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Comprehensive Investigation into the Utility of Amnion Membrane Derived Stem Cells for Orthopedic Regenerative Medicine Applications

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COMPREHENSIVE INVESTIGATION INTO THE UTILITY OF AMNION MEMBRANE DERIVED STEM CELLS FOR ORTHOPEDIC REGENERATIVE MEDICINE APPLICATIONS

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by
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ABSTRACT

Diseases affecting the cartilage and/or bone, including osteoarthritis (OA), are the most prevalent musculoskeletal tissue pathologies. OA is the result of cartilage degradation, altered sub-chondral bone, impaired joint mobility and severe pain - making it one of the leading causes of disability worldwide. While OA is stereotypically described as a physical wear and tear disease, mounting evidence suggests that synovial inflammation significantly contributes to its pathogenesis. In OA, macrophages infiltrate the synovium and secrete supra-physiological levels of pro-inflammatory cytokines, which create a caustic joint environment promoting articular cartilage degradation. Due to the pro-inflammatory characteristics of OA, the immunomodulatory potential of stem cells likely represents an under investigated therapeutic alternative.

The purpose of this research was to investigate if stem cells from the amniotic membrane (a tissue routinely discarded after the birth of term pregnancies) represent an efficacious alternative cell source for future OA therapies. This was achieved by directly comparing the abilities of human amniotic membrane derived stem cells and a commonly employed stem cell, human adipose derived stem cells, with regards to osteogenic and chondrogenic differentiation potential as well as the ability to mitigate OA disease progression both ex vivo and in vivo.

Our results demonstrate stem cells from the amniotic membrane exhibit heightened differentiation potential, higher yields, enhanced immunomodulatory properties, and the ability to induce pro-regenerative (M2) phenotypes within macrophages, in OA experimental models. Additionally, amnion stem cells appeared to

offer accelerated treatment time lines compared to adipose derived stem cells. For these reasons, we believe amnion membrane derived stem cells are an efficacious stem cell source for OA therapeutic approaches.

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TABLE OF CONTENTS

Page

TITLE PAGE i

ABSTRACT ii

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

I. Background & Significance 1

1.1 Stem Cells & Orthopedic Regenerative Medicine 1
1.1.1 The Need for Orthopedic regenerative Medicine 2
1.1.2 Stem Cells: The Future for Orthopedic Regenerative Medicine 4
1.2 Basic Stem Cell Characteristics 5
1.3 Review of Common MSC Isolation Techniques 6
1.3.1 BMSC Isolation Techniques 6
1.3.2 ADSC Isolation Techniques 10
1.3.3 AFSC Isolation Techniques 11
1.3.4 AMSC Isolation Techniques 13
1.3.5 CMSC Isolation Techniques 14
1.4 Review of MSC Yields 16
1.4.1 BMSC Yields 17
1.4.2 ADSC Yields 18
1.4.3 AFSC Yields 19
1.4.4 AMSC Yields 20
1.4.5 CMSC Yields 20
1.5 Obtaining “Clinically Relevant” Stem Cell Populations 21
1.6 FDA Regulation of Stem Cells 25
1.7 Stem Cell Differentiation 26
1.8 Bone and Cartilage Formation: Endochondral Ossification 30

Table of Contents (Continued)

Page

1.9 Articular Cartilage: Structure & Function 33
1.10 Introduction to Osteoarthritis (OA) 37
1.10.1 Prevalence & Pathogenesis 37
1.10.2 Synovial Inflammation & OA 40
1.10.3 Synovial Macrophages & OA 43
1.10.4 Notable Pro-Inflammatory Mediators in OA 46
1.10.5 In Vitro Models of OA 49
1.10.5.1 OA Chondrocyte/Cartilage Explant Culture 49
1.10.5.2 Chemical Doping of OA Explants 50
1.10.5.3 OA Explant Co-culture 50
1.10.6 In Vivo (Pre-clinical) Models of OA 52
1.10.7 OA Treatment Options 56
1.10.7.1 Available Treatments 57
1.10.7.2 Clodronate (A Pre-clinically Investigated Treatment 58
1.10.8 Stem Cells as an OA Therapeutic 60
1.10.8.1 Pre-Clinical and Clinical Stem Cell Therapies 60
1.10.8.2 Potential Mechanisms behind OA Mitigation 63
1.10.9 Amniotic Membrane Derived Stem Cells & OA 64

II. Project Approach 67

2.1 Significance 67
2.2 Specific Aims 68
2.2.1 Aim I: To directly compare the abilities of amnion and adipose derived stem cells to differentiate towards osteogenic and chondrogenic lineages 68
2.2.2 Aim II: To validate a human joint explant joint tissue co-culture model of OA 69
2.2.3 Aim III: To evaluate the ability of MSCs to mitigate OA progression in this validated ex vivo model 69
2.2.4 Aim IV: To compare the therapeutic effects of hAMSCs and hADSCs to attenuate OA progression in vivo 69

Table of Contents (Continued)

Page

III. Aim I: To Directly Compare the Abilities of Amnion and Adipose Derived Stem Cells to Differentiate towards Osteogenic and Chondrogenic Lineages....71
3.1 Introduction....71
3.2 Materials & Methods....73
3.2.1 Amniotic Membrane Harvest....74
3.2.2 Isolation and Enrichment of hAEC and hAMSC Populations....74
3.2.2.1 Histological Confirmation of Cell Isolation....74
3.2.2.2 Flow Cytometric Analysis for Stem Cell Markers....75
3.2.3 Confirmation of a Mixed Amnion Cell Population....75
3.2.4 hADSC and Amnion Derived Cell Osteogenic Differentiation Potential....75
3.2.4.1 Culture Conditions for Stem Cell Differentiation....75
3.2.4.2 Gene Transcript Analysis....76
3.2.4.3 Alizarin Red Histological Staining for ECM Calcifications....77
3.2.5 hADSC and Amnion Derived Cell Chondrogenic Differentiation Potential....77
3.2.5.1 Culture Conditions for Stem Cell Differentiation....77
3.2.5.2 Alcian Blue Histological Staining for ECM Glycosaminoglycan Content....77
3.2.5.3 Immunohistochemistry for Collagen Type 2....78
3.2.6 Microscopic Imaging....78
3.2.7 Statistical Analysis....78
3.3 Results....79
3.3.1 Amniotic Membrane Harvest....79
3.3.2 Confirmation of Enriched hAEC and hAMSC Populations from Amniotic Membrane....80
3.2.3 Confirmation of a Mixed Amnion Cell Population....81
3.2.4 hADSC and Amnion Derived Cell Osteogenic Differentiation Potential....81
3.2.5 Comparison of hADSC and Amnion Derived Cell Chondrogenic Differentiation Potential....83
3.4 Discussion....87

Table of Contents (Continued)

Page

IV. Aim II: To Validate a Human Explant Joint Tissue Co-Culture Model of OA 94
4.1 Introduction 94
4.2 Materials & Methods 95
4.2.1 Joint Tissue Harvest and Culture Initiation 96
4.2.1.1 In Vitro Co-culture 97
4.2.1.2 In Vitro Cartilage Only Culture 98
4.2.1.3 In Vitro Synovium Only Culture 98
4.2.1.4 In Vitro Macrophage Depleted Co-Culture 98
4.2.2 Synovial Macrophage Phenotype 98
4.2.3 Live/Dead Staining 99
4.2.4 Histopathological Assessment of Explant Tissue 99
4.2.5 Assessment of Cartilage Degradation 100
4.2.5.1 Cartilage Glycosaminoglycan (GAG) Content 100
4.2.4 Collagen Leaching to Culture Media 100
4.2.6 Co-culture Media Pro-Inflammatory Profile 100
4.2.7 Microscopic Imaging 101
4.2.8 Statistical Analysis 101
4.3 Results 101
4.3.1 Joint Tissue Harvest and Culture Initiation 101
4.3.2 Synovial Macrophage Phenotype 101
4.3.3 Live/Dead Staining 103
4.3.4 Histopathological Assessment of Explant Tissue 105
4.3.5 Assessment of Cartilage Degradation 106

4.2.6 Co-culture Media Pro-Inflammatory Profile 108
4.4 Discussion 109

V. Aim III: To Evaluate the Ability of MSCs to Mitigate OA Progression in this Validated Ex Vivo Model 116
5.1 Introduction 116
5.2 Materials & Methods 117
5.2.1 Joint Tissue Harvest and Culture Initiation 118
5.2.1.1 hAMSC Culture 118
5.2.2.1.1 OA+hAMSC (Direct Culture) 119

Table of Contents (Continued)

Page

5.2.2.1.2 OA+hAMSC (Indirect Culture) 119
5.2.1.2 OA+hADSC Culture 119
5.2.2 Synovial Macrophage Phenotype 119
5.2.3 Live/Dead Staining 120
5.2.4 Histopathological Assessment of Explant Tissue 120
5.2.5 Assessment of Cartilage Degradation 121
5.2.5.1 Cartilage Glycosaminoglycan (GAG) Content 121
5.2.4 Collagen Leaching to Culture Media 121
5.2.6 Co-culture Media Pro-Inflammatory Profile 121
5.2.7 Microscopic Imaging 122
5.2.8 Statistical Analysis 122
5.3 Results 122
5.3.1 Joint Tissue Harvest and Culture Initiation 122
5.3.2 Synovial Macrophage Phenotype 123
5.3.3 Live/Dead Staining 126
5.3.4 Histopathological Assessment of Explant Tissue 128
5.3.5 Assessment of Cartilage Degradation 130
5.2.6 Co-culture Media Pro-Inflammatory Profile 134
5.4 Discussion 138

Aim IV: To Compare the Therapeutic Effects of hAMSCs and hADSCs to Attenuate OA Progression In Vivo 143
6.1 Introduction 143
6.2 Materials & Methods 144
6.2.1 Stem Cell Preparation 144
6.2.2 Stem Cell Intra-Articular Injections 145
6.2.3 Cartilage Surface Macro Architecture 146
6.2.4 Cartilage Surface Micro Architecture 146
6.2.5 Assessment of Cartilage Degradation 147
6.2.6 Histological Confirmation of Synovitis 148
6.2.7 Microscopic Imaging 148
6.2.8 Statistical Analysis 148

VII. Conclusions 158

VIII. Recommendations 160
5.1 What Worked Well 160

Table of Contents (Continued)

Page

5.2 Future Suggestions for Aim I 161
5.3 Future Suggestions for Aim II 162
5.4 Future Suggestions for Aim III 163
5.5 Future Suggestions for Aim IV 164

APPENDICES 166

A: Additional Data/Figures 167
B: Comprehensive Inflammatory Assessment of OA Explant Cultures 181

REFERENCES 204

LIST OF TABLES

Table		Page
1	Verified Stem Cell Criteria	7
2	Calculated and Reported MSC Yields	21
3	Summary of Stem Cell Therapies for Osteoarthritis	61

LIST OF FIGURES

Figure		Page
1	Pictorial Representation of BMSC Isolation	9
2	Pictorial Representation of ADSC Isolation	10
3	Pictorial Representation of AFSC Isolation	12
4	Pictorial Representation of AMSC Isolation	13
5	Pictorial Representation of CMSC Isolation	15
6	Calculated AFSC, AMSC and CMSC Yields	22
7	Largest Reported BMSC, ADSC, AFSC and AMSC Yields	23
8	Tissue Required To Obtain $1x10^6$ Stem Cells	25
9	Growth Factor-Induced Stem Cell Differentiation	27
10	Osteogenic Differentiation	28
11	Chondrogenic Differentiation	29
12	Endochondral Ossification	31
13	Sox-9 & Runx-2 Involvement in Endochondral Ossification	32
14	Cartilage Structure	34
15	Osteoarthritis (OA)	38
16	Activated Chondrocytes	39
17	Toll-Like Receptor (TLR) Activation	42
18	Macrophage Polarization	44
19	M1 Macrophage Infiltration in OA	46
20	Summary of In Vitro OA Models	51

List of Figures (Continued)

Figure		Page
21	Summary of Common In Vivo OA Models	54
22	Guinea Pig Model of OA	56
23	Amniotic Stem Cell Harvest, Isolation and Characterization	79
24	Osteogenic Gene Transcript Expression of Human Amnion Derived Cells and hADSCs	82
25	Osteogenic Calcified Matrix Expression of Human Amnion Derived Cells and hADSCs	83
26	Chondrogenic Gene Transcript Expression of Human Amnion Derived Cells and hADSCs	84
27	Chondrogenic Gene Transcript and Cartilage Matrix Expression of Collagen -2 in Human Amnion Derived Cells and hADSCs	86
28	Chondrogenic Matrix Component Expression of Human Amnion Derived Cells and hADSCs	87
29	Methods Schematic	97
30	Confirmation of Macrophage Depletion	102
31	Cell Viability Assessment	104
32	OARSI Histopathological Evaluation of Cartilage Microarchitecture	105
33	Biochemical Evaluation of Cartilage	107
34	Cytokine Analysis over 15 Days in Culture	108

List of Figures (Continued)

Figure Page

35 Methods Schematic Demonstrating the Described Comparative Analysis between Control OA Explant Co-culture ("OA)" and OA Co-cultures Treated With Stem Cells Applied Directly or Indirectly to the Cartilage Surface 118

36 Methods Schematic Demonstrating the Described Comparative Analysis between Control OA Explant Co-culture ("OA)" and OA Co-cultures Treated With either hAMSCs or hADSCs 119

37 Semi-Quantitative Analysis of M1 Polarized Macrophages in hAMSC and hADSC-Treated Cultures..... 123

38 Semi-Quantitative Analysis of M1 Polarized Macrophages in Direct and Indirect-Treated Cultures 124

39 Semi-Quantitative Analysis of M2 Polarized Macrophages in hAMSC and hADSC-Treated Cultures..... 125

40 Semi-Quantitative Analysis of M2 Polarized Macrophages in Direct and Indirect-Treated Cultures 126

41 Semi-Quantitative Analysis of Chondrocyte Viability in hAMSC and hADSC-Treated Cultures..... 127

42 Semi-Quantitative Analysis of Chondrocyte Viability in Direct and Indirect-Treated Cultures 128

43 Average OARSI Scores in hAMSC and hADSC-Treated Cultures..... 129

44 Average OARSI Scores in Direct and Indirect-Treated Cultures..... 130

45 Average Cartilage GAG Content in hAMSC and hADSC-Treated Cultures..... 131

List of Figures (Continued)

Figure Page

46 Average Cartilage GAG Content in Direct and Indirect-Treated Cultures 132

47 Average Cartilage Media Hydroxyproline Content hAMSC and hADSC-Treated Cultures 133

48 Average Cartilage Media Hydroxyproline Content in Direct and Indirect-Treated Cultures 134

49 IL-1β Analysis in hAMSC and hADSC-Treated Cultures 134

50 IL-1β Analysis in in Direct and Indirect-Treated Cultures 135

51 TNF-α Analysis in hAMSC and hADSC-Treated Cultures 136

52 TNF-α Analysis in in Direct and Indirect-Treated Cultures 136

53 MMP-13 Analysis in hAMSC and hADSC-Treated Cultures 137

54 MMP-13 Analysis in in Direct and Indirect-Treated Cultures 138

55 Indirect Contact hAMSCs May Be In Direct Contact with Synovium 140

56 Methods Schematic of Cartilage Harvest 147

57 Cartilage Surface Macro Architecture 150

58 Cartilage Surface Micro Architecture of Controls 150

59 Cartilage Surface Micro Architecture of Experimental Groups 151

60 Cartilage GAG Content 151

61 Cartilage Collagen Content 152

List of Figures (Continued)

Figure Page

62 Histological Assessment of Synovitis 153

63 Subject-Matched Histological Assessment of Synovitis 154

CHAPTER ONE

Background & Significance

1.1 Stem Cells & Orthopedic Regenerative Medicine

Musculoskeletal disorders represent the largest disease subset in the U.S. population, as recent reports estimate that 50% of the U.S. population (approximately 110 million individuals) suffers from at least one musculoskeletal tissue pathology.[1] Due to increases in life expectancy these estimates are expected to rise.[1,2] Direct costs associated with musculoskeletal disease management have been estimated upwards of $510 billion.[1] Additionally indirect costs, including lost wages, are estimated near $350 billion.[1] Moreover, the societal costs covering the care of such conditions (hospitals, physicians, therapists, caregivers, etc.) near $1 trillion.[1] Though physical therapy and non-invasive treatment options are often initially pursued, many patients require further intervention.

Orthopedic surgical intervention typically occurs when the conservative management of musculoskeletal tissue pathologies has failed. Such surgeries usually involve the replacement of native tissue(s) with biological or metal implants.[3,4] Musculoskeletal tissue grafts have demonstrated only limited success.[3] Metal-based orthopedic replacements fail prematurely with the potential for biological incompatibility due to the generation of metal wear debris particles.[3,4] In light of these shortcomings, recent research in the field of orthopaedic regenerative medicine has turned to the use of biologic therapies, including stem cell-based therapies.[3–7] Clinically, these cells could be used alone or in combination with either synthetic or natural scaffolds in order to mitigate progression of disease or to promote the repair or regeneration of damaged musculoskeletal tissues. Numerous stem cell sources have been evaluated, yet currently

no single stem cell source has been identified as being ideal for any specific orthopaedic application (i.e. regeneration of bone, cartilage, muscle, tendon, etc.…).

However, evidence is starting to emerge which may suggest preferential tissue-specific lineage differentiation of adult and perinatal stem cells.[6,8,9] While it may stand to reason that adult stem cells derived from the target tissue (i.e. the tissue to be repaired / regenerated) may be optimal; the possibility that these stem cells have 1) also succumb to damage or disease and 2) are only available in limited quantities make them less than ideal for clinical use.[5,10–12] It has been widely accepted that stem cells must be administered in large quantities to demonstrate clinical efficacy. However, only recently has clinical evidence emerged from the orthopedic community suggesting therapeutic benefit is only achieved when stem cell are administered in high doses.[13] In light of this need for large quantities of cells, it has been suggested that the ex vivo expansion of mesenchymal stem cells (MSCs) makes them suited for orthopedic applications.[4,5,14] However, ex vivo expansion exposes cells to increased possibilities of contamination, alterations to their phenotype and differentiation potentials, and a potentially delayed administration.[15–17] Thus, a stem cell source requiring limited (or no) ex vivo expansion in order to achieve clinically significant numbers would be preferred. Accordingly, stem cells with both demonstrated orthopedic applications and high yield isolations, both discussed herein, would be most clinically relevant.

1.1.1 The Need For Orthopedic Regenerative Medicine

According to the American Academy of Orthopedic Surgeons, over 2 million musculoskeletal tissue repairs are performed in the US, annually.[18] Many of these repairs rely on tissue grafts or metal implants in order to reduce pain and restore tissue function.

Tissue grafts have been shown to fail due to incomplete filling of the defect, failed resorption (bone), or lack of host tissue integration.[3,19] Tendon grafts, specifically, do not adequately reproduce the tendon-bone interface, limiting the strength and functionality of the graft.[4] Additionally, there have been reports of allogeneic musculoskeletal tissue graft rejection.[3,4] Implanted biomaterials have finite lifespans, increasing patient morbidity through the resulting necessary re-operation.[4] Metal implants in particular, have the potential to elicit an immune response through metal debris particles and metal implant infection typically necessitates a re-operation to remove the implant.[3,4]

Alternative methods address not only the restoration of musculoskeletal tissue function but also aim towards host tissue integration and regeneration. For example, in osteochondral defect repair, the micro-fracture technique punctures the sub-chondral bone allowing bone marrow to leak into the repaired area, accelerating healing.[4,5] Autologous cartilage transplantation has also been utilized for enhanced cartilage defect repair.[4] Additionally, tissue-engineered materials (ex. calcium sulfate pellets, methyl methacrylate, collagen and hydroxyapatite (HA) have been utilized as bone fillers in bone defect repair.[19] A developing area of research over the past twenty years has been platelet-rich plasma (PRP). PRP therapies involve the autologous administration of the patient's own concentrated plasma.[20,21] As platelets play a significance role in natural wound healing (via cell recruitment and growth factor secretion), the administration of PRP has the potential to enhance healing of various orthopedic injuries.[21] Growing evidence supports accelerated healing and/or enhanced tissue regeneration in bone, muscle and tendon injuries after PRP injection therapy.[20,21]

1.1.2 Stem Cells: The Future for Orthopedic Regenerative Medicine?

Stem cells have been proposed as an intriguing alternative for orthopedic regenerative medicine due to their musculoskeletal tissue differentiation and immunomodulatory capacities. Most musculoskeletal tissues have limited capacity for self-renewal.[6] Therefore, stem cells could be considered as alternative healthy cells within target tissues, potentially leading to replenished host cell populations and tissue regeneration. Additionally, as some musculoskeletal tissue disorders, such as arthritis, have an identified immune component; paracrine or juxtacrine stem cell effects could assist in disease modulation.[4,7,11]

In addition to the hypothesized benefits of stem cell therapies, experimental evidence supports their beneficial use in orthopedics. As previously mentioned, the micro-fracture technique in osteochondral defect repair introduces bone marrow to the defect repair area, promoting tissue healing by exposing the area to bone marrow stem cells.[4,5] MSCs have also been used successfully as a therapy for delayed fracture union, non-unions, arthrodesis, and bone defects.[6] The autologous chondrocyte transplantation technique has been employed with not only autologous chondrocytes, but chondrocyte precursor cells, periosteum and stem cells, as well.[4] Preliminary cohort studies have demonstrated that MSC administration is at least as effective as autologous chondrocyte transplantation.[22] High dose stem cell therapies have also been employed in the treatment of rheumatoid arthritis and other rheumatic diseases, with positive outcomes.[4] Though beyond the scope of our review, there are also numerous animal studies employing stem cells that show mitigation of musculoskeletal disease progression and/or tissue regeneration.[3–6] Interestingly, animal studies comparing the efficacy of current orthopedic

regenerative medicine techniques (periosteal graft, mosaicplasty, and autologous chondrocyte transplantation) to stem cell therapy, show stem cell therapy is superior to periosteal grafts and mosaicplasty.[23]

1.2 Basic Stem Cell Characteristics

Stem cells, by definition, must demonstrate potency (the ability to differentiate into target tissue cell types) and self-renewal (the ability to both proliferate and generate progeny stem cells).[9,24] The minimal criteria defining MSCs was determined by the International Society for Cellular Therapy in 2006. MSCs must: 1) demonstrate plastic adherence, 2) test positively (>95%) for CD105, CD73, and CD90 and negatively (<2% positive) for CD45, CD34, CD14 or CD11b, and CD79 or CD19, and 3) be capable of differentiating into adipogenic, chondrogenic and osteogenic lineages.[25] To ensure isolated cells meet such requirements, experiments are conducted demonstrating 1) plastic adherence through serial passaging on plastic tissue culture flasks, 2) appropriate cell phenotype through flow cytometric analysis (cell marker identification via antibody tagging) with serial passaging, and 3) in vitro (and in some cases in vivo) differentiation experiments where cells are exposed to signals (ex. exogenous growth factors or other chemicals, mechanical stimulation) and evaluated for morphologic and gene/protein phenotypic likeness to target cells.

Large variability in MSC isolation, propagation and characterization techniques exist.[7,9,14,24–26] Some authors have attributed such variability to the noted discrepancy between in vitro experiment success and in vivo experiment (or clinical trial) failure.[7,14] As such, there has been a push to employ more than “minimal” criteria when proposing

to use MSCs.[14,24] Additional suggested criteria include in vivo (as opposed to the common practice, in vitro) differentiation potential, the ability to form colonies in vitro and evaluation of teratoma formation in vivo. It has also been suggested that the definitions of potency and self-renewal should reflect the overarching goal of use in human; thus restricting MSC criteria to in vivo (not in vitro) demonstrations.[24,27]

Bone marrow derived stem cells (BMSCs), adipose derived stem cells (ADSCs), amniotic fluid stem cells (AFSCs), amniotic mesenchymal stem cells (AMSCs), and chorionic mesenchymal stem cells (CMSCs), have been previously evaluated and meet the minimal MSC criteria defined by the International Society for Cellular Therapy.[28–42] Table 1 reports the verified stem cell criteria for each cell type, including reports of cell surface marker expression (phenotype). Cell phenotype is passage dependent and can also vary depending on the isolation method employed.[9] Though ≥95% and ≤2% are the threshold values assigned to positive and negative surface marker expression, variable expression has been reported with positive values as low as 80%.[9]

1.3 Review of Common MSC Isolation Techniques

1.3.1 BMSC Isolation Techniques

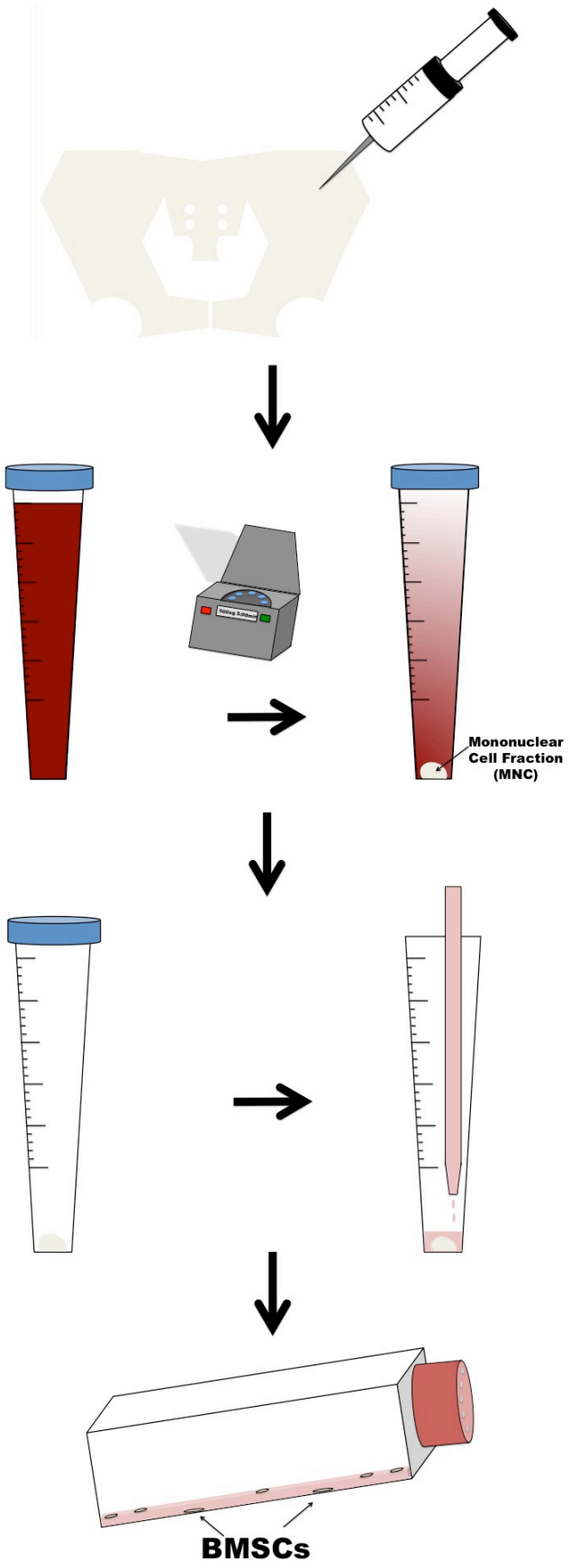
Bone marrow (BM) aspiration is typically required for BMSC isolation. For several decades, the most common site of aspiration has been the iliac crest, though the femoral shaft has also been utilized.[43,44] As illustrated in figure 1, BM is collected through an aspiration needle system at varying depths of insertion into the iliac crest. Typically, the BM aspirate is separated into its constituent components using Ficoll gradient centrifugation. This technique employs centrifugation to separate BM components by density, resulting in

Table 1: Verified Stem Cell Criteria

Stem Cell	Plastic Adherence	Positive for CD105, CD90, CD73	Negative for CD45, CD34, CD14 (or 11b), CD79 (or19)	Differentiation Capacity	Other Marker Expression (+)	Other Marker Expression (-)
BMSC	Yes[34,39,41,45–47]	Yes[34,47,51,52]	Yes[34,47,51,52]	Osteogenic[34,41,46,51] Chondrogenic[34,41,45,47] Adipogenic[41,46]	CD13(+)[41,45] CD29(+)[40,45,46] CD44(+)[34,41,45–47] CD166(+)[34,41,45,47] HLA-ABC(+)[34,47]	CD106(+/-)[41] CD14(-)[46] CD117(-)[34] CD133(-)[46] HLA-DR(-)[34,47]
ADSC	Yes[34,39,41,45–47]	Yes[34,51,52]	Yes[34,51,52]	Osteogenic[34,41,46,51] Chondrogenic[34,41,46,51] Adipogenic[41,46]	CD9(+)[41] CD13(+)[41] CD29(+)[41,46] CD44(+)[34,41,46] CD49(+)[41] CD54(+)[41] CD106(+)[41] CD146(+)[41] CD166(+)[34,41] HLA-ABC(+)[41]	CD14(-)[46] CD117(-)[34] HLA-DR(-)[34,41]
AFSC	Yes[45,48,49]	Yes[45,48,49]	Yes[45,48,49]	Osteogenic[45,49] Chondrogenic[45] Adipogenic[45,49]	CD29(+)[45,48,49] CD44(+)[45,48] CD49(+)[48] CD166(+)[48] CD117(+)[50]	CD10(-)[49] HLA-DR(-)[49]

AEC	**Yes**[54,58]	**Yes**[54,58]	**Yes**[54,58]	Osteogenic[4,58] Chondrogenic[4,58] Adipogenic[4,58]	CD29(+)[45,52,53] CD31(-)[37] CD44(+)[34,47,52–54] CD117(-)[34] CD49(+)[52] HLA-DR(-)[29,34,47] CD71(+)[29] CD166(+)[34,47,52,53] HLA-ABC(+)[29,47]
AMSC	Yes[29,34,37,41,45,46,48,50,52,53]	Yes[29,34,41,45,48,52–54]	Yes[29,34,41,45,48,52–54]	Osteogenic[29,34,41,45,51–54] Chondrogenic[29,34,41,45,51–54] Adipogenic[29,41,45,52–54]	
CVSC	Yes[41,55]	Yes[55]	Yes[55]	Osteogenic[41,45,55] Chondrogenic[41,45,55] Adipogenic[41,45,55]	CD13(+)[55] CD31(-)[55] CD44(+)[55] HLA-DR(-)[55] HLA-ABC(+)[55]
CMSC	Yes[29,41,45,49,52,55,56]	Yes[41,48,51,54,56,57]	Yes[41,48,51,54,56,57]	Osteogenic[29,41,48,51,56,57] Chondrogenic[29,41,48,51,56,57] Adipogenic[45,47,52,54]	CD13(+)[45] CD3(-)[45] CD29(+)[45,52] CD117(-)[34] CD44(+)[34,45,47,52,54] HLA-DR(-)[29,34,47] CD49(+)[52] CD54(+)[45] CD166(+)[34,45,47,52] HLA-ABC(+)[29,47]

Table 1: Verified Stem Cell Criteria for human BMSCs, ADSCs, AFSCs, AECs, AMSCs, CVSCs and CMSCs. Criteria selections have corresponding references supporting the stem cells' ability to meets each specific criterion. The far right column represents additional phenotypic analysis for each stem cell type.



the separation of solid components. The BMSCs are located within the resulting cell pellet, which is known as the mononuclear cell (MNC) fraction. The MNC contains not only BMSCs but populations of cells with phenotypic expression profiles dissimilar to those of a defined stem cell.[33,43,59] The BMSCs can be isolated from the other cells of the MNC by re-suspending (i.e. adding media and gently mixing to re-distribute the cells throughout the suspension) the MNC pellet and culturing on plastic (at least overnight), with BMSCs found amongst the adherent cells. Flow cytometric analysis and multipotency assays, like those previously described, to confirm the

Figure 1: Left, Pictorial Representation of BMSC Isolation beginning at the top with bone marrow aspiration from the iliac crest, followed by centrifugation to separate the MNC, supernatant aspiration, re-suspension in media and plating, where the adherent cells are termed BMSCs.

presence of mesenchymal stem cells are also suggested.[59]

1.3.2 ADSC Isolation Techniques

Adipose tissue is relatively abundant, and it is typically removed in large quantities through gross resection, conventional liposuction and ultra-sound assisted liposuction procedures.[60] Laser-assisted liposuction is less commonly employed.[61] Adipose tissue is most commonly removed from the abdomen, and there does not appear to be a relationship between body mass index or gender and ADSC yield.[62] To obtain the ADSCs, the adipose tissue is usually washed, minced, and enzymatically digested (typically in collagenase) to obtain what is commonly referred to as the

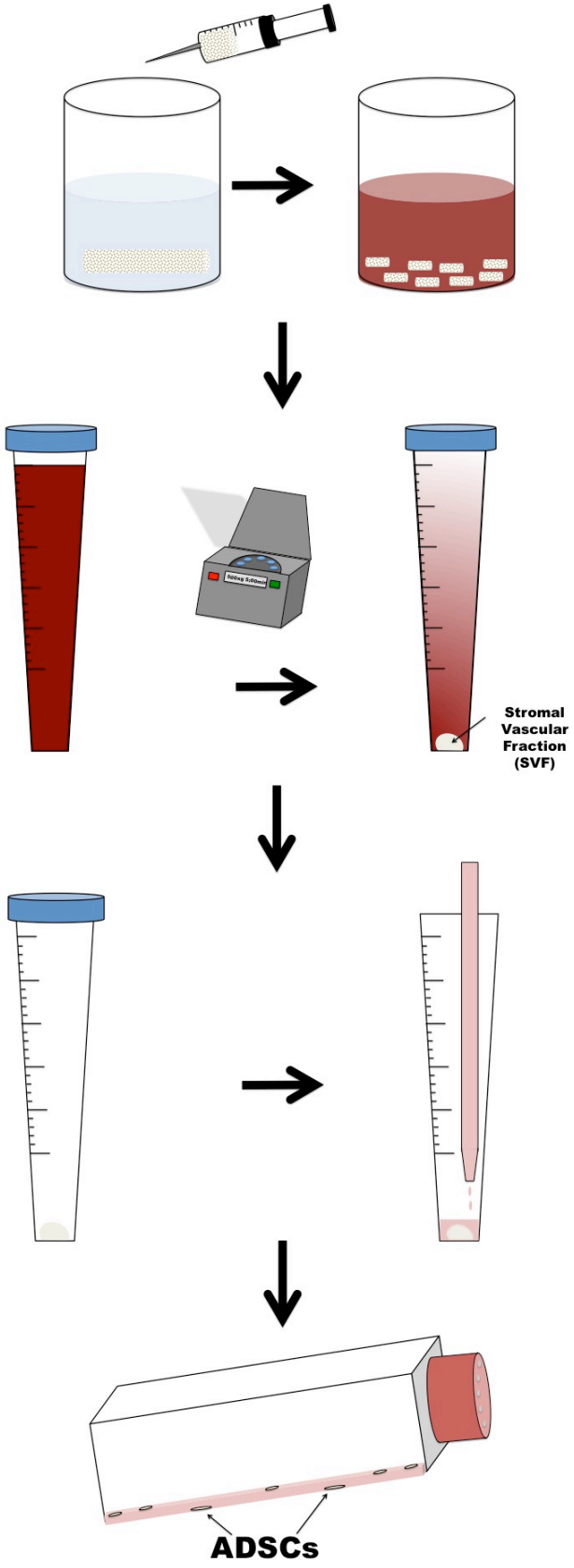
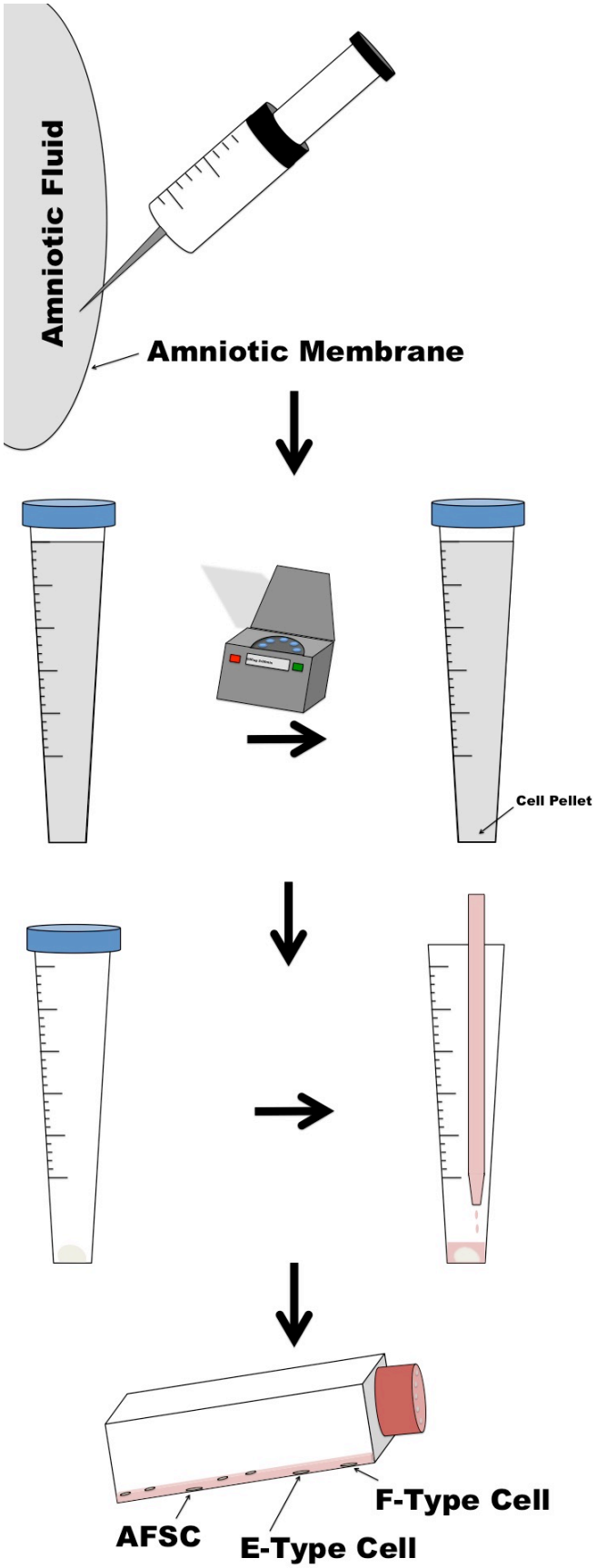


Figure 2: Left, Pictorial Representation of ADSC Isolation beginning at the top, human lipoaspirate is washed and minced prior to centrifugation to separate the SVF, supernatant aspiration, re-suspension in media and plating, where the adherent cells are termed ADSCs.

stromal vascular fraction (SVF).[7,9,60,63] The SVF is comprised of stromal and stem cells (<0.1%), endothelial cells (10-20%), lymphocytes (10-15%), monocytes and macrophages (5-15%), pericytes (3-5%), among other cell types.[9] ADSCs can be separated from the SVF via Ficoll gradient centrifugation.[7,9] As with BMSCs, in vitro culture (at least overnight) and flow cytometric analyses for the previously mentioned surface markers are commonly employed to ensure stemness of the isolated cells.

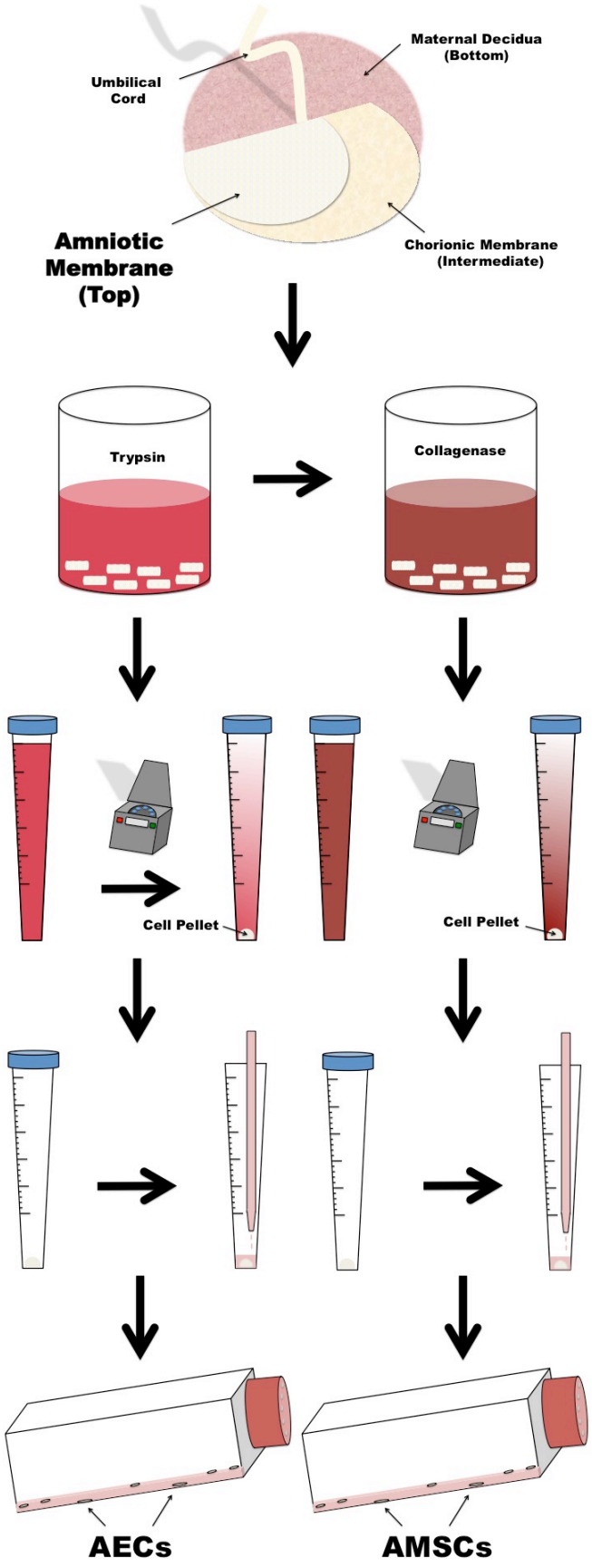
1.3.3 AFSC Isolation Techniques

Amniotic fluid (AF) is typically obtained during the second trimester by ultrasound-guided needle puncture (trans-abdominal or trans-cervical) during an amniocentesis procedure, though it can be obtained with minimal risk from week 14 through the duration of the pregancy.[34,57] In't Anker et al collected AF via trans-abdominal and trans-cervical amniocentesis from second and third trimester pregnancies. AFSCs free of maternal contamination were successfully isolated in n=10/10 trans-abdominal and in n=4/10 trans-cervical second trimester amniocentesis samples (mean AF volume: 8.7±1.7mL trans-abdominal; 32.3±13.9mL trans-cervical). AFSCs free of maternal contamination were successfully isolated in only n=2/10 third trimester samples (mean AF volume: 10.7±4.8mL).[57] This highlights the utility of trans-abdominal amniocentesis and the known change (decrease in AFSC) in the AF cellular profile throughout pregnancy. Higher volumes of AF can be obtained via trans-cervical amniocentesis, but these samples do not reliably yield pure AFSC populations. Trans-abdominal amniocentesis seems to routinely yield pure AFSC populations throughout the second trimester, but lower volumes of AF are typically taken using this procedure. Though groups such as Kaviani et al describe taking as much as 22mL of AF during



amniocentesis, it has been reported that as little as 1-2mL of AF is required to successfully isolate AFSCs.[39,51] As illustrated in figure 3, the AF is centrifuged, the resultant cell pellet is re-suspended and cultured on plastic (at least overnight), with the adherent cells termed AFSCs.[38,40,52,64] The danger of plastic adherence-based isolation of AFSCs is the known plastic adherence exhibited by three distinct AF cells types: E-type, F-type, and AFSCs.[65] Thus stem cell yields calculated from plastic adherence-based isolation are likely inflated values. Plastic adherence-based isolation followed by flow cytometric analysis for CD117, a

Figure 3: Left, Pictorial Representation of AFSC Isolation beginning at the top, human AF is typically obtained via amniocentesis prior to centrifugation (to obtain the cell pellet), supernatant aspiration, re-suspension in media and plating, where the adherent cells are either AFSCs, E-Type or F-type cells. Flow cytometric analyses are typically required to further separate AFSCs.



tyrosine kinase receptor of stem cell factors, is preferred.[34,40,50,66] However, more meticulous isolation methods such as that described by Phermthai et al would also remove this bias in yield reporting.[67]

1.3.4 AMSC Isolation Techniques

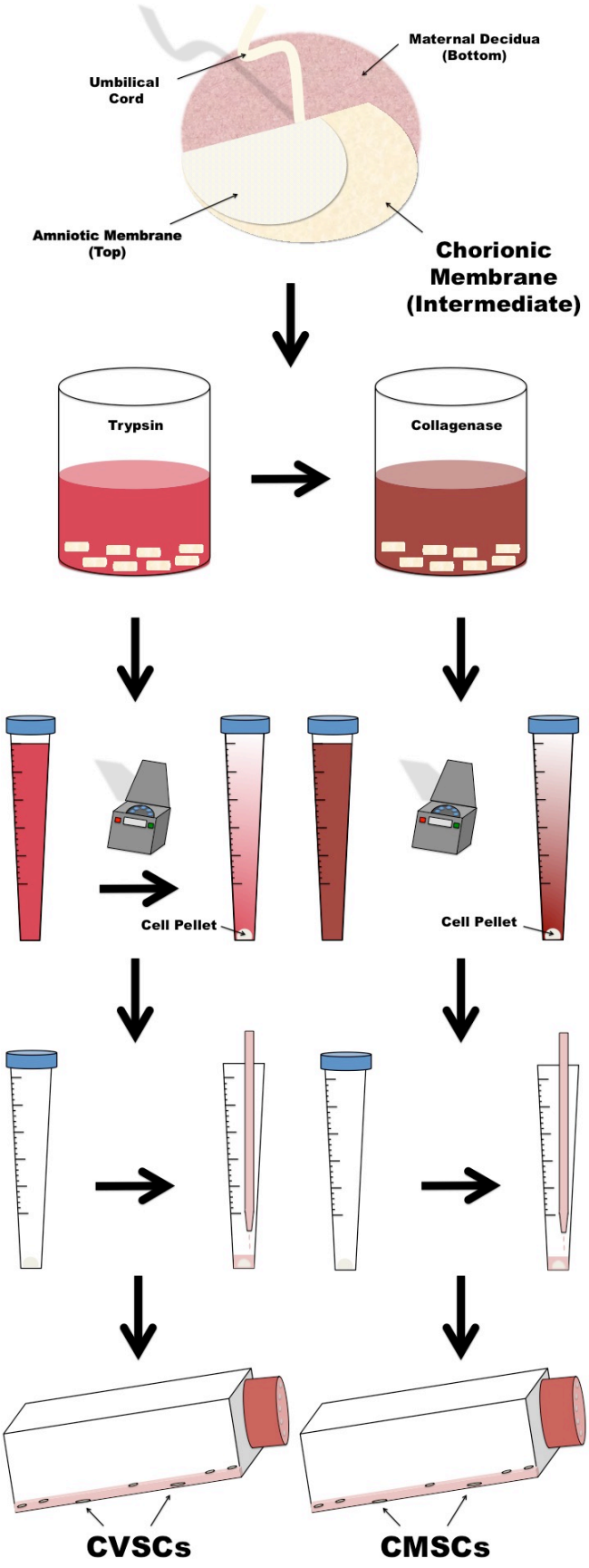
Amniotic membrane (AM) is typically obtained after term cesarean section.[54] The AM contains two distinct populations of cells that exhibit stem cell markers: 1) amniotic epithelial cells (AECs) and 2) amniotic mesenchymal stem cells (AMSCs). The isolation, characterization, and potential application of amniotic cells have been previously reviewed by our group.[68] In't Anker et al successfully

Figure 4: Left, Pictorial Representation of AMSC Isolation beginning at the top, human AM is mechanically peeled from the CM and digested in trypsin/EDTA (to selectively remove AECs) followed by digestion in collagenase (to remove AMSCs). Similar processing steps are utilized as previously described. Adherent cells from the trypsin digest are termed AECs while adherent cells from the collagenase digest are termed AMSCs.

isolated AMSCs from n=8/10 second trimester and n=7/10 term AMs.[57] The most traditional method to isolate out each amniotic cell population is via serial digestion with trypsin/EDTA (to selectively remove the AECs from the epithelial layer) followed by collagenase or dispase digestion (to liberate the AMSCs from the stromal tissue layer).[41,54] To obtain AMSCs, the collagenase-digested sample is centrifuged; the resultant cell pellet is re-suspended and cultured on plastic (at least overnight), with the adherent cells termed AMSCs. It should be noted that initial collagenase digestion of the amniotic membrane (without a trypsin digestion step) results in a mixed (AEC+AMSC) population of cells. In our experience, similar results can be obtained by using diluted volumes of trypsin during the initial digestion step, resulting in only the partial removal of AECs and consequent removal of the remaining AECs and AMSCs during collagenase digestion. Yields reported using such methods are thus inflated, as they do not depict a pure AMSC population. Flow cytometry for epithelial markers (to ensure no AEC contamination) and mesenchymal stem cell markers are suggested employed to further distinguish AMSCs. In fact, Marongiu warns that standard trypsin isolation concentrations (0.05% weight/volume) do not reproducibly yield pure AMSC populations and recommends purification through a density separation method.[47]

1.3.5 CMSC Isolation Techniques

The chorionic membrane contains two primary stem cell populations that retain mesenchymal stem cell characteristics: 1) chorionic mesenchymal stem cells (CMSCs) harvested from the stromal chorionic layer and 2) chorionic villous stem cells (CVSCs) isolated from the trophoblastic chorionic villi (As such, CVSCs are also referred to as chorionic trophoblastic cells). Placentas for CMSC isolation are typically obtained after



term cesarean section. Nazarov et al reported the ability to isolate plastic-adherent CMSCs in all n=10/10 and Bačenková et al reported success in all n=6/6 isolation attempts under such conditions.[46,69] Jones et al compared first and third trimester CMSCs, finding similar phenotypes and differentiation potentials despite the developmental discrepancy. Additionally, first trimester CMSCs exhibited heightened kinetics, smaller sizes and unique surface expression profiles more characteristic of embryonic stem cells.[70] However, as the author acknowledged, therapeutic use of first trimester CMSCs would require pregnancy termination.

Figure 5: Left, Pictorial Representation of CMSC Isolation beginning at the top, human CM is removed form the maternal decidua and digested in trypsin/EDTA (to selectively remove CVSCs) followed by digestion in collagenase (to remove CMSCs). Similar processing steps are utilized as previously described. Adherent cells from the trypsin digest are termed CVSCs while adherent cells from the collagenase digest are termed CMSCs.

These authors could find no report of CVSC use in orthopedic regenerative medicine, though there are reports of using general chorionic (CMSCs+CVSCs) stem cells in musculoskeletal tissue engineering and regenerative medicine investigations. Additionally, as previously indicated their removal is necessary to ensure pure CMSC populations and we have thus briefly included them in our review. CVSCs can be obtained throughout pregnancy through trans-cervical chorionic villous samplings.[34] The primary concern in CVSC isolation is maternal contamination, which is frequently reported.[54,71] The most traditional methods by which to isolate out each chorionic cell population is via serial digestion with trypsin (to selectively remove the trophoblastic cells) followed by collagenase and or dispase digestion (to liberate the CMSCs from the stromal tissue layer), and subsequent in vitro culture to confirm plastic adherence.[31,70–72] There are also reports of CVSC isolation by the explant culture method.[35,73] Briefly, chorionic villi are attached to plastic culture dishes maintained in culture medium for approximately 14-20 days, at which time the migrated cells (CVSCs) are harvested.

1.4 Review of MSC Yields

Beyond orthopedics, the importance of high dose stem cell administration has been established in therapeutic strategies combating wound healing, fistula and heart failure.[74–76] Moreover, in recent proof of concept clinical trials examining the potential therapeutic efficacy of using stem cells to mitigate musculoskeletal tissue pathologies (in this instance osteoarthritis), it has been demonstrated that only study groups receiving high doses ($10x10^7$ autologous ADSCs) show statistically significant improvement

compared to low dose stem cell administration and non-stem cell treated controls.[13] Such reports further the relevance of stem cell yields in musculoskeletal regenerative medicine approaches, as sources exhibiting the highest yields represent the most practical (readily useable) stem cell.

