less fluorescence than MSCs when treatments were imaged in a tube prior to IA injection (data not shown). It's interesting to note that the change in radiant efficiency in the first 0.25 days post-IA injection was greater for CECs than MSCs (Fig 4.4A-C). It's possible that the difference in cell size is contributing to the fluorescent difference seen between treatment groups at 0.25 days post-IA injection. For example, the decreased diameter of the CECs may allow them to diffuse further into the perimeter of the joint compared to the larger MSCs, increasing the fluorescent intensity. Alternatively, it's possible that MSCs are being trafficked out of the joint faster than the CECs, resulting in decreased fluorescence at 0.25 days post IA injection. The difference in radiant efficiency between cell types observed at 0.25 days post IA injection needs to be explored with a larger sample size to determine if the difference in fluorescence is significant.

A preliminary curve fit was also performed (red line; Fig 4.5A-C). The decay over

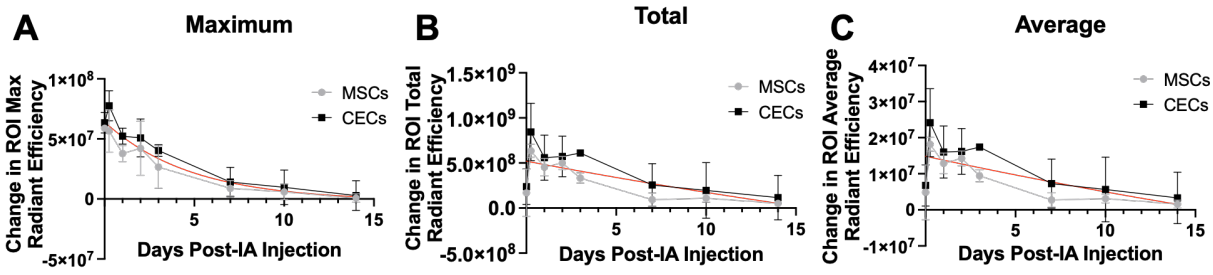

Figure 4.5 Decay in ROI radiant efficiency over time. A) maximum B) total C) average. Data are mean +/- S.E.M.

time appears to be exponential for the maximum radiant efficiency data (Fig 4.5A) and linear for the total and average radiant efficiencies (Fig 4.5B-C). Additional animals are required to understand the relationship between the two treatment groups and their decay over time.

Something to consider when analyzing this data is setting the initial region of interest to determine fluorescent range values. In the above figures, fluorescent ranges for each rat were generated based on the image immediately post IA injection. However, it may be beneficial to determine the fluorescent range (auto generated minimum and maximum) for each rat based on the image with the greatest value in the region of interest post IA injection. This change is suggested because the radiant efficiency increased in three out of four rats at the 6hr post IA injection timepoint. Prior to analysis, the data should still be normalized to the baseline radiant efficiency (immediately prior to IA injection).

Organs were harvested and imaged immediately post euthanasia. There was no fluorescence detected in the organs on day 14 post-IA injection. Representative images for liver, spleen, kidney, heart and lungs are shown in Fig 4.6A-D. It's possible that 1) fluorescent cells are not trafficked out of the joint or 2) fluorescent cells were trafficked to specific organs but had already been cleared at the time of harvest. In future studies, organs should be harvested at earlier timepoints to determine if the cells are trafficked to specific organs.

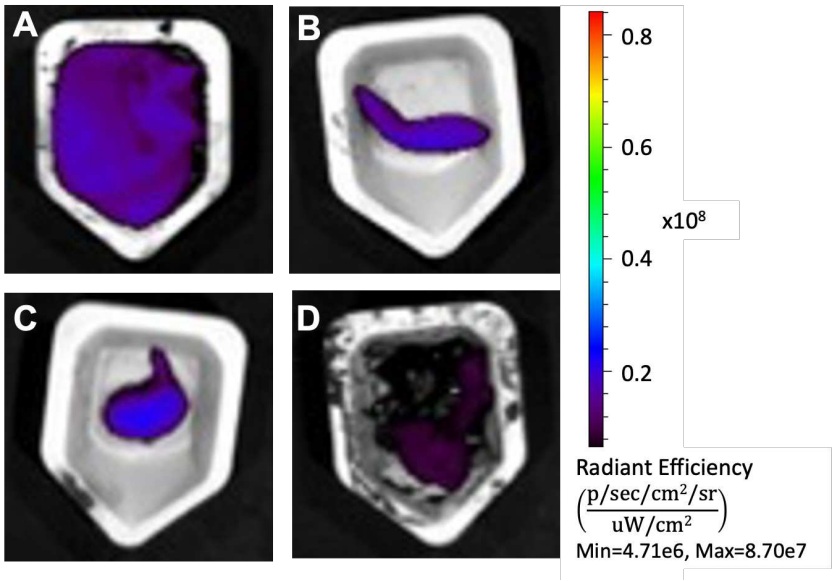

Figure 4.6. Representative images of organs harvested 14 days post-IA injection. A) liver B) spleen C) kidney D) heart and lungs. Radiant efficiency scale is specific to individual donor.

4.5 Anticipated Analyses

***Statistics*.** Based on the summary statistics gathered from these rats (n=2/group), a power analysis was repeated. The experimental sample size (10 rats/group) was calculated used GPower (version 3.1.1). This power analysis resulted in an effect size of 1.37 and a power of 82.5% when using a 95% confidence interval. Therefore, it is anticipated that an additional 8 animals per group are required to reach statistically significance differences.

When the study has been completed, statistical tests will be performed using R software (version 4.0.5). For joint radiant efficiency, values will be normalized to each animal's baseline value as described above. Data will be evaluated for normality using the Shapiro-Wilk test and homoscedasticity using the Levene's test. There are two

types of analyses that will be explored. First, a two-way ANOVA with Tukey adjustment will be utilized with p-values calculated for group (CEC and MSC), time (day 0, immediately before IA injection, immediately after IA injection and 0.25 days post IA injection, and days 1, 2, 3, 7, 10, and 14 post- IA injection), and group/time interactions. Packages lme4, nlme, lmerTest, and emmeans will be used to complete these analyses. Second, the data will be fitted with a curve using linear regression. Based on the shape of the curve from the n=4 rats (Fig 4.5), the linear model will most likely need to be fit with a quadratic term to best fit the model. Then, the curve fits can be compared statistically with the H_0 = one curve fits both CECs and MSCs and H_A = one curve does not fit both CECs and MSCs. P-values at a significance level of 0.05 will be considered statistically significant. Data will be presented as mean $\pm$ standard error.

***Histology*.** Cellular resolution and anatomical detail are shortcomings of the IVIS imaging system. In other words, it is unable to determine where the fluorescent cells are located within the joint. Therefore, histology is an alternative approach that will complement the cell fate tracking data.

Limbs were collected and fixed in formalin. Limbs will be decalcified, embedded in paraffin, and sectioned for future histological analysis. Samples will be stained with the following: unstained +/- 4',6-diamidino-2-phenylindole (DAPI), hematoxylin and eosin (H&E), and toluidine blue or safranin-O. Unstained samples will allow visualization of persistent fluorescent CECs/MSCs within the joint space. DAPI, a blue fluorescent DNA stain, could be added and merged with the unstained fluorescent image to determine the location of the persistent fluorescent CECs/MSCs. H&E staining will allow visualization of the joint architecture as well as immune cell infiltrates. Specifically, at 14

days post IA injection (21 days post-injury), there may be noticeable differences in synovitis between the two treatment groups. Toluidine blue or safranin-O staining will allow visualization of proteoglycans. This can be used to assess the osteoarthritic damage (OARSI score) present within the joint.[52,53] However, to understand the efficacy differences between CECs and MSCs over time, limbs should be collected at all time points.

4.6 Future Cohorts

Additional cohorts are planned for summer 2022 to further investigate cell persistence after IA injection. Based on the results from n=4 rats shown above, collaborators have discussed repeating the experiment with more targeted imaging timepoints: immediately prior to IA injection, immediately post-IA injection, as well as 0.25 days, 2 days, 7 days, and 14 days post-IA injection. These timepoints should capture the differences at early timepoints as well as the approximate time of when the fluorescence between the two cell types converges.