1.4.1 BMSC Yields

Pittenger suggests that only 0.001 – 0.01% of isolated MNCs are stem cells.[59] This was corroborated by Pasquinelli et al who reported 0.001 – 0.1% of MNC suspensions were BMSCs as indicated by stem cell surface marker profiles.[33] Recently, Li et al evaluated the number of colony forming unit fibroblasts (CFU-F; i.e. BMSCs) from repeated BM aspirations in healthy patients and those with leukemia.[77] On average after the first aspiration, the mean number of CFU-F's per 1 x 10^6 MNC's was 84; this indicates roughly a 0.008% yield, which is within the range established by Pittenger's findings. Furthermore, Li found the average number of MNC's isolated during the first aspiration was 12 x 10^6/ mL BM, resulting in an average normalized stem cell yield of approximately 1 x 10^3 BMSC/mL of BM aspirate.[77] Wexler et al had previously assessed the frequency of stem cells in the CFU-F population to be one BMSC in every 3.4 x 10^4 cells.[44] Baer et al cites that "a bone marrow transplant contains approximately 6 x 10^6 nucleated cells per mL."[78] Taken together with the percentage range (0.01-0.001%) of BMSCs per nucleated cell established by Pittenger et al; 60 - 600 BMSCs can be theoretically obtained per milliliter of BM aspirate, which is in accordance with the values determined by Li et al. Lannert et al found no significant difference in BMSC yield when varying bone marrow aspiration methods, resulting in an average of 0.11 - 0.34 x 10^8 total nucleated cells per liter of BM.[43] Considering that only a small portion of

these cells are actually BMSCs; the theoretical stem cell yield per mL aspirate would be 11 - 340 BMSC/mL, which represent slightly lower values than those determined by other authors**.**

1.4.2 ADSC Yields

Chung et al determined that traditional suction assisted liposuction yields a mean stem cell count of 5.4×10^5 cells per mL with roughly 81% cell viability (i.e. 4.4×10^5 viable ADSCs/mL processed lipoaspirate).[61] Baer et al cite that the SVF of adipose tissue contains approximately $0.5 - 2 \times 10^6$ cells per gram.[78] Jurgens et al suggest that approximately 5% of the total cell count found in the SVF from liposuction of the abdomen is ADSCs, though Bourin suggests it can be as low as 0.1%.[9,79] In line with this finding, Baer cites others who indicate that 1-10% of the cells in the SVF are stem cells.[78] Using a conservative estimate of 5% and provided the total number of cells estimated per gram of SVF by Baer, the calculated theoretical ADSC yield is $2.5 \times 10^4 - 1 \times 10^5$ ADSCs/g adipose tissue (or $5 \times 10^3 - 2 \times 10^5$ ADSCs/g, if you assume 10% of the SVF are stem cells). Oedayrajsingh-Varma et al compared whole tissue resection, tumescent liposuction, and ultrasound-guided liposuction to determine if there was a difference in cell yields between these methods. These methods yielded 7×10^5, 5×10^5, and 6×10^5 SVF cells (+/- 1×10^5)/g of harvested tissue, respectively.[60] Using the 5% estimate, the total ADSC yield by these methods was approximately 3.5×10^4, 2.5×10^4, and 3×10^4 (+/- 1×10^5) ADSC/g. This suggests similar effectiveness between isolation methods, and is in accordance with previously calculated ADSC yield values. Christodoulou et al isolated a mean SVF yield of 312×10^6 cells from 173.8g of adipose tissue.[80] Applying the 5% estimate, approximately 8.9×10^4 ADSC/g were obtained, again in accordance

with previously predicted values. However, it is noteworthy that all of these calculated values are larger than the 5 x 10^3 ADSCs/g cited by Hass et al.[32] Zuk et al suggests that 2 – 6 x 10^8 nucleated cells can be found in the SVF obtained from 300 mL of raw lipoaspirate, which would yield 6.7 x 10^5 – 2 x 10^6 cells per mL.[63] Again, assuming 5% of these cells are stem cells (in accordance with Jurgen et al[79]) this would result in approximately 3.4 x 10^4 – 1 x 10^5 ADSCs/ mL of lipoaspirate, which is in agreement with Baer's citations (which includes Zuk et al and others).

1.4.3 AFSC Yield

Though the cellular profile of AF has been shown to change with fetal age, the majority of cells isolated from AF are terminally differentiated and do not have the proliferative capacities characteristic of mesenchymal stem cells.[48,52] It is estimated that only 1% of the cells obtained during amniocentesis are AFSCs.[34,40] It has been reported that the mean AF volume is relatively constant (207±92mL at 16 weeks, 258±97mL at 18 weeks and 365±88mL at 20 weeks), but during the second trimester the number of cells within the AF varies from 10-1,000 cells/μL.[65] Based on these reports, the theoretical number of cells in the total volume of AF at 20 weeks is between 3.65 x 10^6 – 3.65 x 10^8 cells. Following reports that AFSCs constitute only 1% of the overall AF cell population; the theoretical number of AFSCs in the entire AF at 20 weeks is 3.65 x 10^4 – 3.65 x 10^6 AFSCs (or 100-10,000 AFSCs/mL AF). However, it must be noted that the entire AF volume could never be utilized without compromising pregnancy. Pappa et al reported an average of 0.9-1.5% AFSCs isolated from 10-15mL of AF taken at 15-18 gestational weeks, which resulted in 2.7 x 10^5 total AFSCs (or 1.8 x 10^4 – 2.7 x 10^4 AFSCs/mL AF), within the previously calculated theoretical range.[29] Unpublished observations by Ekblad

also fall within this theoretical range, as 1,500±3,250 AFSCs/mL were obtained by pooling 2-3 AF samples (mean sample volume: 6.7mL) and combining n=7 pooled samples together.

1.4.4 AMSC Yields

Expected yields using the previously described serial enzymatic digestion technique are 4-5 x 10^6 AMSCs/AM.[55] Soncini et al reports a typical isolation of 24 ± 10 x 10^6 AMSCs/AM.[71] Using a similar method, Casey et al isolated 1 x 10^6 AMSCs/g of AM (mass of amnion tissues: 5-15 grams).[53] Published data by Bilic et al states that a mean of 1.7 x 10^6 AMSCs/g could be harvested.[49] Unpublished results by Bilic et al (reviewed by Parolini et al[54]) state that "typically 4 x 10^6 AMSCs per 100 cm^2 of starting material" can be isolated from the term amnion. While Alviano et al noted 1.3-1.5 x 10^6 AMSC/4cm^2 AM (3.2-3.75 x 10^5 AMSC/cm^2). They extrapolated that with an average amnion area of 1300cm^2, 4 x 10^8 AMSCs can be isolated/AM.[37] Unpublished data by our group seems to align with previous reports, as we routinely isolate an average of 1.6 x 10^6 AMSCs/mL and as many as 3 x 10^8 AMSC/AM.

1.4.5 CMSC Yields

We are not the only authors to remark at the extremely limited information available regarding CMSCs.[34,46,81] Witkowska-Zimny et al cite Soncini et al who report that 21 x 10^6 CMSCs can be isolated from the enzymatic digestion of a single chorionic membrane.[34,71] While Bačenková reported 11 x 10^6 CMSCs were isolated from 6-pooled 10x10cm chorion segments (11 x 10^4 CMSC/cm^2).[31] Abumaree successfully isolated 11.55 ± 1.23 x 10^3 CVSCs/40mg chorion (wet weight) when employing the standard explant culture technique (2.89 x 10^2/mg) and 24.66 ± 2.67 x 10^3 CVSCs/40mg chorion

(wet weight) when employing trypsin (6.17 x 10^2/mg), with confirmation that the CVSCs were free of maternal contamination.[73] Both Zhang and Igura et al reported 1 x 10^4 CVSCs/5mm^3 chorion using the explant culture method (2 x $10^3/cm^2$).[35,81] All of the previously reviewed stem cell yields are succinctly reviewed below in table 2.

Table 2: Calculated and Reported MSC Yields

Stem Cell	[Stem Cells/ mL tissue]	[Stem Cells/ g tissue]	% Stem Cells in Cellular Fraction	Other	Reference(s)
BMSC	$1x10^3$, <u>60-600</u>, <u>11-340</u>	NA	0.001-0.01%, 0.001-0.1%, 0.008%	NA	33,43,59,78,79
ADSC	$4.4x10^5$, <u>$3.4x10^4$-$1x10^5$</u>	<u>$2.5x10^4$-$1x10^5$</u>, <u>$3.5x10^4$</u>, <u>$2.5x10^4$</u>, <u>$3x10^4$</u>, <u>$8.9x10^4$</u>, <u>$5x10^3$</u>	5%, 1-10%	NA	48,56,59,60, 62,78,79
AFSC	<u>$1x10^2$-$1x10^4$</u>, <u>$1.8x10^4$-$2.7x10^4$</u>, <u>$1.5x10^2$-$3.25x10^2$</u>	NA	1%	NA	29,34,40
AMSC	$1.7x10^6$	NA	NA	$4\text{-}5x10^6$/AM, 24 ± 10^6/AM, $4x10^8$/AM, $4x10^4/cm^2$, $3.2\text{-}3.75x10^5/cm^2$	37,53,54,72,81
CVSC	NA	$6.17x10^5$	NA	$2x10^3/cm^2$	77
CMSC	NA	NA	NA	$21x10^6$/CM, $11x10^4/cm^2$	31,34,72

Table 2: Reported and Extrapolated MSC Yields reviewing the previously described yields obtained or calculated (underlined values) based on the current literature. This table highlights the numerous ways stem cell yields are reported, limiting immediate cross-comparisons between MSC types. Underlined data points indicate calculated values based on the 0.001-0.01% (BMSC), 5% (ADSC) and 1% (AFSC) ranges

1.5 Obtaining "Clinically Relevant" Stem Cell Populations

It has been widely accepted that stem cells must be administered in large quantities to demonstrate clinical efficacy.[4,5,14] Commonly, this is expressed as needing to obtain "clinically relevant" stem cell populations, presumably through ex vivo expansion.[82,83] With limited past precedence within orthopedics, we define clinically relevant populations as those commonly employed in orthopedic clinical trials. Upon

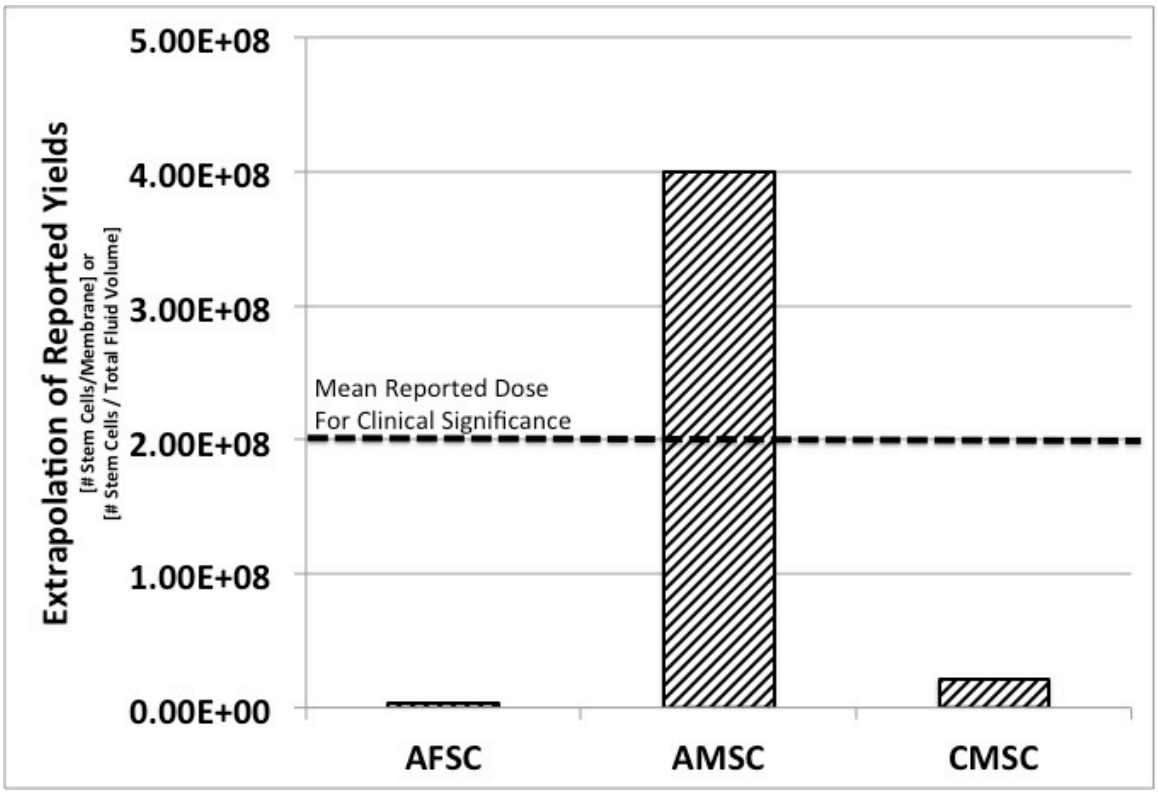


Figure 6: Calculated AFSC, AMSC and CMSC Yields with the dashed lined indicating the mean reported dose of current orthopedic clinical trials employing MSCs.

review of the United States National Institutes of Health clinicaltrials.gov website, between 1 x 10^6 -1 x 10^9 stem cells are commonly employed in various clinical trials for orthopedic applications, seeming to imply that 1 x 10^6 is the minimum number of stem cells required for clinical relevance in stem cell-based orthopedic therapies. Of note, no orthopedic trials were discovered utilizing AFSCs, AMSCs, CMSCs or CVSCs. (Note: The reported range excludes studies with dosages reported as mL BM aspirate. The lowest BM aspirate reported is 6mL, which based on our calculations would yield 3.6 x 10^2 - 3.6 x 10^3). However, this claim can only be verified upon study conclusion and evaluation of ongoing trials.

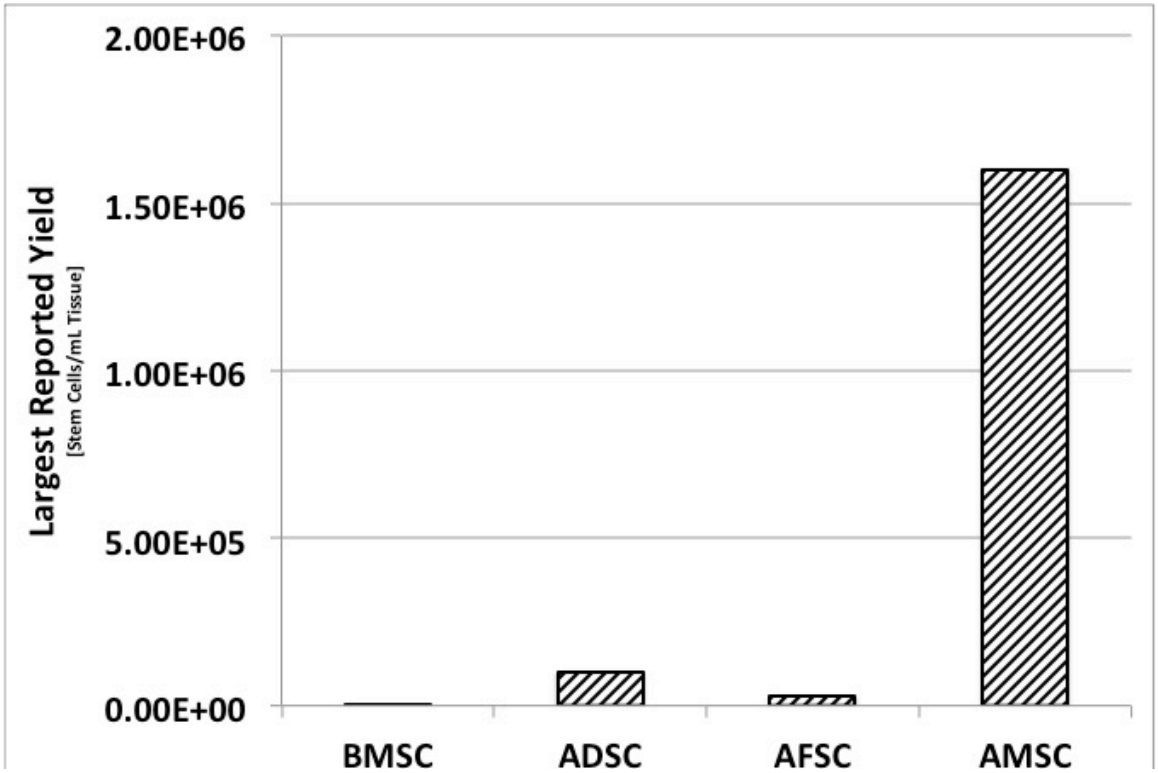


Figure 7: Largest Reported BMSC, ADSC, AFSC and AMSC Yields highlighting the exponentially greater yields of AMSCs compared to more standard stem cell sources.

Based on our summation of the literature and extrapolation of stem cell yields, AMSCs yields are the highest of all stem cell types examined. An averaged size AM can yield up to 4 x 10^8 AMSCs, whereas the largest yield reported from the chorionic membrane is only 21 x 10^6 CMSCs, approximately 20x less than the amniotic membrane. AFSC isolation using the entire AF volume at 20 gestational weeks yields 3.65 x 10^4 – 3.65 x 10^6, approximately 100x less mesenchymal stem cells than the amniotic membrane. These relative yields are represented in figure 6. Additionally, it cannot be ignored that being able to utilize the entire AF volume represents an unrealistic therapeutic scenario. We were unable to determine average lipoaspirate or BM aspirate volumes removed during standard procedures from the literature, but calculated yields

indicate 3.4 x 10^4 – 1 x 10^5 ADSCs/mL lipoaspirate, nearly 500x greater than the calculated BMSC yield of 60-600BMSC/mL BM aspirate, can be obtained. Still, hAMSCs can be isolated in as much as 40x greater quantities than ADSCs (1.6 x 10^6 AMSC/mL AM vs. 3.4 x 10^4 – 1 x 10^5 ADSCs/mL lipoaspirate). These relative yields are represented in figure 7.

In order to obtain the previously identified (minimal) number of stem cells required for clinical relevance (1 x 10^6 stem cells) without ex vivo expansion of cells, at least 1.6L BM, 10mL lipoaspirate, 55mL AF, 0.625mL AM, or 500cm^2 CM would be required (illustrated in figure 8). This highlights the utility of stem cell sources such as the amnion, as one amnion would be substantial to obtain multiple therapeutic doses.

The highest reported clinical trial dosage of 1 x 10^9 is not possible without ex vivo expansion of any of the listed cell types. However, the mean reported dosage (2 x 10^8 stem cells, also the dosage termed "clinically relevant" by Schallmoser et. al.[83]) could only be accomplished without ex vivo expansion through using AM. As previously indicated, ex vivo expansion exposes cells to increased possibilities of contamination as well as alterations to their phenotype and differentiation potentials.[15–17] Thus, AMSCs may be an under-used stem cells source in orthopedic regenerative medicine.

It should be noted that reported values for stem cell yields vary within each tissue due to the differing isolation techniques employed. Furthermore, it is difficult to make direct comparisons between stem cell yields within the same tissue as different investigators utilize varying techniques for quantification. The dosage range of current orthopaedic clinical trials employing stem cell therapies seems to indicate that at least 1 x 10^6 stem cells are required for clinical significance. Based on our summation of the

literature and extrapolation of stem cell yields, AMSCs yields are the highest of all stem cell types examined. AMSCs are also the only cell type examined that can be isolated in quantities equal to the mean dosage of stem cells currently employed in orthopedic clinical trials.

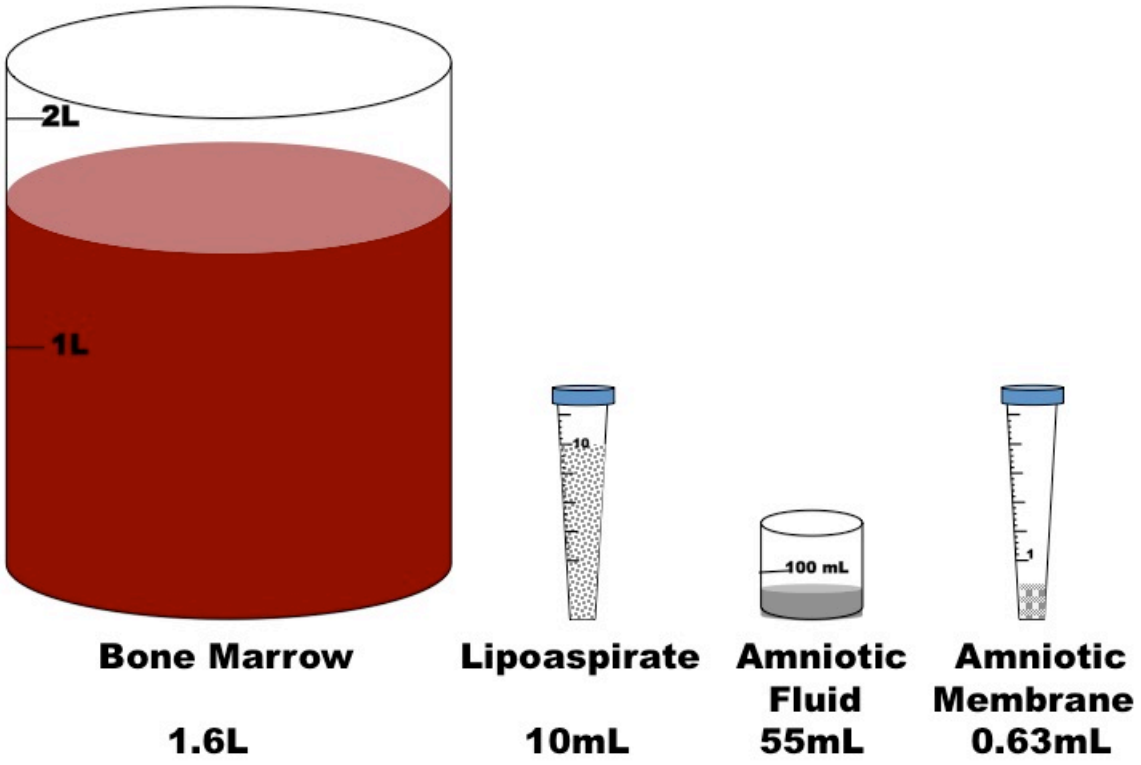


Figure 8: Tissue Required To Obtain 1x10^6 Stem Cells, the minimum number of stem cells required for clinical significance, without ex vivo expansion.

1.6 FDA Regulation of Stem Cells

MSCs currently fall under the jurisdiction of the U.S. FDA's Center for Biologics, Evaluation and Research's (CBER) Office of Cellular, Tissue and Gene Therapies (OCTGT). Stem cells are considered as human cells, tissues or cellular and tissue-based

products. As such, their use must meet current FDA regulatory codes and good manufacturing practices, specifically Title 21, Part 1271.[7,68] The FDA has two clinical regulatory pathways governing the use of MSCs and MSC-based products. It is possible, *but not likely*, for an MSC-based product to be regulated under Section 361 of the Public Health System's Act if the product meets the following criteria: 1) the product has been minimally manipulated, 2) the product is intended for homologous use only, 3) the product was not combined with any other biologic or synthetic article, 4) the product does not have a systemic effect or depend on the activity of living cells for its primary function and 5) the product is for autologous use.[7,68] Regulation by Public Health system Act Section 361 grants products sanctioned use for investigational trials without formal FDA approval. While MSCs are HCT/Ps, to the best of the authors knowledge they do not meet the criteria listed above an accordingly are regulated the PHS act under Section 351 as a biologic drug which must follow current good manufacturing and tissue practices (cGMP and CGTP, respectively) and require investigational device exemptions and clinical trial data to provide evidence of safety and efficacy to gain approval prior to marketing. To date are no FDA approved MSC-based products for orthopaedic applications. For more information on FDA regulations, please see our previous review.[68]

1.7 Stem Cell Differentiation

Stem cells, especially stem cells combined with biomaterial scaffolds, have the potential to differentiate into target cells, establishing new populations of healthy, tissue-forming, cells in diseased areas.[11,84,85] In some instances it has been demonstrated that stem cell differentiation can occur "spontaneously." That is, differentiation can occur without the addition of exogenous growth factors. In such instances the stem cells are

typically seeded onto a scaffold. It is hypothesized that the physical and/or chemical properties of the scaffold provides the stimulatory cues necessary to promote stem cell differentiation, though the exact mechanisms behind such differentiation are not yet fully understood.[86–88]

However the primary means to achieve stem cell differentiation involves manipulation not currently allowed by FDA regulation, the addition of exogenous growth factors to culture media (see figure 9).[32,86,89–97] As different cell types require different

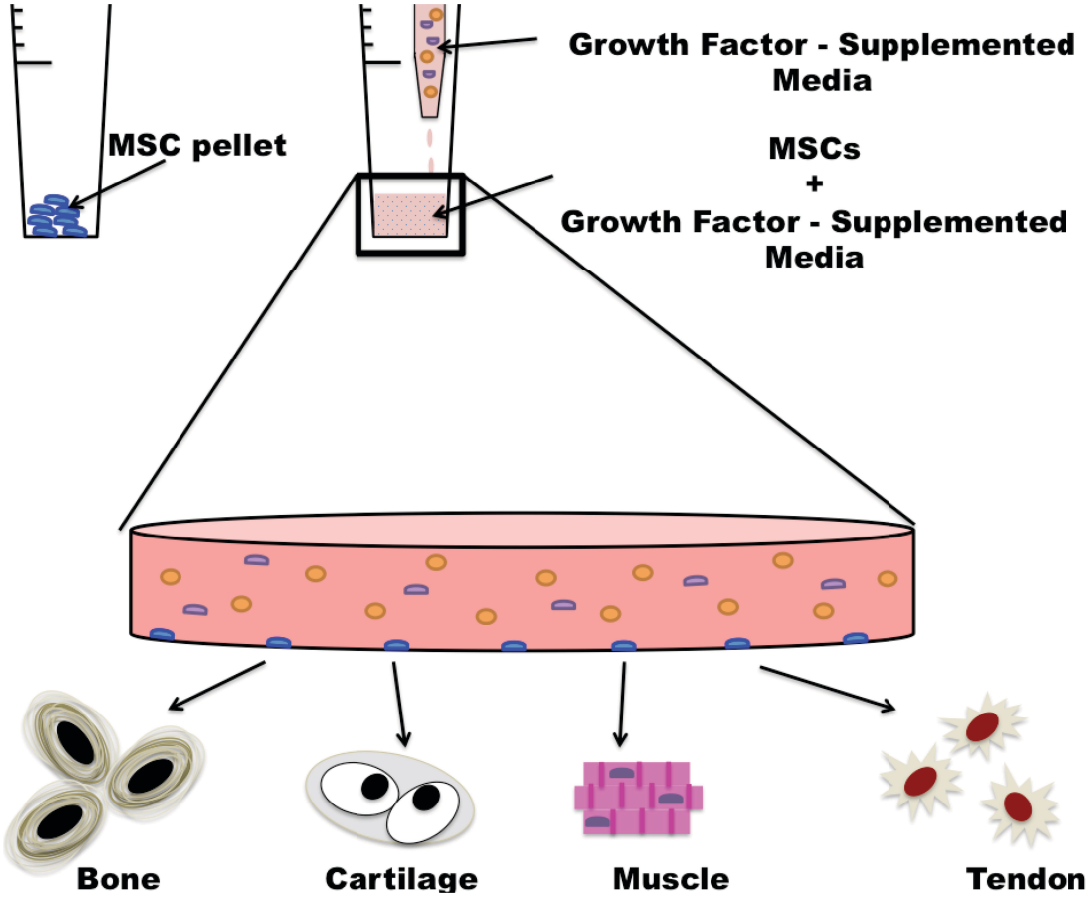


Figure 9: Growth Factor-Induced Stem Cell Differentiation is the most common technique to achieve stem cell differentiation into desired tissue types. This involves the supplementation of cell culture media with growth factors. Individual growth factors are best suited to specific lineages of differentiation.

growth factors to sustain their phenotype and functionality, a variety of chemical cocktails are employed to achieve stem cell differentiation into multiple cell lineages. As

previously indicated, osteogenic, adipogenic and chondrogenic differentiation are necessary characteristics to be defined as a stem cell.[25] Therefore, these lineages are the most widely investigated. For the purposes of this review, only those with musculoskeletal tissue relevance (osteogenic and chondrogenic) will be explained in further detail.

Osteogenic differentiation has been achieved through the addition of dexamethasone, β-glycerophosphate, ascorbic acid or bone morphogenic proteins (BMPs) to culture media.[32,49,89,90,92–95,98] Differentiation is typically evaluated through enhanced

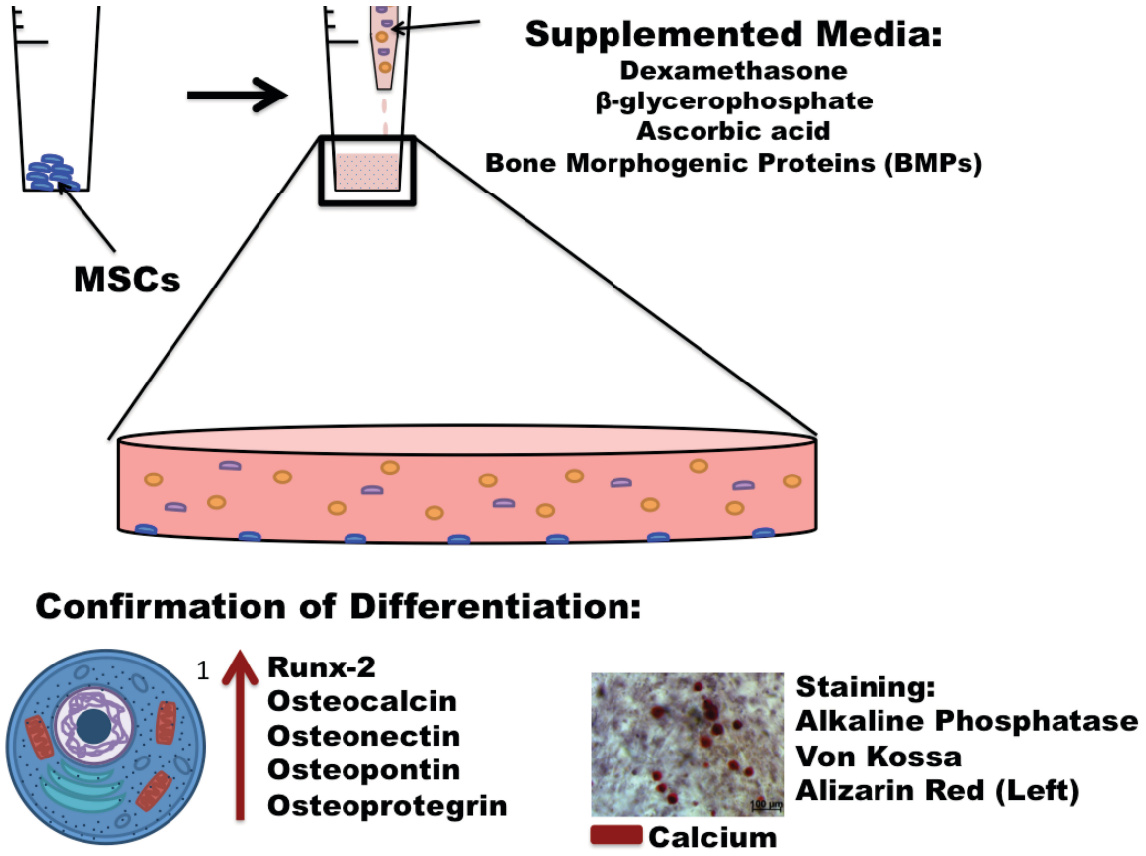


Figure 10: Osteogenic Differentiation is typically accomplished via media supplementation with dexamethasone, β-glycerophosphate, ascorbic acid or bone morphogenic proteins (BMPs). Differentiation is confirmed through gene expression of key osteogenic markers and through protein deposition of bone matrix elements, notably, calcium. [1]Photo credit: OrthoX colleague, Sandra Siatkowski

gene expression of master osteogenic transcription factor, runx-2, and other osteogenic markers: osteocalcin, osteonectin, osteopontin and osteoprotegrin.[32,89,94,98] At the protein level, differentiation is also evaluated through alkaline phosphatase expression and calcium deposition (visualized through von Kossa or Alizarin Red staining).[32,49,89,90,92,93,95,98,99]

Chondrogenic differentiation has been achieved through the addition of transforming growth factor β1, ascorbate-2-phosphate and dexamethasone.[32,93–97] Growth

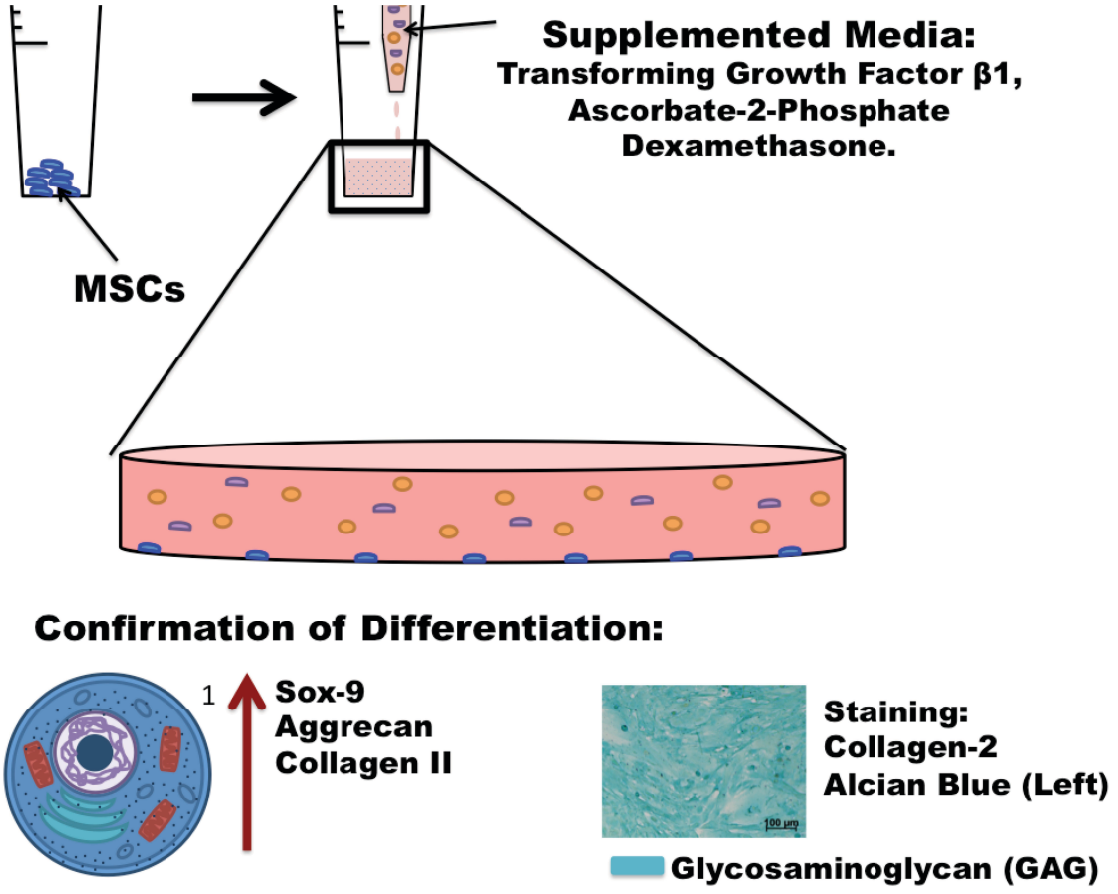


Figure 11: Chondrogenic Differentiation is typically accomplished via media supplementation with transforming growth factor β1, ascorbate-2-phosphate and/or dexamethasone. Differentiation is confirmed through gene expression of key chondrogenic markers and through protein deposition of cartilage matrix elements, notably, glycosaminoglycans (GAGs). [1]Photo credit: OrthoX colleague, Sandra Siatkowski

and differentiation factor-5, fibroblastic growth factor-2 and BMP-6 have also been described.[32] Studies requiring the use of serum-free media (typically those aiming to achieve clinical relevance of results through the use of human cells without animal serum effects), typically employ insulin transferrin selenium (ITS).[86,94,97] Differentiation is typically evaluated through enhanced gene expression of master chondrogenic transcription factor, sox-9, and other chondrogenic markers: aggrecan and collagen-2.[32,93,95–97] At the protein level, differentiation is also evaluated through matrix staining of glycosaminoglycans (GAGs; visualized through Alcian Blue staining).[32,95]

In addition to plated culture, chondrogenic differentiation of MSCs is accomplished through pellet culture. In this approach, stem cells remain in a pellet after centrifugation (as opposed to being re-suspended in culture media and plated). This provides the cells a 3D environment, allowing cell-to-cell interactions that more accurately mimic the natural condensation of MSCs during chondrogenesis to occur.[100,101]

1.8 Bone and Cartilage Formation: Endochondral Ossification

Bone formation (osteogenesis) can occur through mesenchymal condensation differentiation into osteoblasts; this process forms the membranous elements of the vertebral skeleton during development.[102] However, most of the vertebral skeleton forms through endochondral ossification[100,102], the central biochemical pathway responsible for both chondrogenesis and osteogenesis. In endochondral ossification, cells differentiate into chondrocytes, proliferate and undergo hypertrophy until they reach terminal differentiation (ossification).[87,100,102–104] Simultaneously some cells differentiate into osteoblasts and osteoclasts.[102,103] Together these cells degrade the cartilage matrix

(osteoclasts) and replace it with newly formed bone matrix (osteoblasts) through a process known as remodeling.

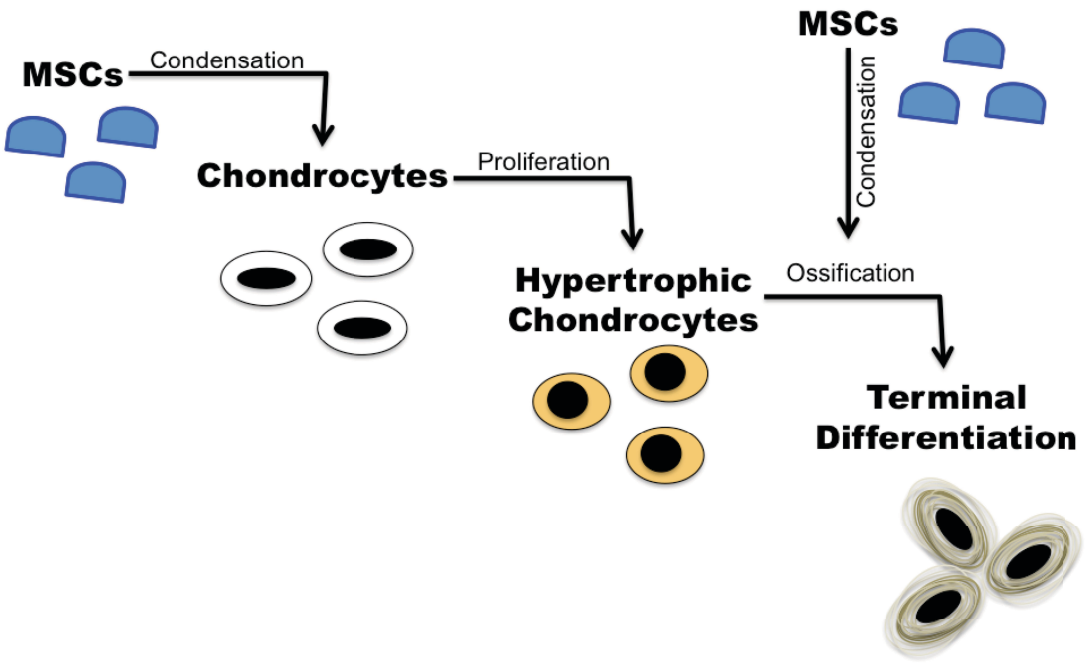


Figure 12: Endochondral Ossification is the primary pathway through which osteogenesis and chondrogenesis occurs; it is also the pathway of MSC differentiation into cells of chondral and osteo lineages. Initially, MSCs condense, forming either osteoblasts/clasts or chondrocytes. These chondrocytes proliferate, ultimately undergo hypertrophy and terminally differentiate through ossification.

Master chondrogenic transcription factor, sox-9, is required for mesenchymal cell condensation formation.[102,103] All chondroprogenitor cells but not all chondrocytes express sox-9.[102] Hypertrophic chondrocytes, in particular, and osteoblasts do not express sox-9, further implicating sox-9 in the proliferative as opposed to the later phases of endochondral ossification.[102,103]

Sox-5 and sox-6 are regulators of key chondrocyte matrix components, collagen 2 and aggrecan.[102,103] Sox-5 and sox-6 are not required for mesenchymal cell condensation but are required immediately following chondrocyte maturation where high levels of surrounding matrix are produced.[102] There does seem to be a redundancy in this pathway

whereby mice deficient in either sox-5 or sox-6 mature with limited skeletal abnormalities.[102] However, mice deficient in both sox-5 and sox-6 die in utero.[102]

As previously indicated, runx-2 is a transcription factor controlling the rate of expression of osteocalcin and other osteogenic matrix components. Runx-2 is required for osteogenic differentiation. Through chemical induction, mesenchymal stem cells can up-regulate runx-2 in order to promote osteogenic differentiation.[102] Natively, runx-2 is expressed by hypertrophic chondrocytes and osteoblasts.[102]

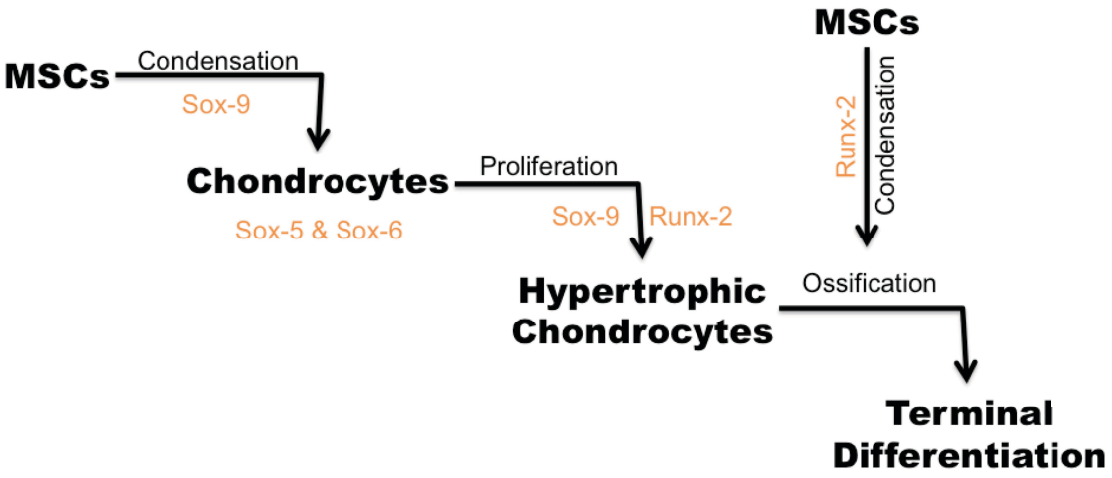


Figure 13: Sox-9 & Runx-2 Involvement in Endochondral Ossification is extensive. Sox-9 is primarily responsible for condensation and chondrocyte proliferation. Runx-2 is primarily implicated in condensation of terminally differentiated osteoblasts/clasts and the transition from proliferative to hypertrophic chondrocytes.

The TGF superfamily is another group of regulators influencing cell growth, differentiation and apoptosis in many cell types, including chondrocytes and osteoblasts.[103] The TGF superfamily includes the BMPs 2-8 as well as the TGF-βs.[103,105] TGF binding has been shown to influence chondrogenesis through two primary molecular interactions, MAPK and SMAD pathways.[103]

As previously indicated, many members of the TGF superfamily are used as exogenous growth factors to induce differentiation in stem cells. Previously it has been demonstrated that TGF-β supplemented medium induces chondrogenesis more

effectively in adult but not fetal BMSCs; conversely, medium supplemented with BMP-2 induces chondrogenesis more effectively in fetal but not adult BMSCs.[95] TGF-β receptor binding leads to the phosphorylation of SMAD2 and SMAD3.[95,105–107] Whereas, BMP-2 receptor binding leads to the phosphorylation of SMAD1 and SMAD5.[95,105–107] Each of these SMADs serve specific (though not currently completely defined) functions. BMP-pathway deficient mice have underdeveloped growth plates due to inhibition of chondrocyte proliferation.[106] Whereas, TGF-β-pathway deficient mice tend to develop elongated growth plates due to maintenance of chondrocytes in the proliferative phase.[106] TGF-β-related SMAD signaling has also been correlated with increased likelihoods of osteoarthritis and osteophyte formation throughout cartilage.[106] While it has been established that both signaling pathways are necessary for proper development[106], there appear to be chondrogenic advantages (less potential for bone formation and maintenance of chondrocytes in the proliferative phase) in BMP-induced chondrogenesis. The biochemical process of endochondral ossification describes the formation of cartilage and bone cells. These cells, together with specified matrix components, create the distinctive tissue structures known as cartilage and bone.

1.9 Articular Cartilage: Structure & Function

Cartilage is a roughly 2-4 mm thick connective tissue composed of a unique host cell type, chondrocytes, and the extracellular matrix (primarily composed of water, collagen and proteoglycan) surrounding the chondrocytes.[108–110] Despite having minimal components, cartilage is a complex tissue with the cells and matrix fibers organized in multiple patterns (referred to as zones). This heterogeneous structure results in varying

cell phenotypes, gene and protein expression.[108,109] Therefore each individual zone offers a unique function to the cartilage unit.

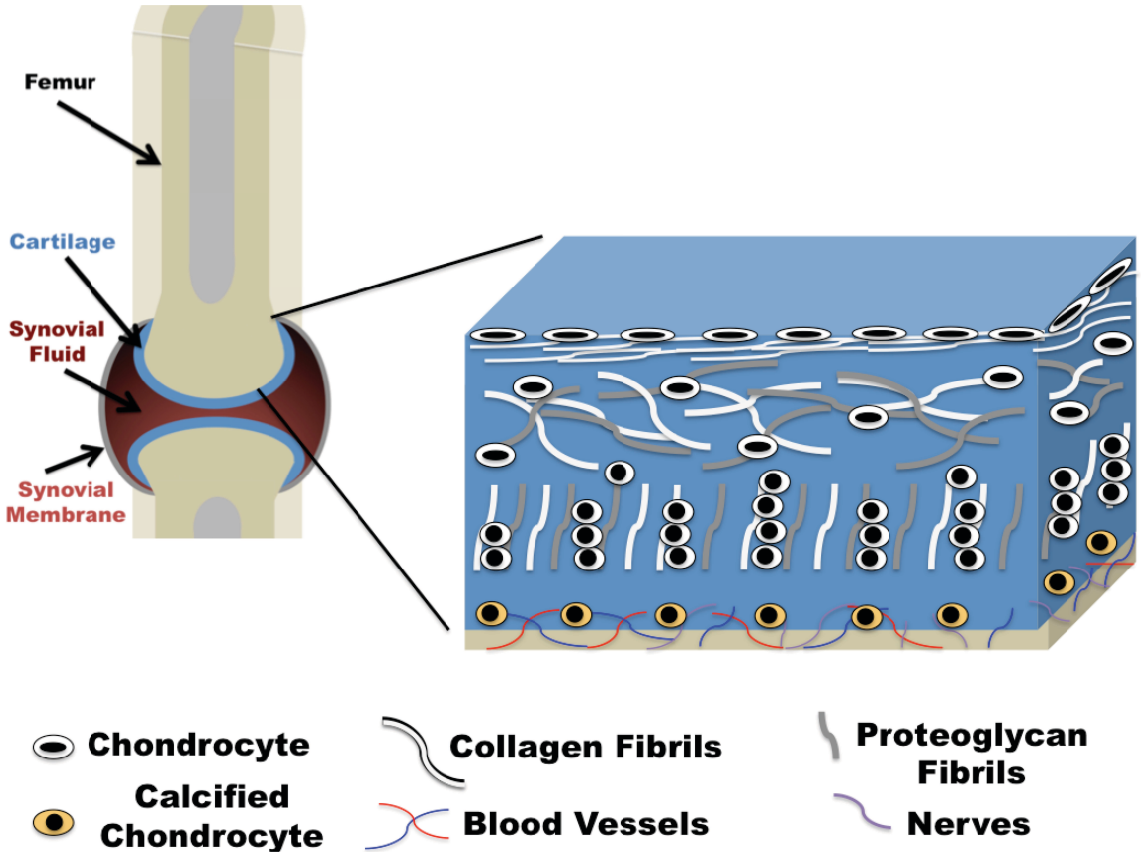


Figure 14: Cartilage Structure is heterogeneous; the variable composition (cellular shape/phenotype and matrix) of each zone yields specific functions. These zones combine together to serve one primary function: the absorption and dissipation the mechanical loads of the joint. Cartilage lines the surface of joints, including the knee joint (pictured at left).

In the superficial zone (figure 14), flattened chondrocytes arrange with their lacunae oriented parallel to the cartilage surface.[108] In a similar manner, type II and IX collagen fibers align parallel to the surface.[109] Type II collagen is the most abundant extracellular matrix component (approximately 90-95% dry weight).[109,111] Collagens I, IV, V, VI and XI are also present, but in much lower proportions; these collagens function primarily to stabilize the type II collagen network.[109] Though this zone is the thinnest cartilage layer (roughly 15% of total cartilage thickness), this parallel orientation functions to protect deeper cartilage layers from mechanical stresses.[109] Thus, the

integrity of the superficial layer is paramount to overall cartilage function.[112] This zone also exhibits high levels of lubricin and hyaluronic acid (HA) secretion, lubricating the joint surface (allowing fluid, pain-free movement).[108] Water is also most abundant in the superficial zone (approximately 80% wet weight), filling intrafibrilar spaces and facilitating nutrient exchange.[109]

In the intermediate zone (figure 14), a combination of more rounded chondrocytes can be found sparsely thought an oblique network of extracellular matrix fibers.[109] These fibers, primarily thicker collagen segments and proteoglycans, function to resist compressive forces.[109] Proteoglycans (up to 15% wet weight) are crucial to the compressive resistance exhibited by cartilage. Aggrecan, the most abundant cartilage proteoglycan, has unique osmotic properties, making it specially suited to resist compressive loading.[109]

The deep zone (figure 14) provides the primary resistance to compressive forces.[109] In the deep zone, chondrocytes arrange in a columnar orientation perpendicular to the cartilage surface. Dense networks of large diameter collagen fibers and proteoglycans arrange parallel to chondrocyte columns (perpendicular to the surface). The deep zone contains the highest proteoglycan and lowest water contents.[109]

Together the superficial, intermediate and deep zones make up the non-mineralized portion of cartilage.[108] These non-calcified zones do not contain blood vessels. Thus the chondrocytes in these regions live under hypoxic conditions, absorbing nutrients from neighboring joint tissues.[108,109,113] Though the exact mechanism is not understood, it has been suggested that the high levels of collagen-2 and aggrecan observed in these zones are a result of the hypoxic environment, as chondrocytes cultured

under normoxia do not display the appropriate biochemical profile.[108] Additionally, hypoxia produces a more chondroprotective environment, reducing synthesis rates of matrix metalloproteinases one and thirteen (MMP-1 and MMP-13, respectively; both responsible for cartilage extracellular matrix degradation and fragmentation).[108] These regions are separated from the deep calcified cartilage by the tide mark (a line roughly parallel to the cartilage surface visualized through hematoxylin staining).[108]

Calcified cartilage (figure 14) has a particularly unique composition that drastically changes with age. Over time, nerves and blood vessels arise from the subchondral bone, infiltrating the calcified cartilage.[108] Additionally, the chondrocytes in this zone express markers of hypertrophy (ex. runx-2, MMP-13 and type X collagen).[104,108,112]

Together, the primary functions of cartilage are to absorb and dissipate mechanical loads.[108] In fact, mechanical stresses are required to maintain cartilage homeostasis, as loading produces fluid movement within the joint.[108,113] This (synovial) fluid facilitates nutrient exchange as well as lubrication and will be addressed in more detail in the subsequent section entitled, "Introduction to Osteoarthritis." Biochemically, mechanical loading decreases matrix metalloproteinase 3 (MMP-3; a cartilage extracellular matrix degrading enzyme) and increases aggrecan expression in chondrocytes.[108] There is also evidence that mechanical loading prevents the secretion of inflammatory mediators, including interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α).[108]

In normal adults cartilage is in a quiescent state.[108,109,112,113] This is largely possible due to the extremely long turn over rates of the primarily components: collagen

(400 years) and proteoglycans (25 years).[109] Chondrocytes, themselves, do not routinely proliferate.[108–110,113] Each chondrocyte is responsible for the turnover of the extracellular matrix in its immediate vicinity. This essentially traps the chondrocytes in place, preventing cell clustering and cell-to-cell signal transduction.[109] Additionally, the extracellular matrix does not repair and replenish the previously described collagen and proteoglycan networks.[108,109,114] Numerous pathologies, including osteoarthritis, disrupt this previously described homeostatic regulation through the activation of chondrocytes.

1.10 Introduction to Osteoarthritis (OA)

1.10.1 Prevalence & Pathogenesis

OA is the most common form of arthritis, affecting over 30% of the U.S. population over the age of 65.[11,12,110,111,113–118] OA is the result of degraded cartilage, impairing joint mobility and causing severe pain - making it one of the leading causes of disability worldwide.[12,110,111,113,119,120] Costs associated with OA have been described as accounting for up to 2% of gross national product in the U.S., the United Kingdom, Canada, France and Australia.[12,120,121] Additionally, is estimated that the prevalence of OA will double by 2020.[122]

Articular cartilage degradation is the result of multiple physical and biochemical processes including, general wear and tear (the result of years of joint use), inappropriate mechanical loading and inflammation.[11,12,104,108,111,114,115,117,119,122] Both systemic (trauma, obesity and genetic predisposition) and local inflammation (primarily synovial inflammation) have been implicated in OA.[11,108,110,123–126]

Though commonly associated with the degeneration of cartilage, OA is a disease of the entire joint space.[12,85,108,113,117,118,126–128] In OA, the subchondral bone, synovium, ligaments and meniscus interact with the cartilage via non-homeostatic mechanisms.[12,85,108,113] Specifically, the subchondral bone can exhibit sclerosis, and osteophyte formation is noted at the junction of the synovium with the periosteum.[115,127] Synovial fluid - the fluid surrounding and lubricating the joint, becomes filled with pro-inflammatory mediators and cartilage degradation products.[11,104] Currently, the exact mechanisms of such processes are unknown, obscuring our understanding of OA etiology.

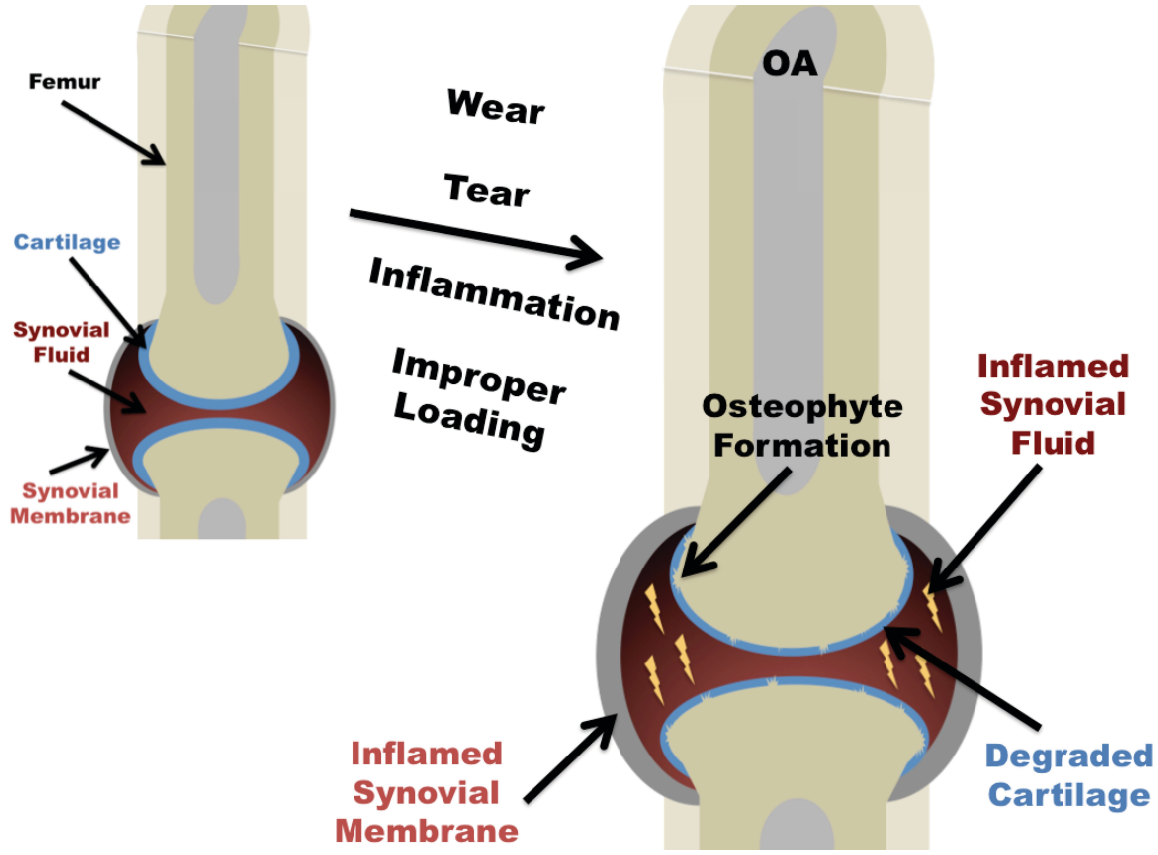


Figure 15: Osteoarthritis (OA) is the result of numerous physical and biochemical processes. Though commonly associated with cartilage injury, OA is also affiliated with osteophyte formation within the cartilage or at the synovial/periosteal interface and chronic inflammation of the synovial membrane and the synovial fluid.

In OA, chondrocytes exist in an activated ("senescent" as opposed to quiescent) state, resulting in rapid cell proliferation, cluster and matrix formation and increased secretion of matrix degrading enzymes (ex. MMPs and A Disintegrin And Metalloproteinase with Thrombospondin Motifs, ADAMTS).[104,108,110,113,129]

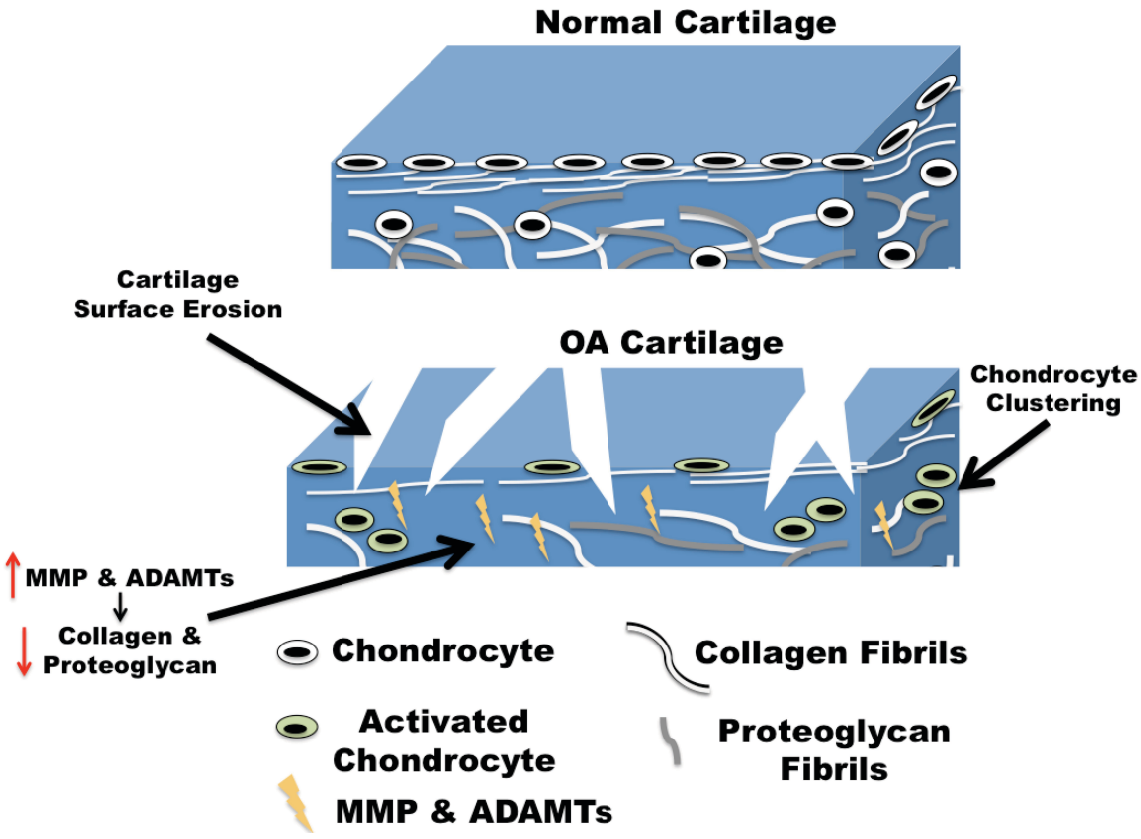


Figure 16: Activated Chondrocytes contribute to the microscopic changes observed in OA through increased proliferation, clustering and the secretion of potent cartilage matrix degrading enzymes, MMPs and ADAMTs. These enzymes contribute to the overall decrease in collagen and proteoglycan observed in OA cartilage.

Primary MMPs involved in OA pathogenesis include MMP-13, MMP-3 and to a lesser extent MMP-2. MMP-13 is a collagenase (collagen degrading enzyme) with a particular affinity for type II collagen.[12,104] In OA, MMP-13 expression is increased within both the synovium and cartilage.[115] In addition to being an effective aggrecanase (aggrecan degrading enzyme), MMP-3 is an upstream regulator and promoter of downstream MMP synthesis.[12,108] MMP-2 cleaves numerous types of collagen and also

has a regulatory role, promoting the synthesis of other MMPs.[115] The ADAMTS are a family of potent aggrecanases. Those most often implicated in OA are ADAMTS 4 and ADAMTS 5.[12,108] In fact, ADAMTS 5 knockout mice have been shown to be protected against OA progression.[12,108,130] However MMP-13 knockout mice prevent collagen (but not aggrecan) depletion.[12,108]

Activated chondrocytes also express unique surface receptors, promoting the binding of inflammatory cytokines and chemokines (specifically those released from the synovium), which activate downstream inflammatory cascades further promoting cartilage destruction.[104,108,131]

1.10.2 Synovial Inflammation & OA

Under normal conditions, the synovial membrane (synovium) is composed of fibrous extracellular matrix approximately 2-3 cell layers thick. The synovium acts as a semi-permeable membrane, facilitating cartilage nutrient exchange through the regulation of synovial fluid composition.[113] The host cells of the synovium, synovial cells, are responsible for secreting synovial fluid components. Two primary components include lubricin and HA, which, as previously indicated, help protect and maintain the surface of articular cartilage.[108,109,113] Lubricin, specifically, is responsible for reducing the deposition of pathologic proteins on the cartilage surface.[113] These molecules are not permeable, allowing high concentrations to be retained within the synovium. In OA, the concentrations of lubricin and HA are lowered, limiting their intrinsic chondroprotective roles. This depletion is attributed to a change in synovial membrane permeability. Clinically, high serum HA concentrations and low synovial fluid HA concentrations have been used to confirm synovitis.[113]

Synovitis is a broad term used to describe (inflammatory) changes within the synovium, which are characteristic of arthritic diseases. Classically, synovitis refers to membrane compositional and organizational changes observed histologically.[113] These include thickening, increased populations of leukocytes and angiogenesis (indicated by increased VEGF concentrations within the synovial fluid) within the synovial membrane.[132,133] However, the synovial biopsy required for such histological examination is not always available. Thus, gross appearance during surgery or magnetic resonance imaging (MRI) have also become acceptable observations.[113,132] It has been reported that as many as 50% of OA cases have significant synovitis that is visible through MRI.[132,134] In comparison to other inflammatory-based arthritis diseases (ex. rheumatoid arthritis), OA synovitis is a low-grade chronic inflammation within the membrane.[113,119,133,135,136] Though synovial inflammation is more frequently observed in end-stage OA[135], the synovial lining is twice as thick (inflamed) in early stage OA compared to late stage OA.[119] Additionally, it has been shown that the degree of synovitis positively correlates with patient pain.[113,137] Synovitis is also directly related to cartilage degradation.[118,132,138,139]

Numerous inflammatory pathways can promote the development and maintenance of synovitis.[104] Recently it has been suggested that the most likely pathway begins when cellular stresses (as a result of injury or non-local inflammation) result in the release of matrix degradation products. These matrix fragments activate toll-like receptors (TLRs) along the cells of the synovial membrane through damage-associated molecular patterns (DAMPS).[108,113,135] The downstream consequence of TLR activation in the synovium is nuclear-factor κB (NF-κB) activation, which is a transcription factor regulating pro-

inflammatory chemokines (ex. IL-8) and cytokines (ex. IL-1, IL-6 and TNF-α).[104,113,135] Synovial cell TLR activation is also responsible for the downstream up-regulation of MMPs and aggrecanses.[113,128,135,140] Similar NF-κB dependent pathways are also responsible for the inhibition of transcription factors controlling chondrogenesis (ex. sox-9).[11]

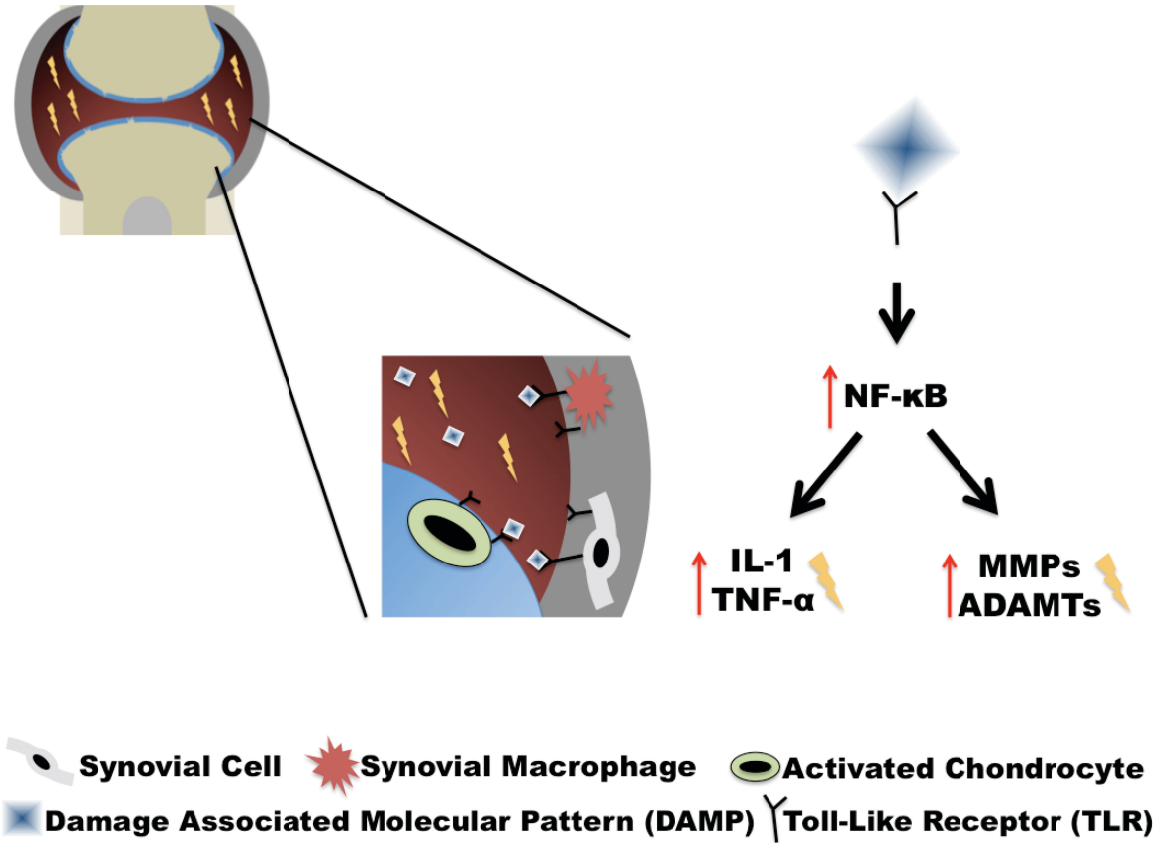


Figure 17: Toll-Like Receptor (TLR) Activation occurs through the binding of cartilage matrix fragments (i.e. DAMPs) to both cells within the synovium or chondrocytes. This binding results in the up-regulation of pro-inflammatory transcription factor NF-κB. Downstream consequences of this up-regulation include increases in pro-inflammatory cytokines and cartilage matrix degrading enzymes.

Notably, in addition to synovial TLR activation, chondrocytes have exhibited TLR activation in response to DAMPs.[12,113] Though it has been suggested that this

activation pathway does not lead to the production of an activated form of IL-1,[108] this pathway still results in the up-regulation of MMPs and ADAMTS. Specifically, TLR-2 and TLR-4 are up-regulated in regions of cartilage erosion.[104,113,135] Up-regulation of these specific TLRs has been linked to downstream increases in master MMP regulator, MMP-3.[113,135] Additionally, TLR-4 activation has been shown to recruit and activate macrophages to/within the synovium.[113] Specific DAMPs have also been isolated from OA synovial fluid; the concentration of DAMPs present was able to predict future cartilage destruction.[134]

This multi-focal activation of TLRs is one reason OA is beginning to be described as a feed-forward pathology, where the cartilage and the synovium act against one another, each fueling the other's disease progression. However, it is generally assumed that cartilage injury initiates the propagation of OA.[125,135] An alternate depiction of TLR activation can be found in appendix A.

1.10.3 Synovial Macrophages & OA

Though commonly associated with host defense, macrophages are involved in many other homeostatic and tissue remodeling activities.[141–143] Tissue microenvironments promote macrophage differentiation into functional phenotypes.[143,144] The two most prominent phenotypes are M1 (pro-inflammatory) and M2 (pro-regenerative/regulatory) polarized macrophages.[133,142,143] M1 macrophage differentiation is commonly induced by interferon-γ (INFγ) or other pro-inflammatory cytokines (ex. TNF-α).[141,142,144] M1 macrophages secrete potent pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12.[144] Contrastingly, anti-inflammatory mediators (ex. IL-4, IL-10 and IL-13) secreted during times of tissue remodeling and hypoxia induce M2 differentiation.[142,144]

M2 macrophages do not express but rather down-regulate IL-1β expression.[144] While M1 macrophages exhibit increased chemokine CCR7 expression, M2 macrophages display an up-regulation of mannose receptors[144], making both (CCR7 and mannose receptors) functional biomarkers for M1 and M2 phenotypic analysis, respectively. Despite their investigation in numerous pathologies, M1 and M2 macrophage phenotypes have not been thoroughly studied in OA.[133]

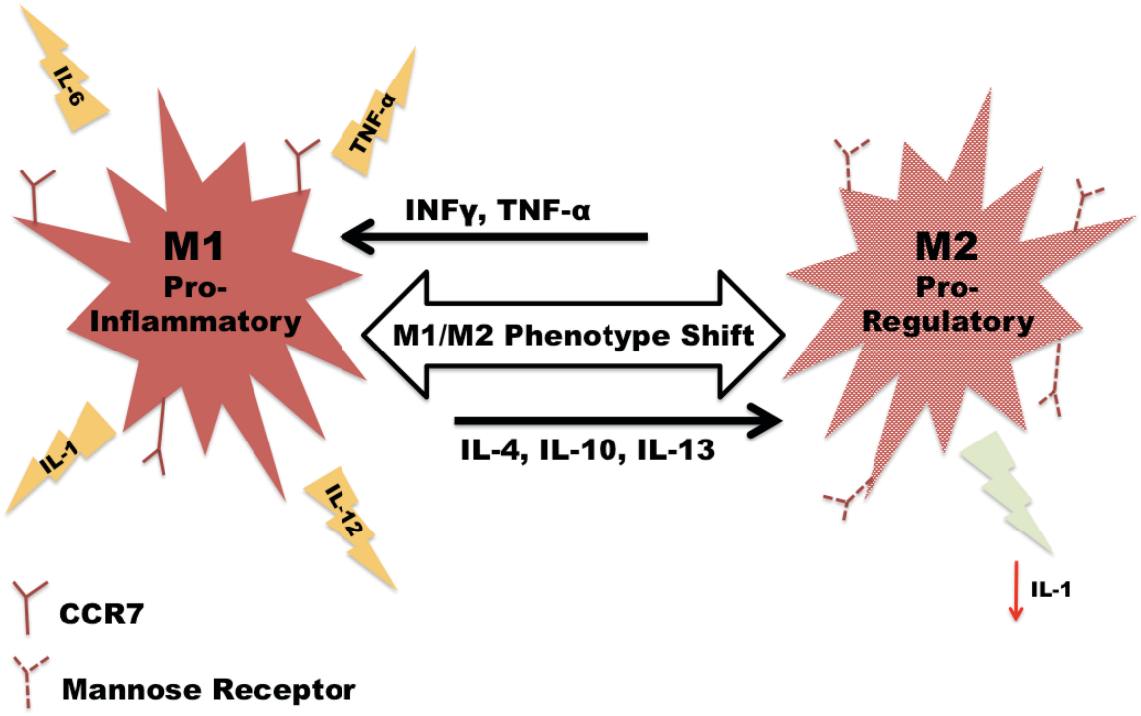


Figure 18: Macrophage Polarization refers to the variable phenotypes of tissue macrophages, the most common being M1 (pro-inflammatory) and M2 (pro-regenerative) macrophages. Pro-inflammatory mediators (ex. INFγ or TNF-α) promote M1 differentiation; while anti-inflammatory mediators (IL-4, IL-10 and IL-13) promote M2 differentiation. M1 macrophages secrete potent pro-inflammatory mediators. The most significant downstream effect of M2 macrophage secretions is the down-regulation of IL-1.

Within the normal human joint space, the body maintains necessary populations of M1 and M2 macrophages according to specific host needs. Therefore, normal

synovium exhibits varying proportions of M1 and M2 synovial macrophages.[145,146] As previously described, in OA the synovial membrane becomes inflamed. This involves the migration and accumulation of numerous macrophages within the synovial tissue.[115,134] Macrophages and T-cells have been reported as the most prominent cells types within OA synovial tissue.[133] Though macrophage populations remain above physiologically normal levels through all stages of OA, evidence suggests there are more CD68+ cells (i.e. macrophages) present in synovium from early stage OA compared to late stage OA tissue samples.[12,119] However, in studies comparing late stage OA synovial fluid to that of (healthy) controls, statistically higher levels of macrophage-related inflammatory mediators are observed.[147]

Clinical studies have demonstrated infiltration of macrophages into the synovium of patients with OA.[110] The presence of synovial macrophages largely determines the degree of synovitis and has been shown to positively correlate with OA cartilage damage.[127] The potent pro-inflammatory cytokines and chemokines expressed by macrophages (specifically M1 macrophages) feed the previously described inflammatory cascades, advancing the stage of the disease. Macrophages can produce an array of MMPs and ADAMTS.[110,115] However, they are also capable of secreting factors that regulate and promote the synthesis of cartilage degrading enzymes.[110,115,127,131,134] It is also thought that growth factors secreted by macrophages (ex. TGFβ and BMP) can directly promote osteophyte formation.[127]

Macrophages have been further implicated in OA pathology through experimental models where synovial macrophages were systematically depleted. In such models, OA progression was halted in macrophage depleted cultures.[127,134] Additionally, culturing OA

chondrocytes with macrophage-conditioned medium was shown to most accurately reflect the in vivo human pathology (i.e. increased type X collagen production, increased MMP activity, increased ADAMST 4 activity, decreased cartilage proteoglycan content) compared to all other models tested.[110]

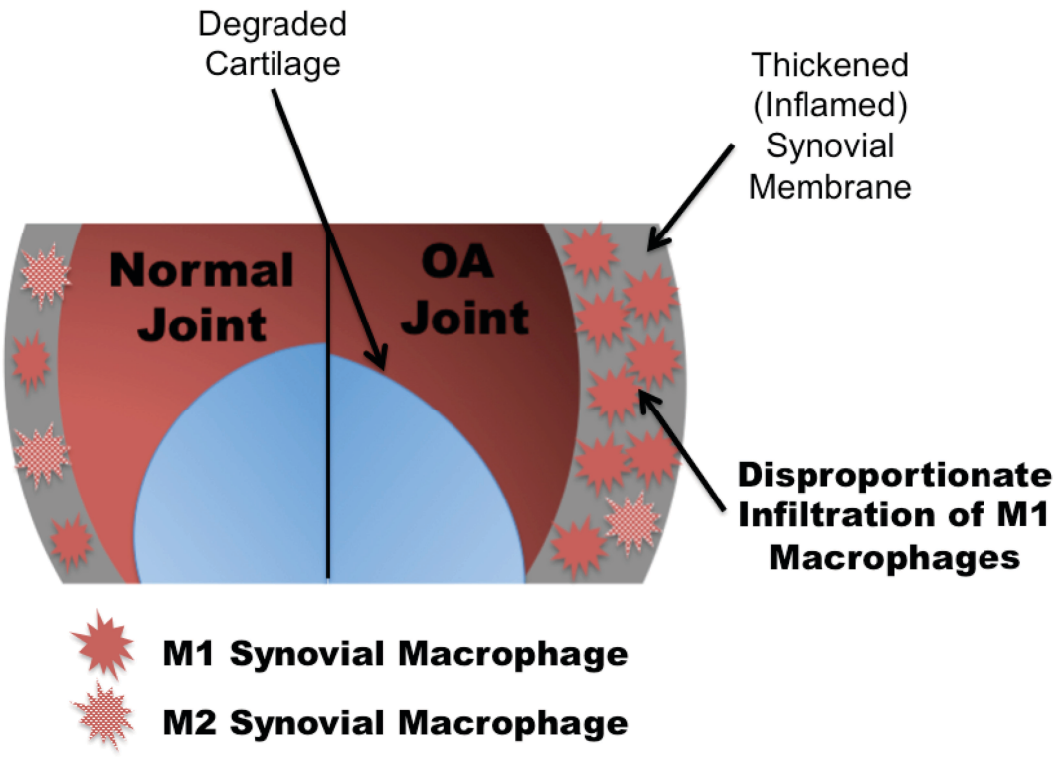


Figure 19: M1 Macrophage Infiltration in OA contributes to the state of chronic inflammation observed in OA joints. Under normal conditions, the body maintains physiologically relevant levels of M1 and M2 macrophages within the synovium. In OA, large populations of M1 macrophages infiltrate the synovium, disrupting homeostasis and creating a pro-inflammatory milieu.

1.10.4 Notable Pro-Inflammatory Mediators in OA

IL-1β is a monocyte, macrophage and chondrocyte-secreted pro-inflammatory cytokine suppressing aggrecan and collagen synthesis.[110,113,118,135] Downstream consequences of IL-1β production also include the up-regulation of ADAMTS-4, MMP-1, MMP-3 and MMP-13 as well as IL-6, IL-8m MCP-1 and CCL5 (also known as

RANTES).[113,118,135] Clinically, blocking only IL-1 activity does not alleviate OA-induced pain.[113,122,147] There is also evidence suggesting a crucial role of this cytokine in normal the regulation of cartilage homeostasis.[118] Unlike other inflammatory based arthritis diseases, IL-1β is not consistently elevated in OA patients.[113,128,136,148] Additionally, it is becoming more widely understood that IL-1β's primary role is in early stage OA.[147] When reported, IL-1β concentrations in late stage OA synovial fluid are typically <4.8 pg/mL,[136,147,149] though there are isolated reports of concentrations as high as 250 pg/mL.[148]

Contrastingly, TNF-α is readily detectable in the synovial fluid of patients with OA.[113,118,136] However, there are varying reports on disease stage-dependent expression, with late stage OA concentrations synovial fluid concentrations reported between 0-20 pg/mL.[113,136,147] TNF-α, like IL-1β, is implicated in cartilage degradation via the stimulation of collagenases and aggrecanases as well as the suppression of aggrecan and type II collagen synthesis.[110,118] Like IL-1β, downstream consequences of TNF-α production also include the up-regulation of MMP-1, MMP-3 and MMP-13 as well as IL-6, IL-8m MCP-1 and CCL5 (also known as RANTES). Similar to IL-1, trials blocking only TNF-α do not uniformly result in the alleviation of patient pain.[113,147]

While the singular elimination of IL-1β or TNF-α has not shown clinical efficacy, it has been demonstrated that simultaneous blocking of both cytokines results in OA mitigation.[150] This likely represents a redundancy in the OA pro-inflammatory cascade; whereby suppression of both pro-inflammatory mediators is required to completely modify (halt the progression) of the cascade. Studies utilizing bovine and porcine OA explants suggest both IL-1β and TNF-α lead to increased expression of ADAMTS-4;

however, neither cytokine affected the expression of ADAMTS-5, which is thought to be the primary ADAMTS involved in OA.[118]

Other cytokines with more obscure connections to OA include IL-7 and IL-15. IL-7, produced by chondrocytes, stimulates the production of MMPs and aggrecanases.[113] IL-7 is routinely reported in OA synovial fluid.[128] IL-15 is regulated by TLR-2 and TLR-4 stimulation. It is elevated in early stage OA, and its expression has a positive correlation with expression of MMP-1 and MMP-13.[113,118,135]

IL-6 has a less understood role in OA.[118] Though commonly (but not always) detected in patients with OA[119,128,135,137], IL-6 has been shown to have a chondroprotective effect in early stage OA while promoting disease progression (via osteophyte formation) in late stage OA. It is routinely detected in the synovial fluid of OA patients.[118] High levels of circulating IL-6 have been associated with increased likelihood of cartilage degradation.[118] In an IL-1β-dependent manner, IL-6 up regulates the expression of MMP-1 and MMP-13 and is thus associated with reduced cartilage matrix component expression.[118] Additionally, IL-6 concentrations have been positively correlated with leukocyte counts.[132]

Chemokines mediate the recruitment and regulation of inflammatory cells. Chemokines can also induce production of master MMP regulator, MMP-3.[113] Chemokines also induce IL-6 production.[118] Specific chemokines that have been linked to worsening symptoms include CCL19, IL-8, MCP-1 and RANTES and CCR7.[113,118] As previously described, CCR7 is expressed by M1, pro-inflammatory, macrophages.

While cytokine and chemokines play crucial roles in the development and maintenance of OA, it must be emphasized that individual biomarker results offer little

research utility and clinical value. Many variables including circadian and diurnal variation as well as differences between joint and circulating values influence biomarker concentrations.[118] Combined, many biomarkers and/or data regarding cartilage structure, synovial inflammation and clinical data (symptom, pain levels, etc.) provide much more meaningful insight into OA.

1.10.5 In Vitro Models of OA

Investigators have studied the pathogenesis of OA and the efficacy of potential OA therapies via the use of in vitro co-culture models. These in vitro co-culture models range in complexity and actual likeness to the disease state. Unfortunately many in vitro studies are completed using non-human tissue samples.[110] These results likely offer limited mechanistic insight into OA due to the known differences in inflammatory-driven matrix degradation, specifically the activation and regulation of ADAMTS 4 and ADAMTS 5, between species.[108,110,151,152]

1.10.5.1 OA Chondrocyte/Cartilage Explant Culture

Monolayer culture of human OA chondrocytes remains the most common model to study OA.[110,114,129,131] However, monolayer culture has been shown to alter cell phenotype limiting the translational applicability of such results.[128,153,154] Specifically, hypoxia and cell-matrix interactions can be altered/absent in monolayer cultures and are better accomplished through 3-D culture.[110] To overcome such barriers, human OA chondrocytes have also been seeded onto tissue engineered scaffolds.[110]

OA cartilage and synovium explants have been successfully cultured (separate from each other) in vitro for up to 21 days.[128] Such cultures tend to show no differences in proteoglycan loss and cell viability over time.[128] It has also been suggested that the

cytokine profiles obtained from individual explant co-culture media are less reflective of the levels described in vivo.[128]

1.10.5.2 Chemical Doping of OA Explants

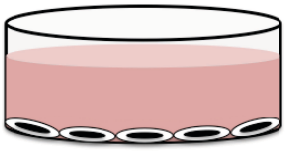
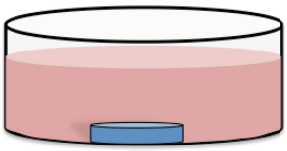
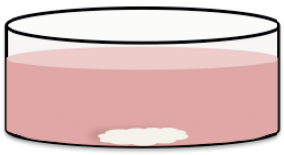
As our understandings of the inflammatory nature of OA have progressed, groups have begun mimicking OA progression through the addition of pro-inflammatory mediators, such as IL-1β and TNF-α, to OA cartilage explant cultures.[128] There are also reports of adding chemical/cytokines to chondrocyte-seeded scaffolds (or unseeded OA chondrocytes) in order to model OA.[110] While, IL-1β and TNF-α are central to OA pathology, there are many other inflammatory mediators influencing OA disease progression. Such chemical doping models fail to represent this complexity, and are therefore not fully representative of OA pathology.[110] Specific examples include the failure to replicate a loss of type II collagen within the cartilage matrix and to demonstrate hypertrophy within the chondrocytes.[110] Additionally, chemical doping leads to concentrations of pro-inflammatory mediators 10-1,000x greater than physiologically reported concentrations, rapidly accelerating the time course of disease progression.[128]

1.10.5.3 OA Explant Co-culture

As previously discussed, OA is a multifactorial disease involving multiple tissues in the joint. Therefore, greater mechanistic insight could likely be obtained from multi-tissue culture systems (i.e. co-culture). There are few reports of OA chondrocytes in co-culture with OA synovial cells.[128,155] These systems have the benefit of more accurately modeling the human anatomy and involved tissues. However, they succumb to the same previously mentioned limitations of cells in monolayer culture. Namely, monolayer culture has been shown to alter cell phenotype limiting the translational applicability of

such results.[128,153,154]

Co-culture models involving joint tissue explants have also been described.[128,140,156–158] The majority of such research has been completed using non-human tissues to study rheumatoid arthritis.[156–158] However, Beekhuizen was among the first to describe similar co-culture models for OA.[128] In joint tissue explant co-culture, OA cartilage explants are placed in the bottom of a tissue well plate. Synovial tissue is added to the culture via a permeable well plate insert. Thus, the cartilage and synovium share the same microenvironment without physically touching (similar to the anatomy of a human joint). Beekhuizen was able to confirm the ability to culture OA cartilage and synovial explants for up to 21 days without greatly compromising viability (viability

In Vitro Model	Pro(s)	Con(s)
Chondrocyte Culture [107, 111, 124, 125, 127] 	• Comparative ease of tissue source • Easy culture maintenance	• Alters cell phenotype • Produces inaccurate cytokine profile • Inability to measure cartilage matrix content
Cartilage Explant Culture [124] 	• 3D culture	• Produces inaccurate cytokine profile • Minimal change in cell viability • Minimal change in cartilage matrix composition
Synovium Explant Culture [124] 	• 3D culture	• Produces inaccurate cytokine profile • Minimal change in cell viability • Minimal change in cartilage matrix composition

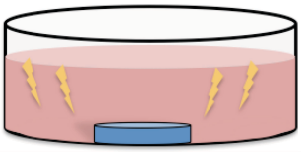
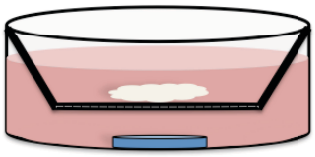
Chemical Doping [107, 124] 	• 3D culture • Model accounts for inflammatory involvement • Results in cartilage matrix degradation	• Produces inaccurate cytokine profile • Rapid OA progression
Explant Co-Culture [124, 134] 	• 3D culture • Mimics native architecture of joint • Model accounts for inflammatory involvement • Results in cartilage matrix degradation	• Limited peer-reviewed references

Figure 20: Summary of In Vitro OA Models highlighting the advantageous and shortcoming of each approach.

assessments were made using a LDH cytotoxicity assay but were not included in the manuscript; exact viability data is unavailable).[128] They were also able to confirm the co-culture system modeled certain aspects of OA disease, including the presence of CD68+ cells (i.e. macrophages) after 21 days in culture, a reduction in cartilage proteoglycan production and similar cytokine profiles to those reported in vivo.[128]

Interestingly, there are reports of chemically doping joint tissue explant co-culture models in an attempt to gauge the therapeutic efficacy of different HA products as compared to progressing OA.[159] However, as previously indicated, the un-natural acceleration of disease progression observed in such studies does not lend itself to translational applications.

1.10.6 In Vivo (Preclinical) Models of OA

The purpose of in vivo, preclinical, models are to reproduce the scale and progression of OA in a controlled manner such that the disease itself can be studied and new therapies can be developed.[117,121,160,161] Ideal preclinical models are of low cost,

reproducible and display likeness to the human pathology under investigation.[117,160] In the case of OA, one particularly important pathological consideration is the proper progression of the disease (too rapid progression is not representative of the slowly degenerative nature of OA and prevents the observation of subtle changes in disease characteristics with time).[117,121] One specific advantage of animal models is the ability to establish intermediate and terminal end points of tissue collection, allowing assessment of the disease in various stages of development.[121] Genetically engineered, experimentally induced and spontaneously occurring OA animal models are described in detail below.

Though not specific to OA, cartilage defect models have been employed to study consequences of cartilage injury and efficacy of potential treatment options.[162] Genetic knock-out mice have been employed to study mechanisms of OA pathogenesis and progression.[163,164] However, genetic models are typically high cost and can produce lethal genetic deletions.[121] Additionally, OA can be induced through the intra-articular injection of cartilage matrix degrading enzymes including collagenase, papain, and chondroitinase, among others.[117,161,165] These models have the advantage of being very rapidly progressive, minimally invasive and easy to implement. However correlations between this progression and human OA have yet to be established.[121]

Experimentally, OA can also be induced through surgical procedures aimed at producing mechanical instability in the joint. These models have the advantages of reflecting the inflammatory characteristics of OA and exhibiting quick disease progression.[117,161] However, due to the traumatic nature of OA induction these models are not fit for studies researching degenerative OA pathogenesis.[117,121] The most commonly reported procedure to induce traumatic OA is anterior cruciate ligament transection.[121]

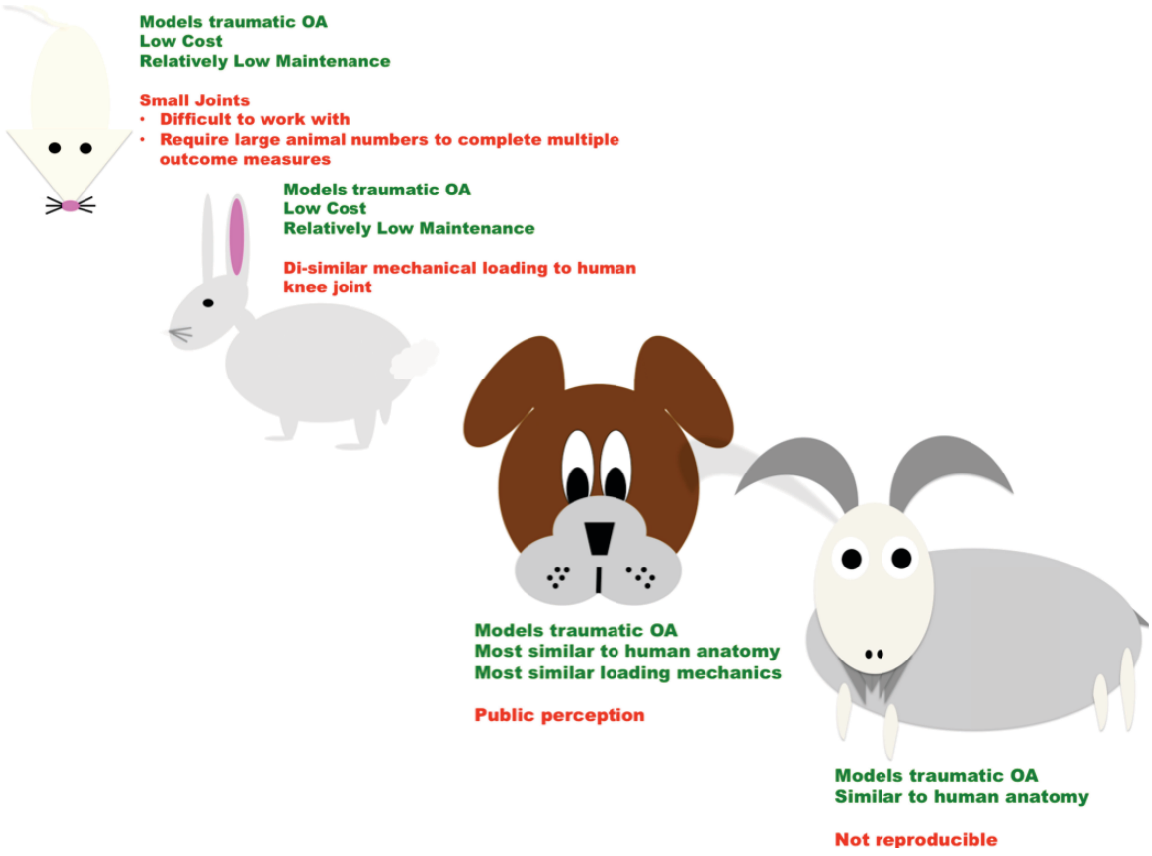


Figure 21: Summary of Common In Vivo OA Models (arranged by size) highlighting the advantages and limitations of each approach.

This approach has been successfully utilized in murine[110,117,121,160,161,166,167] (however the small nature of the joints limit research and applicability to humans[160]), rabbit[84,117,121,160,161,168,169] (however, it should be noted that rabbits primarily load the lateral stifle and are therefore not reflective of human knee joint biomechanics[121,160,161]), canine[117,121,160,161] (anatomically and biomechanically, canine models are considered to most closely mimic humans; however, public perception of canine use in research has limited their abailability[160]), capra[85,117,121,160] (while capra joints are similarly sized to humans they are not prone to spontaneous OA, and the ability to reproducibly induce OA

through surgical transection is under debate[160]) and cow[121] models, with terminal points varying from as little as six weeks to as long as two years. Other surgically-induced OA models include medial meniscectomy, medial cruciate ligament transection, and combinations thereof.[117] Additionally, there are thirteen published studies assessing randomized blinded placebo trials in traumatically-induced equine models of OA.[116] The specific advantages and disadvantages of these preclinical models have been succinctly reviewed elsewhere.[117]

In some animals, OA occurs spontaneously during animal development. Spontaneous OA has been noted in the knee joints of mice, though as previously indicated the small nature of the joints limit the number of possible outcome measures.[161] Non-human primates also exhibit spontaneous OA; however there are stringent guidelines limiting their use in research.[161] The most researched animal exhibiting spontaneous OA is the guinea pig. Similar to the human condition, spontaneous models have the advantage of being naturally occurring after 7 months of age, variable depending on genetic and environmental factors such as weight and reflective of long-term degenerative OA.[117,121,160,170]

Anatomically, the guinea pig knee is very similar to the human knee, though it is much smaller.[160] Additionally, guinea pigs exhibit the multi-factorial nature of bone growth and growth plate fashion observed in humans.[160] Notably, guinea pigs primarily load the medial stifle, making the medial compartment most prone to OA development (similar to humans).[160] The histopathology of the guinea pig has been extensively evaluated and deemed similar to human OA.[160] Drawbacks of the guinea pig model include an extensive time course (natural disease progression takes months-years to

develop) incurring moderate-high costs.[117,121,160] Though it has been suggested that more advanced OA can be achieved through communal caging.[117]

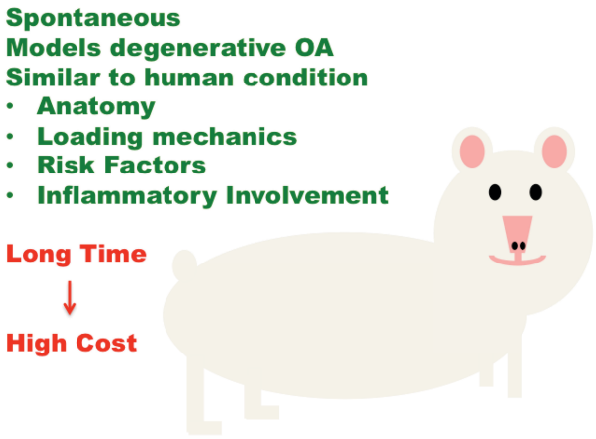


Figure 22: Guinea Pig Model of OA relies on the spontaneous development of degenerative OA by guinea pigs, with age. Other advantages include anatomical, biomechanical and pathological similarities to the human condition. However, these models require long study periods, heightening associated costs.

The male Dunkin-Hartley guinea pig model has been well characterized and offers the following similarities to the pathogenesis of non-traumatic, idiopathic human OA: 1) its susceptibility to common OA risk factors (i.e. advanced age and body mass index)[171,172], 2) spontaneous development of progressive OA in the medial compartment of the knee[161], 3) evidence of IL-1β, MMP-1, -3 and -13 involvement in OA[173–175], 4) early-stage OA with synovial inflammation, chondrocyte cell death, and proteoglycan loss[160,176] 5) its likeness in histopathology.[177] Both male and female DHGPs develop spontaneous OA. However, the accelerated weight gain and maturation of males allows them to develop more consistent changes consistent with OA.[161] Additionally, the DHGP model illustrates bilateral symmetry of the disease thus allowing for patient-matched degenerative controls.[161] Furthermore, recent studies indicate no adverse reaction of these animals following implantation of human stem cells in this model.[178]

1.10.7 OA Treatment Options

Currently there is no cure for OA; meaning, there is no treatment option halting the progression of the disease.[11,116,118,128,129,140,160] Patients suffering from OA are offered

palliative treatment options or surgery for symptomatic relief.[11,117,120,122,128,129] These options, as well as those currently under pre-clinical investigation, are described in detail below.

1.10.7.1 Available Treatments

Common treatments for OA include physical therapy/strengthening programs[12,120,126,179], viscosupplementation[12,122] and glucosamine/chondroitin sulfate supplementation.[12,120,122] All of the previously described treatment options have demonstrated only minimal improvement compared to placebo treatment.[12] The only pharmacologic treatment recommended by the American Academy of Orthopedic Surgeons is non-steroidal anti-inflammatory drugs (NSAIDs).[11,12,118,120,126,179] Narcotics such as Tramadol have also been utilized to alleviate pain in patients with severe OA symptoms.[12] Notably, the intra-articular injection of symptomatic slow acting drugs (ex. HA) has met scrutiny as to whether the benefits meet the minimum clinically important difference (MCID).[126]

Arthroscopic surgery (total joint replacement) has been employed to alleviate pain and to help restore joint mobility to patients suffering from OA.[11,12,120] However, a recent comprehensive series reviewing of the success of such procedures (by the New England Journal of Medicine) concluded that arthroscopic surgery is only minimally effective.[12,180–182] Another common surgical practice for the treatment of smaller chondral defects is autologous chondrocyte implantation.[11,12,87,112,120] Though there are reports of increased Knee Injury and Osteoarthritis Scores, this method suffers from major draw backs, including, the inabilities to rapidly expand chondrocytes, to maintain chondrocyte phenotypes and to produce articular cartilage upon implantation.[12,87,114] There is also data

suggesting other experimental approaches, including one-step stem cell therapies, are more effective than autologous chondrocyte transplantation at repairing osteochondral lesions.[183]

Specifically for knee OA, the AAOS utilizes a guideline rating the level of evidence and grade of recommendation for varying treatment modalities (patient education & lifestyle changes, rehabilitation, mechanical intervention, pain relievers, intra-articular injection, etc.).[184] Those recommendations receiving "A" grades (i.e. most positive endorsement) include: encouraging weight loss among overweight patients, encouraging participation in low-grade aerobics, encouraging glucosamine or chondroitin sulfate not be prescribed and recommending against arthroscopic debridement/lavage.

1.10.7.2 Clodronate (A Pre-clinically Investigated Treatment)

Di-chloromethylene bisphosphonate [Cl_2MDP] (Clodronate) is a bisphosphonate with known anti-bone resorption and anti-inflammatory properties.[185] As a non-nitrogenous bisphosphonate, clodronate prevents the binding of specific transcription factors to DNA, resulting in the production of a non-functional ATP competitor, ultimately resulting in cell apoptosis.[185–187] Though clodronate is primarily cited for inducing apoptosis in macrophages, there is also evidence suggesting the induction of apoptosis in osteoclasts and monocytes (pre-differentiated macrophages), both in vitro and in vivo.[185,188,189] Clodronate is water soluble, limiting its ability to freely cross the phospholipid bi-layer of most cellular membranes. Thus, clodronate is commonly delivered to the intracellular space through liposomes, taking advantage of the phagocytic properties of the usual target cells: the osteoclast and the macrophage. In osteoclasts, clodronate induces osteoclast cell apoptosis diminishing the overall number of

osteoclasts, slowing the resorption of bone. This is a common therapeutic technique used in osteoporosis.

In macrophages, clodronate induces macrophage apoptosis resulting in overall macrophage depletion. Downstream consequences of this depletion include a reduction in IL-1 and TNF- concentrations and overall cartilage destruction (i.e. reduced proteoglycan and collagen loss) in experimental arthritis models.[187,190,191] Macrophage depletion has been used to model various inflammatory-based (ex. OA) as well as various growth and development-based conditions. Historically, liposomes have been used to deliver a wide array of genes, antigens, antimicrobials, etc. to macrophages.[185,192] Liposome-encapsulation has been shown to increase clodronate efficacy by greater than 10x when compared to free clodronate.[187,193,194] This efficacy is typically analyzed through immunohistochemical analysis of pan-macrophage marker CD68 and monocyte marker CD14, as well as assessment of cartilage destruction at the time of surgery through Visual Analogue Scales.[185]

Previously, clodronate has been shown to be somewhat effective as a therapeutic agent in both rheumatoid arthritis and adjuvant arthritis preclinical models.[185,195–197] With mounting evidence suggesting a primary role of macrophages in OA pathogenesis, clodronate (among other bisphosphonates) are beginning to be investigated for their therapeutic efficacy of mitigating OA progression.[122,198] Clodronate intra-articularly injected into human knee joints resulted in improved VAS pain scores.[199] Additionally, some oral antiresorptive drugs resulted in decreased pain (assessed by WOMAC scores) and less subchondral bone abnormalities.[200]

There is also evidence suggesting mesenchymal stem cells can be cultured with

clodronate-encapsulated liposomes without adverse responses.[201] Therefore, future investigations could maximize therapeutic potential through a combined approach.