We hypothesized that, due to chondrocytes inherent ability to thrive in suspension, viable CECs will persist in the joint space after IA injection longer than MSCs. The preliminary results presented here show promise. Although radiant efficiencies for CECs were the same as MSCs after approximately 7 days post-IA injection, there may be a difference between the two cell types early on. This needs to be investigated further as increased persistence at early timepoints could result in greater disease- and/or symptom-modifying effects to alter or slow the progression of OA. This novel therapeutic approach could result in a translatable and impactful means to improve OA in both humans and animals.

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CHAPTER 5: CONCLUSIONS

5.1 Summary

Osteoarthritis (OA) is a highly prevalent and debilitating joint disease in horses, dogs, and humans for which there is no cure. Therefore, treatments that produce lasting symptom- and disease-modifying effects are a medical priority. To advance this line of research, the overarching goal of the work presented herein was to characterize how culture-expanded chondrocytes (CECs) changed as a function of time. An additional goal was to determine how CECs may be the same as bone marrow derived mesenchymal stromal cells (MSCs), such as immunomodulatory potential and molecular fingerprint, or different, such as persistence after intra-articular (IA) injection to determine their potential as an OA therapy. To study this, chapters 2 and 3 addressed CEC characterization *in vitro* while chapter 4 addressed CEC persistence *in vivo*.

Chapters 2 and 3 support that CECs become MSC-like over time. Specifically, CECs acquire more robust immunomodulatory properties with extensive expansion beyond 19 PDs and CD146 expression, a potential marker of therapeutic potency, increases over time. However, CD146 expression only predicted immunomodulatory effects for adult CECs. Largely, CEC dedifferentiation did not coincide with changes in miRNA or growth factor expression. Further, these data do not indicate a benefit of neonatal donors. Chapter 4 provides preliminary evidence that CECs may have greater persistence than MSCs in the first 3 days post IA injection. Taken together, it's possible

that CECs could outperform MSCs as a symptom- and disease-modifying therapeutic for OA. For example, COL2 gene expression, a marker of differentiated chondrocytes, for adult CECs at 19 PDs was 42-fold higher than MSCs (Ch 3), which corresponds to the time point when adult CECs suppressed PBMC proliferation significantly more than previous PDs (Ch 2). Therefore, it's possible that an optimal PD exists where CECs have retained chondrocyte "memory" but also acquired enhanced immunomodulatory properties. This should be evaluated to see if these *in vitro* results correlate to enhanced CEC persistence *in vivo* and/or increased symptom- and disease-modifying effects. Additional studies, some of which are described below, are required to fully capitalize on the potential of CECs as an allogeneic therapy for OA.

5.2 Future Directions

The work described within this dissertation is just the beginning to characterize CECs and how they may be potentially different from MSCs derived from conventional tissue sources. Future *in vitro* experiments should include additional immunomodulatory assays, redifferentiation (chondrogenesis), and angiogenic assays while *in vivo* experiments should include cell dose/timing studies and symptom- and disease-modification studies in rodents before moving into large animal models, such as equine, with spontaneous OA.

5.2.1 *in vitro* experiments

Immunomodulation: The CEC+PBMC and MSC+PBMC co-culture data reported within this dissertation (Ch. 2) was a starting point to understand the immunomodulatory properties of CECs and if they differ from MSCs. However, to fully understand the CECs

potential as a cellular therapeutic, it must be determined how CECs interact with immune cells within the environment, if those interactions change over time and if these aspects are different from MSCs. Therefore, next steps for immunomodulatory CEC *in vitro* experiments should study the anti-inflammatory pathways involved (Fig 6.1) and characterization of the anti- and/or pro-inflammatory milieu when co-cultured with PBMCs.[1–3]

There are many anti-inflammatory pathways, and it has been reported that different cell types and different species may predominantly utilize different mechanisms of action.[4–6] For example, commonly studied factors include indoleamine 2,3-deoxygenase (IDO), prostaglandin E_2 (PGE2), and nitric oxide (NO). We showed in Ch. 2-3 of this dissertation that CECs transition from a chondrocyte phenotype to an MSC-like phenotype with expansion culture. Therefore, CECs should be studied over time to determine which immunomodulatory pathways are employed. One way to explore this would be to plate CECs (5-33 PD, n=5 donors/time point) and MSCs (19 PD, n=5 donors) in monolayer culture, and stimulate cells with an inflammatory factor such as interferon gamma (IFNg), tissue necrosis factor alpha (TNFa) or interleukin 1 beta (IL-1b). Non-stimulated wells would serve as controls. The supernatants could be analyzed for PGE2 (ELISA microplate) and NO (colorimetric microplate), while the cell lysate could be analyzed for IDO (fluorescent microplate). Additionally, intracellular NO levels in live cells could also be detected using flow cytometry. Conversely, these anti-inflammatory aspects could be studied using a more robust -omics approach to characterize all of the proteins and metabolites present. A weakness of this type of

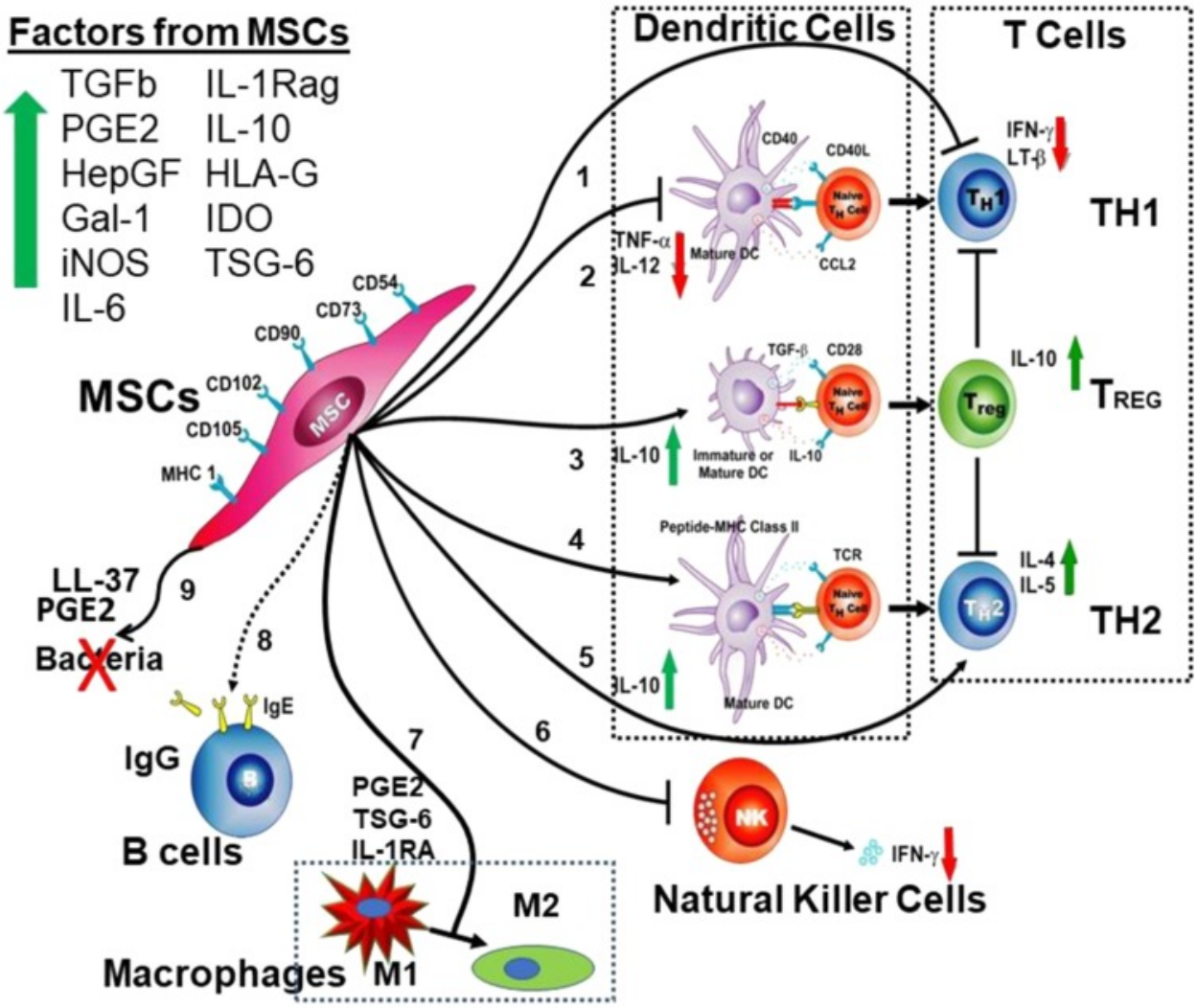

Figure 5.1. MSC—Immune cell interactions. Initial studies envisioned autologous use of MSCs. However, studies with immune cells demonstrated that MSCs are not immediately rejected by T cells and other immune cells, prompting the study of allogeneic MSCs in mutiple therapies. MSCs produce at least 11 factors known to affect immune cells. When interacting with T cells (pathways 1 and 5) MSCs cause a reduction in inflammatory T H1 and an increase in T Regs and T H2 cells with the concomitant decrease in IFNγ, increase in IL-10, IL-4 and IL-5. When MSCs interact with dendritic cells (pathways 2, 3,and 4) there is a decrease in proinflammatory mature DC1 with a decrease in TNF-α and IL-12, and an increase in immature DC and DC2, with increased expression of IL-10. When MSCs interact with natural killer cells (pathway 6) there is a decrease in the expression of IFNγ. When macrophages interact with MSCs (pathway 7), there is a decrease in the proinflammatory M1 phenotype and an increase in the anti-inflammatory M2 phenotype, with increased PGE2, TSG-6 and IL-1RA. MSCs can also reduce the secretion of antibodies from B cells (pathway 8) and inhibit bacterial growth by a direct or indirect mechanism (pathway 9). Taken from Pittenger et al., 2019.[2]

study is that there are multiple cell types and cytokines/chemokines contributing to the OA environment, so this would be an over-simplified *in vitro* model. However, the simplicity of the design could also be considered a strength because it could be determined how CECs specifically contribute to the anti-inflammatory environment.

Another aspect that should be explored is the therapeutic potency of CECs. In an effort to define matrix responses of MSCs to inflammatory cues, Chinnadurai et al., reported secretome-based profiles characterizing the MSC and PBMC responses to each other in humans.[1] This approach would provide a greater level of detail regarding the CEC immunomodulatory profile that was initially described in Ch. 2 and should be completed in the horse. Briefly, the co-culture experiment from Ch. 2 would be repeated. CECs and MSCs from five timepoints (5-33 PDs, n=5 donors/ cell type/time point) would be co-cultured with PBMCs for 96 hrs. Samples would be stimulated with ConA or remain unstimulated. The supernatants would be collected for multiplex analysis and analyzed for 23 common cytokines/chemokines (Equine Milliplex, EMD Millipore). Due to the co-culture used in this approach, a potential weakness is it would not be possible to determine which cytokines were released from which cell types (CECs/MSCs vs PBMCs). A strength of this approach would be determining the anti- or pro-inflammatory profile of the milieu. Not only would this approach allow for characterization of present/absent cytokines, but it would also allow to compare how the cytokine profile changes over time. Additionally, it would be possible to compare cytokine profiles between CECs and MSCs.

The experiments described above could lead to some impactful outcomes. For example, determining how CECs specifically contribute to the anti-inflammatory

environment and which immune cells they may target. Additionally, as discussed in Ch. 2, CECs that were >19 PDs significantly suppressed PBMC proliferation more than CECs that were <19 PDs, but the amount of PBMC suppression did not change between 19-33 PDs. If anti-inflammatory cytokine(s) of interest were significantly greater at 26 PDs compared to 19 PDs, for example, it could be concluded that the therapeutic potency increases with expansion. Taken together, these analyses may help determine the ideal PD target for expansion to maximize therapeutic benefits for treating OA.

It should be noted that these proposed studies focus on only a small subset of immune cells. It has been reported that MSCs regulate a variety of immune cells, such as macrophages, dendritic cells, and natural killer cells.[2,3,6] Therefore, how CECs interact with other immune cells should be explored. For example, as reviewed by Sun et al., MSCs suppress inflammation by blocking the activation of M1 (classically activated) macrophages and induce polarization towards the M2 (selectively activated) phenotype, making MSCs a promising therapeutic for OA.[7] It should be determined if CECs possess the same ability to induce macrophage polarization towards and anti-inflammatory phenotype.

Redifferentiation (chondrogenesis): Trilineage differentiation potential was not examined within this dissertation. However, based on the results from the studies described within this body of work, redifferentiation (chondrogenesis) of CECs should be explored. As described in Ch. 1 and 3, chondrocytes are known to dedifferentiate in expansion culture. In the past, dedifferentiation has been considered a negative when working with chondrocytes for cartilage tissue engineering. However, it's possible that there is an optimal phenotype during dedifferentiation that has gone unrecognized for

allogeneic therapies in regenerative medicine. For example, COL2 gene expression for adult CECs at 19 PDs was 42-fold higher than MSCs ($P<0.05$; Ch 3) and it is unknown if this increased COL2 expression is biologically significant. Therefore, redifferentiation (chondrogenesis) should be studied in CECs over time and compared to MSCs.

To complete this study, a standard chondrogenic culture would be performed. Briefly, CECs (0-33 PD, n=5 donors/time point) and MSCs (19 PD, n=5 donors) would be encapsulated in 1.5% (w/v) agarose at $10x10^6$ cells/ml and cultured for 14 days in chondrogenic medium (high glucose DMEM supplemented with 10mM HEPES, 2% FBS, 1% ITS+ Premix, 0.5% antibiotics/antimycotics, 37.5 µg/ml ascorbate-2-phosphate, 100 nM dexamethasone and 5 ng/ml recombinant human transforming growth beta 1 for 14 days, as previously described.[8] Outcome measures would include glycosaminoglycans (GAG) and hydroxyproline content normalized to the DNA content. The amount of GAG and hydroxyproline could be compared over time (0-33 PDs) and between cell types (CECs and MSCs) to determine if differences exist and how those differences could be utilized for a more targeted therapeutic.

Angiogenesis: Angiogenesis is an important aspect of OA as it contributes to increased inflammation within the joint and chronic synovitis.[9–12] Vascular endothelial growth factor (VEGF) is a major contributor of angiogenesis. As described in Ch. 3, VEGFA gene expression from CECs did not result in a discernable change over time, nor did it differ from MSCs for many (4 out of 6) of the time points. However, gene expression does not always correlate to protein expression. Therefore, it is warranted to study VEGF protein expression. This could be accomplished by seeding CECs (5-33 PD, n=5 donors/time point) or MSCs (19 PD, n=5 donors) in monolayer culture, with

both stimulated and non-stimulated groups, as described above for the immunomodulatory assays. The supernatant could be collected and analyzed via ELISA. Again, it is important to characterize the change in VEGF secretion over time and between cell types (CECs and MSCs).

5.2.2 *in vivo* experiments

Dose and dosing regimens: Based on the results from the completed study in Ch 4, cell dosing studies should be performed to determine the optimal cell concentration per IA injection and/or the optimal number of IA injections. For example, if the CEC fluorescence for the first 7 days post-IA injection is greater than MSCs, increasing the number of cells per injection may increase the CEC persistence beyond day 7. Alternatively, the number of IA injections could be increased. In this approach, additional IA injections could be given as cell fluorescence decreases. For example, if the CEC fluorescence for the first 7 days post-IA injection is greater than MSCs but then converges, an additional dose of CECs could be administered on day 5 to maintain the fluorescent difference, and therefore persistence, between cell types. Depending on the results from the completed study described in Ch 4, both dosing strategies could be explored independently or in combination.

It should be noted that using transgenic GFP donor rats could be considered as an alternative approach for future studies. For example, a GFP rat strain with a Sprague Dawley genetic background is available from Genoway (https://www.genoway.com/catalog/om/rats/gfp.htm), which has a CAG-eGFP inserted within the rat intronic *Rosa26* locus using CRISP/Cas9 technology. eGFP is expressed in all main organs and tissues, which allows for the generation of eGFP cell lines.