However, it should be noted that therapies targeting macrophage depletion suffer from significant drawbacks. Firstly, for a sustained effect, clodronate therapy would need to be administered (likely via intra-articular injection) routinely. Sustained macrophage depletion is not possible in vivo due to recruitment of new monocytes/macrophages through blood supply. In fact, it has been shown that the re-population of macrophages occurs after 2 weeks.[185] Secondly, there is conflicting evidence suggesting that mitigating disease progression implies a clinical alleviation of symptoms.[122] Thus, patients may need a palliative drug regimen in addition to clodronate therapy. Thirdly, the regulatory aspects surrounding bisphosphonate use in OA are strenuous and demanding. To date, no such treatment has been approved through the appropriate regulatory channels.[122]

1.10.8 Stem Cells as an OA Therapeutic Agent

With the evolving view of OA as an inflammatory condition driven by macrophages and their pro-inflammatory secretions, immunomodulatory therapies have come to the forefront of investigation. Due to their trophic, anti-inflammatory and immunomodulatory properties, stem cells may prove to be uniquely suited agents for OA therapies.[11,12,84,87,134,202]

1.10.8.1 Pre-Clinical and Clinical OA Stem Cell Therapies

Numerous stem cell therapies have been described in animal models of OA. The stem cells are typically delivered via intra-articular injection into the knee joint. In comparison studies, stem cell therapies have been shown to yield better outcomes than autologous chondrocyte transplantation.[12] There is also evidence that the stem cells are

still located within guinea pig joint tissues after 1 week[120,170] and up to 8 weeks post-implantation in rats.[120] There are also reports of the stem cells exhibiting signs of proliferation and differentiation. [120,170]

Twenty weeks post injection of $10x10^6$ autologous BMSCs (within an HA carrier) into goat knees, less osteophyte formation and cartilage degeneration were noted.[85] Twelve weeks post injection of $9x10^5$ BMSCs (within an HA carrier) into porcine knees, increased type II collagen and better healing was observed.[162] After 6 months, the injection of $2x10^6$ BMSCs (in an HA scaffold) into rabbit knees resulted in decreased MMP activity and regenerated cartilage with increased type II collagen expression.[168]

The injection of $1\text{-}2x10^5$ allogeneic ADSCs resulted in decreased MMP-1 and TNF-α concentrations.[169] ADSCs have also been shown to decrease synovitis and proteoglycan loss in mice with collagen-induced arthritis.[12] Scaffold-free infrapatellar fat pad-derived stem cells injected into rabbit knees showed less cartilage degeneration and osteophyte formation after 12 weeks.[203]

Table 3: Summary of Stem Cell Therapies for Osteoarthritis

Primary Author	Model	Stem Cell	Length of Study	Results
Murphy[85]	Capra	$10x10^6$ BMSCs	20 Weeks	• Decreased osteophyte formation • Decreased cartilage degeneration
Lee[162]	Porcine	$9x10^5$ BMSCs	12 Weeks	• Increased collagen II production within cartilage
Grigolo[168]	Rabbit	$2x10^6$ BMSCs	6 Months	• Decreased MMP activity • Increased collagen II expression • Evidence of cartilage regeneration

Toghraie[203]	Rabbit	1-2x10^5 Infrapatellar fat pad-derived SCs	12 Weeks	• Decreased osteophyte formation • Decreased cartilage degradation
Frisbie[116]	Equine	Comparative ADSC v. BMSC	70 Days	• No significant differences between groups
Sato[170]	Guinea Pig	7x10^6 Human MSCs	5 Weeks	• No signs of immune rejection of MSCs • Evidence of cartilage regeneration & collagen II production

Table 3: Summary of Stem Cell Therapies for Osteoarthritis noting the animal model employed, the type and dosage of stem cells as well as significant study results. Orange text indicates the stem cells were administered via an HA carrier.

Equine studies compared the efficacy of BMSC and ADSC intra-articular injections through arthroscopically inducing OA in the middle carpal joint.[116] There were no significant differences reported between groups, though it was noted that the BMSCs appeared to have a more beneficial impact. [116] Additionally, one study injecting 7x10^6 commercially available human stem cells into guinea pig knees reported no immune response after 5 weeks.[178] Stem cell therapies have also been experimentally utilized in humans. Autologous BMSCs (8x10^6-4x10^7) have been intra-articularly injected with patient follow-up for up to 2 years. [120,204–206] Such methods have resulted in decreased patient pain as reported by WOMAC and VAS pain scales.[120,204–206] Autologous ADSCs (1x10^7-10x10^7) were injecting in a dosing study, which reported that high doses resulted in improved joint function and decreased pain.[13] Infrapatellar fat pad-derived stem cells seeded onto PRP scaffolds have resulted in improved VAS, Lysholm and OA index scores after 2 years.[207] Injection of allogeneic BMSCs (5x10^7) 10 days after meniscectomy resulted in decreased pain.[208] Similar reports in France utilize autologous

ADSCs.[84]

1.10.8.2 Potential Mechanisms behind OA Mitigation

Hyaluronan facilitates granulation tissue formation, the initial phase of tissue regeneration, during natural wound healing.[85,209] Stem cells also enable wound healing through facilitating and accelerating granulation tissue formation. Additionally, many stem cell therapies are injected in the presence of a hyaluronan (or HA) carriers.[85,168,206] Such approaches likely amplify and accelerate natural tissue repair mechanisms.

As previously indicated, stem cells, especially stem cells combined with biomaterial scaffolds, have the potential to differentiate into target cells, establishing new populations of healthy cells in diseased areas.[11,84,85] For the purposes of OA, stem cells exhibiting enhanced chondrogenic differentiation potential would prove advantageous, as these cells would more readily differentiate into chondrocytes upon implantation into OA joint spaces. Though it has been demonstrated that stem cell implantation into rabbit femoral condyles with cartilage defects resulted in the production of new cartilage, this cartilage was not continuous with the host tissue.[210,211] As previously indicated, integrity of the surface layer of cartilage is paramount to its health and function. Thus, the intended use of stem cells as chondrocyte progenitor cells remains questionable in translational regenerative medicine approaches.

A more intriguing therapeutic mechanism of action is stem cell paracrine effects. Stem cells secrete mediators which attract and home host stem cells to target areas.[11,84] Focal inflammatory cells express monocyte chemoattractant protein-1 (MCP-1). Stem cells express the MCP-1 receptor, CCR2, resulting in their recruitment to the area.[84] Interestingly, stem cell therapies rarely lead to new chondrocyte formation (i.e.

differentiation of stem cells); rather the majority of stem cells home to the synovium.[140]

Stem cells release a variety of growth factors and anti-inflammatory mediators which could help modulate diseases characterized by immune-dysregulation.[11,84,119,129,207] Such immunomodulatory regulators can inhibit the activation and recruitment of inflammatory cells. Those most researched include: indoleamine 2,3-dioxygenase (IDO), interleukin receptor antagonist (IL-1ra), prostaglandin E2 (PGE2) and IL-10.[11,84,134] These anti-inflammatory mediators have been proposed as a primary mechanism through which stem cells establish immune-suppressive local environments, aiding in their immune-privileged status.[11] Notably, stem cells also have the documented ability to induce anti-inflammatory (M2) phenotypes in macrophages.[134,212,213]

It has been demonstrated that stem cells must be "primed" in order to exhibit anti-inflammatory and immunomodulatory characteristics.[134,214] Typically this involves the stimulation of stem cells through potent pro-inflammatory cytokines such as INFγ.[84,214] Though the exact timeline has not been established, the need for priming does represent an inherent delay in the efficacy of OA stem cell therapies. This could be one explanation for delays in patient progress post initiation of stem cell therapy.

1.10.9 Amniotic Membrane Derived Stem Cells & OA

Though adult stem cells have been widely investigated as a potential OA therapeutic, there are drawbacks associated ADSCs and BMSCs that are less attributable to alternative stem cell sources (such as perinatal stem cells). Examples include the inability of BMSCs isolated from OA patients to proliferate and differentiate as effectively as BMSCs from healthy donors.[10–12,85] Adult trabecular bone mesenchymal stem cells have exhibited similar deficiencies.[12] OA BMSCs exhibit an increased

potential for osteogenic and a decreased potential for chondrogenic differentiation.[10,11,112] Though it has been suggested that additional exogenous growth factor supplementation can curb these abnormal effects.[12,215,216] Human periosteal mesenchymal stem cells exhibit spontaneous chondrogenic differentiation in younger donors (< age 30).[12] However, the majority of OA cases occur in patients above age 65. Additionally, implantation of pre differentiated BMSC (into a chondrogenic phenotype) in a capra OA model resulted in non-maintenance of the chondrocyte phenotype (cell demonstrated an increased likelihood of hypertrophy an decalcification).[87,217]

Additional drawbacks of using adult stem cells include low stem cell yields and painful harvest procedures.[87] More recently perinatal stem cells have illustrated promise as an alternative stem cell source for regenerative medicine.[68,218] Previously it has been shown that cord blood MSCs differentiate more readily into chondrocytes compared to ADSCs and BMSCs.[87,219] Moreover, cord blood MSCs have the capacity to differentiate into chondrogenic phenotypes in pro-inflammatory environments comparable to OA; whereas BMSCs do not.[87] Furthermore, cord blood MSCs exhibit increased expression of anti-inflammatory mediators (compared to BMSCs) in OA co-culture models.[87]

An equally intriguing, though less researched, perinatal stem cell source are those stem cells derived from the amniotic membrane (a tissue routinely discarded as medical waste following the birth of full-term babies). Advantages of amniotic membrane derived stem cells (hAMSCs) include 1) their capacity for chondrogenic differentiation and generation of cartilage[91,97,220], 2) their availability of large cell yields at harvest[49,54,71], 3) their ontogenically youthful status limiting their exposure to detrimental age-related changes[221], 4) their proven immunomodulatory and immunosuppressive nature.[55,68,220,222–

[224] Furthermore, recent evidence suggests that perinatal stem cells exhibit superior chondro-protective effects in an inflammatory environment, exhibiting the ability to induce a pro-regenerative (M2) phenotype within synovial macrophages.[73,87] There is also evidence suggesting hAMSCs do not require inflammatory priming prior to initiating therapeutic benefit.[225] Notably, such characteristics do not appear to be affected by freeing or heating cycles, a necessary property owing to the likely need for tissue banking of such a stem cell source.[225]

CHAPTER TWO

PROJECT APPROACH

2.1 Significance

Stem cells are being investigated as alternative therapeutics for numerous orthopedic regenerative medicine approaches, largely due to their musculoskeletal differentiation, anti-inflammatory and immunomodulatory capacities.[4,6,7,11] In order for stem cell therapies to be effective, high numbers of stem cells must be utilized. Therefore, stem cells exhibiting musculoskeletal differentiation, anti-inflammatory and immunomodulatory capacities as well as high yields would be an ideal stem cell source for orthopedic regenerative medicine approaches.

The largest subset of musculoskeletal tissue pathologies involves bone and/or cartilage. Currently, no side-by-side comparative analyses have been conducted in order to determine which stem cell source(s) most robustly differentiates into osteogenic and chondrogenic lineages. Such information would allow clinicians to utilize the best-suited stem cells source in future therapeutic approaches.

While stem cells have shown promise in prevalent musculoskeletal tissue pathologies, including osteoarthritis, few stem cell sources have been evaluated in such therapeutic approaches.[85,116,162,168,178,203] Amnion membrane derived stem cells have yet to be investigated as an OA therapeutic. Additionally, no side-by-side comparisons have been conducted in order to determine the relative efficacy of amnion-based approaches with those currently under pre-clinical investigation (adipose derived stem cells and bone marrow derived stem cells). These authors also know of no (ex vivo or in vivo) studies comparing the differential therapeutic effects of stem cell administration location.

Stem cells from the amniotic membrane (a tissue routinely discarded after the birth of term pregnancies) exhibit numerous characteristics of an ideal stem cell source, including, heightened differentiation potential, availability in high yields, heightened immunomodulatory properties, the ability to induce pro-regenerative (M2) phenotypes within macrophages.[49,54,55,68,71,73,87,91,97,220,222–224] Additionally, perinatal stem cells may offer accelerated treatment time lines as they do not require pro-inflammatory priming prior to initiating therapeutic benefit.[225] Thus, amnion membrane derived stem cells are likely an under investigated stem cell source for orthopedic tissue engineering and regenerative medicine strategies.

2.2. Specific Aims

Our goal is to investigate the utility of amnion derived stem cells for orthopedic regenerative medicine. Through standardized ex vivo comparative analyses, we aim to compare the relative efficacy of amnion derived stem cell differentiation and therapeutic relevance with a commonly utilized stem cell source, adipose derived stem cells.

2.2.1 Aim I: To directly compare the abilities of amnion and adipose derived stem cells to differentiate towards osteogenic and chondrogenic lineages

Our goal is to compare the efficacy of amnion and adipose stem cell differentiation towards osteogenic and chondrogenic lineages, offering initial insight into the utility of amnion derived stem cells in orthopedics. In order to accomplish this, an amnion stem cell harvest procedure and optimized differentiation protocols must be developed. Utilizing such methods a controlled ex vivo comparison of differentiation

should be conducted evaluating both genes and proteins characteristic of the respective lineages being examined.

2.2.2 Aim II: To validate a human explant joint tissue co-culture model of OA

Our goal is to validate an ex vivo platform for testing potential future OA therapeutics, including amnion derived stem cells. Such a platform (i.e. a validated model of osteoarthritis) should demonstrate likeness to the human condition by demonstrating disease progression, pathophysiological levels of pro-inflammatory cytokines and synovial macrophage involvement. Macro and micro-architecture, inflammatory as well as biochemical assessments should be employed in order to verify such characteristics.

Aim III: To evaluate the ability of MSCs to mitigate OA progression in this validated ex vivo model

Our goal is to investigate the utility of amnion derived stem cells as an OA therapeutic, ex vivo, and to compare their efficacy against a field standard stem cell, adipose derived stem cells. This controlled comparison (where all experimental variables excluding one (i.e. stem cell type or administration location) are held constant) should investigate differential therapeutic effects between routes of administration and allow for meaningful insights regarding the efficacy and mechanism of action of MSCs in OA. As before, macro and micro-architecture, inflammatory as well as biochemical assessments should be employed in order to examine these claims.

Aim IV: To compare the therapeutic effects of hAMSCs and hADSCs to attenuate OA progression in vivo

Our goal is to investigate the differential therapeutic effects of amnion and adipose derived stem cells in an established animal model of OA. This pilot study will

provide foundation in vivo evidence into the utility of amnion membrane derived stem cells as an efficacious therapeutic for OA. This approach involves demonstrating OA disease progression in non-treated control animals as well as showing mitigation of disease progression in treated animals. As before, macro and micro-architecture, inflammatory as well as biochemical assessments should be employed in order to examine these claims.

CHAPTER THREE

AIM I: TO DIRECTLY COMPARE THE ABILITIES OF AMNION AND ADIPOSE STEM CELLS TO DIFFERENTIATE TOWARDS & CHONDROGENIC LINEAGES

3.1 Introduction

Cartilage defects represent debilitating lesions, which cause patients significant pain and oftentimes lead to patient immobility.[226,227] A majority of these are classified as full thickness cartilage defects, which involve not only the articular cartilage but also the sub-chondral bone.[227–229] Cartilage in particular lacks the intrinsic ability to repair and regenerate itself.[226,229] This is in part due to the senescent phenotype of the host cells, chondrocytes, preventing the generation of healthy extracellular matrix (ECM) required to fill defect areas. Surgically, such defects are currently addressed by micro fracture or autologous chondrocyte transplantation.[227–229] Both of these techniques function by introducing populations of healthy, reparative cells to the defect area in the hopes that the cartilage ECM will be regenerated and joint function will be improved.[227–229] Mesenchymal stem cells represent a recently investigated alternative approach to cartilage defect repair largely due to their ability to differentiate into both cartilage and bone phenotypes.[226,227,229,230]

Adult mesenchymal stem cells are considered alternative cell sources for use in orthopaedic regenerative medicine. Typically derived from bone marrow (hBMSCs) or adipose tissue (hADSCs), these cells have the ability to self renew, exhibit immunomodulatory properties and differentiate towards numerous tissue lineages.[63] Both cell types have been extensively investigated in preclinical osteochondral defect models as well as in human clinical trials, demonstrating significant clinical improvement (i.e.

reduced pain, decreased symptom intensity and/or radiographic evidence of cartilage damage) as well as various degrees of cartilage regeneration.[226,227,229,230] Additionally, early results indicate greater therapeutic efficacy using such approaches as compared to autologous chondrocyte transplantation.[22,229]

Recently, researchers have begun investigating the potential efficacy of perinatal stem cell populations (isolated from the amnion harvested after the birth of term pregnancies) for orthopaedic applications.[68] Based on the minimal criteria established by the International Society for Cellular Therapy; 1) the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages, 2) >95% positive expression of CD73, CD90 and CD105, and 3) <2% positive staining for CD45, CD34, CD14 or CD11b, and CD79 or CD19)[25], there exists two primary cell types within the amnion exhibiting stem cell characteristics: amniotic epithelial cells (hAECs) and amniotic mesenchymal stem cells (hAMSCs). Developmentally, these cells arise from the pluripotent epiblast.[218,221] This may imply that these cells retain embryonic stem cell-like characteristics, potentially offering more robust therapeutic advantages.

While some have attempted to validate the use of perinatal cells in orthopaedic applications, none have conducted side-by-side comparisons of human amnion derived cells with adult mesenchymal stem cells in regards to their capacity to differentiate towards musculoskeletal tissue cell types. Significantly, the osteogenic and chondrogenic differentiation capacities of hADSCs and hBMSCs have been well established to occur at approximately 21-28 days.[231–234] However, there is evidence suggesting the differentiation of perinatal stem cells may occur earlier than this, indicating perinatal

stem cells may be an alternative therapeutic with an accelerated timeline to clinical benefit.

It has been widely established that numerous experimental conditions impact stem cell properties, including their differentiation capacity.[54,89,92] Experimental variables including stem cell passage number, media type and growth factor supplementation, cell seeding method or densities and gas exchange (i.e. hypoxic conditions), are often held consistent within studies. However various researchers employ different experimental methods making it difficult to directly compare results across studies found throughout published literature. Herein, studies were undertaken to compare the osteogenic and chondrogenic differentiation potential of human amnion and adipose derived stem cells under identical, standardized culture conditions with the goal of providing meaningful insight into the potential clinical efficacy of these cell types for the management of osteochondral defect repair/regeneration.

3.2 Materials & Methods

hADSCs were purchased from Invitrogen (R7788-110). Trypsin was purchased from Fisher scientific (MT-25-053CI). Collagenase was purchased from Worthington Biochemicals (LS004196). Ambion Trizol Reagent (15-596-026) and Turbo DNA Free Kit (AM10907) were purchased from Fisher Scientific. Ambion RETROscript Reverse Transcription Kit was purchased from Life Technologies (AM1710). Qiagen QuantiTect Primer Assays employed in this work included: runx-2 (QT00020517), osteocalcin (QT00232771), sox-9 (QT00001498), aggrecan (QT00001365), collagen-2 (QT00049518) and GAPDH (QT00079247). QuantiTect SYBR Green PCR Kit was also purchased from Qiagen (204143). Alizarin Red (A5533-25G) and Alcian Blue (A3157-

10G) stains were purchased from Sigma. Normal Horse Serum (S2000), VECTASTAIN Elite ABC Kit, Rabbit IgG (PK-6101) and DAB Substrate Kit (SK4100) were purchased from Vector Laboratories, Inc. Triton X-100 and other basic chemicals were purchased from Fisher Scientific. Antibodies employed in this work included: mouse anti CD105 (BD Biosciences, 555690), mouse anti CD73 (BD Biosciences, 550256), mouse anti CD45 (BD Biosciences, 555480), mouse anti CD90 (BD Biosciences, 555593), mouse anti EpCAM (BD Biosciences, 347198), rabbit anti collagen-2 (Abcam, Ab85266), and goat anti-mouse FITC secondary antibody (abD Serotec STAR117F).

3.2.1 Amniotic Membrane Harvest

Human placentas were obtained from consenting patients immediately following delivery via elective cesarean sections of full-term babies (Pro00031185-Greenville Health System). Amniotic membrane derived cells were isolated within 4 hours of delivery.

3.2.2 Isolation and Enrichment of hAEC and hAMSC Populations

Placentas (n=3) were placed with the umbilical cord facing upward such that the fetal (amniotic) surface was accessible (Figure 23A). The amniotic and chorionic membranes were identified and mechanically peeled from each other (Figure 23B). Enriched hAEC and hAMSC cell isolation methods were adapted from Barbati et al.[55] Briefly, amnions were digested twice in 0.25% trypsin for 30 minutes at 37°C with agitation to completely liberate hAECs followed by complete digestion in two digestions of collagenase [2mg/mL collagenase (249 U/mg)] for 30 minutes at 37°C with agitation each to subsequently liberate hAMSCs.

3.2.2.1 Histological Confirmation of Amnion Cell Isolation

Amnion sections were secured within a tissue processing cassette, fixed in 10% phosphate buffered formalin overnight at room temperature prior to undergoing standard tissue processing, paraffin embedding and sectioning to 5 μm thickness. Sections were stained with Hematoxylin and Eosin (H&E) for visualization of ECM and cell nuclei.

3.2.2.2 Flow Cytometric Analysis for Stem Cell Markers

Cells were incubated in primary antibody (either CD105, CD73, CD45, CD90, all at 5μg/mL dilution or EpCAM at 0.3μg/mL dilution) for 30 minutes at room temperature. After 30 minute room temperature incubation in goat anti-mouse FITC secondary antibody (at 5μg/mL dilution), samples were read on a Guava easyCyte™ Single Sample Flow Cytometer.

3.2.3 Confirmation of a Mixed Amnion Cell Population

Briefly, the amnion (n=1) was digested twice in 0.125% trypsin for 30 minutes at 37°C with agitation to incompletely liberate hAECs followed by complete digestion in collagenase (two 2mg/mL collagenase (249 U/mg) digestions for 30 minutes at 37°C with agitation) to liberate the remaining hAECs and hAMSCs, termed the "Mixed" cell population. Cells were analyzed histologically and via flow cytometry as previously described.

3.2.4 hADSC and Amnion Derived Cell Osteogenic Differentiation Potential

3.2.4.1 Culture Conditions for Stem Cell Differentiation

In preparation for in vitro differentiation studies, all cell types were expanded in standard culture medium under standard culture conditions (37°C with 5% CO_2) with media changes every 3 days. The standard culture expansion medium for hAECs consisted of DMEM supplemented with 10ng/mL epidermal growth factor, 10% fetal

bovine serum and 1% antibiotic/antimitotic. The standard medium for hAMSCs and Mixed cells consisted of DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimitotic. The standard (manufacture recommended) culture medium, MesenPro, was used for expanding hADSCs following the manufacturer's instructions. All cells were used at passage 2. To induce osteogenic differentiation, cells were seeded at a density of $2.1x10^4/cm^2$ into tissue treated 12-well plates and cultured in monolayer for up to 28 days in osteogenic differentiation media (DMEM, 10% FBS, 1%AB/AM, 0.1uM dexamethasone, 50μM ascorbate-2-phosphate, 10mM β-glycerophosphate). Negative controls were maintained in standard culture media (DMEM+10%FBS+1%ABAM). Differentiation capacity was assessed via histological staining (n=2/condition, described below) and gene transcript expression (n=4/condition, described below).

3.2.4.2 Gene Transcript Analysis

Total RNA from all differentiated and control conditions were isolated using Trizol reagent according to the manufacture's instructions. RNA integrity and quantification was assessed using a BioTek Epoch reader according to the manufacture's instructions. A total of 600μg-1mg of RNA was reverse transcribed using the Ambion RETROscript kit. Resulting cDNA was amplified using a Rotogene 3000 thermocycler. Reaction products were detected using human QuantiTect primers in conjunction with a QuantiTect SYBRgreen polymerase chain reaction (PCR) kit. Gene expression ratios were calculated using the $2^{-}\Delta ct$ method with GAPDH serving as a housekeeping gene. Gene expression is reported as fold increase. For native (baseline) gene expression, fold increase was calculated by the following equation: $2^{-}\Delta ct_{amnion\ cell\ group} / 2^{-}\Delta ct_{hADSCs}$. For

induced gene expression, fold increase was calculated according to the following equation: $2^{-}\Delta ct_{induced}/2^{-}\Delta ct_{control}$, where control gene expression values were derived from controls of the same cell type cultured to the same time-point in the absence of induction medium.

3.2.4.3. Alizarin Red Histological Staining for ECM Calcification

Well plates were fixed in 4% formaldehyde for 30 minutes at room temperature prior to 3-minute Alizarin Red staining (2% aqueous Alizarin Red, pH 4.2) for visualization of calcium deposition. The percentage of the total well-plate area stained positive was quantified via color threshold analysis using NIH Image-J software by two blinded observers.

3.2.5 hADSC and Amnion Derived Cell Chondrogenic Differentiation Potential

3.2.5.1 Culture Conditions for Stem Cell Differentiation

To induce chondrogenic differentiation, cells were seeded in pellet culture ($1x10^5$ cells per pellet) and cultured in chondrogenic differentiation media (DMEM, 1% FBS, 1% ABAM, 6.25μg/mL Insulin Transferrin Selenium, 50nM Ascorbate-2-phosphate, 10ng/mL human TGF-β). Controls were plated at a density of $2.1x10^4/cm^2$ into tissue treated 12-well plates (i.e. $1x10^5$ cells per well) and cultured in monolayer with standard culture media (DMEM+10%FBS+1%ABAM). Differentiation capacity was assessed via histological staining (n=2/condition, described below) and gene transcript expression (n=4/condition, previously described).

3.2.5.2 Alcian Blue Histological Staining for ECM Glycosaminoglycan Content

Chondrogenic cell pellets (n=3 pellets/condition) were fixed in 10% phosphate buffered formalin for 30 minutes at room temperature before undergoing manual tissue

processing (utilizing the standard protocol of serial ethanol washes followed by Xylene and paraffin washes), paraffin embedding and sectioning. Sections were mordant in 3% aqueous Acetic Acid solution for 3 minutes prior to 30-minute staining in Alcian Blue (1% Alcian Blue in 3% aqueous Acetic acid, pH 2.5) for visualization of GAG deposition. Sections were counterstained with 0.1% aqueous Nuclear Fast Red for 5 minutes for visualization of cell nuclei. The percentage of the total cell pellet area stained positive was quantified via color threshold analysis using NIH Image-J software by two blinded observers.

3.2.5.3 Immunohistochemistry for Collagen Type 2

Immunohistochemistry (IHC) on rehydrated paraffin sections was performed for detection of collagen type 2 in chondrogenic cell pellets (n=3 pellets/condition). Briefly, antigen retrieval was accomplished via 10mM Citric Acid incubation at 90°C for 20 minutes. Sections were permeabilized with 0.025% Triton X-100 for 10 min and then incubated in normal blocking serum for 45 min at room temperature. Primary antibody (rabbit anti-collagen-2, 5μg/mL dilution) was applied for 1 h at room temperature. Negative staining controls did not receive primary antibody. Blocking of endogenous peroxidases was accomplished via incubation in 0.3% hydrogen peroxide in 0.3% horse normal serum for 30 min at room temperature. Visualization of antibody was accomplished via staining with the Vector ABC peroxidase substrate kit. Sections were counterstained with Hematoxylin prior to microscopic imaging.

3.2.6 Microscopic Imaging

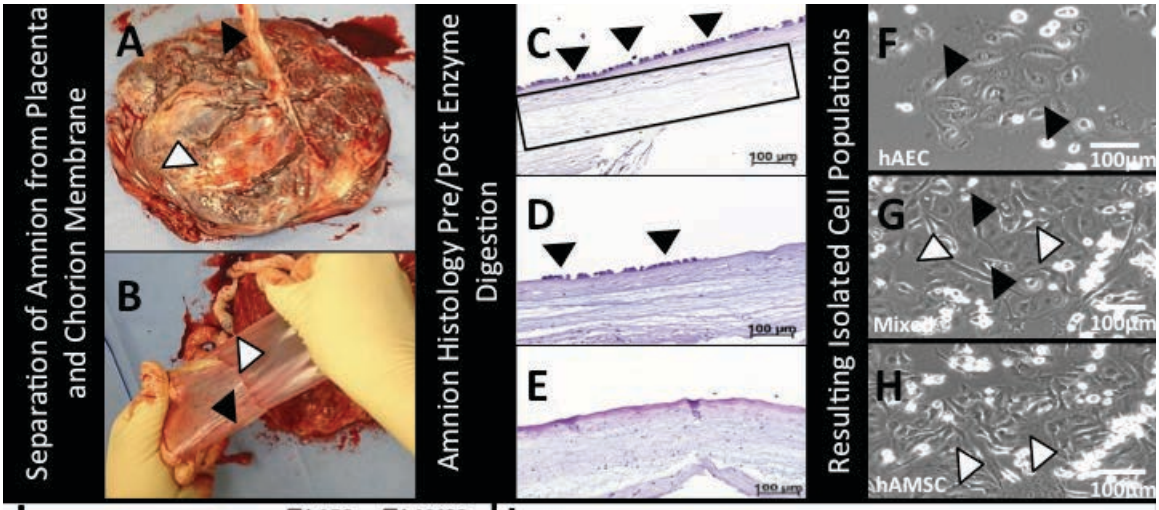
Images were captured on a Zeiss Axiovert.A1 microscope with Axiovision software (Release 4.9.1 SP08-2013).

3.2.7 Statistical Analysis

Results are represented as a mean ± standard error of the mean (SEM). All statistical analyses were performed by two-tailed Student's t-test of unequal variance or analysis of variance (ANOVA) with a Tukey's post-hoc analysis. Significance was defined in all cases as $p<0.05$.

3.3 Results

3.3.1 Amniotic Membrane Harvest



J Percentage of Cells Positive for Surface Markers [%]

	hAEC	Mixed	hAMSC
CD73	97.5±1.5	94.7±1.2	98.1±0.2
CD90	98.6±0.4	94.9±0.9	97.1±0.9
CD105	68.7±10.5	87.5±7.7	97.1±0.4
CD45	3.1±1.1	2.7±0.8	1.6±0.2
EpCAM	96.8±0.1	39.6±1.8	1.4±0.1
Isotype Control	2.1±1.4	7.4±5.1	1.8±0.3

Figure 23: Amniotic Stem Cell Harvest, Isolation and Characterization. A) Representative image of a human placenta with umbilical cord (black arrowhead) and epithelial layer of the amniotic membrane (white arrowhead) facing upwards. B) Image depicting the separation of the amniotic membrane (white arrowhead) from the chorion (black arrowhead). Representative H&E histological sections of C) fresh amniotic membrane exhibiting a continuous layer of hAECs (arrow heads) and intact stroma containing hAMSCs (box), D) amniotic membrane following incomplete removal of hAECs (arrowheads) with 0.125% trypsin and E) amniotic membrane after complete removal of hAECs with 0.25% trypsin. Polarized light microscopy of monolayer culture expanded amnion derived cells; F) hAECs (cuboidal morphology - black arrowheads), G) a mixed population of hAECs (black arrowheads) and hAMSCs (white arrowheads) following incomplete dissociation of hAECs (0.125% trypsin) and H) hAMSCs (spindle morphology - white arrowheads). I) Average viable cell yields from term human amniotic membrane. J) Flow

cytometric analysis of cell surface mesenchymal stem cell, hematopoietic and epithelial markers expressed on passage 2 hAECs, hAMSCs and a mixed population of both cell types (data represented as % positive staining ± SEM).

Human placentas were successfully harvested (Figure 23A) and amniotic membranes were isolated (Figure 23B) within 3 hours of delivery of full-term babies. Histological analysis prior to membrane manipulation revealed a confluent layer of epithelial cells (Figure 23C – arrowheads) and an intact stromal layer (Figure 23C – box) containing mesenchymal cells.

3.3.2 Confirmation of Enriched hAEC and hAMSC Populations from Amniotic Membrane

Histological analysis of amniotic membranes following two serial digestions in 0.25% Trypsin showed that the confluent epithelial layer was completely removed (Figure 23E). Subsequent collagenase digestion released the remaining cells from the stromal layer. Phase contrast microscopy of these plated cells revealed two distinct morphologies: cobblestone- and spindle-shaped (Figures 23F & H, respectively) indicative of epithelial and mesenchymal cells, respectively. Flow cytometric analysis of these cells at passage 2 illustrated that both cell types were positive for mesenchymal stem cell markers (CD73, CD90 and CD105) and negative for the lymphocyte common antigen, CD45 (Figure 3.1J). Cells isolated from the epithelial layer via trypsin digest (hAECs) were positive for epithelial marker, EpCAM, whereas cells isolated from the stroma via collagenase digestion (hAMSCs) were negative. Viable hAEC and hAMSC yields at harvest were determined to be $2.3x10^6 \pm 3.7x10^5$ and $1.6x10^6 \pm 4.7x10^5$ per milliliter of amnion, respectively. Considering the average amniotic membrane size at harvest, approximately $4.2x10^7 \pm 8.2x10^6$ hAECs and $2.8x10^7 \pm 7.2x10^6$ hAMSC were obtained from each membrane.

3.3.3 Confirmation of a Mixed Amnion Cell Population

Histological analysis of amniotic membrane following two serial digestions in 0.125% Trypsin revealed a partially intact epithelial layer (Figure 23D). Subsequent collagenase digestion released the remaining cells from the epithelial and stromal layers. Phase contrast microscopy of these plated cells revealed a mixed morphology of cobblestone and spindle-shaped cells (Figure 23G). Flow cytometry further confirmed this mixed population of cells, as the surface profiles of these cells appeared to be a hybrid of the enriched hAEC and hAMSC populations with roughly 40% of the cell exhibiting positive staining for EpCAM (Figure 23J).

3.3.4 hADSC and Amnion Derived Cell Osteogenic Differentiation Potential

Gene transcript analysis of cells under normal culture conditions (in the absence of osteogenic differentiation media) indicated that hAECs demonstrated a significant increase in runx-2 expression at day 14 compared to their respective day 3 values (Figure 24A). Conversely, runx-2 expression was significantly decreased in hAMSCs compared to their day 3 values. Furthermore, the amnion cell cultures trended towards having increased runx-2 expression compared to hADSCs (dotted line) at all time-points analyzed. With respect to osteocalcin, hAEC expression tended to be higher than hADSCs at all time-points investigated (Figure 24B).

After osteogenic induction, runx-2 expression peaked at earlier time points in the hAECs and mixed cell groups compared to hADSCs (Figure 24C). Additionally, the fold increases in runx-2 expression was significantly greater in the mixed and hAMSC groups compared to hADSCs at day 3 and 7, respectively. Interestingly, hAECs demonstrated a

significantly ($p<0.05$) lower fold change in runx-2 expression as compared to hADSCs at all time-points. The fold increases in osteocalcin observed in all amnion derived cell groups were significantly greater ($p<0.05$) than the changes observed in hADSCs at day 14 (Figure 24D).

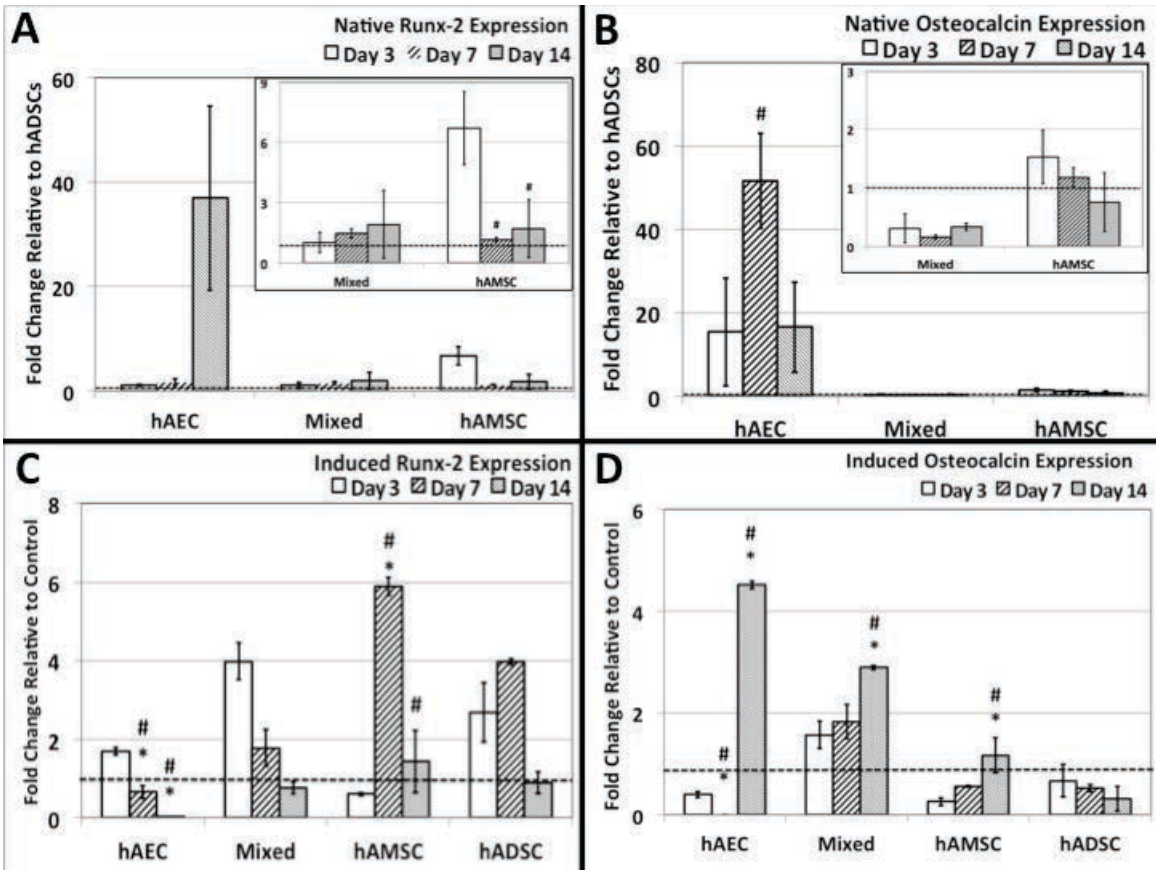


Figure 24: Osteogenic Gene Transcript Expression of Human Amnion Derived Cells and hADSCs. Native (baseline) transcript expression levels of A) runx-2 and B) osteocalcin in hAECs, mixed (hAEC+hAMSC) and hAMSC groups relative to hADSCs (dotted line). C) runx-2 and D) osteocalcin expression of all osteogenically induced cell types as compared to control expression levels (i.e. cells of the same type cultured in non-induction media cultured to the same time-point; dotted line). * indicates statistical difference from hADSC group at same time point ($p<0.05$). # indicates statistical difference from day 3 time point ($p<0.05$).

Semi-quantitative Alizarin Red staining indicated that the hAEC and mixed cell groups deposited significantly ($p<0.05$) more calcium at day 3 compared to hADSC (Figure 25A). Additionally, all amnion groups demonstrated significantly ($p<0.05$) increased presence of calcified matrix as compared to hADSCs at day 14 (Figure 25B).

Interestingly, hAECs cultured under normal conditions (in the absence of osteogenic media) demonstrated calcium deposition after 28 days (Figure 25C), whereas mixed amnion cell groups (Figure 25D) and hADSCs (data not shown) did not.

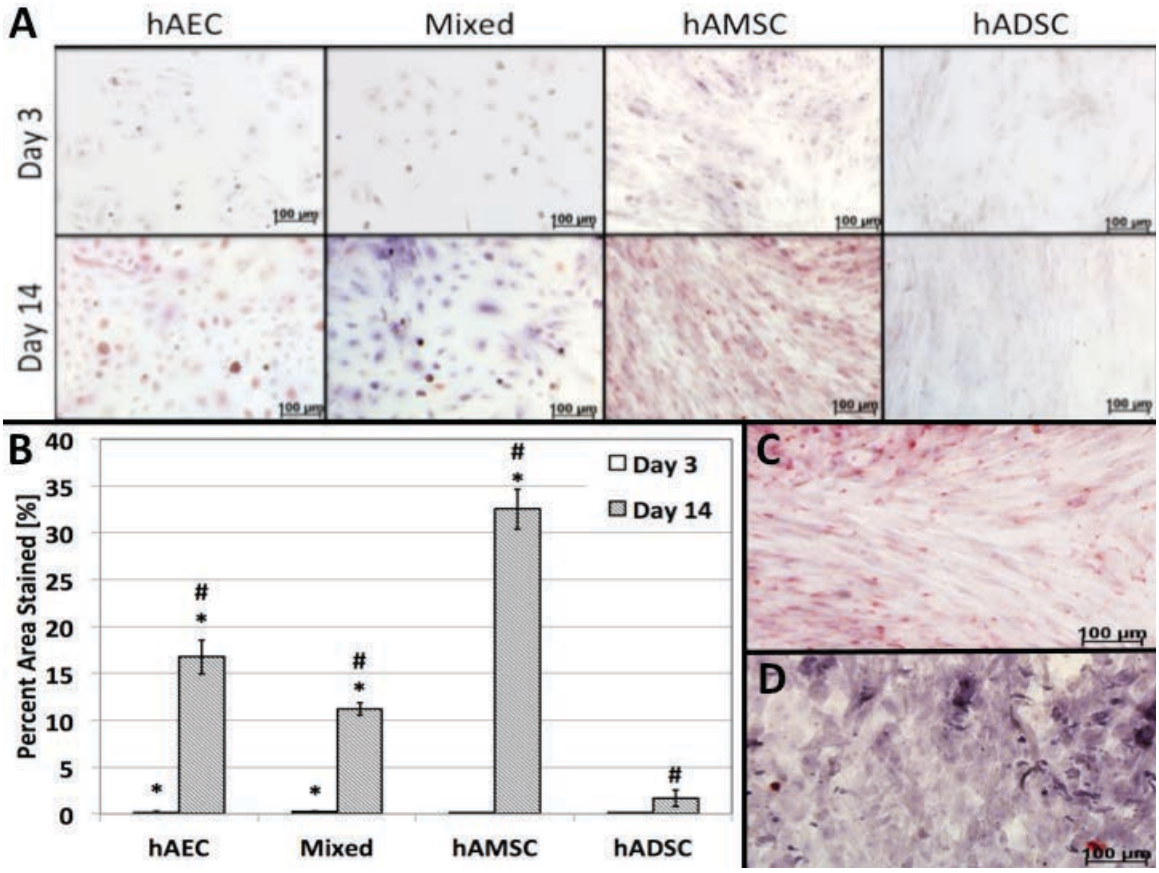


Figure 25: Osteogenic Calcified Matrix Expression of Human Amnion Derived Cells and hADSCs. A) Alizarin red staining of monolayer cultures of osteogenically induced hAECs, mixed (hAEC+hAMSC), hAMSCs, and hADSCs (red=positive staining for calcium deposition) at day 7 and 14. B) Quantitative analysis of percent area of the well stained positive for calcium deposition. Alizarin Red staining of C) hAECs and D) mixed cells cultured in the absence of osteogenic induction medium. * indicates statistical difference ($p<0.05$) from hADSC group at same time point. # indicates statistical difference from day 3 time point ($p<0.05$).

3.3.5 Comparison of hADSC and Amnion Derived Cell Chondrogenic Differentiation Potential

Gene transcript analysis of cells under normal culture conditions (in the absence of chondrogenic differentiation media) showed that hADSCs (dotted line) had higher expression of the master chondro-regulatory transcription factor sox-9 as compared to the

hAMSC and mixed amnion cell groups at day 14 (Figure 26D). Conversely, hAECs tended to express greater levels of sox-9 compared to hADSCs (dotted line) at all time-points. Conversely, hAEC and hAMSC cell groups innately expressed higher levels of aggrecan gene transcript compared to hADSCs by day 14 (Figure 26E). The mixed amnion cell group demonstrated a significant increase in innate collagen type 2 gene expression as the culture period increased and both mixed and hAMSC groups tended to have increased collagen type II gene expression as compared to hADSCs (Figure 26B). Of note, collagen type 2 expression was highly variable in the hAEC study group (data not shown).

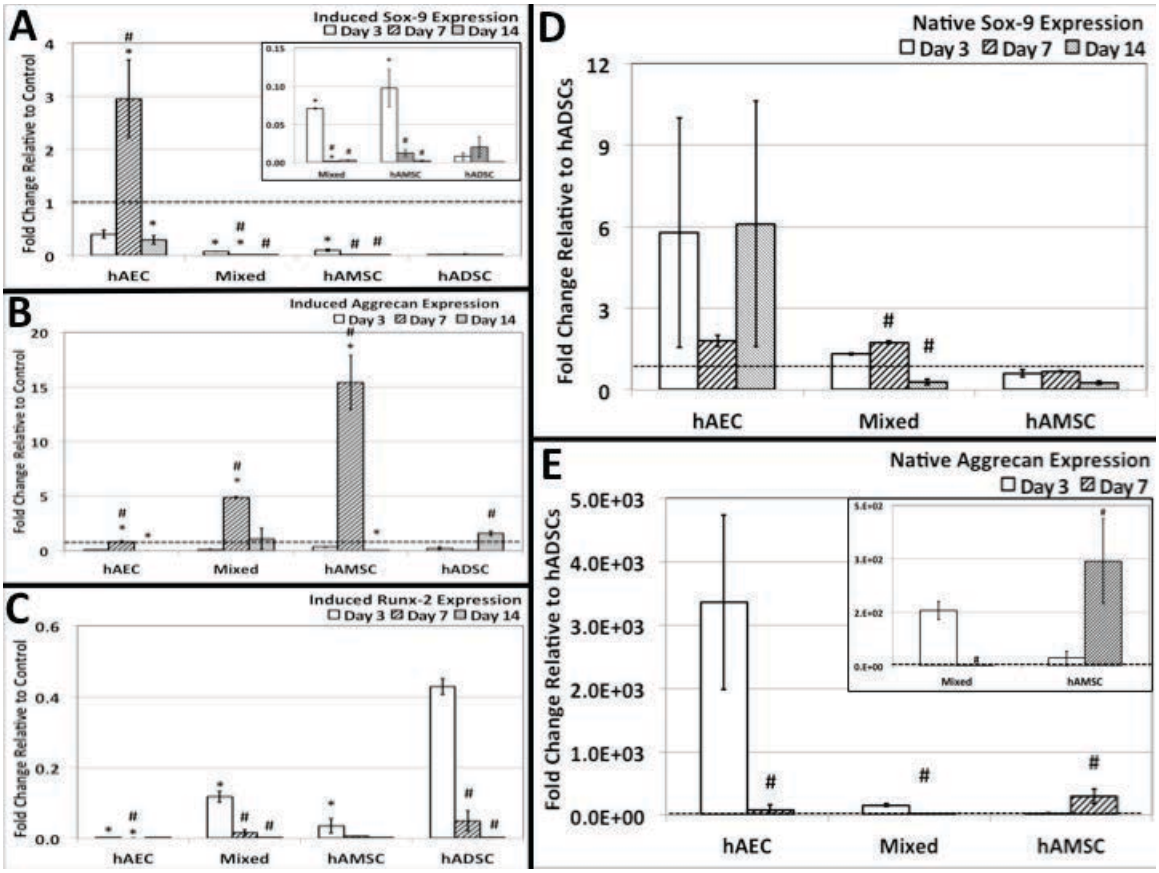


Figure 26: Chondrogenic Gene Transcript Expression of Human Amnion Derived Cells and hADSCs. Native (baseline) transcript expression levels of D) sox-9 and E) aggrecan in hAECs, mixed (hAEC+hAMSC) and hAMSC groups relative to hADSCs (dotted line). A) sox-9, B) aggrecan and C) runx-2 expression of all chondrogenically induced cell types as compared to control expression levels (i.e. cells of the same type cultured in non-induction media

cultured to the same time-point; dotted line). * indicates statistical difference from hADSC group at same time point ($p<0.05$). # indicates statistical difference from day 3 time point ($p<0.05$).

After chondrogenic induction, all amnion derived cells exhibited significantly ($p<0.05$) increased sox-9 gene transcript expression as early as day 3 (in Mixed and hAMSCs cell groups) and day 7 (in hAECs) as compared to hADSCs at the same time points (Figure 26A). As expected, peaks in sox-9 expression tended to precede peaks in aggrecan expression; however these peak values tended to appear earlier in hAMSC and Mixed cell groups as compared to hAEC and hADSCs. The fold change in aggrecan gene transcript expression peaked at day 7 in all amnion derived cell groups; these values were significantly greater than those of hADSCs at day 7 (Figure 26B). Notably, Mixed and hAMSC cell groups exhibited the largest fold change in aggrecan gene transcript expression of all differentiated cell types. The fold change in collagen type 2 gene transcript expression peaked in all differentiated cell types at day 7 (Figure 27C). Additionally, hAMSC and mixed amnion cell groups expressed greater levels of collagen type 2 compared to hADSCs at this time point (Figure 27C). Interestingly, runx-2 gene transcript expression in chondrogenically induced cells tended to be greater in hADSCs as compared to all amnion derived cell groups, though this difference was only significant at day 3 (Figure 26C).

Immunohistochemical staining of cell pellets for collagen type 2 illustrated that Mixed and hAMSC cell groups produced collagen type 2 enriched matrix as early as 7 days (Figure 27A). This continued through 14 days, at which time hADSC groups exhibited minimal collagen type 2 matrix production.

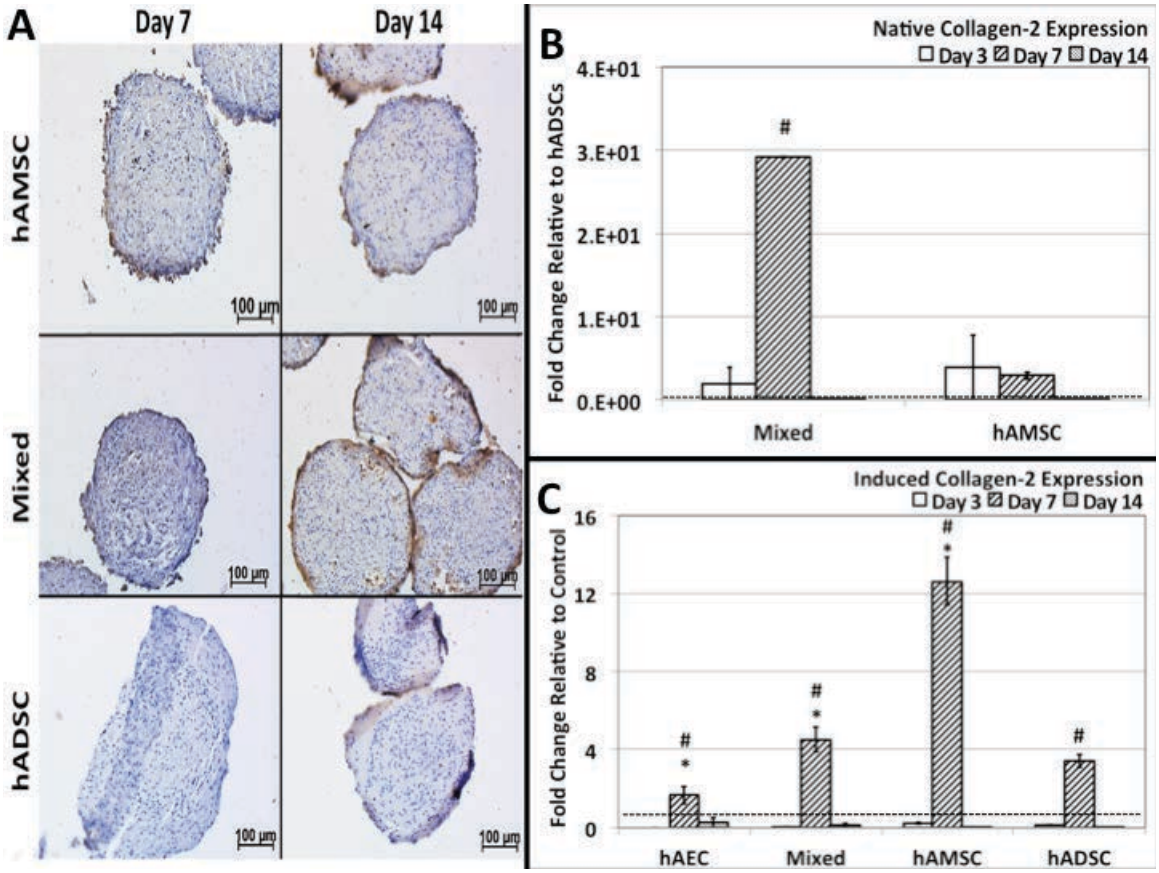


Figure 27: Chondrogenic gene transcript and cartilage matrix expression of collagen-2 in human amnion derived cells and hADSCs. A) Immunohistochemical staining (brown=positive staining for collagen-2 matrix) of chondrogenically induced hAMSC, Mixed (hAEC+hAMSC) and hADSC cell pellets. B) Native (baseline) transcript expression levels of collagen-2 in hAECs, mixed (hAEC+hAMSC) and hAMSC groups relative to hADSCs (dotted line). C) Collagen-2 expression of all chondrogenically induced cell types relative to native (baseline) expression levels (dotted line). * indicates statistical difference from hADSC group at same time point . # indicates statistical difference from day 3 time point ($p<0.05$).

Histological analysis of chondrogenic cell pellets indicated a progressive increase in pellet diameter with time in culture (Figures 28A & C). At 7 days, the Mixed and hADSC cell groups exhibited the largest pellet diameter, whereas hAMSC pellets were the smallest. However, semi-quantitative Alcian Blue staining indicated that pellets formed by Mixed and hAMSC cell groups had significantly increased areal staining of GAG-enriched matrix at all time-points investigated as compared to the hADSC cell group (Figure 28B).