Therefore, it is anticipated that eGFP cells could be isolated from cartilage and bone marrow to produce eGFP CEC and MSC stocks. There are two main benefits to using eGFP cells. First, eGFP cells would not need to be labeled with a fluorescent dye, such as VivoTrack 680 (Ch. 4), to be imaged post IA injection because they constitutively express GFP. This eliminates an *in vitro* step that could introduce variability into the system. Second, it's possible that cell proliferation could be tracked over time because eGFP is passed down to each daughter cell. It may be possible to visualize eGFP daughter cell engraftment into the host tissue (unstained histology under a fluorescent microscope) and/or quantify the number of eGFP cells that remain within the synovial fluid flush (flow cytometer or hemocytometer count under a fluorescent microscope) post-euthanasia.

If GFP donor rats were to be used in future experiments, it would be important to characterize the eGFP cells *in vitro* prior to their use *in vivo*. For example, eGFP chondrocytes could be isolated from cartilage and imaged under a fluorescent microscope and/or examined via flow cytometry to confirm eGFP expression.[13] Then, primary eGFP chondrocytes could be expanded under the same culture conditions described in Ch 4 to produce the allogeneic cellular therapies. The eGFP expression should be examined at each passage to confirm the transgenic gene is being passed down to daughter cells. Lastly, the fluorescent intensity should be characterized while eGFP cells are in monolayer and suspension to determine the fluorescent intensity based on the number of cells present. To do this, eGFP cells would be seeded in monolayer with supplemented alphaMEM and allowed to adhere to tissue culture plastic or seeded in suspension on polyHEME-coated plates with supplemented

alphaMEM+1% methylcellulose, as described in Ch 2, in 2-fold dilutions. The fluorescent intensity would be recorded and analyzed with a curve fit to determine if an exponential increase in cell number resulted in an exponential increase in fluorescence.

Symptom- and disease-modification: As discussed in the Introduction (Ch 1), previous cellular therapies have not provided lasting symptom- and disease-modifying effects. Therefore, once the optimum CEC dose has been determined, the following study should focus on modifying symptoms and disease pathology. Following IA injection, modification of clinical signs could be evaluated by voluntary gait analysis via the Rodent Walkway (Tekscan, Boston, MA) and voluntary movement assessment utilizing overhead cage monitoring and ANYmaze software. Outcome measures for these techniques would include maximum force, force time impulse, maximum peak pressure, symmetry, and distance traveled. These measures would be compared between CECs and MSCs to determine if CECs provided greater symptom modification. Structural disease modification could be evaluated by identifying pathologies via histology and microCT. An independent pathologist could perform histological grading of four serial coronal sections using adapted Mankin criteria based upon species-specific features of OA.[14] Additionally, chondropathy could be scored for the medial femorae and tibial plateaus. For microCT analysis, an independent pathologist or surgeon could evaluate the images for features typical of OA, such as presence and location of osteophytes, subchondral bone sclerosis and cystic changes, articular bone lysis, and intra-articular soft tissue mineralization.[15]

Large animal model: Following initial studies in rats, the disease- and symptom-modifying experiments should be repeated in larger animal models, such as dogs and

horses as both species have naturally occurring OA. Horses are an excellent preclinical model for OA because clinical OA has been extensively documented and a predictable model of OA has been developed to test putative therapies.[16] Therefore, a potential experimental plan could be as follows. First, an established model of OA, such as the carpal osteochondral fragment model, could be used as it mimics clinical equine OA and has relevance to human OA.[16] CECs or MSCs would be injected into the diseased joint and similar to the lab animal studies described previously, outcome measures could be divided into symptom- and disease-modifying effects. Outcome measures could include gait analysis, musculoskeletal examinations (standing pain score, pain in response to flexion, range of motion of the affected joint, and grade of synovial effusion), imaging (radiograph and MRI), synovial fluid analysis, and histology.[16–18] Providing results are favorable in the carpal osteochondral fragment model, the next phase of testing would involve injecting CECs into clinical equine patients with naturally occurring OA and assessing symptom-modification over time. This novel therapeutic approach could be impactful for treating OA in animals, such as dogs and horses, and could be translatable to humans.

5.3 Closing Remarks

The data presented in this dissertation serve to broaden the field's understanding of how the cellular characteristics of CECs change over time and compare them to bone marrow derived MSCs, the current cell of choice for IA injection. There is a critical need to improve upon the effectiveness of MSC therapies for treating OA, and using an alternative cell type, such as chondrocytes, may provide therapeutic benefits. Data from chapters 2 and 3 indicate that chondrocytes retain a strong propensity for

immunomodulation, that increases with expansion and dedifferentiation does not coincide with other changes in gene expression. Further, these data do not suggest there is a benefit of using neonatal donors. Taken together, future research should focus on elucidating the therapeutic mechanisms employed by CECs and how to utilize them to promote repair. Preliminary data from chapter 4 indicate that CECs may have a greater persistence than MSCs in the first 3 days post IA injection, highlighting the need for future research to determine the symptom- and disease-modifying effects in relevant preclinical models, such as the rat, horse, and dog.

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CHAPTER 6: THE PLATELET-RICH PLASMA AND MESENCHYMAL STEM CELL MILIEU: A REVIEW OF THERAPEUTIC EFFECTS ON BONE HEALING[1]

6.1 Summary

Platelet-rich plasma is autologous plasma that contains concentrated platelets compared to whole blood. It is relatively inexpensive to produce, can be easily isolated from whole blood, and can be administered while the patient is in the operating room. Further, because platelet-rich plasma is an autologous therapy, there is minimal risk for adverse reactions to the patient. Platelet-rich plasma has been used to promote bone regeneration due to its abundance of concentrated growth factors that are essential to wound healing. In this review, we summarize the methods for producing platelet-rich plasma and the history of its use in bone regeneration. We also summarize the growth factor profiles derived from platelet-rich plasma, with emphasis on those factors that play a direct role in promoting bone repair within the local fracture environment. In addition, we discuss the potential advantages of combining platelet-rich plasma with mesenchymal stem cells, a multipotent cell type often obtained from bone marrow or fat, to improve craniofacial and long bone regeneration. We detail what is currently known about how platelet-rich plasma influences mesenchymal stem cells *in vitro*, and then highlight the clinical outcomes of administering platelet-rich plasma and mesenchymal stem cells as a combination therapy to promote bone regeneration *in vivo*.

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6.2 Clinical Need for Bone Regeneration

Fractures, tumors, bony defects, trauma, and periodontal disease are common conditions that require bone regeneration in order to heal. Approximately 7.9 million bone fractures occur annually in the USA, of which 5-10% result in delayed/nonunion.[1–3] Incidence of non-union is even higher for the elderly or those with co-morbidities, such as osteoporosis and diabetes.[3,4]

Bone healing is a series of orchestrated events. First, a hematoma forms around the injury site, which contains fracture debris that initiates an acute, pro-inflammatory cascade containing M1 (classical) macrophages and other inflammatory cells.[5–7] Progression to an anti-inflammatory state, populated by M2 (alternatively activated) macrophages that secrete cytokines and growth factors for repair, is critical for healing.[6,7] Bone will regenerate via two distinct pathways: intramembranous or endochondral ossification. Intramembranous ossification is direct bone formation and occurs when mesenchymal progenitor cells differentiate directly into osteoblasts, form trabeculated bone, which is then remodeled into cortical bone.[7] Calvarial fractures tend to heal via intramembranous ossification.[8] Endochondral ossification, which is the most common pathway through which long bone fractures heal, occurs when mesenchymal progenitors differentiate into chondrocytes, forming a cartilage intermediate template.[9–11] Chondrocytes within the fracture callus undergo hypertrophic maturation and then transform into osteoblasts and osteocytes, mineralizing and remodeling the cartilage matrix to bone.[12–14] This highly trabeculated bone is then remodeled to cortical bone in the final stage of healing.[7]

There is a clinical need to accelerate bone regeneration after a fracture, and therefore, numerous grafting materials have been tested in orthopaedics. Autogenous cancellous bone is currently the gold standard. However, it has well-described limitations, including limited volumes that can be harvested from donor sites, donor site pain, and prolonged healing time for the donor sites.[15,16] Other bone grafting materials, such as natural and synthetic polymers, ceramics, metals and composites, have advantages and disadvantages also. For example, as summarized by Garcia-Gareta *et al*., synthetic polymers are biodegradable and versatile, but have low mechanical strength.[17] Calcium-phosphate ceramics are generally biocompatible and osteoconductive, but are brittle compared to bone.[17] These materials have been paired with orthobiologic therapies, which include stem cells, osteoinductive growth factors (such as those provided by platelet-rich plasma), osteoconductive matrices, and anabolic agents to enhance bone healing.[18]

Mesenchymal stem cells (MSCs) are a promising adjunctive therapy for fracture repair. MSCs are multipotent stromal cells that can differentiate into multiple cell types, including chondrocytes and osteoblasts, and play an important role in bone regeneration/fracture repair[19] by providing a source of primary osteogenic cells, encouraging osteoinduction, and initiating osteoconduction.[20] Bone marrow-derived MSCs have been used to reduce healing time and aid in the repair of non-union fractures.[19,21]

6.3 Preparing Platelet-Rich Plasma

Broadly, platelet-rich plasma (PRP) is autologous plasma that contains more platelets than whole blood. Most commonly, PRP is defined as plasma containing

platelets at a concentration approximately two to five times higher than whole blood, depending on the preperation.[22–25] However, protocols can be adjusted to create PRP with higher or lower platelet concentrations. Not all PRP is reported as an increased platelet percentage over baseline. Marx defined PRP as 5ml volume of plasma containing $1x10^6$ platelets/μl because it enhanced bone and soft tissue healing.[25] While PRP is the most common nomenclature used, it is also referred to as platelet-rich concentrate, autologous platelet gel or plasma-rich growth factors.[25,26]

Normal platelet counts in whole blood average $200x10^3 – 250x10^3$ platelets/μl.[22,25] Platelets circulate in peripheral blood until they are activated to form a clot, which concentrates platelets to the injury site. The clotting cascade initiates healing via one of two pathways that converge in the latter steps resulting in platelet activation and release of proteins necessary for clot formation.[27] Following fracture, platelets are essential in the formation of a fracture hematoma, after which they degranulate and release growth factors from alpha granules that are crucial to the healing process.[2,25,28] Seventy percent of the stored growth factors are secreted within 10 minutes of clotting, and 90-95% within 1 hour.[25] Additional growth factors are synthesized and secreted for the remaining 7-8 days of the platelets' lifespan.[25,26] While a naturally occurring hematoma is composed of 95% erythrocytes, a clot that has been formed using leukocyte-depleted PRP is composed of 95% platelets, 4% red blood cells, and 1% white blood cells.[27] While the effect of PRP is largely attributed to the increased concentration of growth factors necessary for wound healing, PRP is a complex, bioactive milieu with other factors, including plasma proteins, delta and lambda granules

from platelets, and other cell types (leukocytes, erythrocytes) that may play a critical role in its biologic activity.[28]

PRP production is not standardized and therefore, numerous PRP protocols have been reported with significant variations in anticoagulant, centrifugation technique and activation method used. For example, common anticoagulants used include ethylenediaminetetraacetic acid (EDTA), acid citrate dextrose (ACD), and sodium citrate. Centrifugation protocols can include one (soft spin) or two (soft spin and hard spin) centrifugations with variable speed and times. Platelets can be activated endogenously via freeze-thaw cycles to disrupt the platelet membrane, however, exogenous platelet activation using either thrombin or calcium chloride is more common. Some authors caution against the use of thrombin because it can lead to the formation of peripheral blood clots and myocardial infarction.[29] Further, it has been reported that if bovine thrombin is used, bovine factor V could cross-react with human factor V leading to coagulopathies.[29] Lastly, commercial preparation kits have been shown to produce variable PRP. Oudelaar at el., reviewed 10 commercial PRP preparation kits and concluded that there was a large amount of heterogeneity between kits in regards to platelet, leukocyte and growth factor concentration.[30]

Another factor that contributes to the variability of PRP is the concentration of other cell types, such as leukocytes and erythrocytes. The centrifugation process used to produce PRP dramatically reduces or eliminates erythrocytes and therefore, erythrocyte content is often not reported. Nonetheless, as reviewed by Oryan *et al*., erythrocytes should be excluded from PRP due to the potential for free radical production and induction of platelet aggregation.[31] Further, under conditions of oxidative

stress, heme (the protein-bound molecules that make up hemoglobin), can become free and cytotoxic.[28]

Whether leukocytes should be retained in PRP is highly debated and the results testing leukocyte-rich versus pure (or leukocyte-poor) PRP are confounding. The majority of commercial PRP kits yield leukocyte-rich PRP in a review performed by Oudelaar *et al*.[30] This review also highlighted that there are both beneficial and adverse effects of leukocyte inclusion, and that leukocyte content should be matched to the specific clinical field and application.[30] Regarding bone regeneration, leukocyte-rich PRP has been shown to stimulate osteogenic differentiation and proliferation of human MSCs *in vitro* in a dose-dependent manner compared to PRP without leukocytes.[32] As reviewed by Oryan *et al*., high concentrations of leukocytes in PRP seem to retard bone healing by inducing an inflammatory reaction that may become chronic, whereas low concentrations of leukocytes may not be able to induce the necessary inflammatory response needed in early bone regeneration.[31] Given these confounding reports, the effect of leukocyte content on bone healing is still unknown.[33]

It is worthwhile to note that the confounding results of leukocyte content in PRP may be due to the fact that simple classification (rich vs poor) is inadequate. Leukocytes are classified as granulocytes (neutrophils, eosinophils, and basophils) or mononuclear cells (lymphocytes and monocytes/macrophages). The role of granulocytes, especially neutrophils, is to combat invading pathogens but they can also incite local tissue destruction.[34] Therefore, if the goal of PRP is to enhance healing, adding neutrophils in excess is likely antagonistic.[28] However, circulating monocytes (likely M2 phenotype) have been shown to suppress inflammation, promote angiogenesis, and support

collagen synthesis through transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), respectively.[28] As these growth factors are also important for bone healing (discussed below), it is reasonable to hypothesize that the leukocyte cell subtype included in PRP may differentiate the ultimate impact on bone repair and reporting this could improve our mechanistic understanding of PRP as well as its clinical efficacy.

6.4 Bone Healing Growth Factors in PRP

There are numerous growth factors in PRP that are important for wound healing: platelet derived growth factor (PDGFaa, PDGFbb, PDGFab), TGFβ$_1$/TGFβ$_2$, VEGF, FGF, epithelial growth factor (EGF), bone morphogenetic protein (BMP), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF).[23,25,35] Upon platelet activation post-injury, growth factors are secreted through the platelet membrane and act upon target cells to stimulate cellular proliferation, vascular invasion (angiogenesis), matrix formation, osteoid production, and collagen synthesis.[23,25,26]

Many of the growth factors within PRP play a direct role in promoting fracture repair. Activated PDGF attaches to transmembrane receptors on osteoblasts, osteoclasts, chondrocytes, fibroblasts and macrophages to stimulate mitogenesis, angiogenesis, bone remodeling, and phagocytosis of damaged tissue during normal wound and fracture healing.[36,37] TGFβ has been shown to regulate proliferation, differentiation, chemotaxis and adhesion of progenitor cells in the wound bed.[38] Further, TGFβ is a potent chondroinductive growth factor that rapidly upregulates type II collagen expression in MSCs.[39] Chondrocytes and osteoblasts are enriched with TGFβ

receptors supporting the idea that TGFβ plays a significant role in the bone healing process and the most intense TGFβ immunostaining occurs during chondrogenic proliferation and endochondral ossification.[40] VEGF, which is expressed by hypertrophic chondrocytes in the fracture callus, as well as the growth plate, plays a critical role in promoting vascular invasion into the avascular cartilage anlagen.[41,42] Further, VEGF acts synergistically with osteogenic proteins, such as BMP4 and BMP2, by enhancing cell recruitment, prolonging cell survival, increasing angiogenesis, accelerating cartilage resorption and enhancing bone mineralization.[43,44]

6.5 PRP History in Orthopaedics

Historically, PRP was used in cell culture beginning in the 1970's [45] and was first used in a clinical setting in 1987.[46] In the early 1990's, Marx used PRP for maxillofacial surgery,[26] which jumpstarted its use in bone repair. Today, PRP has been used as a bioactive agent to restore bone defects or soft tissue in orthopaedic, maxillofacial and plastic surgery. PRP's use as a bioactive therapy has been reviewed extensively for its role in bone, muscle, cartilage, tendon and ligament repair.[29,47–52] In this review, we will briefly discuss the clinical outcomes of PRP alone on bone healing and then focus on the effects of PRP combined with exogenous MSC therapy on intramembranous and endochondral bone regeneration.