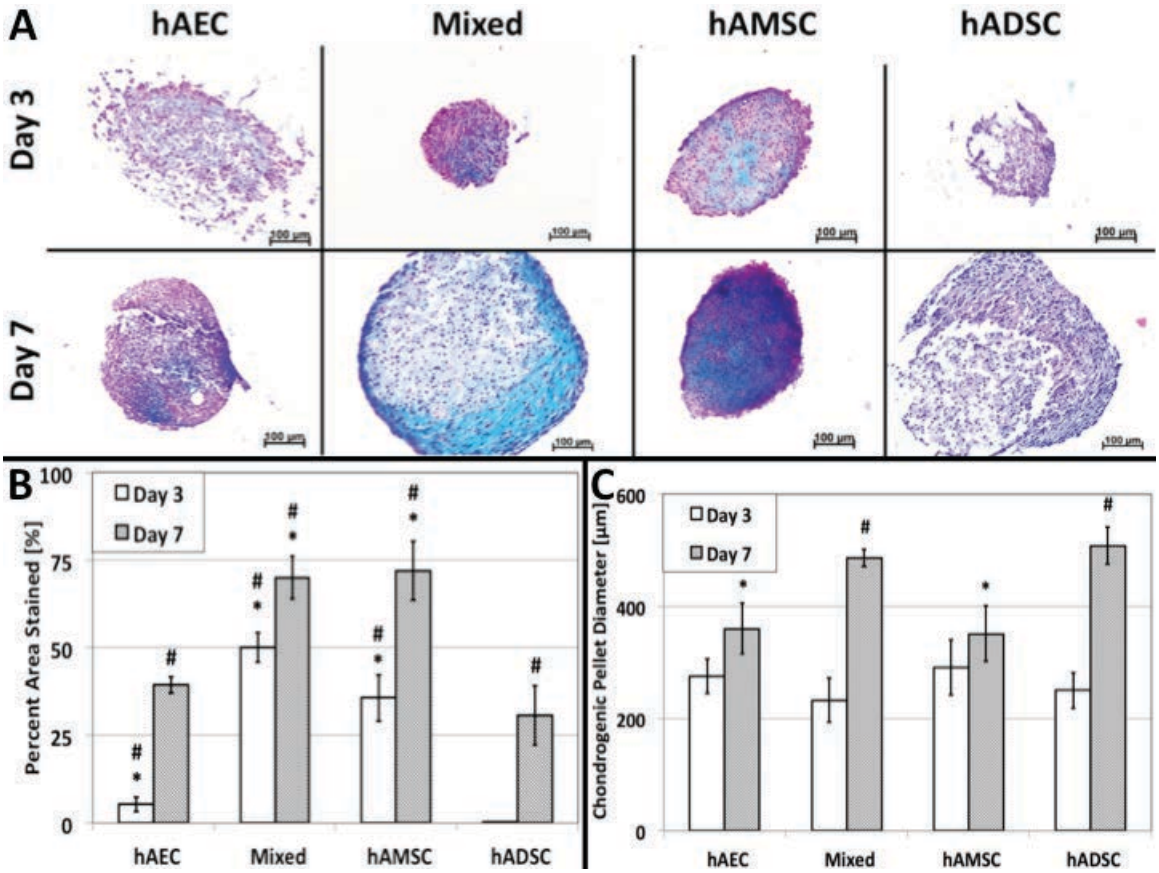


Figure 28 Chondrogenic matrix component expression of human amnion derived cells and hADSCs. A) Alcian Blue staining for glycosaminoglycan of chondrogenically induced hAEC, Mixed (hAEC+hAMSC), hAMSC and hADSC cell pellets (blue=positive staining for GAG) at day 7 and day 14. B) Quantitative analysis of percent area of the cell pellet stained positive for GAG C) Comparison of chondrogenically induced hAEC, Mixed (hAEC+hAMSC), hAMSC and hADSC cell pellet diameter at day 7 and 14. * indicates statistical difference from hADSC group at same time point ($p<0.05$). # indicates statistical difference from day 3 time point ($p<0.05$).

3.4. Discussion

Herein, we demonstrate the ability of amnion derived stem cells to differentiate earlier and more robustly into osteogenic and chondrogenic lineages compared to a commonly used stem cell source; hADSCs. Specifically, our results highlight the utility of hAECs and hAMSCs as well as hAMSCs and mixed (hAEC+hAMSC) cell populations for osteogenic and chondrogenic differentiation, respectively. Furthermore, we confirm the availability of amnion cells to be obtained in large quantitates. The prevalence of osteochondral defects coupled with the progression of such lesions to debilitating musculoskeletal tissue pathologies (i.e. osteoarthritis), highlight the need for

alternative therapeutic strategies, addressing both the bone and cartilage. Therefore, a stem cell source exhibiting enhanced osteogenic and chondrogenic differentiation could prove beneficial.

Histological analysis prior to enzyme digest indicated that the mechanical separation of the amniotic membrane from the chorion during tissue harvest and subsequent transport did not detrimentally alter the native architecture of the amnion as evidenced histologically by an intact amniotic epithelium and underlying stroma. Histological and flow cytometric results demonstrated the ability to isolate both hAECs and hAMSCs via sequential enzymatic digestion with trypsin and collagenase, respectively and that they maintain stemness markers through passage two. These results are in alignment with previous reports which demonstrate that both cell populations exhibit stem cell characteristics.[54,58,92,224,235] In addition to the minimal criteria established by the International Society for Cellular Therapy, hAECs have been shown to express pluripotent stem cell markers such as NANOG and SOX-2, only minimally express HLA-A/B/C, and do not express HLA-DP/DQ/DR surface antigens.[58,236] However, there are mixed reports of hAEC surface expression (particularly CD105), especially with serial passaging.[49,237,238] hAECs have been shown to express similar major histocompatibility complex surface profiles as hAMSCs.[54,57,224,235] Flow cytometric analysis performed within the current study allowed for the confirmation of the isolation of these two distinct cell populations from one another, or in the case of the Mixed cell group, the hybrid isolation of these cell types. The Mixed group was included in this study for two primary purposes. First, it potentially represents a more clinically relevant amnion-based therapeutic approach, where the entire amnion (with its two comprising

stem cell like cell populations) would be utilized. Examples of such previously investigated approaches have been succinctly reviewed elsewhere.[68] However our results appear to indicate lower levels of CD90 and CD73 from cells in this Mixed population state. Second, some isolation methods described throughout literature indicate that in many instances collagenase digestion is performed on the amniotic membrane without prior trypsinization thus yielding a mixed population of amniotic cells. Thus it would be beneficial to determine if the presence of both stem cell types simultaneously have an effect on differentiation potential and so that comparisons to these studies could be drawn.

Isolated amniotic membranes varied in size (16.8mL±3.8mL), with cell yield directly relating to amnion weight [mg] and volume [mL]. Similar to previous reports, our results demonstrated higher numbers of hAECs than hAMSCs isolated from the amniotic membrane.[10,49,54,55,71] We report an average of $2.3x10^6$ hAEC and $1.6x10^6$ hAMSC per mL amniotic tissue, which is significantly greater than the reported $5.4x10^5$ hADSCs isolated per mL from adipose tissue.[61]

hADSCs were chosen as a representative mesenchymal stem cell for comparative analysis due to their proven osteogenic and chondrogenic differentiation potential, the numerous studies confirming their stem cell criteria, and their clinical relevance (relative abundance with minimally invasive harvest procedures).[63] The osteogenic and chondrogenic differentiation capacities of hADSCs and hBMSCs have been well established to occur after 21 days in their respective induction media.[231–234] Considering this, the current study was designed to analyze initial time points at less than 21 days in

order to establish if amnion derived cells were capable of differentiation prior to this time.

Innate gene transcript expression results for the current experiments were consistent with reports that amnion derived cells exist in a multi-differentiated state, meaning in their natural, undifferentiated state they can express genes of each germ layer (i.e. endodermal and mesodermal for hAECs and hAMSCs, respectively).[89] Consistent with previous reports, our analysis also revealed all three cell types investigated could be successfully differentiated into osteogenic and chondrogenic lineages.[42,63,89,93]

With respect to osteogenic gene transcript expression, hAECs demonstrated a trend towards increased innate expression of runx-2 and osteocalcin compared to hADSCs. Following induction, hAMSCs demonstrated the highest runx-2 expression at day 7. Additionally, all amnion derived cell groups demonstrated significantly higher osteocalcin expression at day 14 compared to hADSCs with hAECs demonstrating the highest fold change in expression.

Furthermore, differentiation of amnion derived cells appeared to precede hADSCs and resulted in more robust matrix production. More specifically at nearly every time-point investigated, significantly more calcified matrix was found in the presence of amnion derived cells as compared to hADSCs, especially in cultures with hAMSCs and hAECs suggesting enhanced osteogenesis. Of note, in the absence of osteogenic induction media, hAECs were able to produce calcified ECM by 28 days; a phenomenon that was not observed in hAMSC, mixed or the hADSC group. This is not the first report on the innate capacity of hAECs to differentiate into an osteogenic lineage.as other have also observed this innate capacity for osteogenic differentiation.[239] Interestingly, mixed

cells did not display this innate capacity for osteogenic differentiation in the absence of induction media despite flow cytometric results which indicated that the mixed amnion cell group contained as much as 40% hAECs, yet the ability of the hAECs to spontaneously differentiate appears to be reduced in the presence of hAMSCs. Furthermore, these results indicate that osteogenic differentiation of hAECs can be obtained without the addition of exogenous chemicals, potentially minimizing lab costs and concerns of chemical manipulation and toxicity for clinical application. Thus both hAMSCs and hAECs may be more optimal for bone repair/regeneration applications as compared to hADSCs.

With respect to induced chondrogenic differentiation, hAMSCs and amnion derived mixed cells consistently produced ECM containing more collagen type 2 and glycosaminoglycan as compared to hADSCs at the time-points investigated. Gene expression data demonstrating increased sox-9, aggrecan, and collagen type 2 expression corroborated these findings. Although the chondrogenic cell pellets containing hAMSCs only tended to be smaller and more compact as compared to all other groups by day 7, the percent areal staining for glycosaminoglycan-rich ECM was highest possibly indicating an enhanced recapitulation of condensation observed during chondrogenesis during embryological development. hAMSCs and mixed amnion cell groups also trended towards having innately higher collagen type 2 and aggrecan gene transcript expression in the absence of chondrogenic induction media suggesting that these cells may be more optimal for cartilage repair/regeneration applications as compared to hADSCs. These results also add to previous findings that indicate hAMSCs may be more efficient in chondrogenic differentiation than hAECs.[239]

The pre-mature ossification of mesenchymal stem cells is thought to be one of the greatest drawbacks to their clinical use.[228] Considering this, it has been demonstrated herein that amniotic cell types undergoing chondrogenic differentiation express significantly less osteogenic markers than hADSCs, potentially indicative of a decreased potential for endochondral ossification. Thus, hAMSCs may prove more useful for regenerative therapies requiring continual chondrogenesis as opposed to the typical progression of chondrogenesis through endochondral ossification, however further studies illustrating this point are warranted.

Although care was taken to standardize the experimental conditions during these in vitro investigations, so study limitations were noted. Unavoidably, amniotic membranes and hADSCs were obtained from different donors. It has been shown that donor health and age impact stem cell properties.[5,10,11,98] The most accurate comparative analysis would have been to obtain stem cells from the same donor; however this was not possible in this case for patient safety. Additionally, some reports have concluded that bone marrow stem cells (hBMSCs) are potentially more robust at musculoskeletal tissue differentiation.[233,240] Therefore, future studies will aim to perform similar analyses comparing amnion derived cells to hBMSCs as well. It may also be advantageous to complete chondrogenic differentiation comparative analyses under hypoxic conditions, as numerous reports indicate increased clinical relevance and enhanced differentiation utilizing such approaches.[32,241]

Taken together, this data suggests amniotic cells are an ideal alternative cell source for orthopaedic regenerative medicine approaches. Amniotic stem cell yields are higher per tissue volume compared to hADSCs and they appear to more readily

differentiate into osteogenic and chondrogenic lineages. Moreover, these differentiation capacities make amnion stem cells a candidate stem cell for osteoarthritis therapies.

CHAPTER FOUR

AIM II: TO VALIDATE A HUMAN EXPLANT JOINT TISSUE CO-CULTRE MODEL OF OA

4.1 Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting over 30% of the U.S. population over the age of 65.[11,12,110,111,113–117] OA is the result of cartilage degradation, impaired joint mobility and severe pain - making it one of the leading causes of disability worldwide.[12,110,111,113,119,120] Though commonly associated with the destruction of cartilage, OA is a disease of the entire joint space.[12,85,108,113,117,126–128]

While OA is stereotypically described as a physical wear and tear disease[242,243], mounting evidence suggests that synovial inflammation significantly contributes to its pathogenesis.[243] In OA, macrophages infiltrate the synovium and secrete supra-physiological levels of pro-inflammatory cytokines (including TNF- α and IL1-β), which create a caustic joint environment promoting articular cartilage degradation.[127,243] Degraded matrix fragments activate toll-like receptors (TLRs) within cells of the synovial membrane, activating nuclear-factor κB (NF-κB), a potent pro-inflammatory transcription factor.[108,113,135] TLR activation is also responsible for the downstream up-regulation of MMPs and aggrecanses.[113,128,135,140] In addition to synovial TLR activation, chondrocytes have exhibited TLR activation in response to degraded matrix fragements.[12,113] Ultimately, this TLR cascade results in the recruitment of increased numbers of macrophages and pro-inflammatory cytokine secretion, creating a state of chronic, low-grade inflammation.[113,135,244] Thus, OA is most accurately described as a

feed-forward pathology where the cartilage and synovium exhibit reciprocal pathologic effects on one another.

Investigators have studied this pathogenesis and efficacy of potential future OA therapies via the use of *in vitro* culture models of human cartilage explants exposed to supra-physiological levels of exogenous inflammatory cytokines (i.e. IL-1 and TNF-α) commonly observed in OA in order to accelerate cartilage destruction.[140,245] More recently, researchers have begun co-culturing human joint tissue explants (cartilage together with synovium) in an attempt to more accurately reflect the complexity of the joint space environment as well as the many known mediators of OA resulting from the natural progression of the disease.[128,140] Although such co-culture models have demonstrated likeness to the natural disease state[246], to our knowledge these systems have not been extensively validated in order to determine the degree to which they model: cartilage destruction in the presence of both synovial macrophages and pathophysiologic levels of pro-inflammatory cytokines, gradual progression with time and, significantly, a feed-forward progression.

In an attempt to confirm that such co-culture models reflect human OA pathology and progression, herein an investigation into human joint tissue explant co-culture has been undertaken. This was accomplished by comparing human joint explant co-cultures of cartilage and synovium together with cultures of isolated human cartilage (in order to determine the effects of the synovium on the cartilage) and isolated human synovium (in order to determine the effect of the cartilage on the synovium), ultimately to demonstrate the ability of OA explant co-culture to effectively model the feed-forward nature of OA as well as gradual disease progression with time. Furthermore, the impact/role of

synovial macrophage's contribution to the OA model was investigated by selective depletion via clodronate. Taken together, validation of such a model may highlight its utility in OA research, as it may prove to be an effective system for investigating OA pathology and evaluating potential future therapies.

4.2 Materials & Methods

Insulin Transferrin Selenium was purchased from Life Technologies (41400045). Ascorbate-2-phosphate (59-990-141) and trans-well culture plates (07-200-157) were purchased from Fisher Scientific. Clophosome A was purchased from FormuMax Scientific Inc (F70101C-A-2). Live/Dead Animal Cell Kit was purchased from VWR (89260-208). 1,9 Dimethyl-Methylene Blue (341088-1G), Chondroitin Sulfate (C4384-5G), Hydroxyproline Assay Kit (MAK008-1KT), Safranin-O Stain (S2255-25G) and Fast Green Stain (F7258-25G) were purchased from Sigma. Normal Horse Serum (S2000), VECTASTAIN Elite ABC Kit, Rabbit IgG (PK-6101) and DAB Substrate Kit (SK4100) were purchased from Vector Laboratories, Inc. Triton X-100 and other basic chemicals were purchased from Fisher Scientific. Antibodies employed in this work included: anti-mannose receptor (ab32527) and anto-CCR7 (ab64693). All ELISAs employed in this work were purchased from RayBiotech: Human TNF-alpha (LH-TNFa-1), Human MMP-13 (ELH-MMP13-1) and Human IL-1 beta (ELH-IL1b-1).

4.2.1 Joint Tissue Harvest and Culture Initiation

Human cartilage and synovium were obtained from consenting patients immediately following total knee arthroplasty for OA (IRB# PRO00031185 Greenville Health System). All patients were classified as having Kellgran-Lawrence grade 4 OA. Using aseptic technique, cartilage and synovium tissues were biopsied into 6mm diameter samples in preparation for in vitro culture. Tissue biopsies were placed in 12-well plates (1 cartilage and 1 synovium biopsy /

well) and maintained in 2mL DMEM supplemented with 1% Insulin Transferrin Selenium (ITS), 1% antibiotic/antimitotic (ABAM) and 50nM Ascorbate-2-phosphate for 3 days in standard culture conditions (5% CO_2; 20% O_2; 37° C). Henceforth, this media formulation will be referred to as "explant medium." Care was taken to ensure the cartilage and synovium were not in direct contact. After 3 days, the media was removed and labeled "day 0" media.

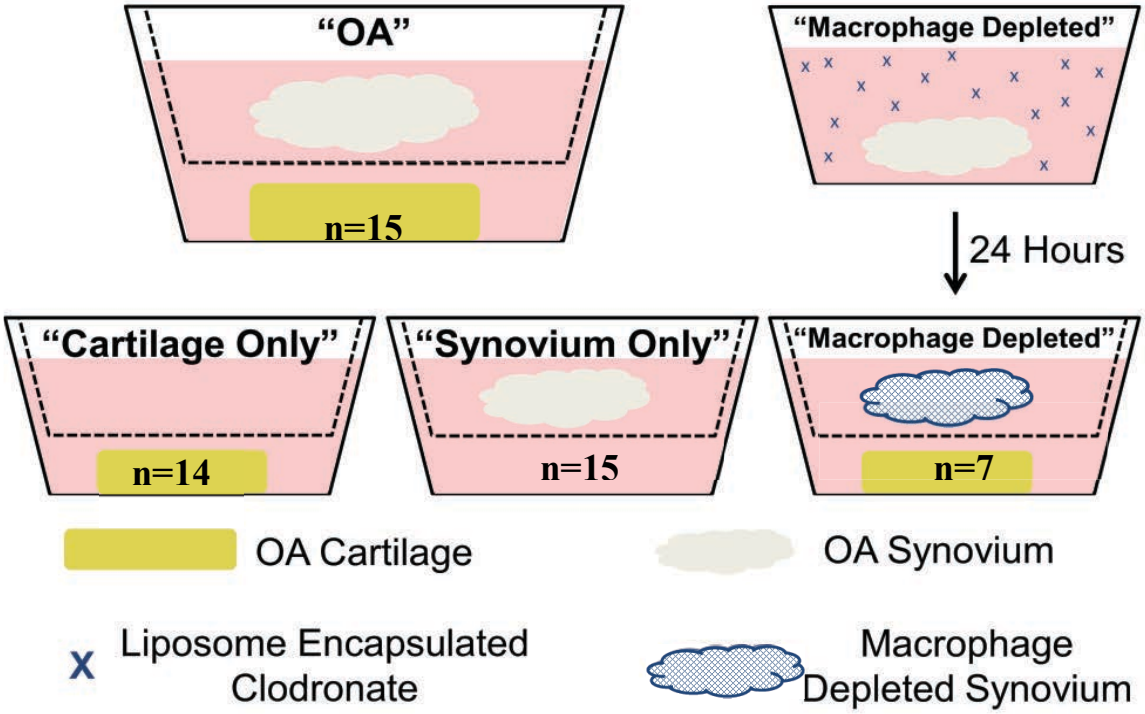


Figure 29: Methods Schematic demonstrating the described comparative analysis between control OA explant co-culture ("OA"), OA tissue cultured in isolation ("cartilage only" and "synovium only") and macrophage depleted OA cultures, after 15 days.

4.2.1.1 In Vitro Co-Culture

Cartilage biopsies (n=15) were placed in the bottom of a 12-well trans-well plate (1 cartilage biopsy/well) and submerged in 1.5mL explant medium. The well insert was returned to the well and patient- matched synovial biopsies were placed within inserts (1 synovium

biopsy/well) with 500µL explant medium. Media was changed every 3 days until the termination of culture at 15 days. This is depicted in figure 29.

4.2.1.2 In Vitro Cartilage Only Culture

Cartilage biopsies (n=14) were placed in the bottom of a 12-well trans-well plate (1 cartilage biopsy/well) and submerged in 1.5mL explant medium. Media was changed every 3 days until the termination of culture at 15 days. This is depicted in figure 29.

4.2.1.3 In Vitro Synovium Only Culture

Synovial biopsies (n=15) were placed within well inserts (1 synovium biopsy/well) and treated as previously described until the termination of culture at 15 days. This is depicted in figure 29.

4.2.1.4 In Vitro Macrophage Depleted Co-culture Culture

Synovial biopsies (n=7) were placed in the wells of a 12 well plate and submerged in 1.5mL explant medium. To deplete synovial macrophage populations, 24 hours prior to the initiation of co-culture each synovial biopsy was treated with 0.2mL Clophosome®-A (liposome encapsulated clodronate). After 24 hours, each synovial biopsy was washed 3x in explant medium prior to co-culture initiation with patient-matched OA cartilage as previously described. This is depicted in figure 29.

4.2.2 Synovial Macrophage Phenotype

Immunohistochemistry (IHC) on rehydrated paraffin synovium sections was performed for detection of synovial macrophages. Briefly, antigen retrieval was accomplished via 10mM Citric Acid incubation at 90°C for 20 minutes. Slides were rinsed twice in TBS for 5 minutes, permeabilized in 0.025% Triton X-100, non-specific binding and endogenous peroxidases were blocked with normal serum and a solution of 0.3% hydrogen peroxide in 0.3% normal serum,

respectively. A rabbit polyclonal antibody towards human CCR7 (0.5μg/mL dilution) or human mannose receptor (1μg/mL dilution) was incubated for 1 hour at room temperature prior to thorough rinsing and incubation at room temperature for 30 minutes with a secondary biotinylated antibody and avidin biotin complex according to manufacturer's instructions (Vectastain®ABC Elite Kit Rabbit IgG - Vector Labs). A DAB substrate kit (Vector Labs: SK4100) was used to visualize positive staining prior to counterstaining with a dilute hematoxylin solution for 30 seconds. Negative controls did not receive primary antibody.

4.2.3 Live/Dead Staining

Live/Dead staining was completed on cartilage and synovium according to manufacture instructions. Briefly, cartilage and synovium were incubated in a Live/Dead working solution (2μM calcein AM and 4μM Ethd-1) and at room temperature for 45 minutes. Tissues were placed on a microscope slide prior to fluorescent imaging.

4.2.4 Histopathological Assessment of Explant Tissue

Cartilage sections were secured within a tissue processing cassette, fixed in 10% phosphate buffered formalin overnight at room temperature prior to undergoing standard tissue processing, decalcification, paraffin embedding and sectioning at 5 μm thickness. Sections were stained with Safranin-O counterstained with Fast Green for visualization of proteoglycan rich cartilage matrix. Briefly, rehydrated sections were differentiated in 1% acid alcohol for 2 seconds prior to room temperature incubation in 0.02% Fast Green for 2.5 minutes. After 30 second incubation in 1% Acetic acid, sections were stained with 1% Safranin-O for 15 minutes. Three images were taken spanning the surface length of the sample. An observer blinded to the condition of the cartilage completed the OARSI histopathological assessment on each image according to the direction of Pritzker et al.[247] These results were averaged to obtain the final sample OARSI score.

4.2.5 Assessment of Cartilage Degradation

4.2.5.1 Cartilage Glycosaminoglycan (GAG) Content

Lyophilized cartilage tissues were digested in 125μg/mL Papain in PBE Buffer, pH 7.5 overnight at 65°C. Tissue digests were assessed for GAG content via Dimethylmethylene Blue Assay (DMMB). Briefly, 200μL DMMB reagent (46μg DMMB, 40mM Glycine, 40mM NaCl, pH 3) was added to 50μL samples. Standards were created using a 1mg/mL stock solution of Chondroitin-6-Sulfate. Sample absorbance was read at 525nm.

4.2.5.2 Collagen Leaching to Culture Media

Culture media was assessed for hydroxyproline collagen content via Hydroxyproline Assay Kit according to manufacture instructions. Briefly, samples were hydrolyzed with hydrochloric acid at 120°C for 3 hours prior to well plate transfer. Wells were evaporated to dryness at 60°C. Equal amounts of Chloramine T/Oxidation Buffer Mixture and Diluted DMAB Reagent followed by 5-minute room temperature and 90-minute 60°C incubations, respectively. Standards were created using a 1mg/mL stock solution of Hydroxyproline Standard Solution. Sample absorbance was read at 560nm.

4.2.6 Co-culture Media Pro-Inflammatory Profile

ELISA was performed according to manufacture instructions. Briefly, 96-well plates were incubated overnight at 4°C with standards and explant co-culture media samples (n=3 per condition). After several washes, wells were incubated with biotinylated antibodies (either TNF-α, IL-1β or MMP-13) for 1 h, followed by incubation in HRP-

conjugated streptavidin for 45 min. Enzymatic reactions were allowed to develop, and the absorbance of each plate was read at 450 nm.

4.2.7 Microscopic Imaging

Images were captured on a Zeiss Axiovert.A1 microscope with Axiovision software (Release 4.9.1 SP08-2013). For all semi-quantitative histological data, three images were taken of each sample. An observer blinded to the condition of the sample manually counted each of these three images in order to determine the relative number of positive cells (i.e. brown cells (IHC), live cells or dead cells (Live/Dead), etc.) per sample. These three results were averaged in order to obtain the final percentage of positive cells for each sample.

4.2.8 Statistical Analysis

Results are represented as a mean ± standard error of the mean (SEM). All statistical analyses were performed by two-tailed Student's t-test of unequal variance or one-way analysis of variance (ANOVA). Significance was defined in all cases as $p<0.05$.

4.3 Results

4.3.1 Joint Tissue Harvest and Culture Initiation

Human joint tissues were successfully harvested and 6mm biopsies of patient-matched cartilage and synovium were obtained. Tissues were maintained in *in vitro* culture for 15 days without any macroscopic signs of tissue deterioration or bacterial infection.

4.3.2 Synovial Macrophage Phenotype

IHC staining of CCR7 (figure 30) showed a relatively constant percentage (84.5% to 76.8%) of M1 polarized macrophages over 15 days in OA co-culture. Over 15 days,

the synovium only culture group exhibited a statistical decrease in the percentage of cells staining positive for macrophage markers (84.6% to 58.6%). IHC staining confirmed the targeted depletion of macrophages in the macrophage depleted group (17.2% positive staining at day 0). This depletion effect remained throughout the culture period (14.8% at day 15). After 15 days in culture, synovium from OA co-culture contained a statistically higher percentage of macrophages compared to the other conditions tested.

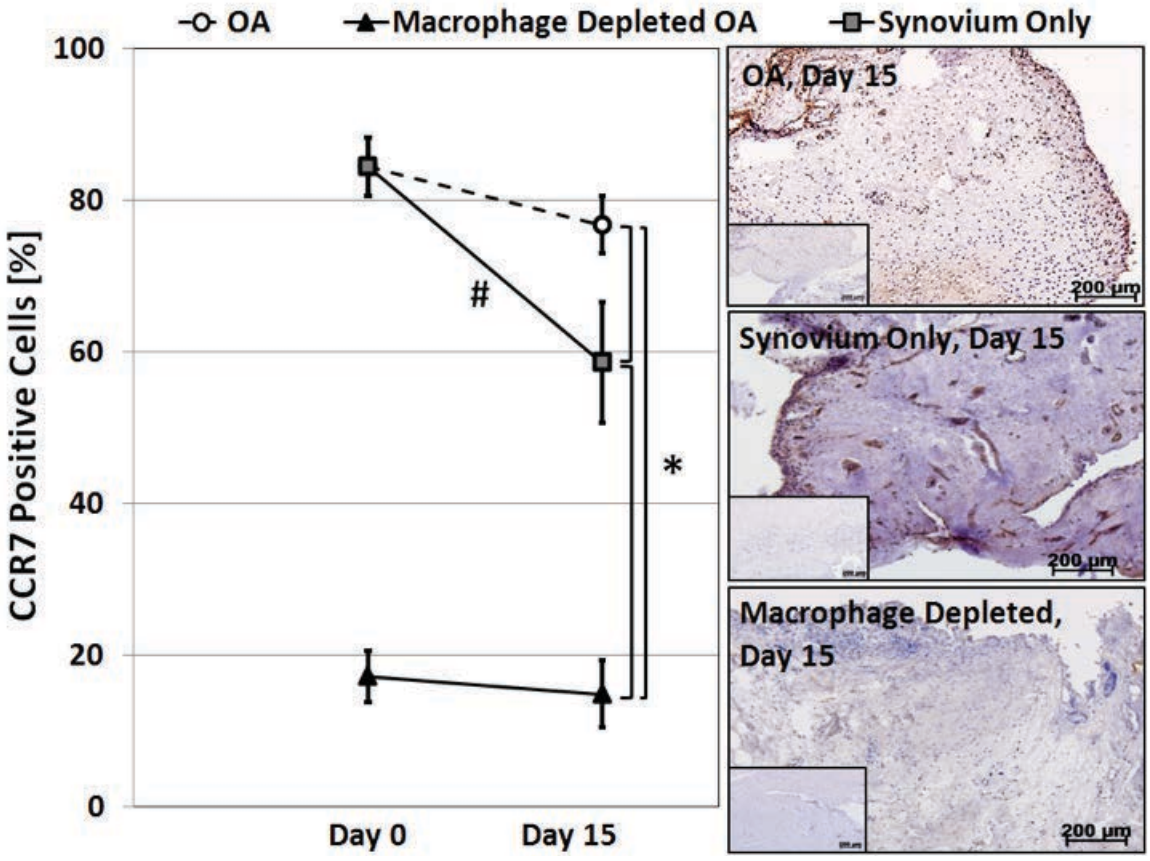


Figure 30: Confirmation of Macrophage Depletion. Semi-quantitative analysis of immunohistochemical stain for synovial M1 macrophages in all study groups over 15 days. At right, corresponding representative images of M1 macrophage staining (brown = positive) across study groups. Image inserts are negative controls. # indicates statistical difference ($P<0.05$) from day 0 to day 15. * indicates statistical difference ($p<0.05$) between study groups within time point.

4.3.3 Live/Dead Staining

Live/Dead staining of cartilage biopsies (figure 31A) revealed a statistical decrease (85.3% to 56.8%) in chondrocyte viability over 15 days in OA co-culture. Conversely, cartilage from the cartilage only culture exhibited relatively constant (85.3% to 74.9%) chondrocyte viability. Over 15 days, the macrophage depleted culture group demonstrated a statistical increase in chondrocyte viability (to 97.7%). By day 15, chondrocyte viability was significantly different ($p<0.05$) between all groups, with macrophage depleted cultures demonstrating the highest viability followed by cartilage only and OA co-culture.

Live/Dead staining of synovium biopsies (figure 31B) revealed a statistical decrease in synovial cell viability over 15 days in both the OA co-culture (72.1% to 44.6%) and the synovium only culture (72.1% to 54.1%). Conversely, the synovium from the macrophage depleted co-culture exhibited a statistical increase in synovial cell viability (to 98.7%). Therefore after 15 days in culture, synovium from the macrophage depleted co-culture contained a statistically greater percentage of viable cells compared to synovium cultured alone or with OA cartilage.

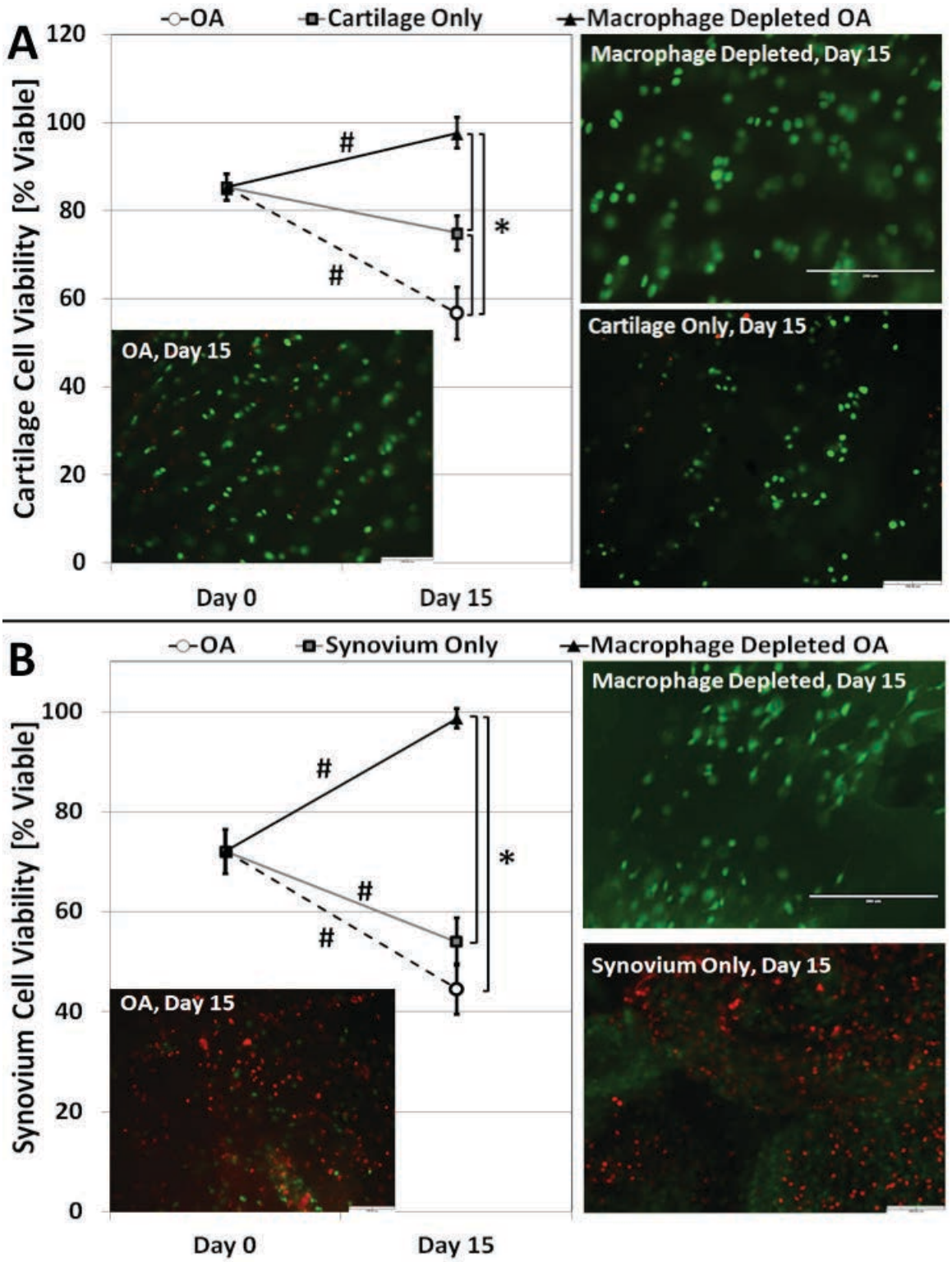


Figure 31: Cell Viability Assessment. A) Semi-quantitative analysis of Live/Dead chondrocyte staining from each group over 15 days with corresponding representative day 15 Live/Dead images of the cartilage. B) Semi-quantitative analysis of Live/Dead synovial cell staining (Red = dead cell; Green = viable cell) from each study group over 15 days with corresponding representative day 15 Live/Dead images of the synovium. White scale bar is equal to 100μm. # indicates statistical difference ($P<0.05$) from day 0 to day 15. * indicates statistical difference ($P<0.05$) between study groups within time point.

4.3.4 Histopathological Assessment of Explant Tissue

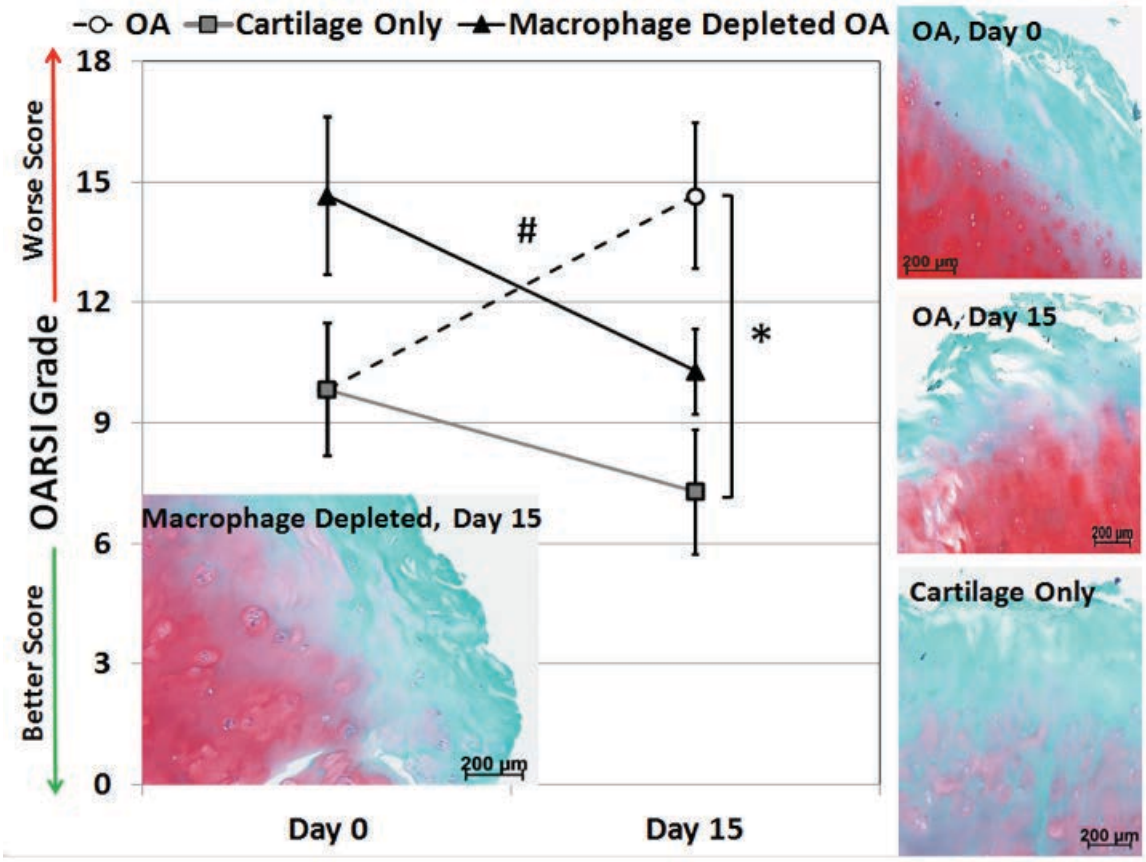


Figure 32: OARSI Histopathological Evaluation of Cartilage Microarchitecture. Graph of average OARSI scores (stage OA x grade OA) for each study group, with greater scores indicating greater cartilage deterioration. Graph insert is a Safranin-O stained cartilage section (Red= Proteoglycan-rich Cartilage; Green= Background) depicting cartilage surface microarchitecture after 15 days in macrophage depleted culture. At right, a patient matched set of Safranin-O stained cartilage sections, showing relative progression of OA in OA co-culture and cartilage only culture groups over 15 days. # indicates statistical difference ($P<0.05$) from day 0 to day 15. * indicates statistical difference ($P<0.05$) between study groups within time point.

Safranin-O stained cartilage evaluated via the OARSI histopathological assessment is represented in figure 3. The cartilage exhibited increased surface fibrillation across greater portions of the cartilage surface, manifesting in a statistically increased (worse) OARSI score when OA joint explants were co-cultured together ($p<0.05$). Conversely, cartilage from the cartilage only culture and macrophage depleted

co-culture trended towards decreased (better) scores. After 15 days, the OA co-culture OARSI score was statistically higher (worse) than the cartilage only culture.

4.3.5 Assessment of Cartilage Degradation

As seen in figure 33, cartilage GAG content evaluated via DMMB assay revealed that OA co-culture resulted in a significant and progressive loss of GAG content (from 141.9μg/mg to 65.7μg/mg). Conversely, over 15 days the cartilage only culture and macrophage depleted co-culture showed no statistical progression. Therefore after 15 days in culture, cartilage from the OA co-culture contained a statistically lower percentage of GAG compared to other tested groups.

Culture media evaluated via hydroxyproline assay revealed that OA co-cultures trended toward an increase in the amount of collagen leached from OA cartilage into the culture media. Conversely, cartilage only culture and macrophage depleted co-culture appeared to remain constant throughout the culture period. After 15 days, OA co-culture resulted in significantly more collagen leached into the media as compared to cartilage only culture.

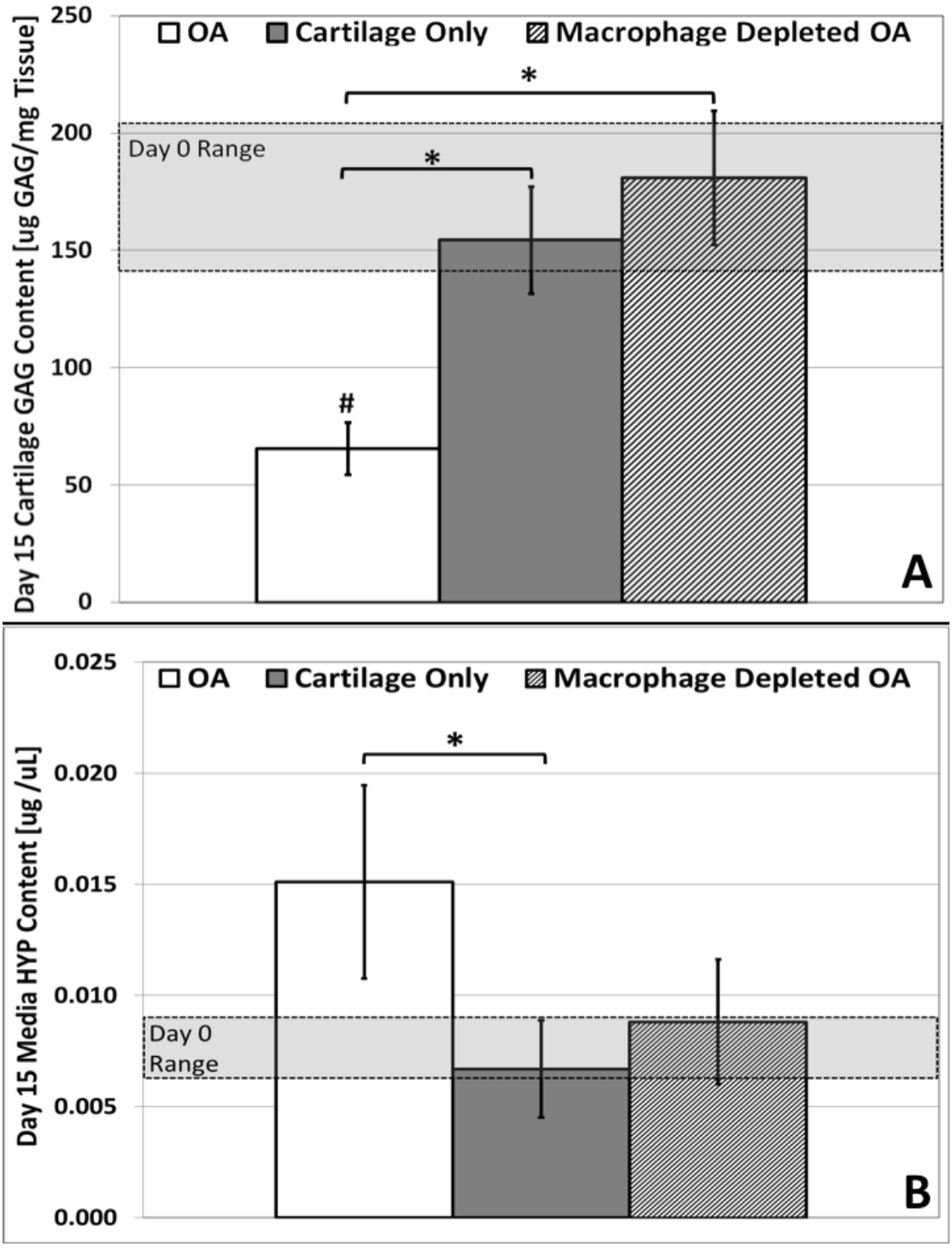


Figure 33: Biochemical Evaluation of Cartilage. A) DMMB glycosaminoglycan (GAG) assay depicting average cartilage GAG content from each study group after 15 days. The range of D0 values is depicted as a shaded box from 140-210ug GAG/mg Tissue. B) Hydroxyproline assay depicting average amount of collagen leached from the cartilage into the culture media from each study group after 15 days. The range of D0 values is depicted as a shaded box from 0.007-0.009μg/uL. # indicates statistical difference ($P<0.05$) from day 0 to day 15. * indicates statistical difference ($P<0.05$) between study groups within time point.

4.3.6 Co-culture Media Pro-Inflammatory Profile

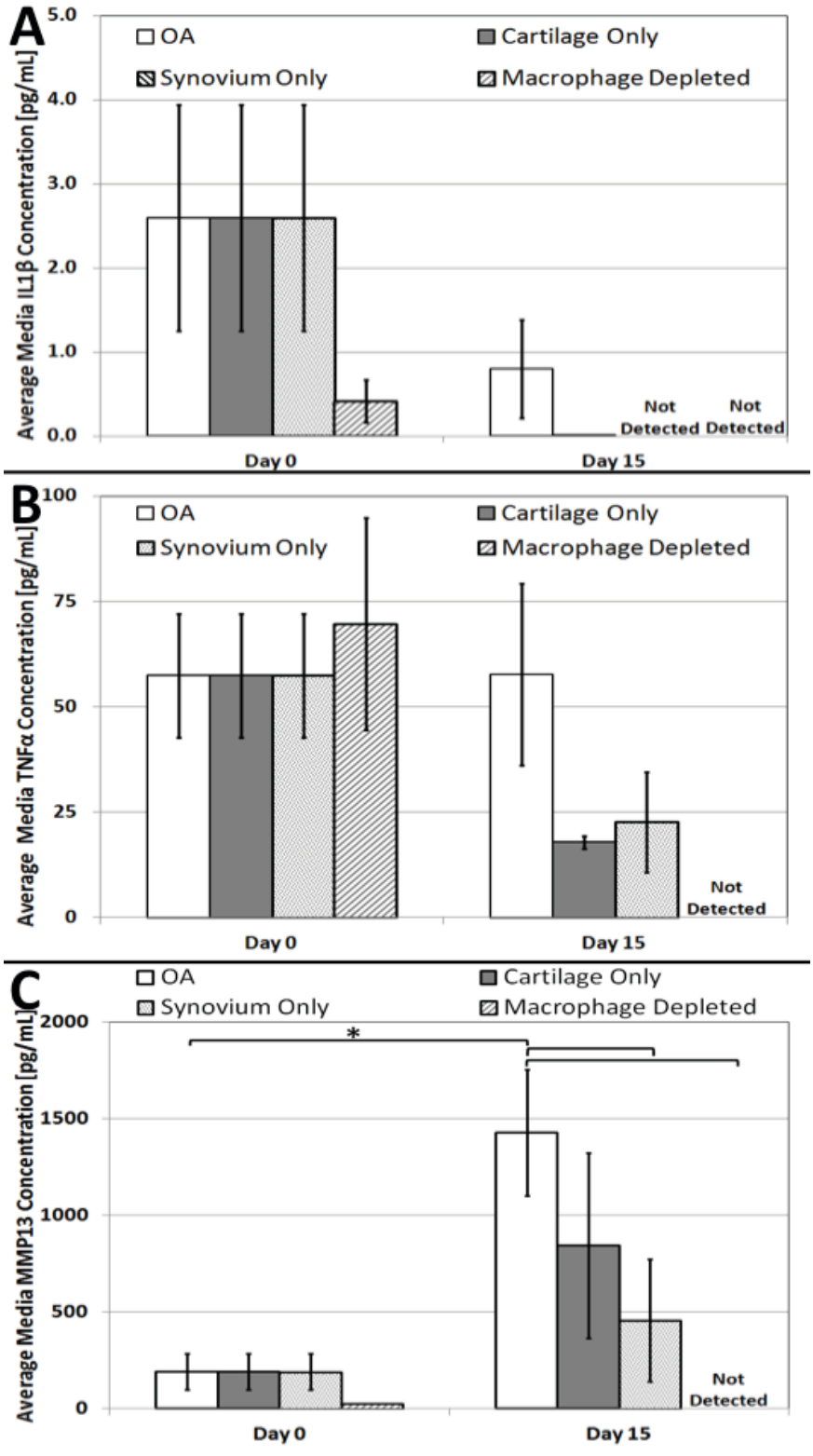


Figure 34: Cytokine Analysis over 15 Days in Culture. A) Average IL1-βconcentration in cell culture media from each study group. B) Average TNF-αconcentration in cell culture media from each study group. C) Average MMP13

concentration in cell culture media from each study group. "Not detected" indicates no sample from the study group contained sufficient cytokine concentrations for detection (Minimum detection limit of IL1-β, TNF-α and MMP13 ELISA kits: 0.3 pg/mL, 30 pg/mL and 6 pg/mL, respectively). * indicates statistical difference ($P<0.05$) between study groups within time point.

Figure 34A illustrates day 0 IL-1β media content tended to be lower in macrophage depleted groups; however this was not found to be statistically significant. By day 15, IL-1β was only detected in media from the OA co-culture group.

TNF-α ELISA performed on cell culture media revealed that macrophage depletion did not significantly influence day 0 TNF-α concentrations. However, figure 34B demonstrates by day 15 TNF-α was no longer detected in macrophage depleted co-cultures. While TNF-α concentrations remained constant in OA co-cultures, cartilage only culture and synovium only culture trended towards reduced concentrations.

MMP-13 ELISA performed on cell culture media revealed that over 15 days in culture, OA co-cultures significantly increased in MMP-13 content ($p<0.05$). MMP-13 was only minimally detected at day 0 and not detected at day 15 in macrophage depleted co-cultures.

A thorough cytokine profile via RayBiotech QAH-CYT-SW-1 cytokine array can be found in appendix a.

4.4 Discussion

We successfully demonstrate human joint tissue explant co-culture mimics OA pathogenesis by modeling: 1) cartilage destruction in the presence of synovial macrophages and pathophysiologic levels of inflammatory cytokines, 2) gradual progression with time and 3) a feed-forward progression, where the cartilage and synovium each contribute to the other's disease progression.

OA is a multifactorial disease affecting the major components of the human joint space: the cartilage, the synovium and the synovial fluid.[242,243,248,249] In OA, the cartilage matrix degrades.[113,135] The synovial fluid provides not only nutrients to joint tissues but also a potent cocktail of pro-inflammatory cytokines (ex. IL-1β and TNF-α), as well.[113,135] The synovial fluid becomes filled with degradation products, primarily cleaved collagen, from the cartilage.[11] The synovium becomes inflamed, a condition known as synovitis in which this traditionally 2-3 cell layer membrane undergoes hypertrophy as macrophages infiltrate the fibrous matrix.[113,135,242]

An ideal in vitro model would mimic the aforementioned characteristics of OA through evidence of cartilage matrix degradation (i.e. loss of proteoglycan and collagen content), maintenance of the pro-inflammatory milieu within the culture (media) environment and maintenance of inflammatory cell populations within the synovium. A model exhibiting progression of the disease would display worsening matrix degradation with time and increasing concentrations of pro-inflammatory cytokines and downstream matrix degrading enzymes.