6.6 Clinical Outcomes of PRP on Bone Repair

Most preclinical investigations show that PRP-treated bone defects exhibit more advanced healing than controls, however this finding has not been universally consistent. Of the 29 PRP-treated animal long-bone studies included in the meta-

analysis by Gianakos *et al*., 16/18 (89%) showed statistically significant increases in bone healing and tissue differentiation rates while 9/11 (82%) showed qualitative improvement of bone regeneration.[53] In studies that quantified healing, a statistically significant increase in the amount of consolidation, bone formation, and cortical bone thickness was reported. In qualitative studies, 7/9 (78%) reported improvement in bone consolidation.[53] Lastly, 6 studies investigated mechanical properties: five studies reported significantly higher torsional stiffness in the PRP-treated group while one study reported a significant increase in three-point load bearing resulting in increased bone strength after PRP treatment.[53] Based on these results, it is reasonable to hypothesize that PRP acts on the intrinsic stem cell niche to promote repair. However, while the majority of studies show a positive effect on bone healing, there are a minority that show no significant benefit.[53] Due to these conflicting published results, some investigators have suggested that PRP may not be sufficient as a sole adjuvant to enhance bone regeneration.

6.7 *In Vitro* Outcomes of PRP + Exogenous MSCs

Because of the osteogenic growth factors in PRP, it was hypothesized that PRP may act synergistically with MSCs to accelerate osteogenesis. Therefore, many studies have analyzed the effects of combining PRP and MSCs *in vitro*. As reviewed by Fernandes and Yang, PRP generally enhanced the proliferation and differentiation of MSCs in multiple species.[29] MSC proliferation was significantly increased with PRP present in the culture media when compared to DMEM controls with ITS+1 (insulin, transferrin, selenium, linoleic-BSA) supplementation only.[54] In fact, cell proliferation

showed a significant dose-dependent increase when MSCs were cultured with increasing concentrations of PRP (2-10%).[55]

In addition to increasing proliferation, PRP has also been shown to increase osteogenesis of MSCs *in vitro*.[56] Wei *et al*., reported that while PRP alone failed to induce osteogenesis of MSCs, adding PRP to osteogenic media dramatically increased mineralization.[57] Further, when MSCs were combined with PRP *in vitro*, there was significantly increased alkaline phosphatase and osteocalcin synthesis indicating enhanced osteogenic differentiation.[58] Taken together, these *in vitro* studies demonstrate that PRP could improve the osteogenic potential of MSCs.

The effect of PRP on MSCs *in vitro* have also been investigated using scaffolds. MSCs had improved osteogenic differentiation when cultured with PRP and nanohydroxyapatite-type I collagen beads.[59] There was significantly increased formation of mineralized nodules (both area and intensity, marked by fluorescent calcein blue) as well as increased osteocalcin, collagen type 1 and 3, and tenomodulin gene expression in MSCs cultured with a PRP gel/calcium phosphate (CaP) composite.[60] Further, in a small case study, when MSCs from nonunion patients were cultured in a collagen matrix/PRP complex, MSCs proliferated and differentiated into osteogenic progenitor cells within the clot.[61]

6.8 Clinical Outcomes of PRP + Exogenous MSCs in Craniofacial Applications

Bone Healing Outcomes in Small Animal Models

PRP combined with MSCs has been studied extensively in periodontics, maxillofacial and calvarial defects that heal via intramembranous ossification (Table

6.1). Using a rabbit model, Hwang *et al*., studied the consolidation period in distraction osteogenesis of the mandible.[62] The PRP+MSCs group had accelerated bone formation compared to the PRP and control groups, suggesting that the concentrated growth factors in PRP worked synergistically with MSCs to regenerate bone.[62] Further, there was significantly more bone formation in a critical-sized calvarial defect when PRP was combined with MSCs in a sandwich-like scaffold than the empty defect, PRP alone or MSCs alone.[63] Although reported platelet counts in whole blood and PRP are lower than published normal ranges for rodents, bone filling in a calvarial defect was significantly greater when PRP was combined with adipose derived stem cells (ASCs) compared to PRP alone or ASCs in a carrier scaffold.[64]

Bone Healing Outcomes in Large Animal Models

Dogs are a common model in periodontal and maxillofacial research (Table 6.1). Yamada *et al*., reported defects filled with MSC+PRP were equivalent to particulate cancellous bone and marrow (PCBM; autogenous bone) and superior to other groups (MSCs alone and empty defects), resulting in new bone formation after 2 weeks and a tubular pattern with abundant vascularization at 8 weeks.[65–68] However, in another canine periodontal defect model, the PRP+ASC co-implantation group had more new bone formation (64%) after two months, but was not significantly different from PRP alone (54%).[69] Further, a pilot study by Yun *et al*., reported that PRP+MSCs in a hydroxyapatite (HA) scaffold had increased bone density (62% at 6 weeks, 72% at 12 weeks) and increased bone-to-implant contact (22% at 6 weeks, 42% at 12 weeks) in adult dogs with three-wall intrabony defects, a periodontal defect, compared to PRP+HA, MSC+HA, or HA alone, but it failed to reach statistical significance.[70]

Table 6.1: PRP combined with MSCs for craniofacial healing.

Reference	Species	Age	Sex	Sample Size	Defect Model	Cell Type	PRP Platelet Concentration	Scaffold	Treatment Groups	Major Outcome
Hwang et al.,[62]	Rabbit	Adult	Male	38	Distraction osteogenesis of the mandible	Rabbit MSC	Not reported	PRP gel	Empty defect, PRP, PRP+MSC	Significantly more new bone in the distraction gap, thus reducing the consolidation period, in the PRP+MSC group compared to PRP and empty defect.
Liu et al.,[63]	Rat	8 weeks	Male	42	Critical-sized (8mm), Calvaria	Rat MSC, +/- genetically modified to express BMP2	Not reported	Nano-calcium sulfate (nCS)	Empty defect, nCS, nCS+PRP, nCS+MSC, nCS+MSC+PRP, nCS+MSC/BMP2, nCS+MSC/BMP2+PRP	Micro-CT and histology revealed nCS+MSC/BMP2+PRP had significantly more bone formation than all other groups. Bone formation in nCS+MSC+PRP was not significantly different from nCS+MSC/BMP2 but was significantly greater than remaining groups.
Tajima et al.,[64]	Rat	11 weeks	Male	50	Calvaria (5mm)	Rat ASC	$180x10^4$/ml	PRP gel or type I collagen gel	PBS, collagen, PRP, ASC+collagen, ASC+PRP	Newly formed bone was significantly greater in the ASC+PRP group compared to all other groups at 4 and 8 weeks after transplantation.
Yamada et al.,[65]	Dog	Adult	Not reported	4	Mandible (10mm)	Dog MSC	$1.3x10^6$ (no unit given)	PRP gel or particulate cancellous bone and marrow (PCBM)	Empty defect, PCBM, PRP, PRP+MSC	PRP+MSC can elicit bone regeneration equivalent to PCBM and significantly more than PRP and empty defect.
Yamada et al.,[66]	Human	53-74 years	Male and female	3	Alveolar ridge atrophy	Human MSC	Not reported	PRP gel	MSC+PRP	Treatment with MSC+PRP resulted in complete coverage of the implant, absence of mobility at 6 months, and marginal bone resorption.
Yamada et al,[67]	Human	43-74 years	Male and female	8	Severe bone resorption of the alveolar arrest in the maxilla	Human MSC	Not reported	PRP gel	MSC+PRP	All implants were clinically stable, and the prostheses functioned during the follow up period. Alveolar bone height significantly increased compared to pre-operative bone height.
Yamada et al.,[68]	Human	19-78 years	Male and female	104	Dental cases requiring bone regeneration	Human MSC	Not reported	PRP gel	MSC+PRP	Post-operative bone density was significantly greater than pre-operation.
Tobita et al,[69]	Dog	8-10 months	Not reported	8	Class III bifurcation defect at P2, P3, P4	Dog ASC	$946x10^3/mm^3$ (3-fold increase over whole blood)	PRP gel	Empty defect, PRP, PRP+ASC	PRP+ASC had more new bone formation but not significantly different from PRP alone.
Yun et al.,[70]	Dog	1 year	Male	4	Three-wall intrabony (periodontic, 4 x 4 x 4mm)	Human MSC	$1x10^6$/ul	Hydroxyapatite (HA)	HA, HA+MSC, HA+PRP, HA+MSC+PRP	Bone density and bone-to-implant contact were greatest in the HA+MSC+PRP group but not significantly different from the other groups.