The OA co-culture system described herein effectively models gradual OA progression with evidence of statistically increased cartilage fibrillation and decreased cartilage GAG content, as well as trends towards increased pro-inflammatory cytokines and collagen concentrations within the culture media. Given the short-term nature of the study, it was unlikely to observe statistical differences in collagen leaching into the culture media due to previous reports that collagenolytic activity in OA tissues peaks at 4 weeks.[250]

Significantly, the control groups in this study allowed us to confirm OA explant co-culture's ability to model the feed-forward pathogenesis of the disease, where the cartilage and synovium appear to feed off each other, working in tandem to further disease progression. Decreases in cartilage GAG and leached collagen contents as well as microscopic surface fibrillation were observed to a lesser extent when OA cartilage was cultured alone, without synovial contributions. The relatively constant percentage of macrophages observed in the OA co-culture group seems to indicate that the macrophages receive continued stimulation within the explant co-culture model; whereas synovium only cultures exhibited a statistical decrease in the percentage of macrophages potentially due to the removal of such stimulatory cues (likely from the cartilage, as OA chondrocyte TLR activation has been extensively described by others[12,113]). This activation pathway results in the up-regulation of MMPs and ADAMTS. Specifically, TLR-2 and TLR-4 are up-regulated in regions of cartilage erosion.[113,135] Up-regulation of these specific TLRs has been linked to downstream increases in master MMP regulator, MMP-3.[113,135] This could be one pathway accounting for the increases in MMP-13 observed in our OA co-culture and cartilage only groups.

Interestingly, though there is a constant presence of macrophages observed during OA co-culture, the percentage of viable cells within OA co-cultured synovium statistically decreases with time. This may indicate that the viability of host synovial (fibroblastic) cells, not macrophages, was compromised. This could be a further indication of the worsening health of the synovial tissue as OA progresses. Though general tissue manipulation and in vitro culture can account for some loss in viability, our viability results generally support a feed-forward inducing effect of cell death when

cartilage and synovium are cultured together. In order to ensure these effects were not the result of culture system overload (i.e. depriving the cells of appropriate levels of nutrients), we seeded an additional $1.5x10^5$ human stem cells onto the surface of the cartilage and monitored viability effects. This data (not shown) demonstrated that cell viability was maintained, and in some cases increased OA chondrocyte viability. Corroborating viability results in joint explant co-culture models used by others were not found in current literature. However, these results could indicate a currently overlooked mechanism whereby OA progression is driven by a loss in specialized tissue cell types in favor of inflammatory cell populations.

In order to specifically assess the impact of synovial macrophages on OA progression in the model, select synovium samples were pre-treated with Clodronate ("macrophage depleted co-culture"), a bisphosphonate known to induce apoptosis in phagocytic cells without affecting non-phagocytic cell viability, prior to the initiation of co-culture.[185,186,188,193,251] Previously it has been shown that such depletion results in downstream reductions in IL-1β and TNF-α concentrations as well as reduced cartilage destruction in experimental models of arthritis.[189,191,193] Results from the current study confirmed similar outcomes in this joint tissue explant co-culture model.

Though macrophages were successfully depleted in our in vitro model, it is worth noting that such sustained macrophage depletion may not likely be an advantageous therapeutic avenue *in vivo* due to macrophage recruitment/replenishment though the native synovial blood supply.[185] However, macrophage depletion more likely represents a mechanistic tool for researchers as opposed to a potential future therapeutic.

Interestingly, clodronate is currently being investigated as an OA therapeutic both in animals and in human clinical trials, albeit with mixed results.[122,198–200]

OA explant co-culture also more effectively models pro-inflammatory cytokine involvement in the human pathology. IL-1β is a macrophage secretion product suppressing aggrecan and collagen synthesis, which is heavily implicated in OA disease pathology and progression.[104,110,113,135] Physiological levels of IL-1β in osteoarthritic synovial fluid have been reported at <4.8pg/mL.[136,147,149] At all culture time-points in this study, values for this cytokine fell within reported *in vivo* ranges, which is significantly lower than the reported 5mg/mL of IL-1β used in studies which chemically induce OA through the addition of supraphysiological levels of cytokines to cartilage explant only culture models.[245,252] This seems to indicate that cartilage-synovial co-culture provides a more natural model of the cytokine profile of OA, though further comparative analyses would need to be completed to corroborate this claim. Others have noted extremely low concentrations of IL-1β in ex vivo cultures.[252–254] This has been attributed to the extreme lability associated with this cytokine, and researchers have cautioned conclusions based solely on IL-1β biochemical analysis.[173,255] Both IL-1β and TNF-α induce signaling pathways resulting in pro-inflammatory stimulation and decreased collagen expression.[104] As TNF-α is a macrophage-secreted product, the dramatic concentration reduction observed in the macrophage depleted groups was expected. In a similar manner, the removal of the synovium ("cartilage only") represents the removal of synovial macrophages and would thus also be expected to lower TNF-α concentrations. It is interesting that the removal of the cartilage ("synovium only") yields a similar effect,

again potentially supporting the concept that OA progresses via a feed-forward, cell mediated destructive mechanism.

In conclusion, we were able to successfully demonstrate human joint tissue explant co-culture mimics OA pathogenesis by modeling: 1) cartilage destruction in the presence of inflammatory cytokines and synovial macrophages, 2) gradual progression with time and 3) a feed-forward progression, where the cartilage and synovium exhibit reciprocal pathologic effects on one another. Additionally, our mechanistic evaluations utilizing macrophage depletion studies indicate synovial macrophages and/or their secretions are likely primary effectors driving disease progression in the model. This work highlights the utility of joint tissue explant co-culture in OA research, as it is an effective system for investigating OA pathology and evaluating potential future therapies.

Though this ex vivo model mimics key aspects of human OA, any *in vitro* model is subjected to limitations. Specifically, this experimental design requires tissue biopsies that demonstrate relatively equal disease status at the time of culture initiation. While care was taken to ensure only the use of cartilage immediately surrounding areas of OA-induced cartilage erosion, there is inherent variability associated with each biopsy. In future studies, we suggest minimizing this variability through halving each sample, using one half as a biopsy-matched day 0 control. An additional limitation is inherent inter-patient variability. We attempted to overcome such variability through increased sample sizes (n=15). Normalization of data to each patient's matched day 0 values is also suggested thus eliminating result biases from inter-patient variability. Lastly, there is extremely limited data describing the use of human joint tissue explant co-culture making it difficult to validate results across different investigators via comparisons with peer-

reviewed literature sources. As more studies are published using such models, comparative data should become available to allow for cross comparisons. However, as of now we caution cross comparisons of this work with those employing smaller sample sizes and tissue samples from different species. As previously indicated, a sample size of at least 14 was required in order to obtain the statistical differences and trends described herein. Additionally, the known differences in inflammatory-driven cartilage matrix degradation between species limit the utility of cross-species comparisons.[108,110,151,152]

CHAPTER FIVE

AIM III: TO EVALUATE THE ABILITY OF MSCs TO MITIGATE OA PROGRESSION IN THIS VALIDATED EX VIVO MODEL

5.1 Introduction

While osteoarthritis (OA) is traditionally viewed as a physical wear and tear disease, mounting evidence suggests that M1 macrophage-driven synovial inflammation significantly contributes to its pathogenesis.[243] Pro-inflammatory, M1, macrophage secretions create a degenerative joint environment, ultimately resulting in the up-regulation of effectors promoting cartilage degradation and causing patients immense pain. Current OA treatments are palliative, failing to halt the progression of the disease. Stem cells offer a potential therapeutic alternative due to their anti-inflammatory and immunomodulatory properties.[54,218] Current stem cell-based therapies focus on the intra-articular injection of adult stem cells including those derived from adipose tissue (hADSCs).[120,207] However, perinatal stem cells, specifically those derived from the amniotic membrane (a tissue routinely discarded as medical waste following the birth of full-term babies), have illustrated promise as an alternative stem cell source for regenerative medicine.[68,218] Though perinatal stem cells have yet to be tested as a therapeutic strategy combatting OA, recent evidence suggests perinatal stem cells exhibit superior chondro-protective effects, exhibiting the ability to induce a pro-regenerative (M2) phenotype within synovial macrophages.[73,87]

Herein, we describe efforts undertaken to establish the ability of stem cells to mitigate OA progression in an established ex vivo co-culture model of naturally progressing OA. We employed standardized comparative analyses utilizing two stem cell

sources, hADSCs and human amniotic mesenchymal stem cells (hAMSCs), in order to determine the relative efficacies of a newly investigated (perinatal) and a clinically established (hADSC) stem cell source as potential OA therapeutics. Furthermore, following the current paradigm of intra-articular administration, it is likely that injected stem cells would be found in a mixed contact scenario with OA cartilage where only some stem cells remain in direct contact with the cartilage (see appendix A). In an effort to better understand the potential efficacy of intra-articular administration of perinatal stem cells as a future OA treatment paradigm, we evaluated and compared the potential differential effects of administering hAMSCs in two different co-culture models (i.e. direct and indirect contact co-culture with OA cartilage in the presence of synovium). Taken together, such results would highlight the utility of a currently under investigated stem cell source (hAMSCs) for OA regenerative medicine approaches and provide significant mechanistic into their clinical use.

5.2 Materials & Methods

Insulin Transferrin Selenium was purchased from Life Technologies (41400045). Ascorbate-2-phosphate (59-990-141) and trans-well culture plates (07-200-157) were purchased from Fisher Scientific. Clophosome A was purchased from FormuMax Scientific Inc (F70101C-A-2). Live/Dead Animal Cell Kit was purchased from VWR (89260-208). 1,9 Dimethyl-Methylene Blue (341088-1G), Chondroitin Sulfate (C4384-5G), Hydroxyproline Assay Kit (MAK008-1KT), Safranin-O Stain (S2255-25G) and Fast Green Stain (F7258-25G) were purchased from Sigma. Normal Horse Serum (S2000), VECTASTAIN Elite ABC Kit, Rabbit IgG (PK-6101) and DAB Substrate Kit (SK4100)

were purchased from Vector Laboratories, Inc. Triton X-100 and other basic chemicals were purchased from Fisher Scientific. Antibodies employed in this work included: anti-mannose receptor (ab32527) and anto-CCR7 (ab64693). All ELISAs employed in this work were purchased from RayBiotech: Human TNF-alpha (LH-TNFa-1), Human MMP-13 (ELH-MMP13-1) and Human IL-1 beta (ELH-IL1b-1).

5.2.1 Joint Tissue Harvest and Culture Initiation

Human cartilage and synovium were obtained from consenting patients immediately following total knee arthroplasty for OA (IRB# PRO00031185 Greenville Health System). All patients were classified as having Kellgran-Lawrence grade 4 OA. Using aseptic technique, cartilage and synovium tissues were biopsied into 6mm diameter samples in preparation for in vitro culture. Tissue biopsies were placed in 12-well plates (1 cartilage and 1 synovium biopsy / well) and maintained in 2mL DMEM supplemented with 1% Insulin Transferrin Selenium (ITS), 1% antibiotic/antimitotic (ABAM) and 50nM Ascorbate-2-phosphate for 3 days in standard culture conditions (5% CO_2; 20% O_2; 37° C). Henceforth, this media formulation will be referred to as "explant medium." Care was taken to ensure the cartilage and synovium were not in direct contact. After 3 days, the media was removed and labeled "day 0" media.

5.2.1.1 hAMSC Culture

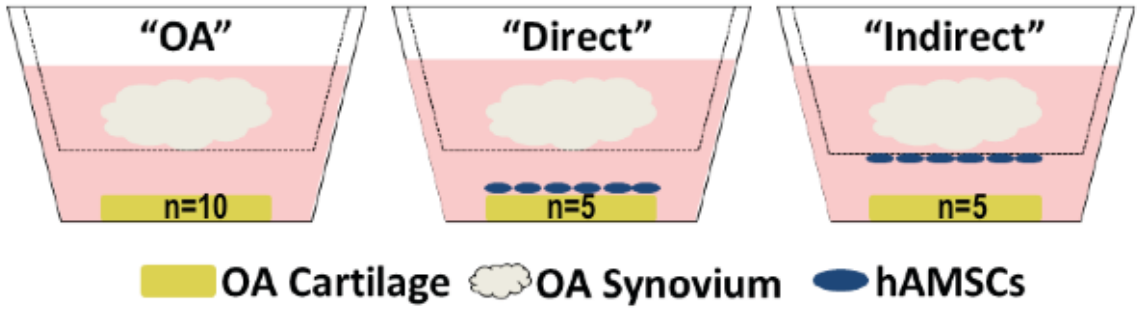


Figure 35: Methods Schematic Demonstrating the Described Comparative Analysis between Control OA Explant Co-culture ("OA") and OA Co-cultures Treated with Stem Cells Applied Directly or Indirectly to the Cartilage Surface after 15 days. This comparative analysis will determine the relative efficacies of two potential clinically relevant routes of administration.

Joint co-culture was initiated as previously described. hAMSCs were isolated from term amniotic membrane as previously described (Aim I). Each stem cell treated co-culture was simultaneously cultured with a patient matched "OA" control.

5.2.1.1.1 OA+hAMSC (Direct Culture)

At day 0, $1x10^5$ hAMSCs were seeded directly on top of cartilage biopsies.

5.2.1.1.1 OA+hAMSC (Indirect Culture)

At day 0, $1x10^5$ hAMSCs were seeded indirectly onto the bottom on a porous trans-well insert.

5.2.1.2 OA+hADSC Culture

Joint co-culture (n=5) was initiated as previously described. hADSCs were purchased from a commercial vendor for comparative analysis. At day 0, $1x10^5$ hADSCs were seeded directly on top of cartilage biopsies. Each stem cell treated co-culture was simultaneously cultured with a patient matched "OA" control.

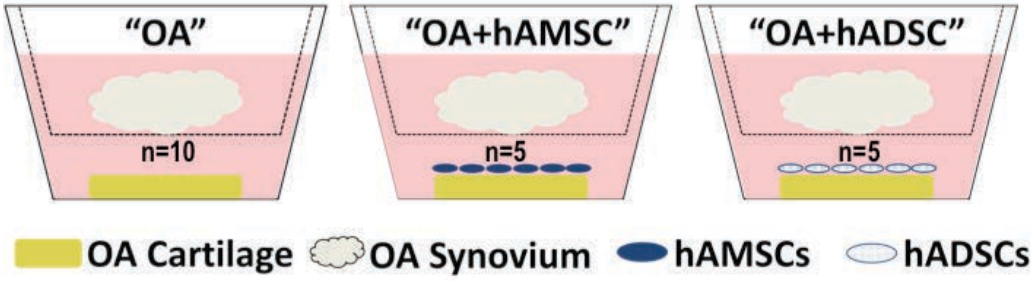


Figure 36: Methods Schematic Demonstrating the Described Comparative Analysis between Control OA Explant Co-culture ("OA") and OA Co-cultures Treated with either hAMSCs or hADSCs after 15 days. This comparison will determine the relative efficacies of a commonly utilized (hADSC) with a newly investigated (hAMSC) stem cell therapy for OA mitigation.

5.2.2 Synovial Macrophage Phenotype

Immunohistochemistry (IHC) on rehydrated paraffin synovium sections was performed for detection of synovial macrophages. Briefly, antigen retrieval was accomplished via 10mM Citric Acid incubation at 90°C for 20 minutes. Slides were rinsed twice in TBS for 5 minutes,

permeabilized in 0.025% Triton X-100, non-specific binding and endogenous peroxidases were blocked with normal serum and a solution of 0.3% hydrogen peroxide in 0.3% normal serum, respectively. A rabbit polyclonal antibody towards human CCR7 (0.5μg/mL dilution) or human mannose receptor (1μg/mL dilution) was incubated for 1 hour at room temperature prior to thorough rinsing and incubation at room temperature for 30 minutes with a secondary biotinylated antibody and avidin biotin complex according to manufacturer's instructions (Vectastain®ABC Elite Kit Rabbit IgG - Vector Labs). A DAB substrate kit (Vector Labs: SK4100) was used to visualize positive staining prior to counterstaining with a dilute hematoxylin solution for 30 seconds. Negative controls did not receive primary antibody.

5.2.3 Live/Dead Staining

Live/Dead staining was completed on cartilage and synovium according to manufacture instructions. Briefly, cartilage and synovium were incubated in a Live/Dead working solution (2μM calcein AM and 4μM Ethd-1) and at room temperature for 45 minutes. Tissues were placed on a microscope slide prior to fluorescent imaging.

5.2.4 Histopathological Assessment of Explant Tissue

Cartilage sections were secured within a tissue processing cassette, fixed in 10% phosphate buffered formalin overnight at room temperature prior to undergoing standard tissue processing, decalcification, paraffin embedding and sectioning at 5 μm thickness. Sections were stained with Safranin-O counterstained with Fast Green for visualization of proteoglycan rich cartilage matrix. Briefly, rehydrated sections were differentiated in 1% acid alcohol for 2 seconds prior to room temperature incubation in 0.02% Fast Green for 2.5 minutes. After 30 second incubation in 1% Acetic acid, sections were stained with 1% Safranin-O for 15 minutes. Three images were taken spanning the surface length of the sample. An observer blinded to the condition of the cartilage completed the OARSI

histopathological assessment on each image according to the direction of Pritzker et al.[247] These results were averaged to obtain the final sample OARSI score.

5.2.5 Assessment of Cartilage Degradation

5.2.5.1 Cartilage Glycosaminoglycan (GAG) Content

Lyophilized cartilage tissues were digested in 125μg/mL Papain in PBE Buffer, pH 7.5 overnight at 65°C. Tissue digests were assessed for GAG content via Dimethylmethylene Blue Assay (DMMB). Briefly, 200μL DMMB reagent (46μg DMMB, 40mM Glycine, 40mM NaCl, pH 3) was added to 50μL samples. Standards were created using a 1mg/mL stock solution of Chondroitin-6-Sulfate. Sample absorbance was read at 525nm.

5.2.5.2 Collagen Leaching to Culture Media

Culture media was assessed for hydroxyproline collagen content via Hydroxyproline Assay Kit according to manufacture instructions. Briefly, samples were hydrolyzed with hydrochloric acid at 120°C for 3 hours prior to well plate transfer. Wells were evaporated to dryness at 60°C. Equal amounts of Chloramine T/Oxidation Buffer Mixture and Diluted DMAB Reagent followed by 5-minute room temperature and 90-minute 60°C incubations, respectively. Standards were created using a 1mg/mL stock solution of Hydroxyproline Standard Solution. Sample absorbance was read at 560nm.

5.2.6 Co-culture Media Pro-Inflammatory Profile

ELISA was performed according to manufacture instructions. Briefly, 96-well plates were incubated overnight at 4°C with standards and explant co-culture media samples (n=3 per condition). After several washes, wells were incubated with biotinylated antibodies (either TNF-α, IL-1β or MMP-13) for 1 h, followed by incubation in HRP-

conjugated streptavidin for 45 min. Enzymatic reactions were allowed to develop, and the absorbance of each plate was read at 450 nm.

5.2.7 Microscopic Imaging

Images were captured on a Zeiss Axiovert.A1 microscope with Axiovision software (Release 4.9.1 SP08-2013). For all semi-quantitative histological data, three images were taken of each sample. An observer blinded to the condition of the sample manually counted each of these three images in order to determine the relative number of positive cells (i.e. brown cells (IHC), live cells or dead cells (Live/Dead), etc.) per sample. These three results were averaged in order to obtain the final percentage of positive cells for each sample.

5.2.8 Statistical Analysis

Results are represented as a mean ± standard error of the mean (SEM). All statistical analyses were performed by two-tailed Student's t-test of unequal variance or one-way analysis of variance (ANOVA). Significance was defined in all cases as $p<0.05$.

5.3 Results

5.3.1 Joint Tissue Harvest and Culture Initiation

Human joint tissues were successfully harvested, 6mm biopsies of patient-matched cartilage and synovium were obtained, and the tissues were maintained in *ex vivo* culture for 15 days without any macroscopic signs of bacterial infection.

5.3.2 Synovial Macrophage Phenotype

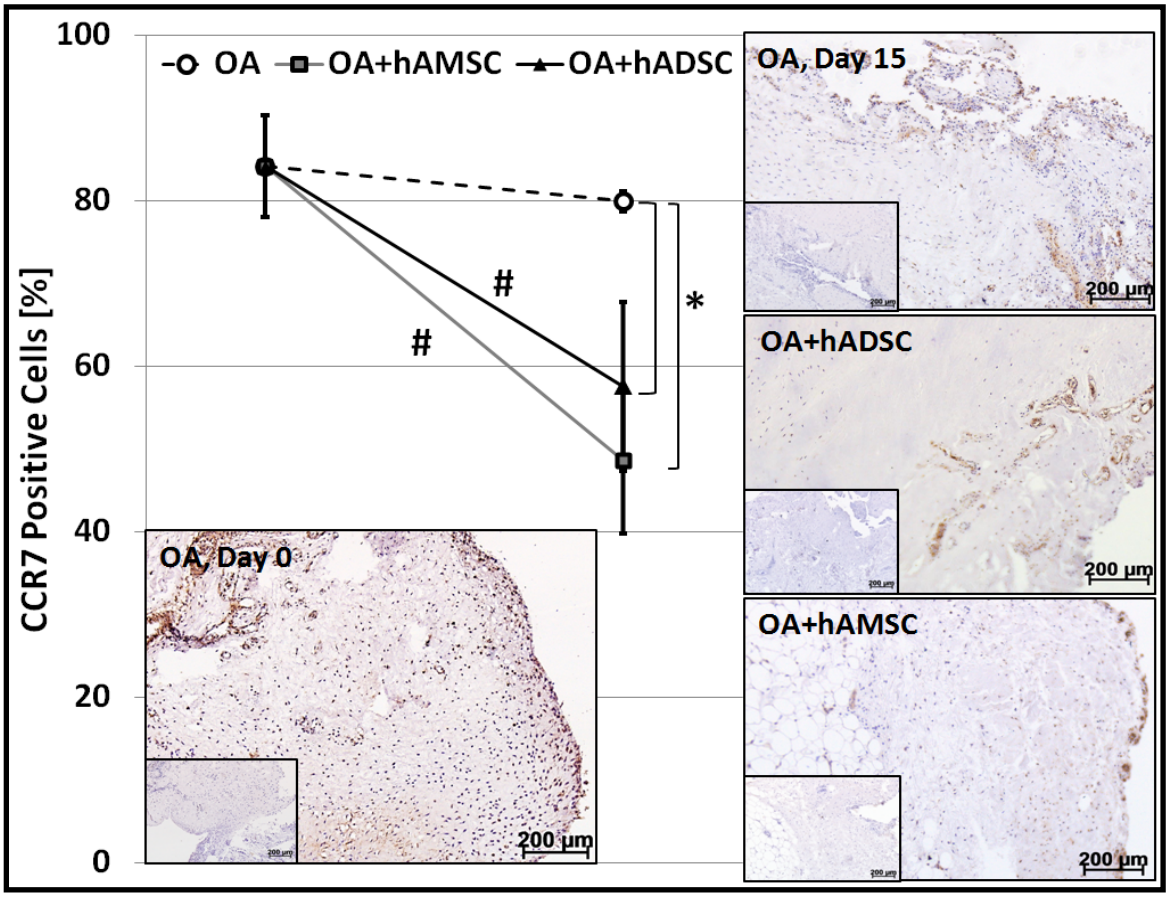


Figure 37: Semi-Quantitative Analysis of M1 Polarized Macrophages in hAMSC and hADSC-Treated Cultures demonstrating stem cell treatment results in a statistical reduction in M1 macrophages within the synovium. Corresponding representative images of macrophage staining (brown = positive) are located to the right of each graph. Photo inserts represent negative controls. # indicates statistical difference ($P<0.05$) from day 0 to day 15 * indicates statistical difference ($P<0.05$) between study groups within time point.

IHC staining of CCR7 (Figure 37) showed a relatively constant percentage (84.1% to 79.9%) of M1 polarized macrophages over 15 days in OA co-culture. Over 15 days, both the OA+hAMSC and the OA+hADSC groups exhibited a statistical decrease in the percentage of cells staining positive for macrophage markers (88.7% to 48.6% and 79.5% to 57.2%, respectively).

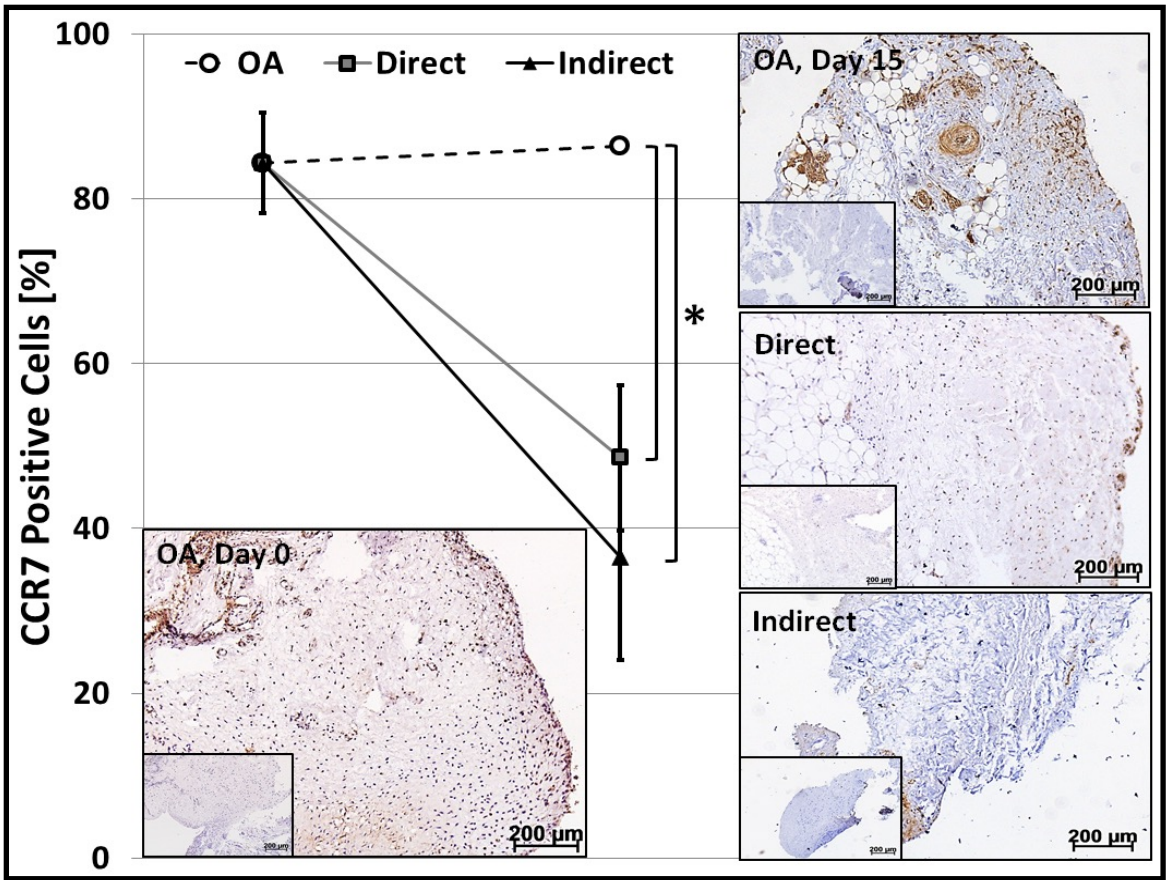


Figure 38: Semi-Quantitative Analysis of M1 Polarized Macrophages in Direct and Indirect-Treated Cultures demonstrating stem cell treatment results in a statistical reduction in M1 macrophages within the synovium regardless of the route of administration. Corresponding representative images of macrophage staining (brown = positive) are located to the right of each graph. Photo inserts represent negative controls. # indicates statistical difference ($P<0.05$) from day 0 to day 15 * indicates statistical difference ($P<0.05$) between study groups within time point.

Similarly, figure 38 shows both the direct and the indirect hAMSC groups exhibited a statistical decrease in the percentage of cells staining positive for macrophage markers over 15 days (88.7% to 48.6% and 79.9% to 36.5%, respectively).

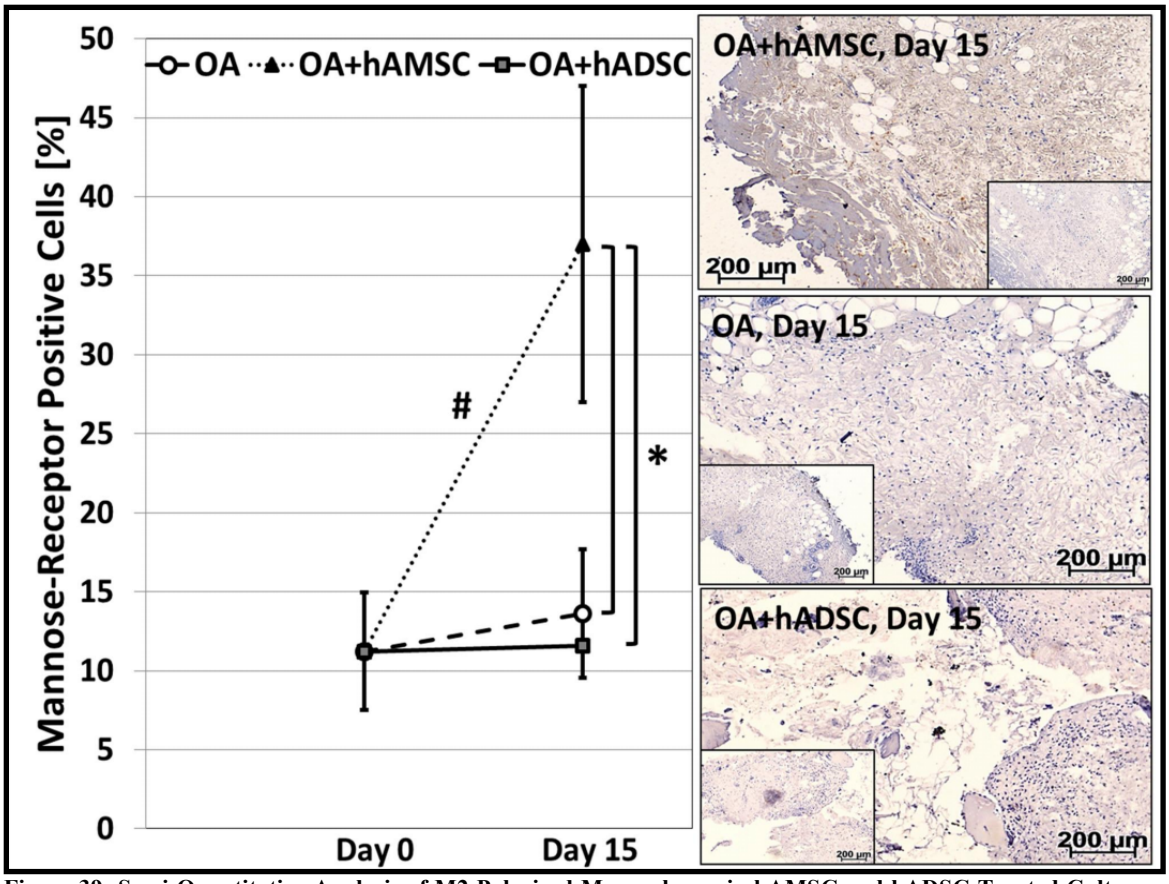


Figure 39: Semi-Quantitative Analysis of M2 Polarized Macrophages in hAMSC and hADSC-Treated Cultures demonstrating hAMSC treatment results in a statistical increase in M2 macrophages. Corresponding representative images of macrophage staining (brown = positive) are located to the right of each graph. Photo inserts represent negative controls. # indicates statistical difference ($P<0.05$) from day 0 to day 15 * indicates statistical difference ($P<0.05$) between study groups within time point.

As illustrated in figure 39, in OA+hAMSC cultures, this decrease in M1 polarized macrophages was concomitant with a statistical increase in M2 polarized macrophages. However, OA and OA+hADSC cultures exhibited almost no change in the percentage of M2 polarized macrophages within the synovium over 15 days. In figure 40, both direct and indirect hAMSC culture produced this statistical increase in M2 macrophages (Interestingly, direct and indirect culture accomplished this equally effectively).

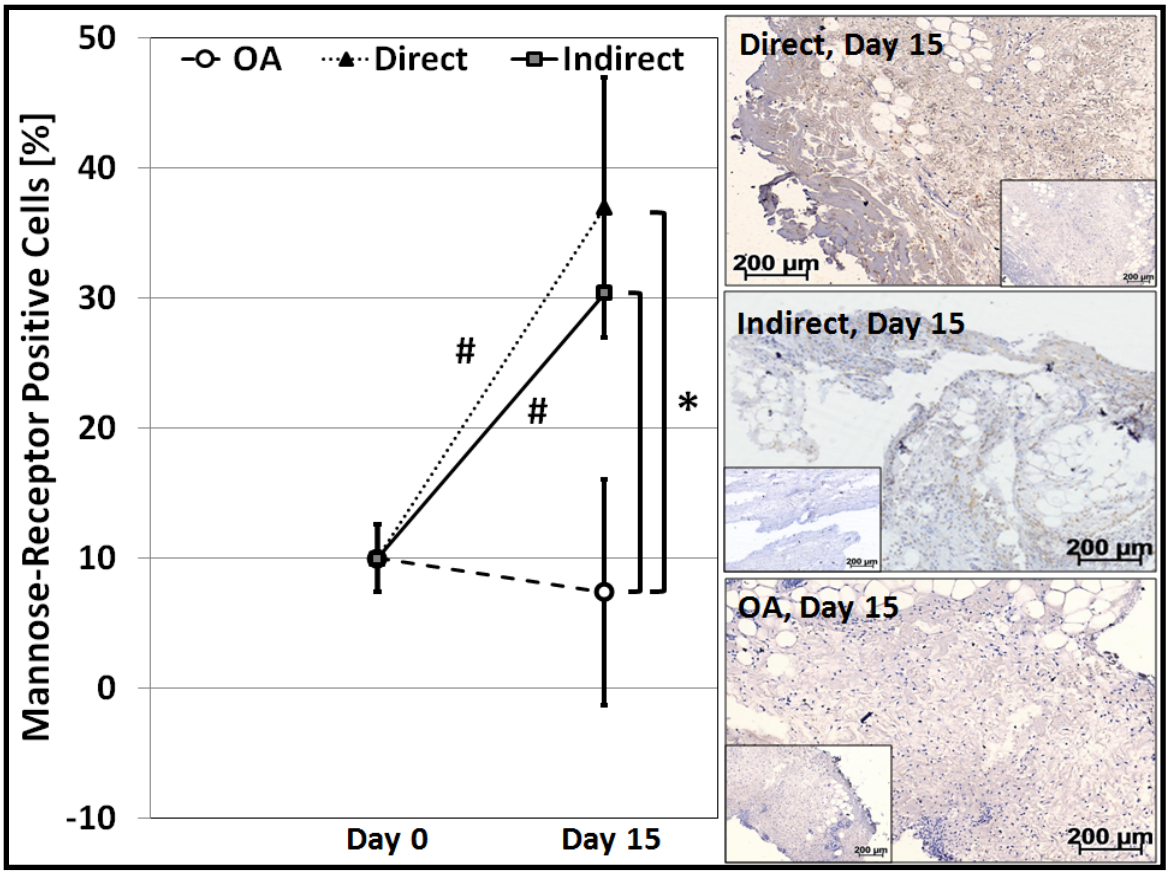


Figure 40: Semi-Quantitative Analysis of M2 Polarized Macrophages in Direct and Indirect-Treated Cultures demonstrating both routes of administration result in a statistical increase in M2 macrophages. Corresponding representative images of macrophage staining (brown = positive) are located to the right of each graph. Photo inserts represent negative controls. # indicates statistical difference ($P<0.05$) from day 0 to day 15 * indicates statistical difference ($P<0.05$) between study groups within time point.

5.3.3 Live/Dead Staining

Live/Dead staining of cartilage biopsies (figure 41) revealed a statistical decrease (87.8% to 59.1%) in chondrocyte viability over 15 days in OA co-culture as well as OA+hADSC culture (87.1% to 49.9%). Conversely, cartilage treated with hAMSCs exhibited relatively constant (88.5% to 89.8%) chondrocyte viability.

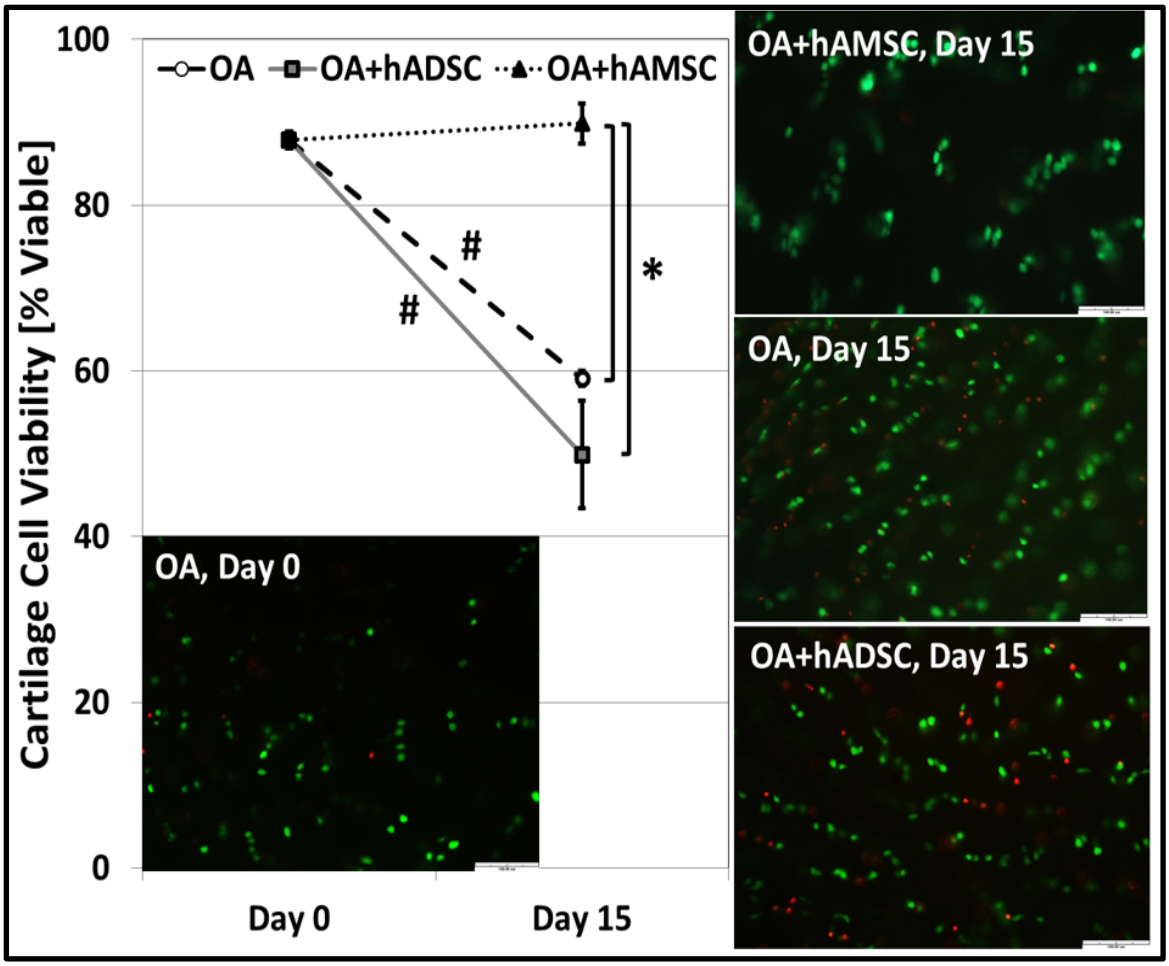


Figure 41: Semi-Quantitative Analysis of Chondrocyte Viability in hAMSC and hADSC-Treated Cultures with representative day 15 Live/Dead images, demonstrating OA+hAMSC ability to most effectively maintain chondrocyte viability. White scale bar is equal to 100μm. # indicates statistical difference ($p<0.05$) from day 0 to day 15. * indicates statistical difference ($p<0.05$) between study groups within time point.

However, figure 42 illustrates that this observed increase in chondrocyte viability is dependent on the route of administration of the hAMSCs.

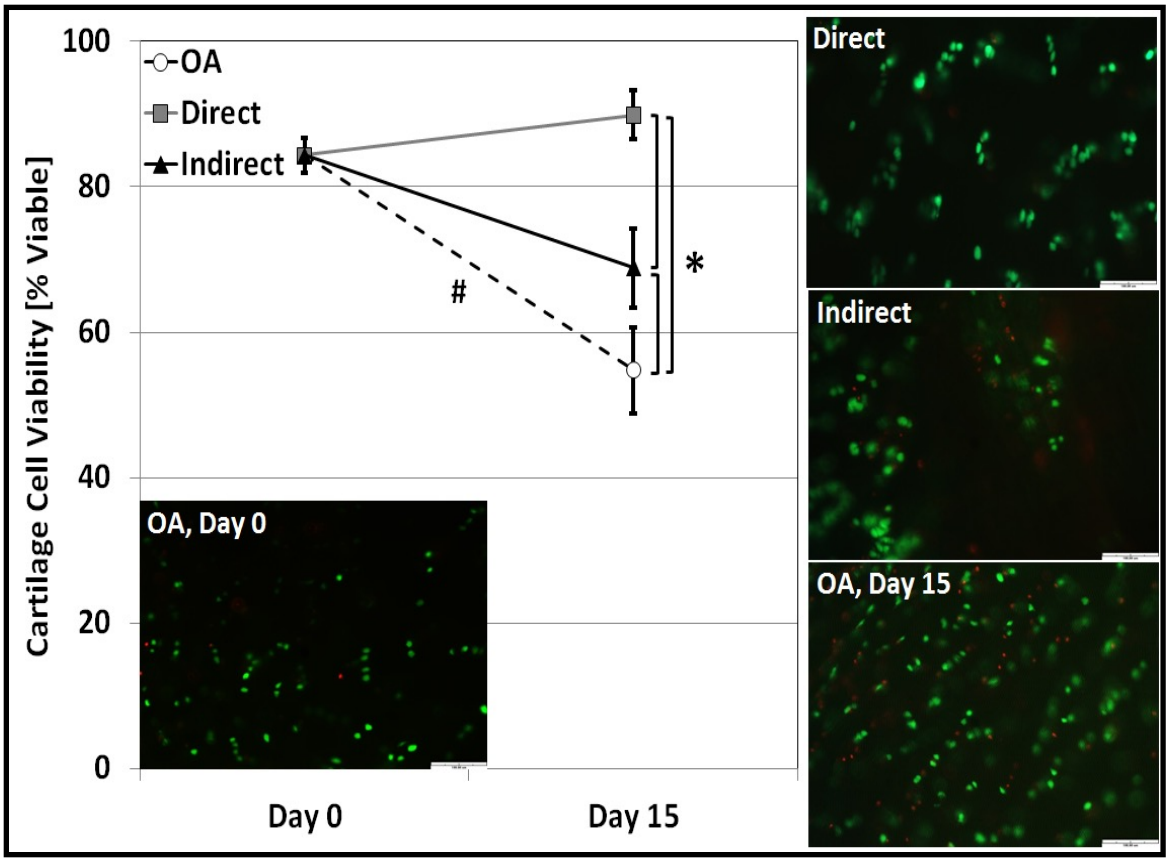


Figure 42: Semi-Quantitative Analysis of Chondrocyte Viability in Direct and Indirect-Treated Cultures with representative day 15 Live/Dead images, demonstrating OA+hAMSC ability to most effectively maintain chondrocyte viability. White scale bar is equal to 100μm. # indicates statistical difference ($p<0.05$) from day 0 to day 15. * indicates statistical difference ($p<0.05$) between study groups within time point.

5.3.4 Histopathological Assessment of Explant Tissue

Safranin-O stained cartilage evaluated via the OARSI histopathological assessment is represented in figures 43 and 44. The cartilage exhibited increased surface fibrillation across greater portions of the cartilage surface, manifesting in a statistically increased (worse) OARSI score when OA joint explants were co-cultured together ($p<0.05$). Conversely, figure 43 demonstrates OA+hAMSC cartilage exhibited less surface fibrillation, manifesting in a statistically decreased OARSI score. While OA+hADSC cultures trended towards a similar decrease.

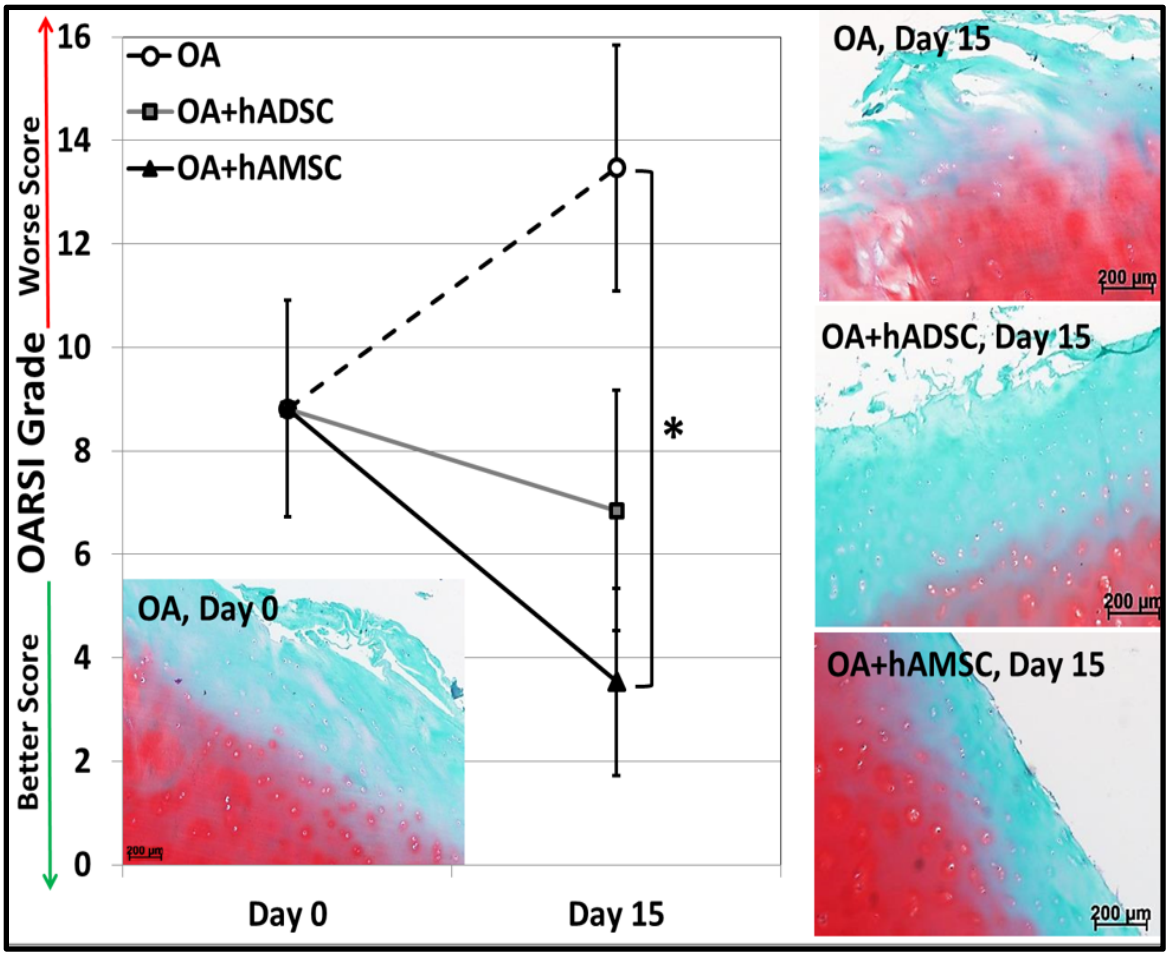


Figure 43: Average OARSI Scores in hAMSC and hADSC-Treated Cultures with corresponding safranin-o stained cartilage sections depicting worsening microarchitecture in un-treated groups as well as the ability of stem cells to deter surface fibrillation.

Interestingly, figure 44 depicts that this chondroprotective effect of the hAMSCs appears to be dependent on the route of administration, as Indirect cultures still exhibited a trend towards worse cartilage surface fibrillation (worsening OARSI score), though this trend was not as dramatic as OA control cultures. Interestingly indirect cultures exhibited statistically decreased number of chondrocyte clusters, another metric employed by researchers to gauge OA progression, compared to OA+hADSC cultures (see appendix A).

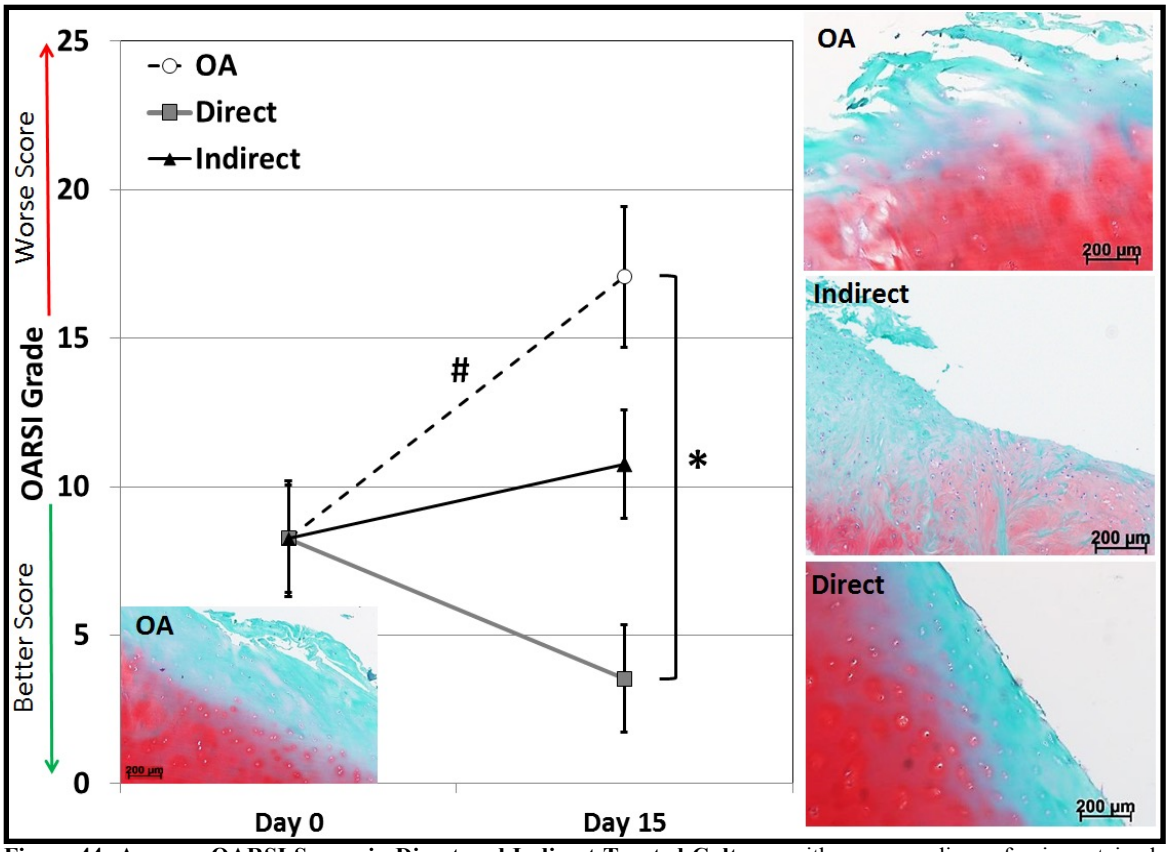


Figure 44: Average OARSI Scores in Direct and Indirect-Treated Cultures with corresponding safranin-o stained cartilage sections depicting worsening microarchitecture in un-treated groups as well as the ability of stem cells in direct contact with cartilage to deter surface fibrillation.

5.3.5 Assessment of Cartilage Degradation

As seen in figure 45, cartilage GAG content evaluated via DMMB assay revealed that OA co-culture resulted in a progressive loss of GAG content (from 181.9µg/mg to 141.7µg/mg). Conversely, over 15 days the OA+hADSC culture showed no progression, and the OA+hAMSC groups exhibited a trend towards increased cartilage GAG content (156.4µg/mg to 247.0µg/mg).