Bone Healing Outcomes in Human Patients

Studies combining PRP with MSCs for humans is mostly limited to dentistry and maxillofacial surgery (Table 6.1). Titanium implants encased by coagulated PRP+MSC have shown some success as indicated by complete coverage of the implant, absence of mobility at 6 months, and marginal bone resorption in three patients with severe maxillary alveolar ridge atrophy.[66] Yamada *et al*., reported the average alveolar bone height was significantly increased at three and six months after osteotome sinus floor elevation combined with PRP+MSCs compared to pre-operative bone height.[67] Further, when PRP+MSC was transplanted in 104 dental cases requiring bone regeneration, a statistically significant increase in bone density was observed at all time points post-operation compared to baseline (pre-operation).[68] Combining PRP with MSCs appears to be a promising therapy for craniofacial applications but controlled studies are lacking.

6.9 Clinical Outcomes of PRP + Exogenous MSCs in Long Bone Applications

Bone Healing Outcomes in Small Animal Models

Rodent models have been used extensively to study the outcome of combining PRP and MSCs on long bone healing (Table 6.2). Wei *et al*., demonstrated a positive effect on tibial bone healing in ovariectomized (OVX) rats. By day 14 post injury, no bone callus had formed in the OVX group, a thin callus formed in the OVX+PRP and OVX+MSC groups and thick callus formed in the OVX+PRP+MSC and sham (not ovariectomized rats) groups.[57] Further, the calluses had completely mineralized by 42 days in the OVX+PRP+MSC and non-OVX groups and lamellar bone had fully formed in the OVX+PRP+MSC rats whereas woven bone remained in the OVX, OVX+PRP and

Table 6.2: PRP combined with MSCs for long bone healing.

Reference	Species	Age	Sex	Sample Size	Defect Model	Cell Type	PRP Platelet Concentration	Scaffold	Treatment Groups	Major Outcome
Qi et al.,[60]	Rat	12 weeks	Male	18	Femur (2.5x5mm)	Rat MSC	$13.2x10^8$/ml (6-fold increase from serum)	PRP gel +/- calcium phosphate composite (CaP)	Empty defect, MSC+PRP, CaP, PRP+CaP, MSC+CaP, MSC+PRP+CaP	MSC+PRP+CaP regenerated significantly more bone tissue at 4 weeks than other groups.
Wei et al,[57]	Rat	Adult	Female	100	Ovariectomized, Tibia (1.5mm)	Rat MSC	Not reported	PRP gel	Non-OVX, OVX, OVX+MSC, OVX+PRP, OVX+PRP+MSC	PRP+MSC aided bone callus mineralization by day 42 and lamellar bone formed in OVX+PRP+MSC and non-OVX groups.
Kawasumi et al,[71]	Rat	9 weeks	Male	91	Limb lengthening, Femur	Rat MSC	High = $4358x10^3$/ul; Med = $1453x10^5$/ul; Low = $48x10^6$/ul	PRP gel or collagen gel	MSC+PPP, MSC+PRP low, MSC+PRP med, MSC+PRP high, MSC+collagen gel	Significantly larger area of mineralized bone in MSC+PRP high group at 4 weeks. No significant difference among other groups.
Lin et al.,[59]	Rabbit	12-16 weeks	Male	36	Femoral epicondyle (0.5x1.0cm)	Rabbit MSC	$105x10^7$/ml (3.7-fold increase over whole blood)	Nanohydroxyapatite-type I collagen beads (CIB) or PRP gel	Empty defect, drilling treatment, PRP, PRP+MSC, CIB+PRP, CIB+PRP+MSC	CIB+PRP+MSC had enhanced osteogenesis and accelerated mineralization compared to other groups.
El Backly et al.,[72]	Rabbit	Not reported	Male	10	Ulna (1.4cm)	Rabbit MSC	$3x10^6$/ul	PRP+MSC gel with nanohydroxyapatite/poly (ester urethane) scaffold	Scaffold+PBS, scaffold+MSC, scaffold+MSC+PRP	Micro-CT quantification revealed MSC+PRP had twice as much bone regeneration as MSC or scaffold alone.
Park et al.,[73]	Rabbit	Adult	Male	30	Femur (2cm)	Rabbit MSC	Not reported	Bone graft	PRP+bone graft, PRP+MSC+bone graft	MSC+PRP enhanced bone formation and increased growth factor production in grafts.
Kasten et al.,[74]	Rabbit	6-9 months	Female	36	Critical-sized (15mm), Radius	Rabbit MSC	$10x10^8$/ml	Calcium-deficient hydroxyapatite ceramic (CDHA)	Empty defect, defect + autogenous bone, CDHA, CDHA+MSC, CDHA+PRP, CDHA+MSC+PRP	PRP and MSC increased bone regeneration individually compared to empty defect, but no additional effect of PRP+MSC combined.
Yu et al.,[58]	Rabbit	10 months	Male	24	Radius (12mm)	Rabbit MSC (osteogenic)	$1056x10^3$/ul (5.3-fold increase over whole blood)	Beta-tricalcium phosphate (bTCP)	MSC+bTCP, PRP+MSC+bTCP	PRP+MSC enhanced osseous callus and increased radiograph score at 8 weeks.
Lucarelli et al.,[75]	Sheep	3-5 years	Female	10	Critical-sized (3cm), Metatarsus	Ovine MSC	$1x10^6$/ml	PRP gel+collagen matrix, Bone allograft	PRP+MSC+bone graft, bone graft	Increased vascular invasion, more bone regeneration, and higher extraction torque test values in PRP+MSC+bone allograft group.
Qiu et al.,[76]	Minipigs	12-18 months	Female	12	Femoral condyle (8x10mm)	Minipig MSC	$1.7x10^9$/ml (4-fold increase over whole blood)	Calcium phosphate cement (CPC)	CPC, CPC+MSC, CPC+MSC+PRP	New bone formation and blood vessel density was: CPC+MSC+PRP > CPC+MSC > CPC, all significantly different from each other.
Nair et al.,[77]	Goat	Adult	Not reported	6	Femur (2cm)	Goat MSC (osteogenic)	$6x10^8$/ml (1.5-fold increase over whole blood)	Triphasic ceramic-coated hydroxyapatite (HASi)	HASi, HASi+MSC, HASi+MSC+PRP	No difference in new bone formation or material degradation between the three groups.
Niemeyer et al.,[78]	Sheep	3 years	Female	20	Tibia (3cm)	Ovine MSC, ovine ASC	$1x10^9$/ml (4-5-fold increase over whole blood)	Collagen sponge	Collagen, MSC+collagen, ASC+collagen, PRP+ASC+collagen	MSC had significantly more bone formation than collagen and ASC groups. PRP+ASC had more bone formation but lacked statistical significance.
Kitoh et al.,[79]	Human	12-20 years	Male and female	46	Distraction osteogenesis, Femur and tibia	Human MSC	$26.7x10^5$/ul	PRP gel	No cellular treatment, MSC+PRP	Patients with MSC+PRP had significantly less time until osseous consolidation and fewer complications than patients without MSC.
Wittig et al.,[61]	Human	27-81 years	Male and female	3	Nonunion fractures; tibia, fibula, femur	Human MSC	Not reported	Collagen sponge	MSC+PRP+collagen sponge	All nonunion fractures had healed within 1-3 years after MSC+PRP treatment.