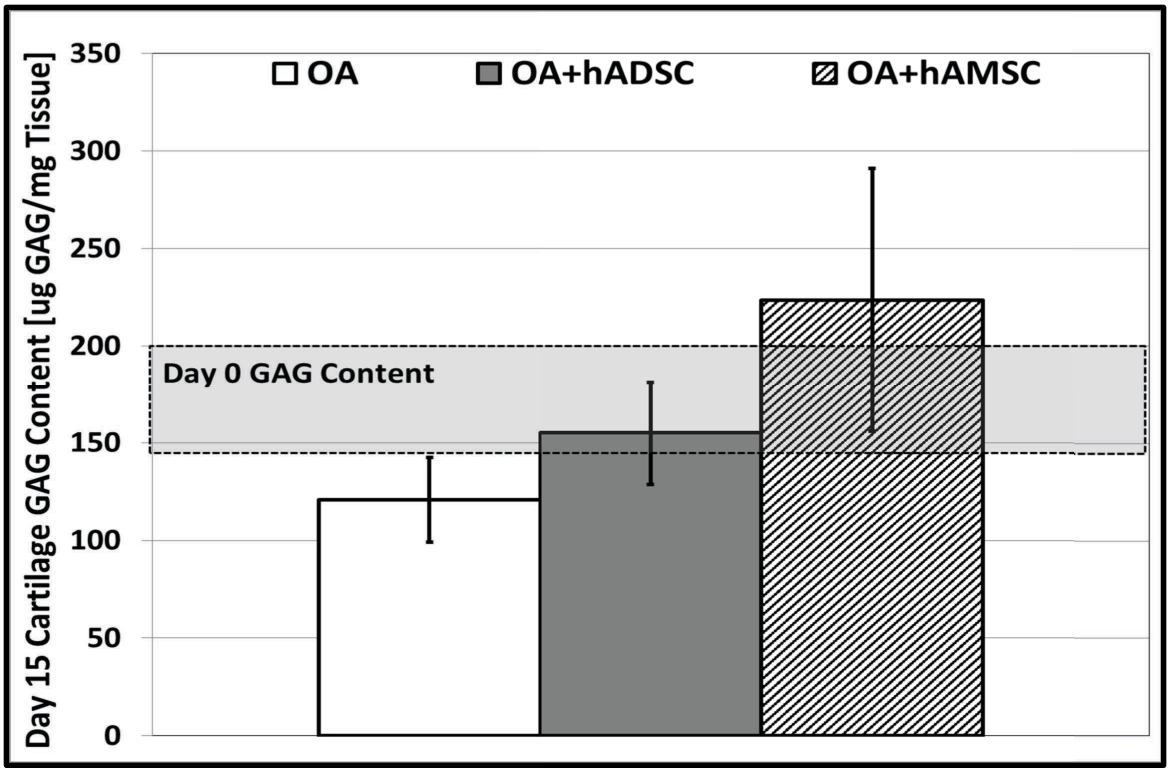


Figure 45: Average Cartilage GAG Content in hAMSC and hADSC-Treated Cultures demonstrating the ability of hAMSCs to mitigate cartilage GAG deterioration. The range of D0 values is depicted as a shaded box from 148-200µg GAG/mg dry weight tissue.

Similar to previous assessment measures, indirect culture of hAMSCs resulted in a trend toward less change in cartilage GAG content (-12.2 ± 21.0% Indirect vs. -40.2 ± 8.9% OA). However, figure 46 illustrates direct contact culture of hAMSCs resulted in a significant positive change in GAG content (30.7% ± 17.1%).

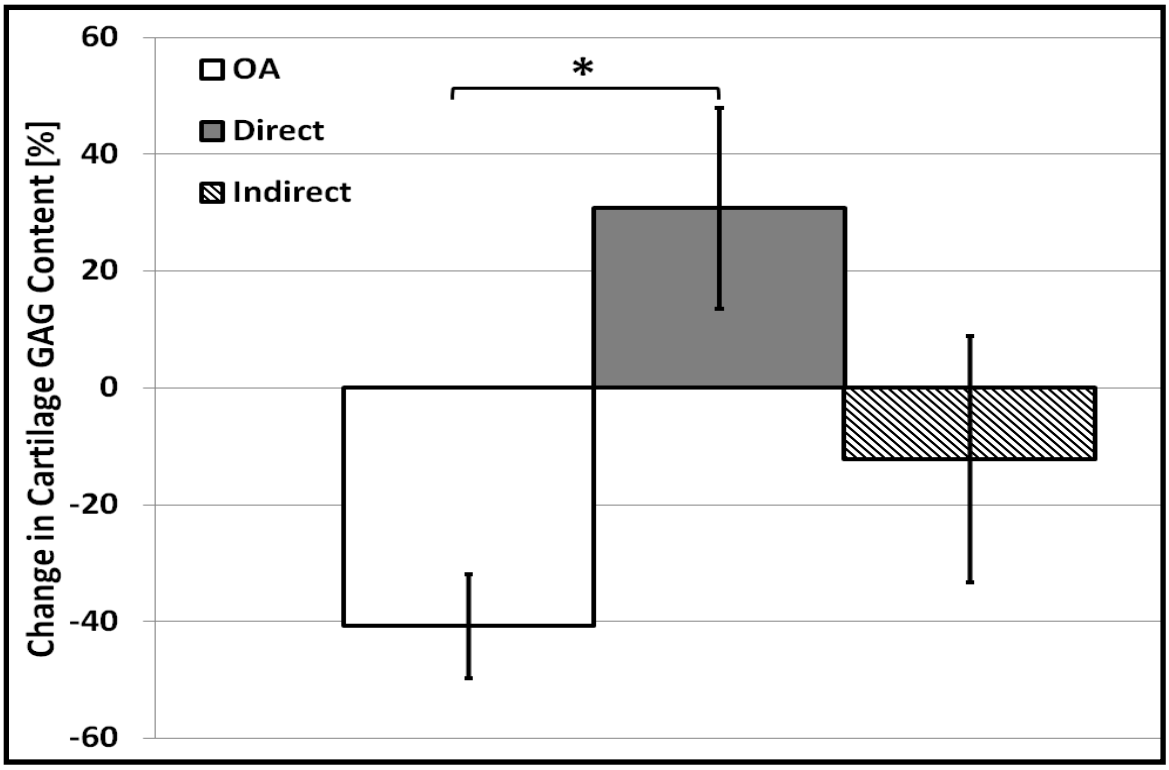


Figure 46: Average Cartilage GAG Content in Direct and Indirect-Treated Cultures demonstrating the ability of hAMSC seeded directly onto the cartilage surface to mitigate cartilage GAG deterioration.

Culture media evaluated via hydroxyproline assay revealed that OA co-cultures trended toward an increase in the amount of collagen leached from OA cartilage into the culture media (Figure 47). OA+hADSC cultures exhibited similar trends. Conversely, OA+hAMSC cultures maintained the amount of collagen leached into culture media.

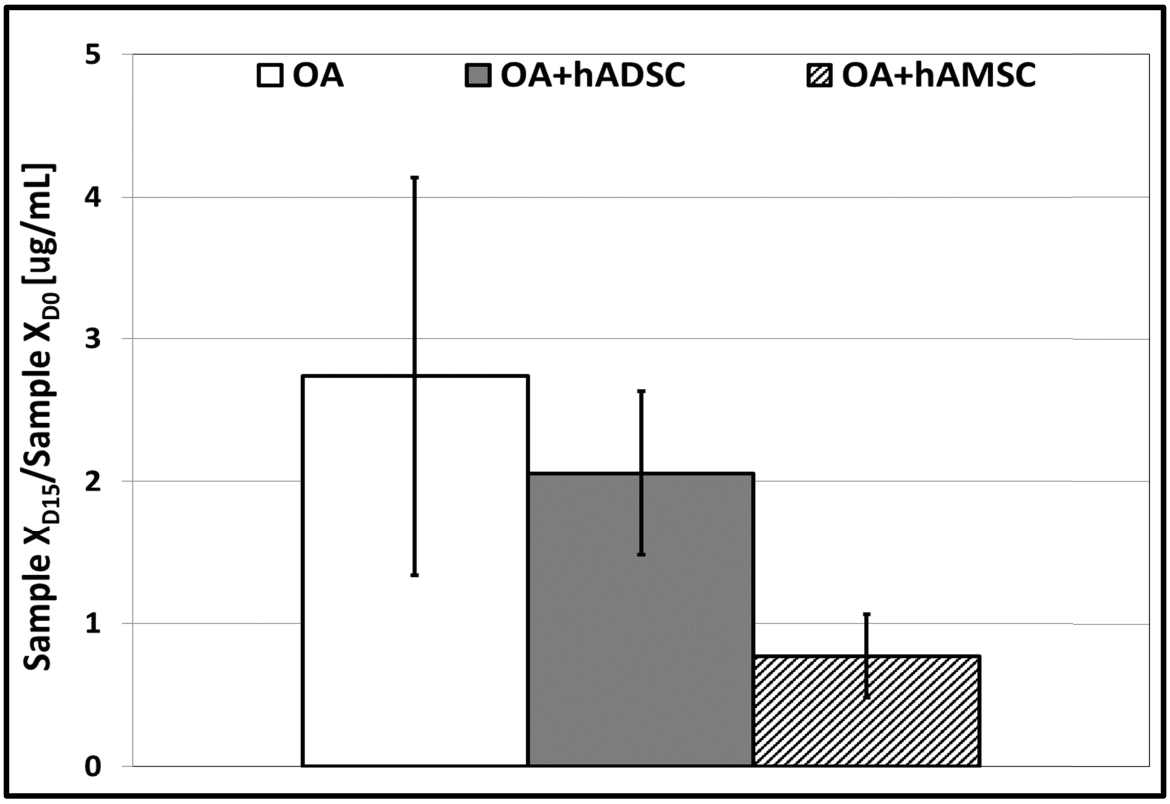


Figure 47: Average Change in Media Hydroxyproline Content in hAMSC and hADSC-Treated Cultures depicting the relative increase in collagen leached from OA cartilage into the culture media in OA control and OA+hADSC groups, while hAMSC treated groups remained constant.

Culture media evaluated via hydroxyproline assay revealed that the ability of hAMSCs to halt the progression of collagen leaching from cartilage appears to be dependent on the route of administration (Figure 48). While indirect cultures exhibited no change in the amount of collagen released into culture media, direct cultures demonstrated a trend towards decreased collagen released into culture media.

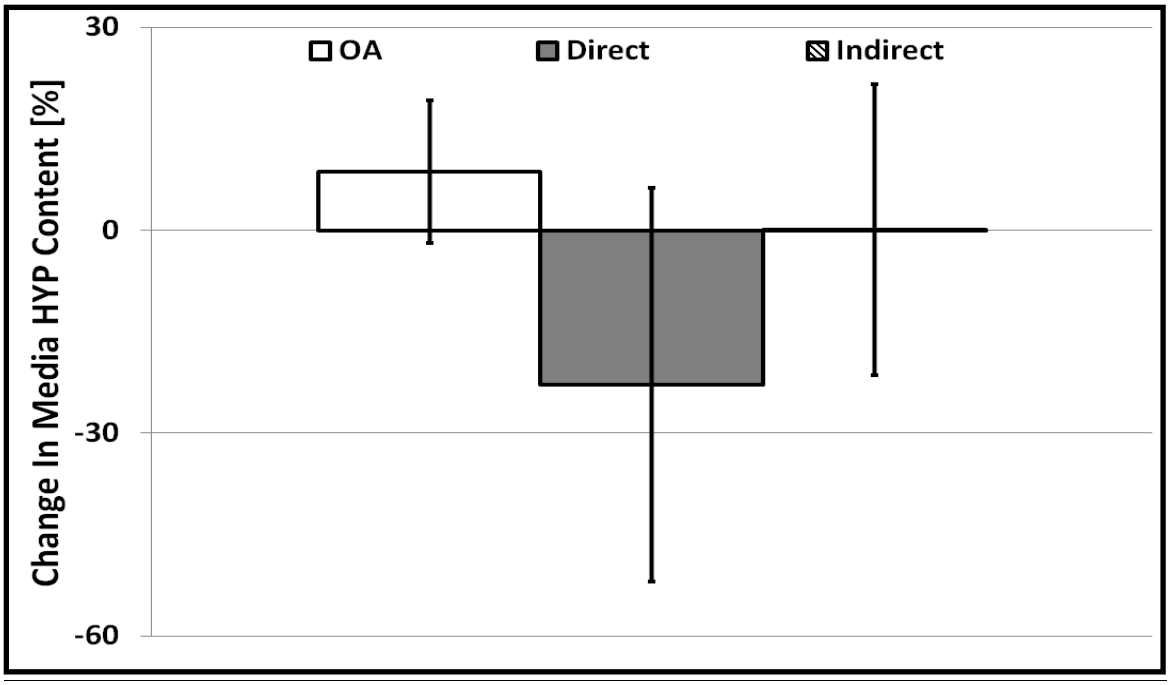


Figure 48: Average Change in Media Hydroxyproline Content in Direct and Indirect-Treated Cultures depicting the relative increase in collagen leached from OA cartilage into the culture media in OA control and OA+hADSC groups, while hAMSC treated groups remained constant.

5.3.6. Pro-Inflammatory Profile Assessment

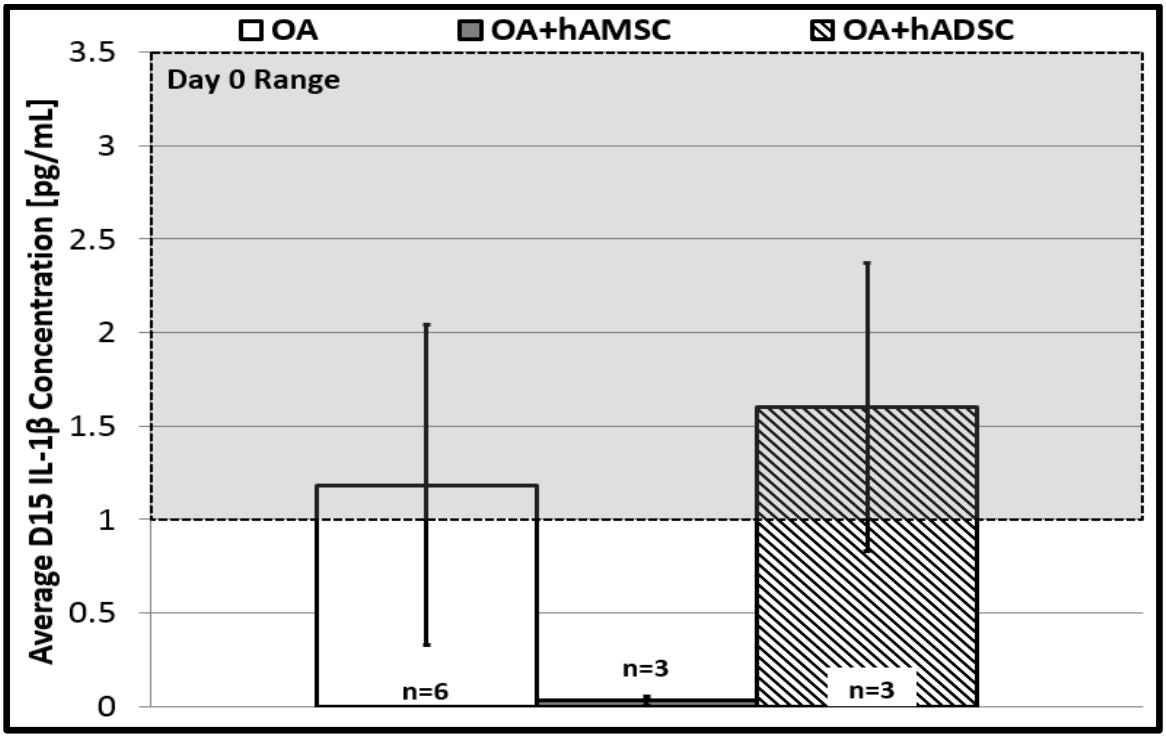


Figure 49: IL-1β Analysis in hAMSC and hADSC-Treated Cultures depicting the average IL-1β concentrations in cell culture media from each study group. (Minimum detection limit: 0.3 pg/mL).

Figure 49 illustrates a trending decrease in IL-1β concentrations in OA+hAMSC treated cultures, while all other cultures remained constant. Figure 50 depicts the relationship between IL-1β concentration and route of administration. The ability of hAMSCs to quell IL-1β secretion appeared to be dependent on the route of administration.

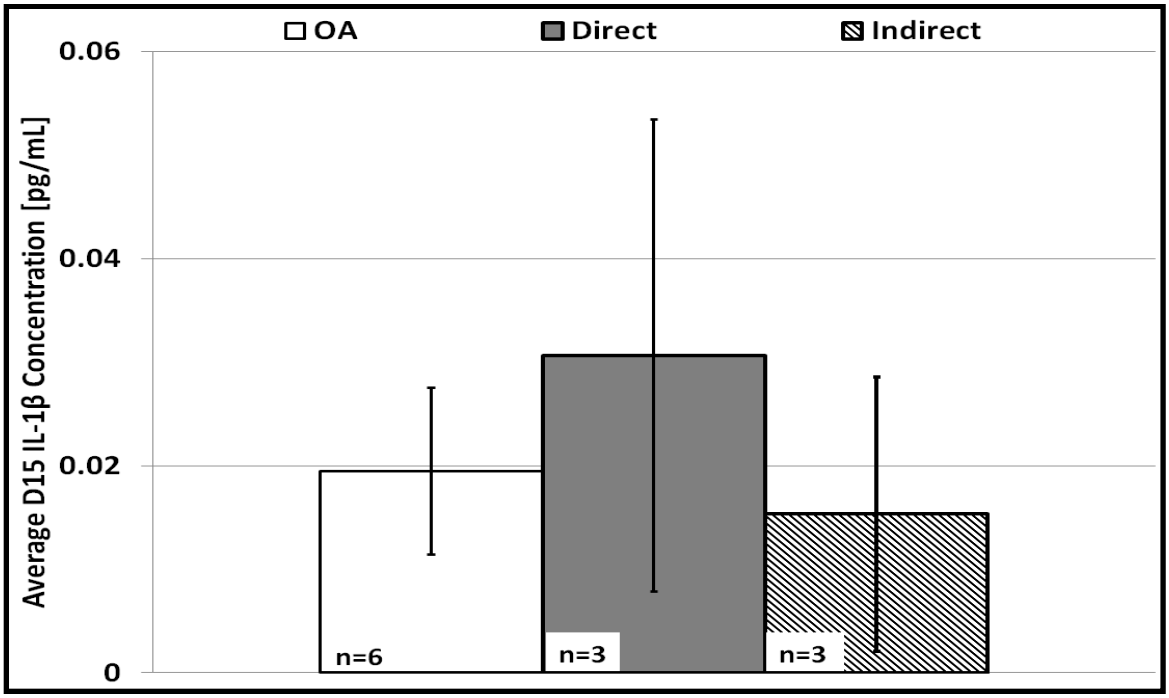


Figure 50: IL-1β Analysis in Direct and Indirect-Treated Cultures depicting the average IL-1β concentrations in cell culture media from each study group. (Minimum detection limit: 0.3 pg/mL).

While TNF-α concentrations remained constant in OA co-cultures, OA+hAMSC and OA+hADSC cultures trended towards reduced concentrations (figure 51).

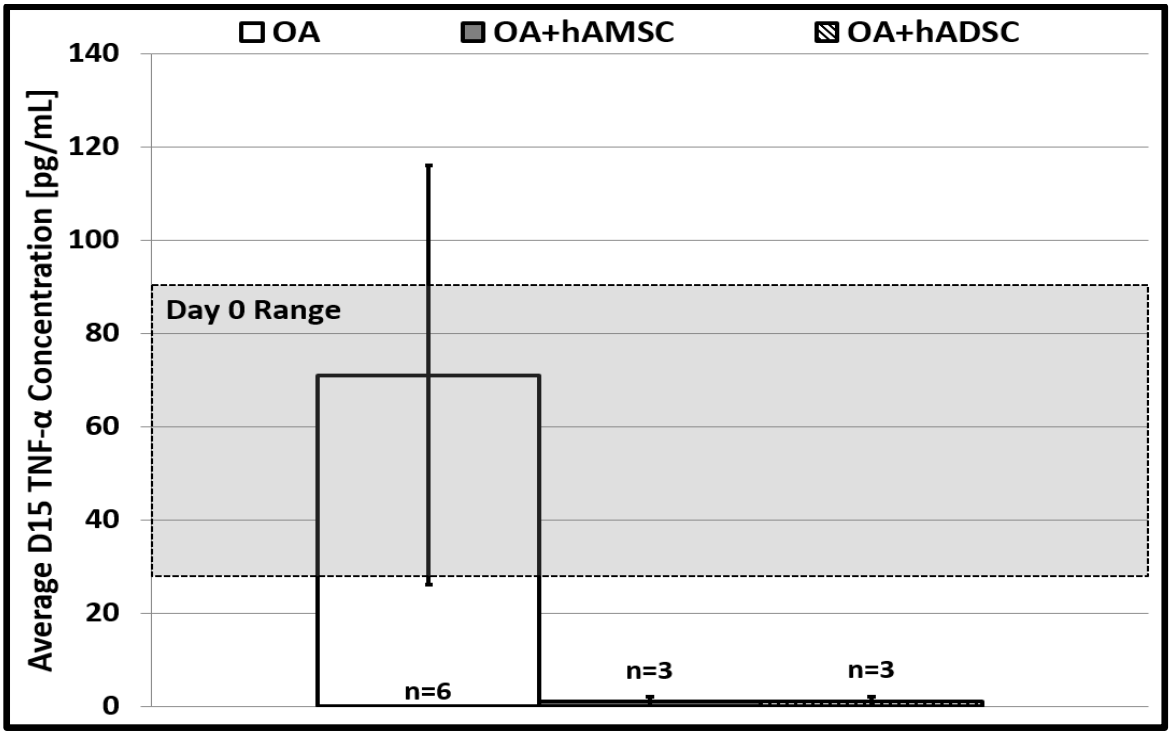


Figure 51: TNF-α Analysis in hAMSC and hADSC-Treated Cultures depicting the average TNF-α concentrations in cell culture media from each study group. (Minimum detection limit: 6pg/mL).

Direct and indirect administration of hAMSCs accomplished this equally effectively.

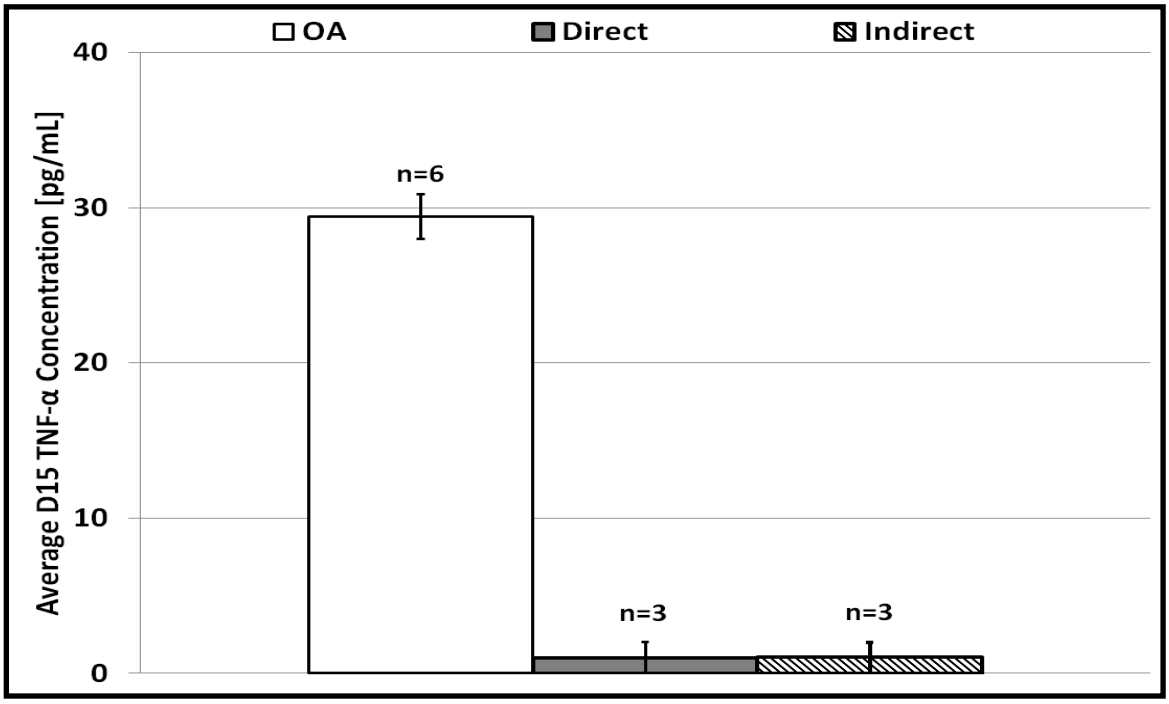


Figure 52: TNF-α Analysis in Direct and Indirect-Treated Cultures depicting the average TNF-α concentrations in cell culture media from each study group. (Minimum detection limit: 6pg/mL).

MMP-13 ELISA performed on cell culture media revealed that over 15 days in culture, there were no significant changes in MMP-13 media concentration. However, the stem cell treated groups exhibited trends towards decreased MMP-13 concentrations (Figure 53).

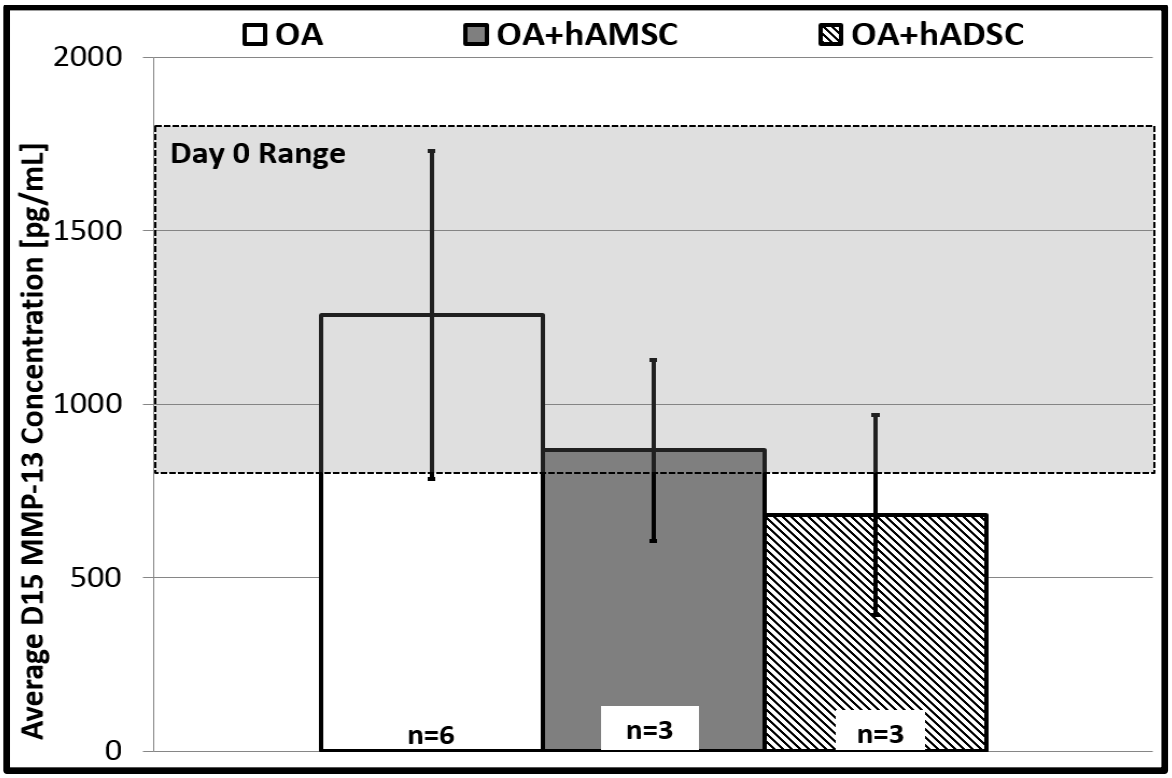


Figure 53: MMP-13 Analysis in hAMSC and hADSC-Treated Cultures depicting the average MMP-13 concentrations in cell culture media from each study group. (Minimum detection limit: 30pg/mL).

Analysis of direct and indirect hAMSC cultures showed no significant changes in MMP-13 concentrations. However, the direct group did exhibit a trend towards decreased MMP-13 while the indirect group exhibited no change compared to OA controls (Figure 54).

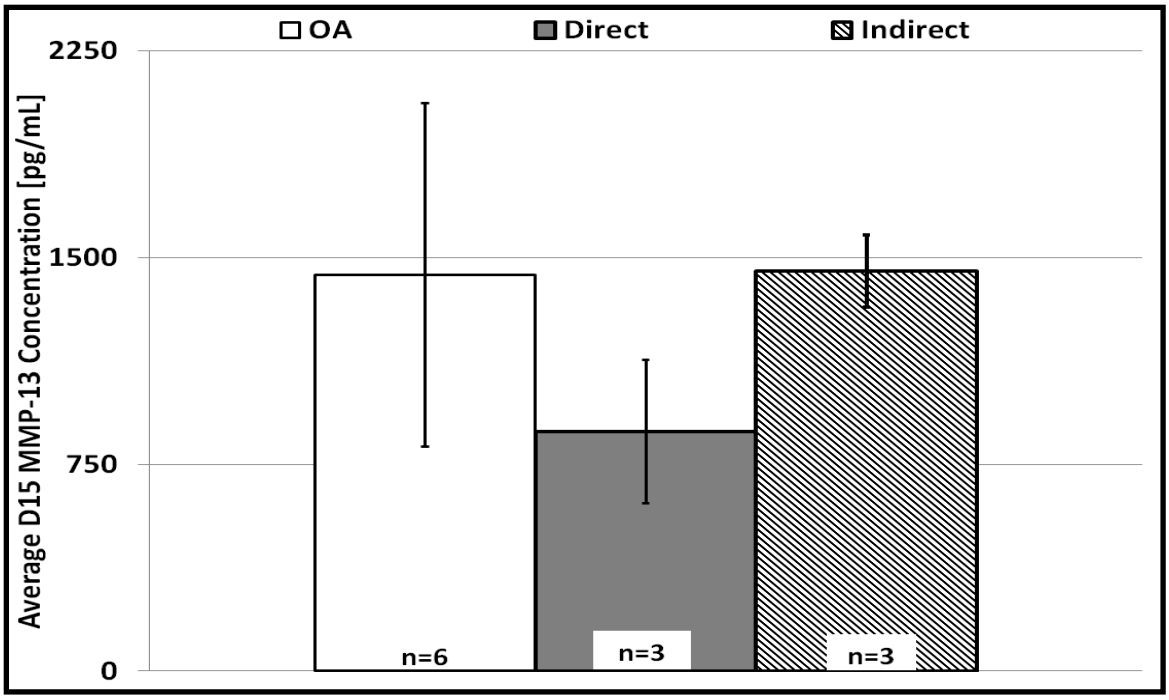


Figure 54: MMP-13 Analysis in hAMSC and hADSC-Treated Cultures depicting the average MMP-13 concentrations in cell culture media from each study group. (Minimum detection limit: 30pg/mL).

In addition to these ELISA data, a comprehensive cytokine array was completed (see appendix B for complete results). Most notably, this showed statistical increases in anti-inflammatory marker IL-13 in OA+hADSCs cultures and anti-inflammatory marker IL-4 in OA+hAMSC cultures.

5.4 Discussion

With the evolving view of OA as an inflammatory condition driven by macrophages and their pro-inflammatory secretions, immunomodulatory therapies have come to the forefront of investigation. Due to their trophic, anti-inflammatory and immunomodulatory properties, stem cells may prove to be uniquely suited agents for OA therapies.[11,12,84,134] In fact pre-clinical animal studies and human trial results support OA mitigation through the intra-articular injection of stem cells.[12,120]

Though two specific stem cell sources have been widely investigated as a potential OA therapeutic, there are drawbacks associated ADSCs and bone marrow derived stem cells (BMSCs) that are less attributable to alternative stem cell sources (such as perinatal stem cells), including 1) the inability of BMSCs isolated from OA patients to proliferate and differentiate as effectively as BMSCs from healthy donors, 2) low cell yields and 3) painful harvest procedures.[11,12,84]

Conversely, perinatal stem cells exhibit significant advantageous qualities including, 1) high cell yields, 2) ontogenetically youthful status limiting their exposure to detrimental age-related changes and 3) superior chondro-protective effects in an inflammatory environment, exhibiting the ability to induce a pro-regenerative (M2) phenotype within synovial macrophages.[68,73,87,221] Significantly, our data further supports this ability of hAMSCs (but not hADSCs).

It has been demonstrated that stem cells must be "primed" in order to exhibit anti-inflammatory and immunomodulatory characteristics.[134,214] Typically this involves the stimulation of stem cells through potent pro-inflammatory cytokines such as INF-γ.[84,214] Though the exact timeline has not been established, the need for priming does represent an inherent delay in the efficacy of OA stem cell therapies. This could be one explanation for delays in patient progress post initiation of stem cell therapy. Interestingly, it has been demonstrated that perinatal stem cells do not require priming prior to initiating therapeutic benefit; this could explain why hAMSCs appear to more effectively mitigate OA progression in our investigation. Additionally, it has been demonstrated that reductions in both IL-1β and TNF-α are necessary to mitigate OA disease progression.[225] Only hAMSCs are able to elicit reduction in both cytokines. IL-13, IL-10 and IL-4 induce

an M2 phenotype within macrophages.[256,257] IL-13 and IL-4 have demonstrated the ability to mitigate cartilage degradation in vitro.[258] While IL-4 and IL-10 have direct effects on chondroprotection through the down regulation of ADAMTSs and MMPs, respectively, IL-13 produces an indirect anti-inflammatory effect through the regulation of PGE_2.[259] There is limited data providing insight into the relative efficacy of IL-4 and IL-13 therapeutic strategies. However, this mechanistic insight seems to offer a potential explanation for the heightened and more rapid mitigation of OA observed in hAMSC-treated cultures. Please see appendix B for more detailed mechanistic insight into stem cell mode of action (accomplished via a comprehensive cytokine analysis).

The intra-articular administration of stem cell therapies delivers stem cells into the joint space, likely resulting in a mixed contact scenario where only some stem cells remain in direct contact with the cartilage. Interestingly there is evidence suggesting stem cells do not tend to stay in direct contact with the cartilage; they home to the synovium[140], furthering the need for researchers to understand the potential mechanistic differences and differential therapeutic effects of both treatment modalities. While stem cells likely generate therapeutic benefit via the secretion of immunomodulatory mediators (thus not requiring direct contact with cartilage)[11,120], potential advantages of direct contact with the damaged cartilage have yet to be established. Interestingly, indirect contact co-culture of hAMSCs (which as figure 54 illustrates may actually be in direct contact with synovium via porosity in the trans-well insert) seem to exhibit similar mitigation of synovial pro-inflammatory M1 macrophages, though this does not translate into an increased chondro-protective effect in the timeframe studied. OA has increasingly become understood as a multi-focal disease, where both the cartilage and the synovium

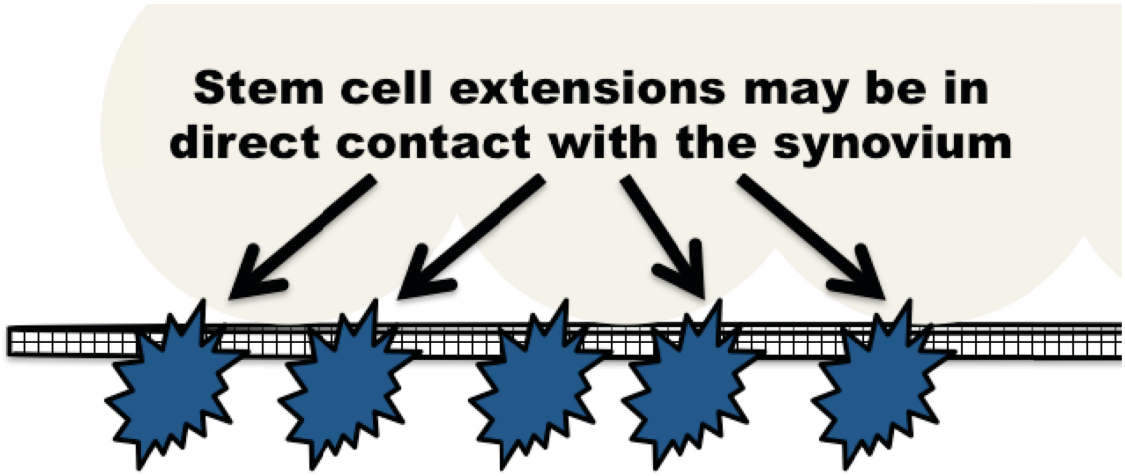


Figure 54: Indirect Contact hAMSCs May be in Direct Contact with Synovium due to the porosity of the insert being large enough to allow passage of extension but not entire cells.

exhibit key surface receptors (toll-like receptors) fueling the pro-inflammatory activation pathways characteristic of OA progression.[12,125,135,260] While the exact etiology of OA is still largely unknown, it is generally assumed that cartilage injury (as opposed to synovial inflammation) initiates the propagation of OA.[125,135] If true, it could be argued that therapies targeting the source of OA pathogenesis, the cartilage, would be most efficacious. Our results seem to indicate that such a targeted approach would be therapeutically beneficial.

In conclusion, we were able to successfully demonstrate that perinatal stem cells are capable of mitigating disease progression in an explant co-culture model of OA. Moreover, perinatal stem cells appear to more effectively mitigate OA disease progression compared to a commonly employed stem cell, hADSCs. The observed reduction in M1 macrophage populations could be a primary mechanism explaining the halt of OA progression observed in stem cell treated groups. Likewise, the increase in M2 macrophage populations observed in hAMSC treated groups could be a primary mechanism explaining the enhanced ability of hAMSCs to mitigate OA progression.

Significantly, both direct and indirect contact co-culture of hAMSCs with human OA cartilage seem to result in the mitigation of OA progression. However, direct contact co-culture with human OA cartilage seems to result in enhanced chondro-protective effects, exhibited by enhanced chondrocyte viability, maintenance of cartilage GAG content and trending reductions in the concentrations of cartilage matrix degrading enzymes and collagen leaching. Taken together, these results (and those highlighted in Appendix B) highlight the utility of hAMSCs for OA regenerative medicine approaches and provide significant mechanistic into their clinical use.

CHAPTER SIX

TO COMPARE THE THERAPEUTIC EFFECTS OF hAMSCs AND hADSCs TO ATTENUATE OA PROGRESSION IN VIVO

6.1 Introduction

With the evolving view of OA as an inflammatory condition driven by macrophages and their pro-inflammatory secretions, immunomodulatory therapies have come to the forefront of investigation. Due to their trophic, anti-inflammatory and immunomodulatory properties, stem cells may prove to be uniquely suited agents for OA therapies.[11,12,84,87,134,202]

Numerous stem cell therapies have been described in animal models of OA. The stem cells are typically delivered via intra-articular injection into the knee joint. In comparison studies, stem cell therapies have been shown to yield better outcomes than autologous chondrocyte transplantation.[12] There is also evidence that the stem cells are still located within guinea pig joint tissues after 1 week[120,170] and up to 8 weeks post-implantation in rats.[120] There are also reports of the stem cells exhibiting signs of proliferation and differentiation. [120,170]

Preliminary results from our group support the enhanced mitigation of OA by amnion compared to adipose stem cell treated groups, ex vivo (chapter V). However, to date amniotic stem cells have yet to be tested in vivo as an OA therapeutic. Additionally, no direct comparative analyses have been conducted investigating the relative therapeutic potential of amniotic stem cells with current clinically investigated stem cell sources (i.e. bone marrow and adipose tissue).

Herein, we describe efforts undertaken in order to determine: 1) if amniotic mesenchymal stem cells are capable of mitigating OA progression in vivo and 2) if this therapeutic approach is at least as beneficial as those utilizing adipose derived stem cells.

6.2 Materials & Methods

hADSCs were purchased from Invitrogen (R7788-110). Trypsin was purchased from Fisher scientific (MT-25-053CI). Collagenase was purchased from Worthington Biochemicals (LS004196). Guinea pigs were purchased from Charles River RMS. Pharmaceutical grade HA was purchased from Lifecore Biomedical (HA700K-1). Indian Ink was purchased from Dr. Martin's Bombay.

6.2.1 Stem Cell Preparation

Human placentas were obtained from consenting patients immediately following delivery via elective cesarean sections of full-term babies (Pro00031185-Greenville Health System). Amniotic membrane derived cells were isolated within 4 hours of delivery. hAMSCs were isolated as previously described (chapter III). Briefly, placentas were placed with the umbilical cord facing upward such that the fetal (amniotic) surface was accessible. The amniotic and chorionic membranes were identified and mechanically peeled from each other. Amnions were digested twice in 0.25% trypsin for 30 minutes at 37°C with agitation to completely liberate the epithelial layer followed by complete digestion in two digestions of collagenase [2mg/mL collagenase (249 U/mg)] for 30 minutes at 37°C with agitation each to subsequently liberate hAMSCs. hAMSCs were expanded until p2 in standard culture medium (DMEM+10%FBS+1%AB/AM), with the media changed every 3 days.

hADSCs were purchased from a commercial vendor and expanded until p2 according to manufacturer's instructions, with the media changed every 3 days.

At p2, all cell types were suspended in sterile HA in order to obtain $1x10^6$ viable stem cells/100μL HA aliquot. HA suspensions were loaded into 21g syringes in preparation for injection.

6.2.2 Stem Cell Intra-Articular Injections

As illustrated in figure 55, n=24 guinea pigs were allowed 14 days upon arrival to our facilities for acclimation. According to approved animal used protocols, at day 0 n=6 guinea pigs were used to practice injections. Briefly, a HA+hAMSCs suspension was injected into the left stifle and a saline injection was administered into the right stifle of these guinea pigs. Ultrasound guidance was utilized similar to methods described by Vazquez et al.[1] Briefly, stifles were placed in flexion, the ultrasound probe was covered with gel and applied to the leg surface near the patella. A 21-gague syringe (loaded HA+hAMSCs) was inserted posterior to the medial edge of the patellar ligament. After initial insertion, the leg was placed in extension to facilitate visualization of the intra-articular space. The needle was inserted until it was in direct contact with the femur, and the contents of the syringe were injected.

Additionally, at day 0 our experimental group underwent their first round of injections. The left stifle received an injection of either an HA+hAMSC suspension, an HA+hADSC suspension or HA alone (the HA group was included to ensure the HA itself did not offer significant therapeutic benefit, confounding our results). Notably, the passage (2), number of stem cells ($1x10^6$/injection) and injection volume (100μL)

remained constant between groups. The right stifle received a saline injection; these limbs served as terminal time point OA controls.

After 7 days, the set of 6 practice guinea pigs were euthanized and their saline injected limbs served as day 0 degenerative controls. After 1.5 months, the remaining guinea pigs (n=18) were administered a second injection according to the methods previously described. Finally after 3 months, the guinea pigs were euthanized. Outcome measures for this study included macro and micro surface architecture and biochemical assessments of the cartilage; as well as synovial inflammation.

6.2.3 Cartilage Surface Macro Architecture

Formalin fixation (48 hours) was followed by 7 days in decalcification solution. After 3x washing in PBS, joints were dissected until the femoral condyles and tibial plateaus were easily accessible. Approximately 100μL of 20% vol/vol solution of Indian Ink in PBS was applied to the exposed cartilage surfaces, excess liquid was blotted away and macroscopic images were taken.

6.2.4 Cartilage Surface Micro Architecture

Guinea pig stifles were secured within a tissue processing cassette, fixed in 10% phosphate buffered formalin overnight at room temperature prior to undergoing standard tissue processing, decalcification, paraffin embedding and sectioning at 8 μm thickness. Sections were stained with Safranin-O counterstained with Fast Green for visualization of proteoglycan rich cartilage matrix. Briefly, rehydrated sections were differentiated in 1% acid alcohol for 2 seconds prior to room temperature incubation in 0.02% Fast Green for 2.5 minutes. After 30 second incubation in 1% Acetic acid, sections were stained with 1% Safranin-O for 15 minutes.

6.2.5 Assessment of Cartilage Degradation

As depicted in figure 56, stifles for biochemical analysis were removed from the guinea pig. The femur and tibial surfaces were identified, and these surfaces were divided into their medial and lateral components. The cartilage was then removed using a curette. As guinea pigs primarily load the medial compartment of their joint; this area is most susceptible to OA. To ensure results were not skewed by the tibial portions, assessments were completed in quartered sections. The results from the medial tibial surface, which is the surface most prone to OA in this model, are described herein.

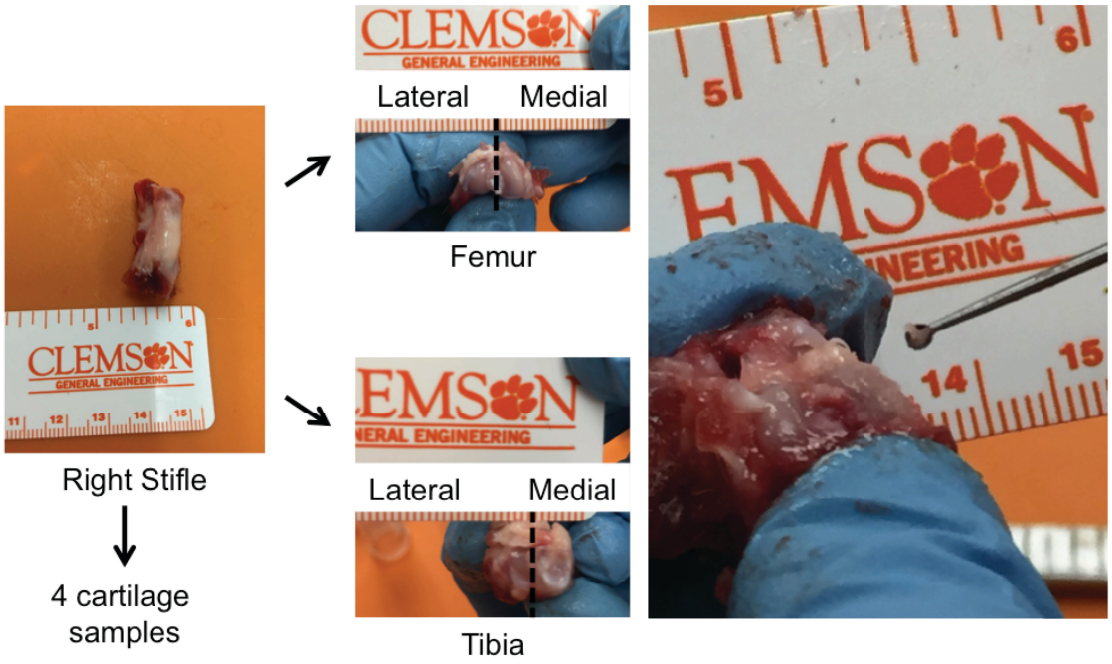


Figure 56: Methods Schematic of Cartilage Harvest for biochemical evaluation. Stifles were dissected to expose the femoral and tibial surfaces. These surfaces were further divided into medial and lateral components. Only results from the medial tibial surfaces are reported, as this is the area most prone to OA development in this animal model.

6.2.5.1 Cartilage Glycosaminoglycan (GAG) Content

Lyophilized cartilage tissues were digested in 125µg/mL Papain in PBE Buffer, pH 7.5 overnight at 65°C. Tissue digests were assessed for GAG content via Dimethylmethylene Blue Assay (DMMB). Briefly, 200µL DMMB reagent (46µg

DMMB, 40mM Glycine, 40mM NaCl, pH 3) was added to 50μL samples. Standards were created using a 1mg/mL stock solution of Chondroitin-6-Sulfate. Sample absorbance was read at 525nm.

6.2.5.2 Collagen Leaching to Culture Media

Cartilage was assessed for hydroxyproline collagen content via Hydroxyproline Assay Kit according to manufacture instructions. Briefly, samples were hydrolyzed with hydrochloric acid at 120°C for 3 hours prior to well plate transfer. Wells were evaporated to dryness at 60°C. Equal amounts of Chloramine T/Oxidation Buffer Mixture and Diluted DMAB Reagent followed by 5-minute room temperature and 90-minute 60°C incubations, respectively. Standards were created using a 1mg/mL stock solution of Hydroxyproline Standard Solution. Sample absorbance was read at 560nm.

6.2.6 Histological Confirmation of Synovitis

Synovial sections were secured within a tissue processing cassette, fixed in 10% phosphate buffered formalin overnight at room temperature prior to undergoing standard tissue processing, paraffin embedding and sectioning to 8 μm thickness. Sections were stained with Hematoxylin and Eosin (H&E) for visualization of cellularity and membrane hypertrophy.

6.2.7 Microscopic Imaging

Images were captured on a Zeiss Axiovert.A1 microscope with Axiovision software (Release 4.9.1 SP08-2013).

3.2.8 Statistical Analysis

Results are represented as a mean ± standard error of the mean (SEM). All statistical analyses were performed by two-tailed Student's t-test of unequal variance or

analysis of variance (ANOVA) with a Tukey's post-hoc analysis. Significance was defined in all cases as $p<0.05$.

6.3 Results

6.3.1 Stem Cell Preparation

Stem cell populations were successfully expanded and prepared for intra-articular injection.

6.3.2 Stem Cell Intra-Articular Injections

Stem cell suspensions were successfully injected into the intra-articular space of guinea pig stifles. See appendix A for more detailed information on these confirmation assessments.

6.3.3 Cartilage Surface Macro Architecture

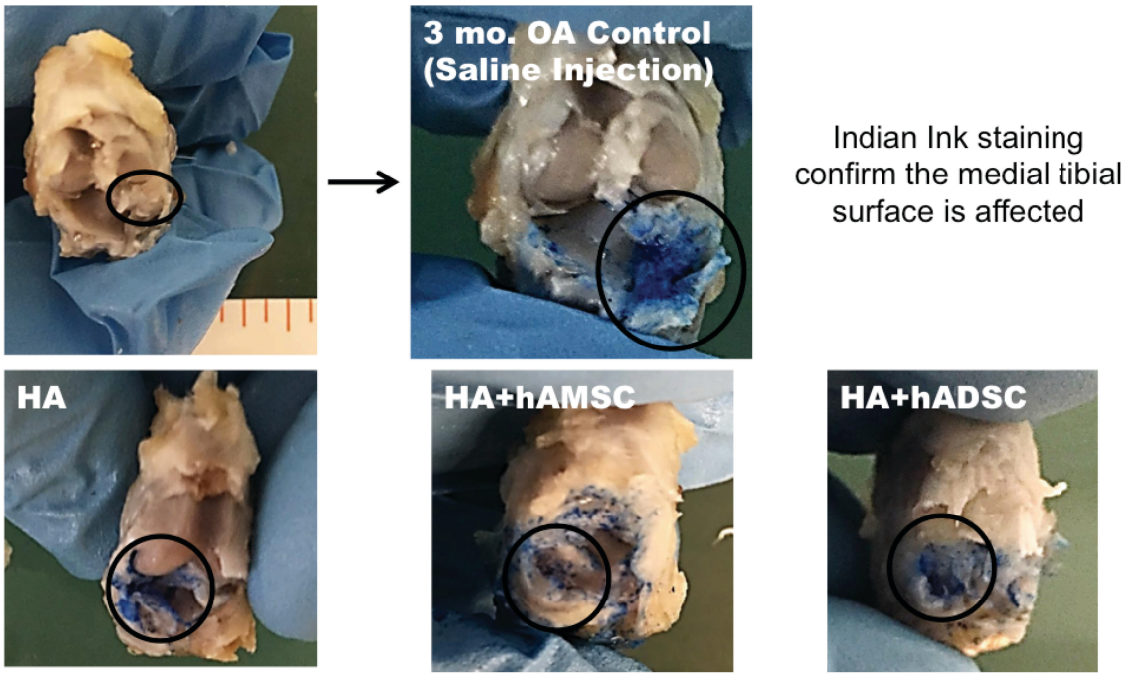


Figure 57: Cartilage Surface Macro-Architecture demonstrating medial tibial surface erosion in 3-month saline and HA injected groups. Varying amounts of surface fibrillation/erosion is noted within stem cell treated groups.