OVX+MSC groups.[57] PRP combined with MSCs has had favorable results in distraction osteogenesis as well. Kawasumi *et al*., utilized a rat limb lengthening model to demonstrate that callus formation was enhanced in a platelet dose-dependent manner within the MSC scaffolds.[71] Further, at 4 weeks post distraction, the high concentration group (1055% platelet concentration of whole blood) was the only group with radiological union and had significantly larger areas of bone mineralization compared to low (117%) and medium (352%) platelet concentrations.[71] In 12-week old rats, femoral defects treated with MSC+PRP gel combined with a CaP composite regenerated dense, cortical bone tissue that was indistinguishable from normal bone.[60] However, when MSC+PRP gel was administered alone, it resulted in thin, woven bone tissue regeneration that was superior to controls, but was still distinguishable from normal bone at 4 weeks post-operation.[60]

Rabbit is another common model for studying PRP+MSC combination therapy for long bone regeneration (Table 6.2). Femur defects filled with nanohydroxyapatite-type I collagen beads combined with PRP and MSCs had improved osteogenesis via accelerated mineralization compared to PRP+collagen beads or PRP alone.[59] In a proof of concept study, El Backly *et al*., combined PRP+MSC in a periosteal engineered membrane which was wrapped around an ulnar defect and demonstrated enhanced bone filling compared to empty and MSC only scaffold controls.[72] Further, treatment with PRP+MSC in a femoral segmental bone defect in male rabbits resulted in enhanced bone formation and gene expression of growth factors in grafted tissues compared to treatment with PRP alone.[73] In a study using PRP and osteogenic-induced MSCs loaded onto a beta-tricalcium phosphate (bTCP) scaffold, Yu *et al*., tested bone

regeneration following a segmental radial defect.[58] At 8 weeks post-operation, the PRP+MSC+bTCP group had enhanced osseous callus around the periphery of the implant and significantly higher radiograph scores than the MSC+bTCP group without PRP.[58] Although most studies report a synergistic response when PRP is combined with MSCs, Kasten *et al*., reported that PRP and MSCs both increased bone regeneration individually, compared to empty defects, however, there was no additional effect on bone healing when PRP and MSCs were combined.[74]

Bone Healing Outcomes in Large Animal Models

PRP and MSC combination therapy studies in large animal models are more limited (Table 6.2). Lucarelli *et al*., reported that the amount of new bone inside allografts was higher with MSC+PRP compared to control, with less space between the host bone and the graft on radiographs, clear signs of ossification such as presence of mature lamellar bone, and 4-5 times the vessel penetration into the graft from the host bone in ovine metatarsal critical sized defects.[75] In another study, combining MSCs and PRP enhanced vascularization and new bone formation. At 12 weeks, calcium phosphate cement (CPC) scaffold combined with PRP+MSCs had significantly more new bone formation in the femoral condyle of minipigs than both the CPC+MSC and CPC groups.[76] Histology confirmed new bone was being deposited by osteoblasts while osteocytes and new blood vessels were surrounded by woven bone matrix.[76] Further, the CPC+MSC+PRP group had significantly greater blood vessel density than CPC+MSC group and was two-fold that of the CPC control.[76]

However, there is conflicting evidence that PRP+MSC may not enhance bone regeneration. In a small study using 6 adult goats, there was no significant difference in

bone regeneration between the osteogenically induced MSCs combined with PRP and osteogenically induced MSCs alone in a segmental femoral defect and scaffold material at two months post-operation.[77] In another study, roughly 25% of the defect was filled with new bone when ASC+PRP were administered to a mid-diaphyseal tibial defect in sheep compared to ASCs alone (~10% filled with new bone), but failed to reach statistical significance.[78]

Bone Healing Outcomes in Human Patients

Studies combining MSCs with PRP to treat long bone defects are extremely limited in humans (Table 6.2). Regarding distraction osteogenesis of human long bones, 16 patients that were treated with PRP+MSCs at the bone lengthening sites experienced significantly less time (34 days vs 73 days) until osseous consolidation and fewer complications (6% vs 23%) than the 30 patients treated with no additional cell therapy.[79] In another small case study, three patients with nonunion femur, tibia and/or fibula fractures were administered autologous MSCs with collagen matrix in a PRP clot at the defect site.[61] Within 3-6 months, radiographs revealed all patients had radiopaque (osteogenic) areas at the site of MSC+PRP implantation and the nonunion fractures had completely healed within 1-3 years.[61] Although these results are favorable for long bone healing in human patients, controlled studies utilizing PRP and MSCs are lacking.

6.10 Conclusions

PRP shows preliminary promise as a biologic adjunct to promote bone repair, particularly when used in combination with MSCs. However, when the body of scientific literature is reviewed as a whole, it is evident that PRP with or without MSCs is not a

proven therapeutic. The optimal method for preparation, final platelet concentrations, activation processes, inclusion/exclusion of other cell types, dose and dose intervals have yet to be determined and optimization may differ between species, donors, and tissue application. Further research is required to understand how platelet, growth factor and specific leukocyte subtype concentrations within PRP can be customized to promote bone repair in specific clinical applications. The balance of data suggests that a combination of PRP and MSCs appears to be superior for bone healing as compared to PRP alone. However, lack of PRP standardization and the MSC tissue source have not been consistent, thereby introducing a great deal of variability in outcomes and conclusions. As an additional confounder, investigators have utilized varying bone healing models (intramembranous versus endochondral ossification) and it is likely that the influence of PRP with and without MSCs may differ in efficacy in each of these bone healing pathways. Therefore, there is a critical need for more controlled studies to elucidate the mechanism of repair when PRP is combined with MSCs in various types of bone healing environments.

6.11 Future Directions

Currently, PRP production and reporting is not standardized. Therefore, PRP can vary widely due to inter- and intra-patient variability. The most common factors that introduce inter-patient variability include the centrifugation protocol/commercial preparation kit used and the inclusion/exclusion of white blood cells. In a systematic review of PRP and long bone healing, Gianakos *et al*., reported that only 55% of studies reported the platelet count or concentration in their PRP preparations and no studies reported the white blood cell count.[53] The lack of consistency makes it hard to draw

conclusions on bone healing between different modalities. Therefore, it is necessary to advocate for consistency in reporting methods, such as platelet concentration, volume, and dose interval.[80] DeLong *et al*., developed a classification system for PRP reporting called PAW which is based on the Platelet concentration, Activation method used, and presence or absence of White cells in the PRP.[33] Further, a more comprehensive classification system called Minimum Information for Studies Evaluating Biologics in Orthopaedics (MIBO) was developed for minimum reporting requirements for clinical studies evaluating PRP.[81] Twenty-three experts compiled a 25-item checklist including details regarding study design, recipient details, injury details, intervention, whole blood processing, whole blood characteristics, PRP processing, PRP characteristics, activation, delivery, postoperative care, and outcome that should be reported for each study.[81] If these systems were adopted, it would encourage standardization, which may provide clarity about the effects of PRP on bone healing. Further, optimal PRP protocols could be developed that are tailored to each specific tissue type and pathological process. The ideal platelet concentration, volume per PRP treatment, number of treatments, and timing of injections still needs to be determined for optimal bone regeneration.

It is also important to determine the optimal scaffold to deliver PRP to bone defects. Fibrin gel is a common scaffold, but additional scaffolds, such as hydroxyapatite, beta-tricalcium phosphate and calcium phosphate cement, are also used and could potentially alter PRP properties, such as growth factor release. A review of PRP combined with MSCs utilizing different scaffolds for bone defects is warranted.

PRP has been supplemented with gene therapy in limited studies. Genetically modified MSCs have been combined with PRP to enhance bone regeneration therapies. Several approaches using MSCs genetically modified to express BMP2,[63] angiopoietin-1,[82] and VEGF[83] have been shown to enhance bone regeneration when combined with PRP.

Lastly, it is important to consider the chronicity of the bone lesions when designing studies. Most PRP studies using animal long bone models surgically create acute long bone defects that are immediately treated with PRP prior to closure.[53] However, in clinical practice, delayed union is a chronic condition.[53] Therefore, it would be ideal if future *in vivo* PRP studies examined chronic bone pathologies.

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