Figure 57 illustrates cartilage surface macro architecture assessments. A macroscopic image of a stifle with the femoral condyles exposed on the top and the tibial surface exposed on the bottom is presented in the top left of figure 57. The creamy and shiny quality of the cartilage is easily noticeable; it is present everywhere except over the medial tibial surface, which is highlighted by the black circle.

Indian ink stains bone as well as fibrillation of the cartilage surface. After 3 months, the medial tibial surface of saline injected stifles, which macroscopically looked quite eroded, stains deep blue (figure 57). The HA group exhibited similar intense blue staining on the medial tibial surface (figure 57). However, the stem cell treated groups showed varying evidence of surface fibrillation and/or erosion (figure 57).

6.3.4 Cartilage Surface Micro Architecture

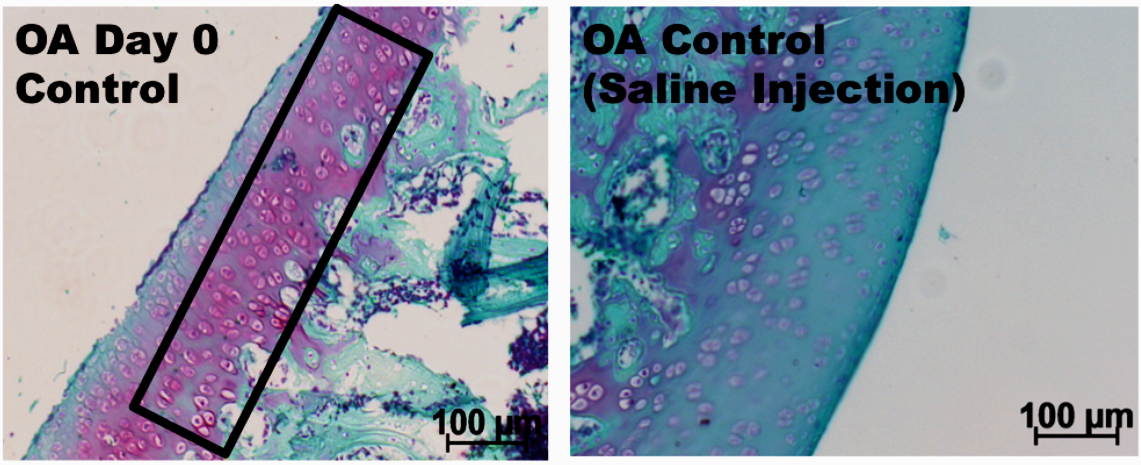


Figure 58: Cartilage Surface Micro Architecture of Controls, indicating high cartilage proteoglycan content at day 0. However by 3 months, the proteoglycan content has been largely degraded.

At day 0, there was a large volume of proteoglycan rich matrix (figure 58). However, after 3 months this rich proteoglycan matrix appeared to have degraded away (figure 58). Comparatively the HA group showed similar if not lower proteoglycan content (compared to 3-month saline injected controls). While stem cell treated groups

showed at least comparable if not more proteoglycan rich matrix (figure 59).

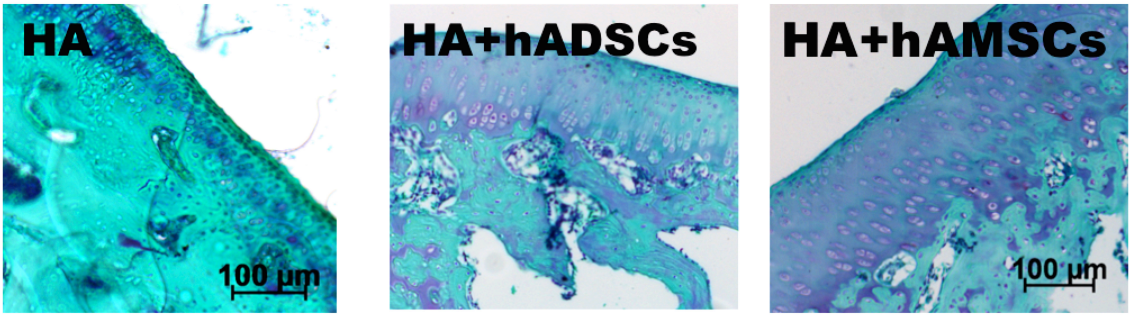


Figure 59: Cartilage Surface Micro Architecture of Experimental Groups, indicating low proteoglycan content in HA-treated groups. However, stem cell treated groups show at least equivalent levels of proteoglycans compare to controls.

6.3.5 Assessment of Cartilage Degradation

hAMSC treatment resulted in significantly greater ($p<0.05$) cartilage GAG content compared to our untreated and HA controls (figure 60).

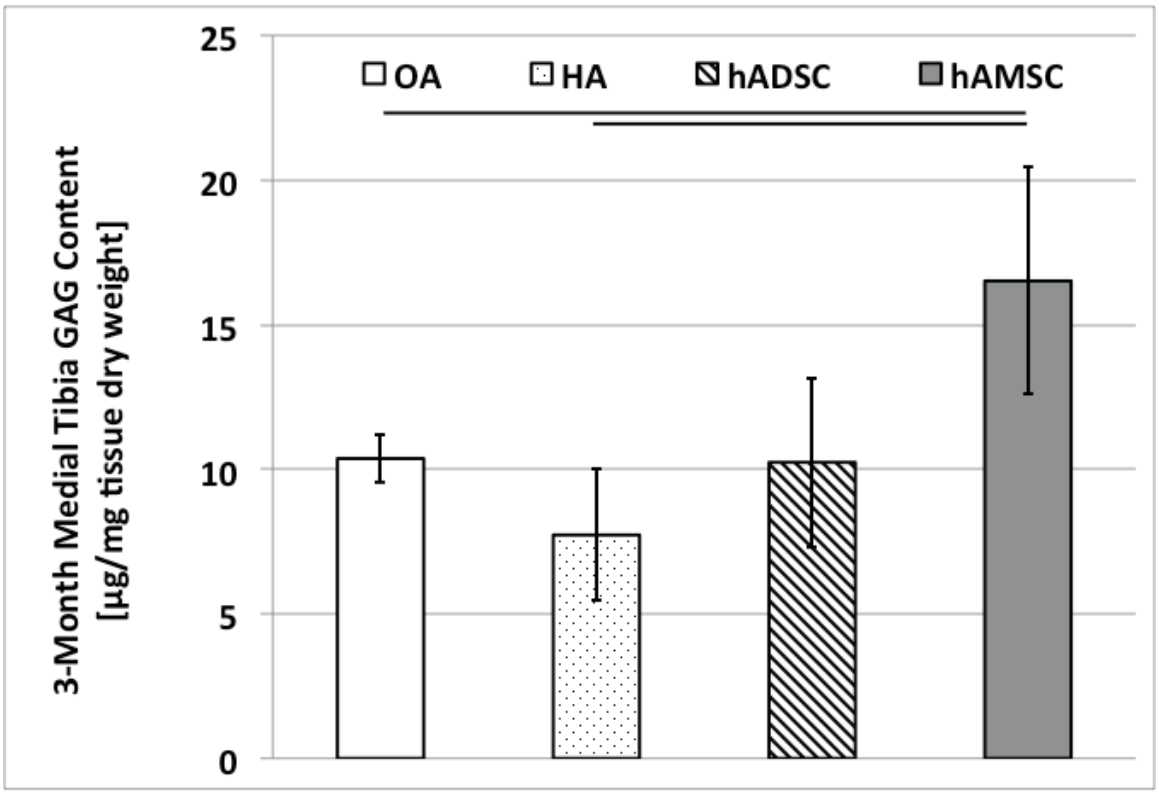


Figure 60: Cartilage GAG Content in all guinea pig groups. hAMSC-treated groups exhibit the highest cartilage GAG content after 3 months.

Additionally, hAMSC treatment resulted in significantly greater ($p<0.05$) cartilage collagen content compared to HA controls (figure 61).

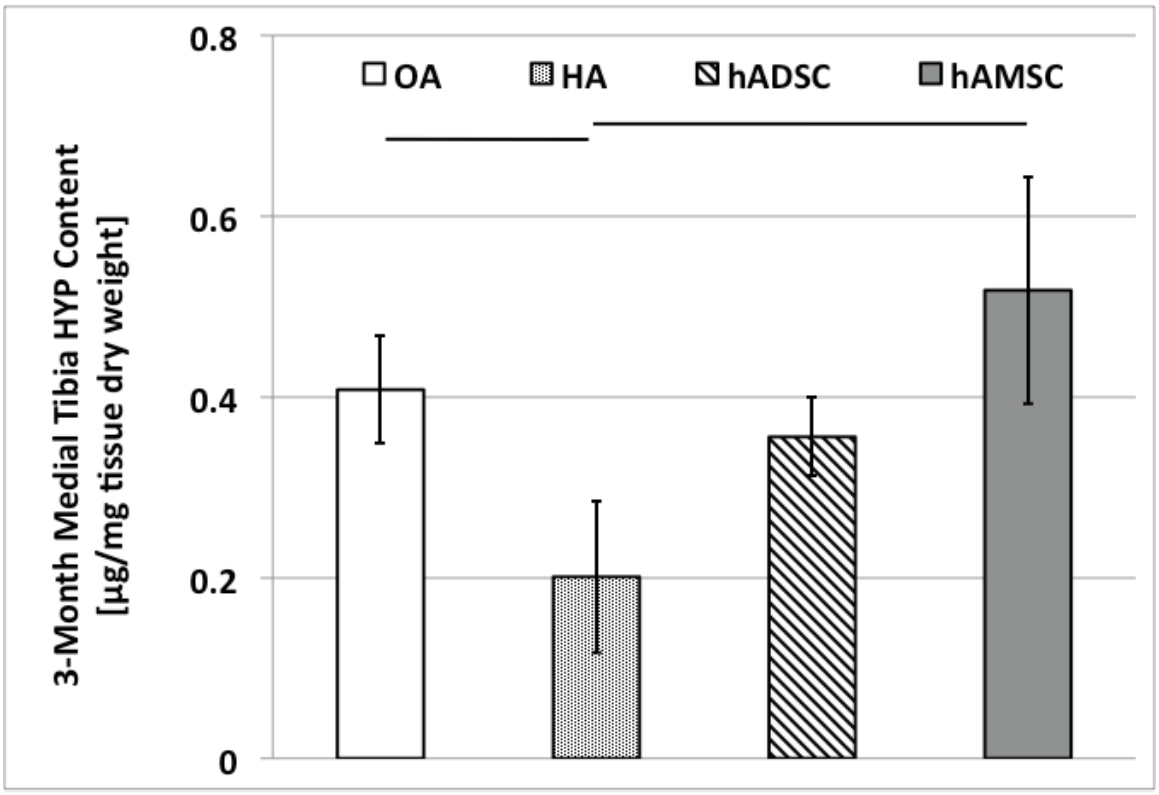


Figure 61: Cartilage Collagen Content in all guinea pig groups. hAMSC-treated groups exhibit the highest cartilage collagen content after 3 months.

6.3.6 Histological Confirmation of Synovitis

Figure 62 shows H&E staining of synovium sections for assessment of synovitis. Beginning with day 0 controls, there is intense cellularity near the lining; therefore the lining does exhibit hyperplasia, with an addition 4+ layers of cells than what would typically be expected in the synovial lining (this is highlighted by the black boxes). After 3 months, the non-treated group (this was the group which received saline injections) showed similar cellularity and membrane hypertrophy; indicating at least equivalent levels of synovitis over this 3-month period. After 3 months, the HA group also showed

high cellularity and extensive membrane hypertrophy, likely indicating the HA treatment had no therapeutic, immunomodulatory effect. Conversely, after 3 months the hAMSC treated group showed moderate cellularity with less hypertrophy. Like the hAMSC group, the hADSC group appeared to contain less cells along the synovial lining.

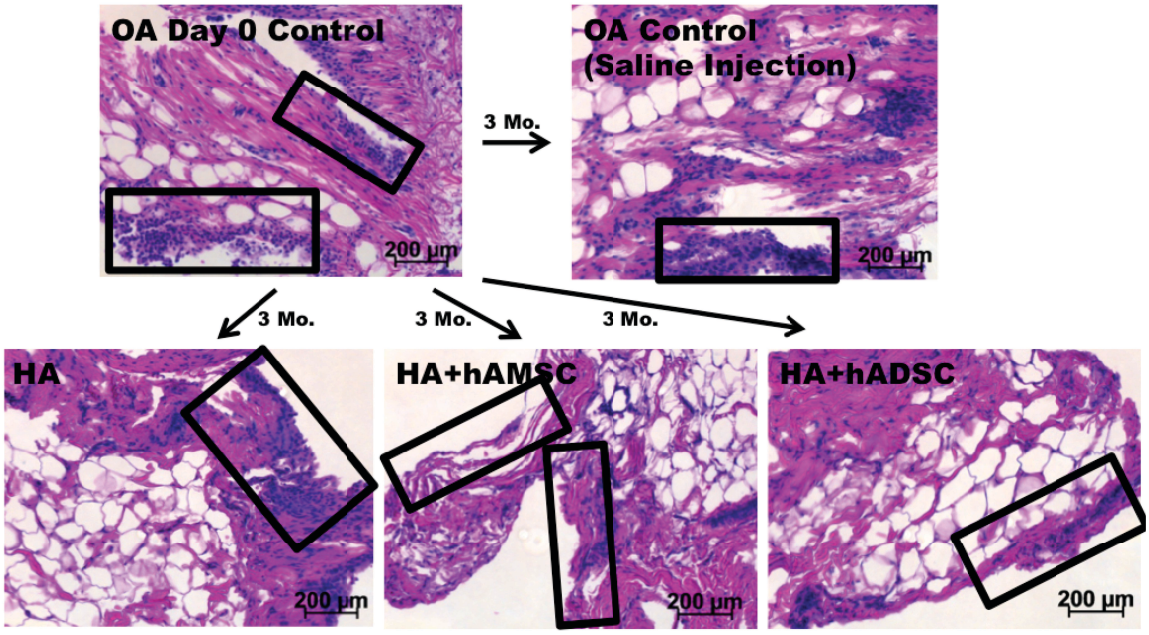


Figure 62: Histological Assessment of Synovitis demonstrating trends between control (untreated) stifles and treated stifles with time. Black boxes highlight the synovial lining.

3-month saline injected right stifles (pictured along the top in figure 63) were then compared with their subject matched 3-month treatment injected left stifles (pictured along the bottom of figure 63). Synovitis appeared to progress in the HA-treated group. The hAMSC treated group appeared to show improvement in both cellularity and membrane hyperlasia. Lastly, the synovitis in hADSC treated guinea pigs appeared to be the same.

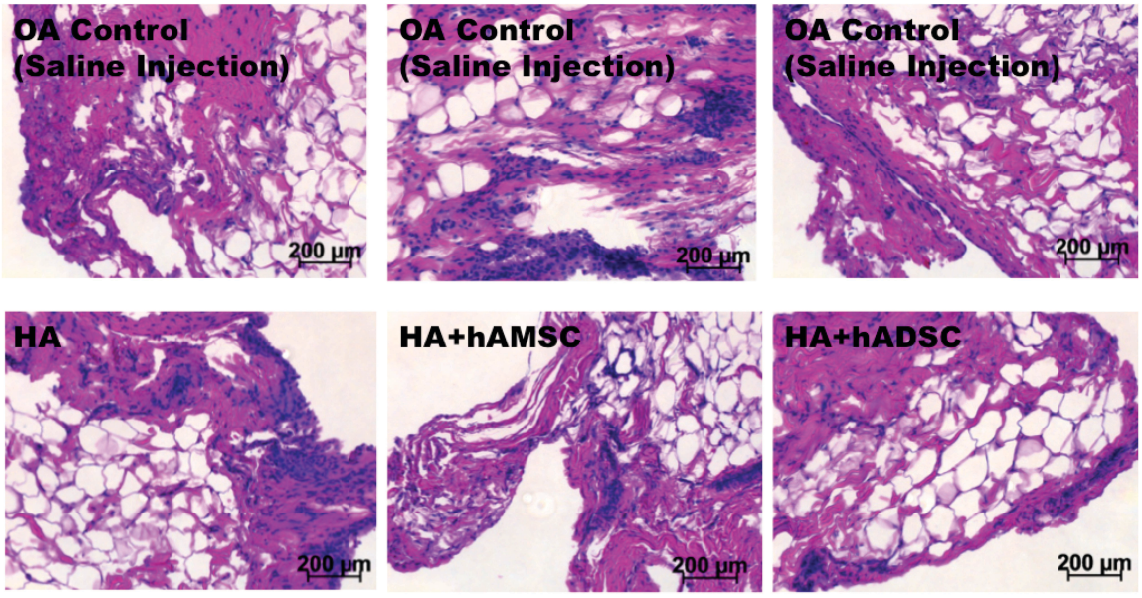


Figure 63: Subject-Matched Histological Assessment of Synovitis demonstrating trends between subject-matched left (treatment) and right (control) stifles.

6.4 Discussion

We were able to confirm and employ our approach of intrtopa-articular injection of stem cells in a pilot guinea pig study (see appendix A). We also demonstrated preliminary trends supporting the therapeutic efficacy of hAMSC and hADSC therapies mitigating OA progression in vivo. While we are continuing to analyze this data, the biochemical data specifically seems to indicate hAMSCs, in particular, are having a beneficial effect.

The Dunkin Hartley guinea pig animal model was employed in this study, because anatomically, the guinea pig knee is very similar to the human knee, though it is much smaller.[160] Additionally, guinea pigs exhibit the multi-factorial nature of bone growth and growth plate fashion observed in humans.[160] Notably, guinea pigs primarily load the medial stifle, making the medial compartment most prone to OA development (similar to humans).[160] The histopathology of the guinea pig has been extensively

evaluated and deemed similar to human OA.[160]

All guinea pigs showed no signs of immunologic rejection throughout the study. There were no abnormal behavioral changes (aggressiveness or lethargy) typically associated with bio-incompatability. However, IHC detection of lymphocytes should be employed in order to confirm this.

Both macro- and microscopically, it appeared the guinea pigs did reliably develop OA of the medial tibial plateau at 6-months of age (animals were 6-months old upon study termination at 3-months). Though the Dunkin Hartley guinea pig model does reliably develop OA in the entire medial compartment by 1-year of age.[2,3] Our results indicate OA was limited to the medial tibial compartment. An extended treatment timeline as well as the use of older animals would likely yield more advanced disease progression.

Synovial inflammation also confirmed OA progression in the guinea pigs with time. The grouping, size and shape of the cells observed in synovial inflammatory assessments appear to be some sort of inflammatory cell, potentially a macrophage, but macrophage-specific staining via IHC would be necessary to confirm this. These assessments proved particularly useful as they also led to the determination that due to small sample sizes, increased control (n=6) vs. experimental (n=2) subject numbers and moreover, large inter-subject variability, the most appropriate reporting of the in vivo data involved comparing the subject-matched treatment and saline injected limbs. Such comparisons appeared to offer more accurate depictions of disease progression/mitigation.

We noted the hAMSC-treated group appears to more successfully mitigate synovial inflammation. Not surprisingly, this group also preliminarily exhibited superior chondro-protection compared to hADSC and HA treated groups. As previously discussed, OA is an inflammatory condition driven by a feed-forward, macrophage-based mechanism. Based on previous results and the preliminary results of this study, hAMSCs me in fact be lowering the number of M1 macrophages or facilitating some sort of macrophage polarization shift. Whether this results or is a result of chondro-protective effects remains to be seen.

There are many more analyses that can be conducted on these samples, including tracking of the stem cells and macrophage/lymphocyte detection. Tracking of stem cells could be accomplished utilizing IHC detection of human mitochondria. In future iterations of such studies, the stem cells could be fluorescently tagged prior to injection as another metric of stem cell tracking. Similar to those results reported in chapter V, it would be interesting to track macrophage phenotype over time. Ex vivo we have demonstrated hAMSC treatment seems to shift macrophage polarization to an M2 phenotype. However, it remains to be seen if similar phenomena occur in vivo. Unfortunately, this will require anti-body development, as the necessary antibodies are not currently available in the guinea pig. IHC detection of lymphocytes will help further claims of biocompatibility of the injected stem cell therapeutics. Though such methods utilizing human cells in this guinea pig model have been previously described with no immune rejection,[4] this should be confirmed in our investigation. In future guinea pig studies, it would be ideal to obtain synovial fluid samples. Cytokine profiling would be a potential alternative method of gaining insight into macrophage polarization.

Additionally, it would be interesting to confirm the IL-4 and IL-13-dependent therapeutic mechanisms proposed in aim III, in vivo.

CHAPTER SEVEN

CONCLUSIONS

In conclusion, we were able to successfully demonstrate stem cells from the amniotic membrane exhibit heightened differentiation potential, availability in high yields, heightened immunomodulatory properties, and the ability to induce pro-regenerative (M2) phenotypes within macrophages, in musculoskeletal tissue-related experiments. Additionally, perinatal stem cells appeared to offer accelerated treatment time lines compared to a field standard stem cell, adipose derived stem cells.

In aim I, we were able to successfully isolate amniotic stem cells in high yields and differentiate them into osteogenic and chondrogenic lineages more readily than hADSCs. These differentiation capacities verified that amnion stem cells a candidate stem cell for osteoarthritis therapies. We therefore, continued to examine the utility of amnion stem cells through investigations into their OA therapeutic efficacy.

In aim II, we were able to successfully demonstrate human joint tissue explant co-culture mimics OA pathogenesis by modeling: 1) cartilage destruction in the presence of inflammatory cytokines and synovial macrophages, 2) gradual progression with time and 3) a feed-forward progression, where the cartilage and synovium exhibit reciprocal pathologic effects on one another. Additionally, our mechanistic evaluations utilizing macrophage depletion studies indicate synovial macrophages and/or their secretions are likely primary effectors driving disease progression in the model. This work highlights the utility of joint tissue explant co-culture in OA research, as it is an effective system for investigating OA pathology and evaluating potential future therapies.

In aim III, we were able to successfully demonstrate that perinatal stem cells are capable of mitigating disease progression in an explant co-culture model of OA. Moreover, perinatal stem cells appear to more effectively mitigate OA disease progression compared to a commonly employed stem cell, hADSCs. Significantly, both direct and indirect contact co-culture of hAMSCs with human OA cartilage seem to result in the mitigation of OA progression. However, direct contact co-culture with human OA cartilage seems to result in enhanced chondro-protective effects. Lastly, we were able to propose a mechanism for the observed mitigation via M2 macrophage anti-inflammatory secreted products.

In aim IV, we were able to successfully complete pilot animal study comparing the relative therapeutic efficacy of hAMSC and hADSC therapies for the mitigation of OA. Evidence, specifically biochemical analyses, appears to suggest hAMSCs offer superior therapeutic benefit.

For these reasons, we believe amnion membrane derived stem cells are an efficacious stem cell source for orthopedic tissue engineering and regenerative medicine strategies.

CHAPTER EIGHT

RECOMMENDATIONS

8.1 What Worked Well

Many of the methodologies employed in this research proved extremely efficacious and/or added validity to the work. I suggest the following methods continue to be implemented in future iterations of this work:

1. Conducting standardized experiments comparing the investigated amnion stem cells against commonly employed stem cells allowed for conclusions regarding the validation of amnion as a stem cell source for orthopedic regenerative medicine (validation requires comparison to established standards; in this case hADSCs). I encourage similar comparative analysis to be conducted with another commonly employed stem cell, hBMSCs, as well as some more widely researched perinatal stem cells, CMSCs and chord blood stem cells. Comparisons between human joint tissue explant co-culture and chemical doping models would also prove beneficial.
2. All histological assessments were conducted by at least one blinded researcher. This/these researcher(s) remained blinded to the condition/treatment of the sample being analyzed. This added significant validity to the obtained results, as it limits peer-review rejection of the results due to researcher bias.
3. Large sample sizes were required in order to overcome inter-patient variability of OA disease characteristics. Based on this work, utilizing sample sizes below 10 is highly discouraged, as it does not seem to accurately reflect the trends and/or statistical differences observed with n=15+ samples.

8.2 Future Suggestions for Aim I

Chondrocytes above the non-calcified zone in cartilage live under hypoxic conditions. While researchers are unsure of the exact mechanism(s), it appears that obtaining the appropriate ratios of aggrecan and collagen-II described in articular cartilage requires hypoxic conditions. Our experiments were conducted under normoxic conditions. Thus, chondrogenic differentiation characteristics could have been altered or more likely, will be enhanced and/or accelerated if completed under hypoxic conditions.

Mechanical stimulation is necessary in order to maintain cartilage homeostasis. This stimulation provides many functions, including facilitating synovial fluid movement and preventing the secretion of pro-inflammatory cytokines. The development of a bioreactor system that could apply appropriate mechanical loading to the cell system may enhance and/or accelerate chondrogenic differentiation of amnion stem cells.

Many musculoskeletal tissue pathologies, including OA, involve the creation and maintenance of a pro-inflammatory milieu within the pathologic tissue. Differentiation of most stem cells, including hADSCs, has been shown to be less effective under such inflammatory conditions. However, there is evidence that perinatal stem cells are able to differentiate under inflammatory conditions. Therefore, the results of our differentiation study (which favor amnion stem cells) could become even more skewed if the experiment were conducted with media supplemented with pro-inflammatory cytokines (ex. INF-γ, TNF-α, IL-1β).

Lastly, there are minor adjustments to the method that could be employed to increase the clinical relevance of the study. Firstly, the amino stem cells could be isolated using the explant culture technique (i.e. without enzymatic digestion). Secondly, the

amnion cells should undergo at least one freeze-thaw cycle prior to use in the differentiation study, as this will more closely mimic the tissue-banking processes that would be necessary with the clinical use of amnion. Finally, during stem cell expansion and differentiation, animal-serum media should be employed.

8.3 Future Suggestions for Aim II

As previously indicated, cartilage homeostasis requires hypoxia and mechanical stimulation. The development of a bioreactor system that could apply appropriate mechanical loading to the co-culture systems would more closely mimic the native anatomy and physiology being modeled.

For similar reasons, the meniscus, a highly inflammatory tissue of the OA joint space, could be added to the co-culture system. As OA progression seems to be inflammatory based, this may further enhance the OA progression observed. Extending the length of the study to at least 28 days would also likely show greater disease progression. However, our low synovium viability results seem to indicate that culture past this time period may not be possible.

While literature-based comparisons are possible based on these results, standardized comparative analyses employing this OA co-culture model and those employing chemical doping of the co-culture media are strongly encouraged. Such results would bolster claims of this model more accurately reflecting the natural and progressive nature of human OA.

8.4 Future Suggestions for Aim III

Significant variability was observed both within and between samples in the OA co-culture studies. In order to be sure such variability does not significantly alter the observed data trends, the 6mm biopsy tissue samples could be cut in half prior to initiating culture. One half of the sample could receive hADSC treatment while the other half of the sample could receive hAMSC treatment. Additionally, a dosing study to determine a the stem cells required to effectively mitigate disease progression would offer significant, clinically relevant, insight.

The model could also be employed in further mechanistic studies in order to further confirm and/or advance studies of OA. For example, M2 macrophage specific clodronate could be introduced into the culture system in order to systematically deplete M2 macrophages. Disease progression similar (if not worsened compared) to OA co-culture controls would be anticipated. Additional biochemical screening could include PGE_2 (to further investigate hADSC mitigation via auxiliary pathways), IL-1Ra (a known competitor of IL-1 binding which could be up-regulated by stem cells) and ADAMTS-5 (the primary ADAMTS involved in OA which could be differentially down-regulated). Additionally, this experimental design could be modified in order to test the regenerative (vs. mitigation) capacity of the stem cells. Creating a reproducible defect within the cartilage biopsies and monitoring the condition of the defect with time/treatment could accomplish this.

In an attempt to make these studies more clinically relevant, minced amnion could be employed as an alternative (therapeutic) study group. There is precedence for the use of amniotic membrane, as numerous amniotic membrane-based products are

commercially available. If minced amnion is an efficacious therapeutic alternative, it may prove easier to obtain FDA backing than stem cell therapies.

8.5 Future Suggestions for Aim IV

Dunkin-Hartley guinea pigs reliably exhibit severe OA by one year of age. Therefore increasing the study duration and/or utilizing older animals would be encouraged in order to ensure each animal exhibited OA prior to stem cell injection.

A stem cell tracking study would provide information regarding the length of time the stem cells remain in the joint space as well as the location of the stem cells. This could provide mechanistic insight into the primary mode of action of the stem cells (differentiation into chondrocytes vs. immunomodulatory). Such a study may also help ease concerns regarding the short lifespan of HA within the joint, as stem cell migration from the HA carrier into the joint space could be monitored. While IHC or immunofluorescence for human mitochondria is one method for tracking stem cells, immunofluorescent tagging of the stem cells is another commonly employed alternative. Though such an approach would require the use of female guinea pigs, the use of male cells in a female animal model would allow the tracking of stem cells via Y-chromosome in situ hybridization.

The inabilities to test for M1/M2 macrophages and to obtain synovial lavages were severe limitations of our pilot guinea pig study described in aim IV. It is strongly recommended to obtain synovial fluid samples in order to obtain further mechanistic insight into the action of the stem cells. Particularly, it would be interesting to see if the up-regulation of IL-13 and IL-4 in hADSC and in hAMSCs, respectively, observed ex

vivo is also observed in vivo.

Lastly, steps could be taken to increase clinical relevance. The addition of more clinically relevant outcome measures would also enhance the validity of further guinea pig investigations. Functional testing through the use of cage platforms (monitoring animal activity levels) or guinea pig pedometers could be employed. Radiographic-based assessments of the joint space would also more accurately reflect clinical grading and assessment of the joint. Additionally, synthetic HA could be investigated as a stem cell carrier due to its more prevalent use compared to natural HA. Furthermore, there is precedence (from a regulatory standpoint) for the use of amniotic membrane as a wound covering. Therefore investigating the use of amniotic membrane as a "band aid" covering the synovial membrane (vs. a stem cell therapy) may be worthwhile. Results from our hAMSCs seeded in indirect contact with the cartilage surface (aim III) seem to support such an approach.

APPENDICES

APPENDIX A

ADDITIONAL DATA/FIGURES

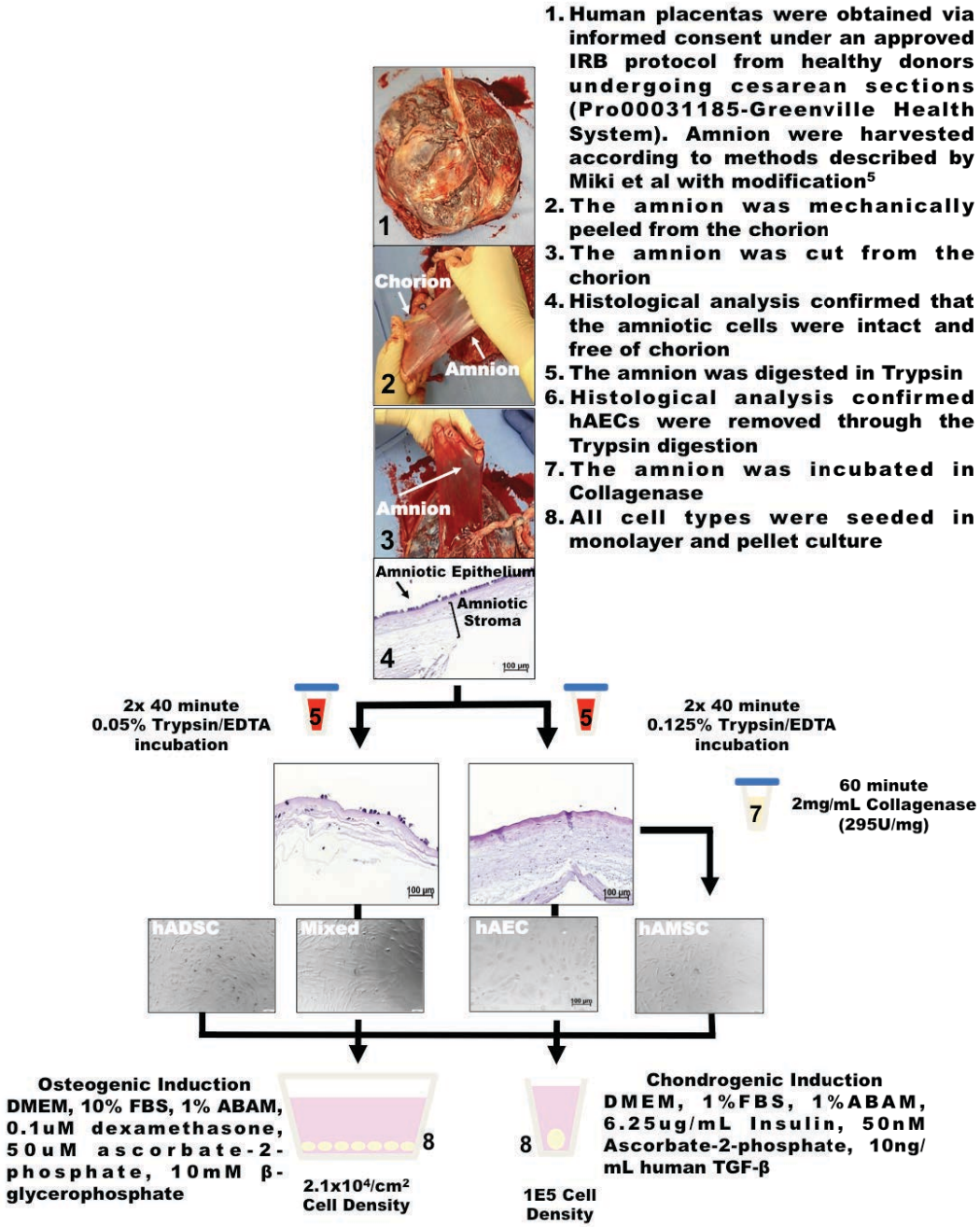


Figure A.1: Detailed Methods Schematic describing the experimental design of the differentiation study described in aim I.

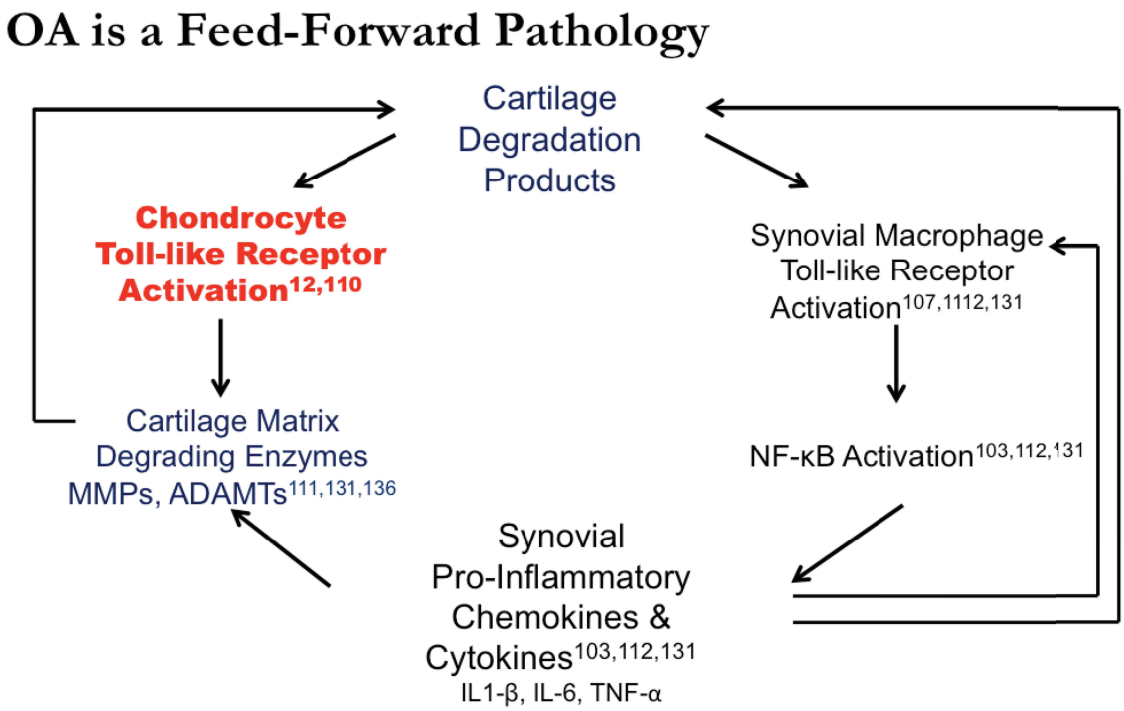


Figure A.2: Alternate depiction of TLR-activation highlighting the ability of chondrocytes (in additional to synovial cells) to contribute to TLR-activation.

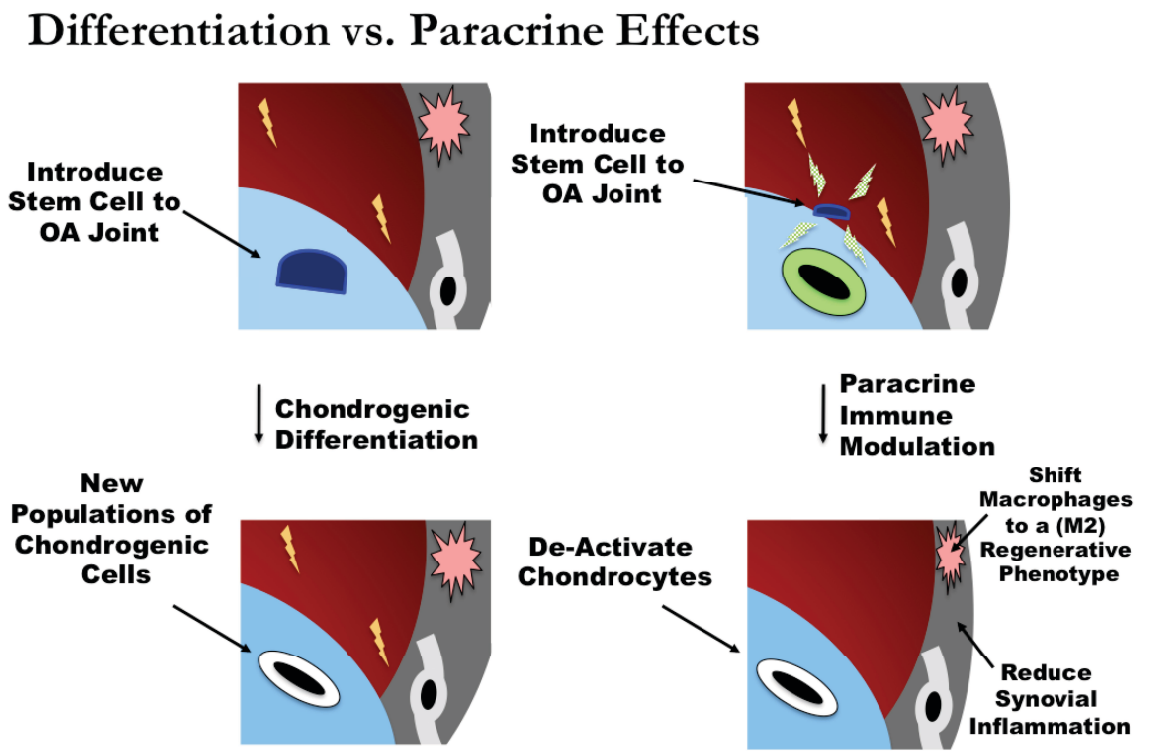


Figure A.3: Pictorial Representation of the Multi-Focal Therapeutic Potential of Stem Cells highlighting two of the proposed mechanisms of action: differentiation and immune modulation.

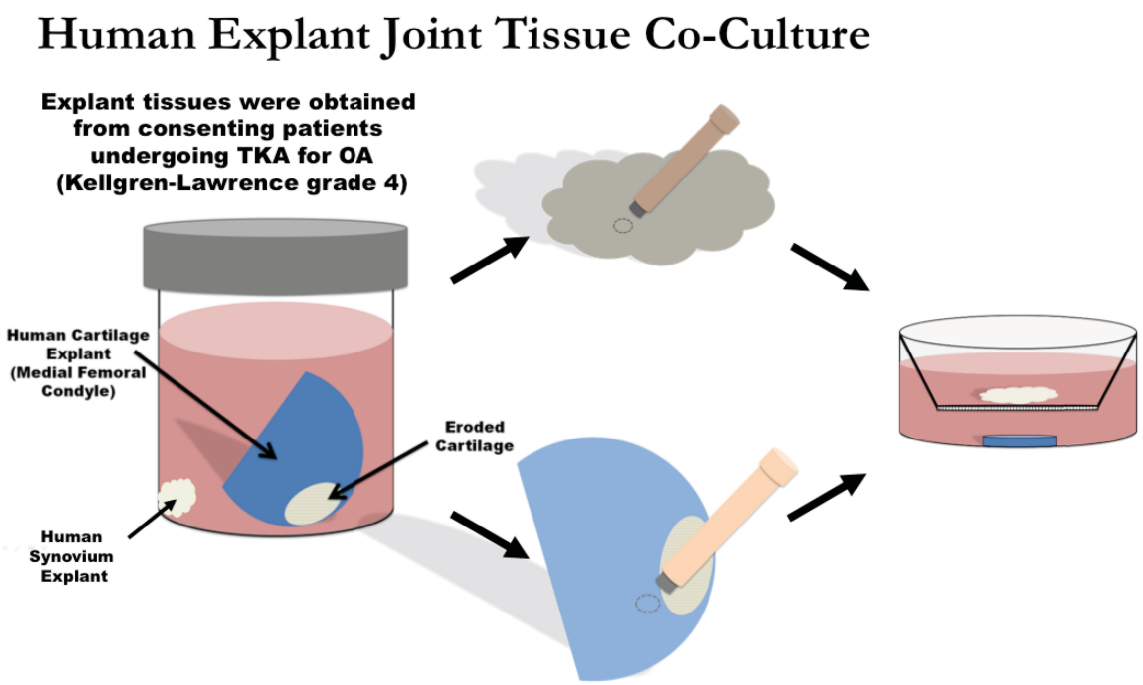


Figure A.4: Detailed Methods Schematic Describing Joint Tissue Explant Tissue Harvest and the initiation of co-culture.

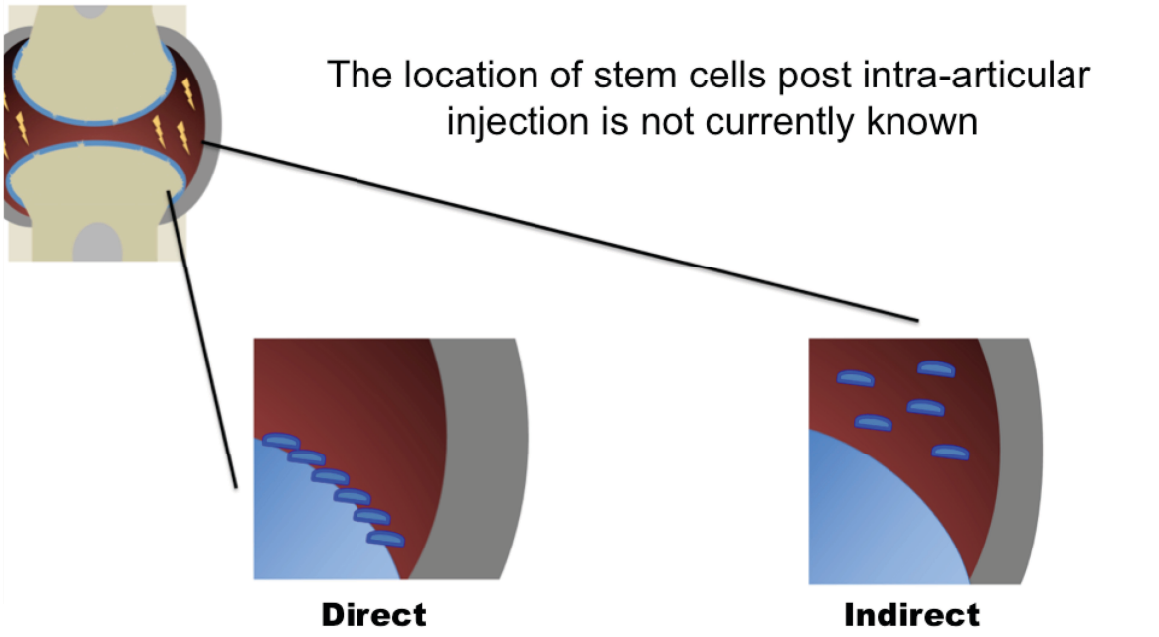


Figure A.5: The Fate of Intra-Articularly Injected Stem Cells is Unknown. Stem cells are typically administered via intra-articular injection, and it is currently not known what tissue(s) the stem cells actually remain in contact with. It is possible the stem cells are directed to that surface via signaling cues and settle (due to gravity) on the surface of the cartilage. It is also possible that the movement of the joint would actually prevent the stem cells from settling and they could remain in indirect contact with the cartilage surface. Therefore we wanted to investigate the effects of varying stem cell administration location on therapeutic potential.

Alternative Method of Achieving Chondrogenic Differentiation: Micro Mass Culture

Methods: hAESCs, hADSCs, and Mixed cell groups (n=4 per cell type per treatment) for use in chondrogenic differentiation were seeded in micro-culture at $8x10^4/10\mu L$ media. After 2 hours, the remaining media was added to the micro-culture. At select time points, cultures were stained with Alcian Blue and underwent semi-quantitative analysis as described previously in chapter IV.

Results: All negative control cell groups exhibited GAG deposition by day 14, and figure A.6 confirms this deposition continued through day 28. Staining of the negative controls appeared to be less extensive than in the test groups (Figure A.7), and semi-quantitative analysis confirmed this in most cases. Additionally it was noted that, in comparison to the hAESCs and hADSCs, the hybrid cell population proliferated much more extensively, and yielded greater staining, by each time point (Figure A.7).

Table A.1: Average % Area Stained by Alcian Blue						
Day 28	hAEC	hAEC Neg.	Mixed	Mixed Neg.	hADSC	hADSC Neg.
	43.02*+	31.28*+	86.70*+#	97.13*+#	26.20*#	24.22*+
Day 14	hAESC	hAESC -	hAMSC	hAMSC -	hADSC	hADSC-
	28.41*+#	26.15*+	30.15*+#	26.39*+#	20.30*#	25.88*+
Day 7	hAESC	hAESC -	hAMSC	hAMSC -	hADSC	hADSC -
	0.01	0.00	0.00	0.00	0.00	0.00
Day 3	hAESC	hAESC -	hAMSC	hAMSC -	hADSC	hADSC -
	0.00	0.00	0.00	0.00	0.00	0.00

Table A.1: Semi-Quantitative Alcian Blue results demonstrating the increased chondrogenic differentiation of amnion groups compared to hADSCs. *Denotes statistical difference ($P<0.05$) between test group and respective control within time point +Denotes statistical difference ($P<0.05$) between cell group and hADSC test group within time point # Denotes statistical difference ($P<0.05$) between cell group and hADSC negative group within time point

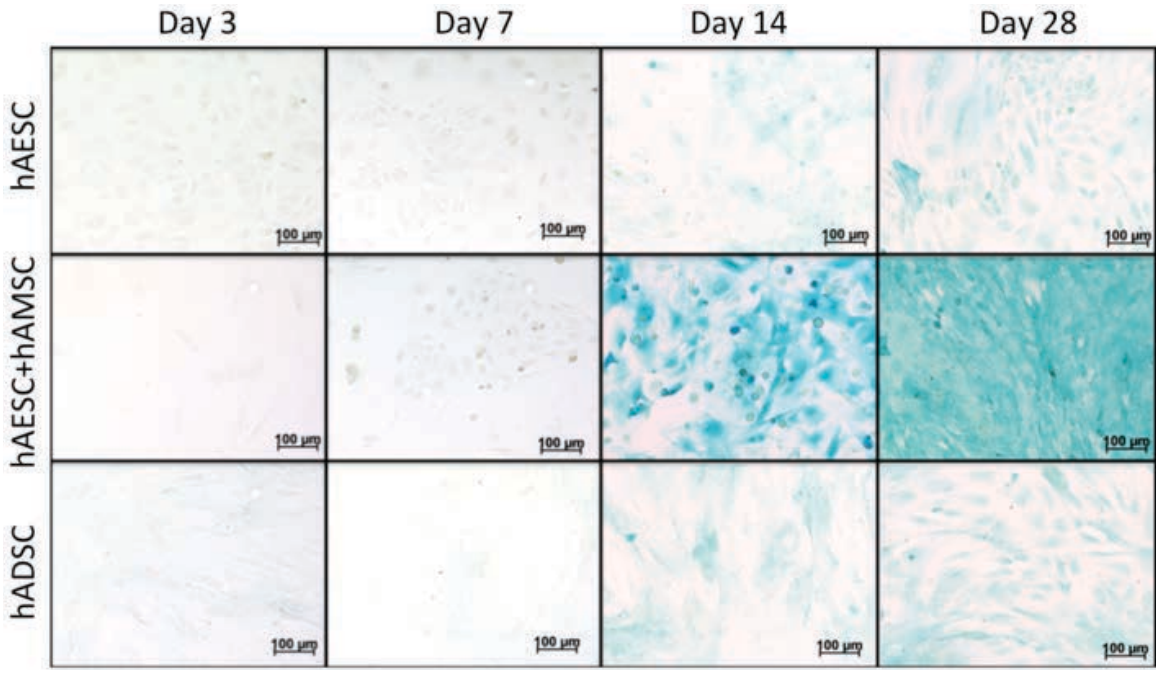


Figure A.6: Micro Mass Chondrogenic Differentiation of Negative Controls with Alcian Blue staining. GAG deposition was apparent across all groups by 14 days. No significant morphological changes were noted.

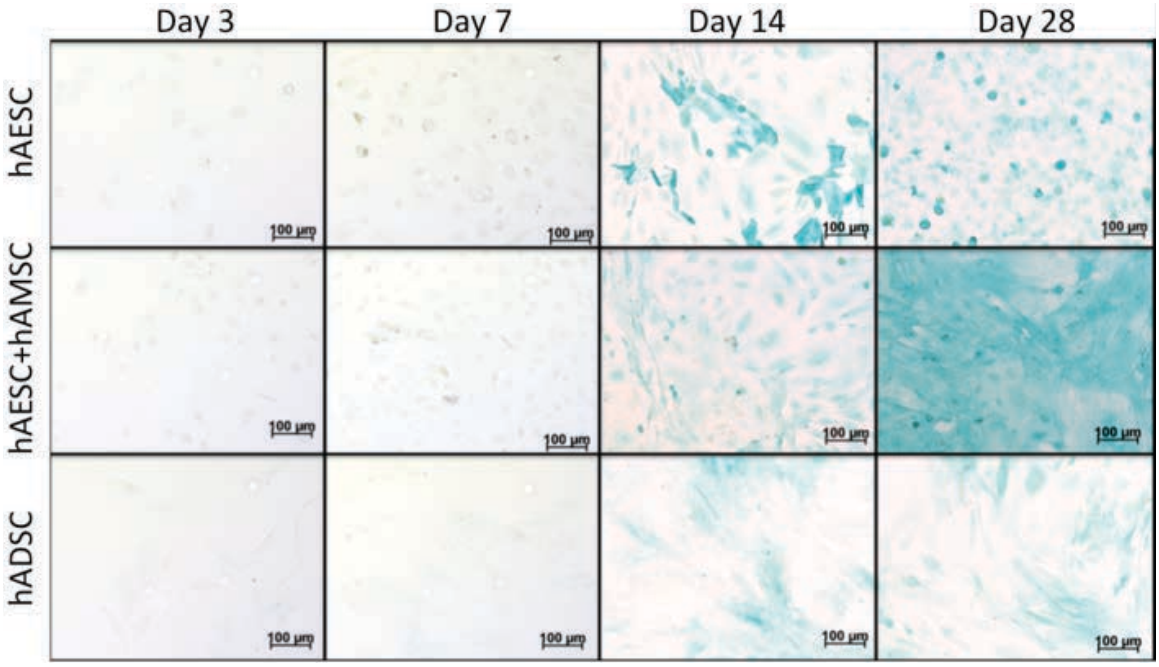


Figure A.7: Micro Mass Chondrogenic Differentiation of Experimental Group with Alcian Blue staining. GAG deposition was visible across all groups by day 14. Morphological changes were apparent in both amniotic membrane-derived cell groups as early as day 3, whereas hADSCs maintained their spindle shape a through 28 days.

Chondrocyte Clustering in OA Co-culture

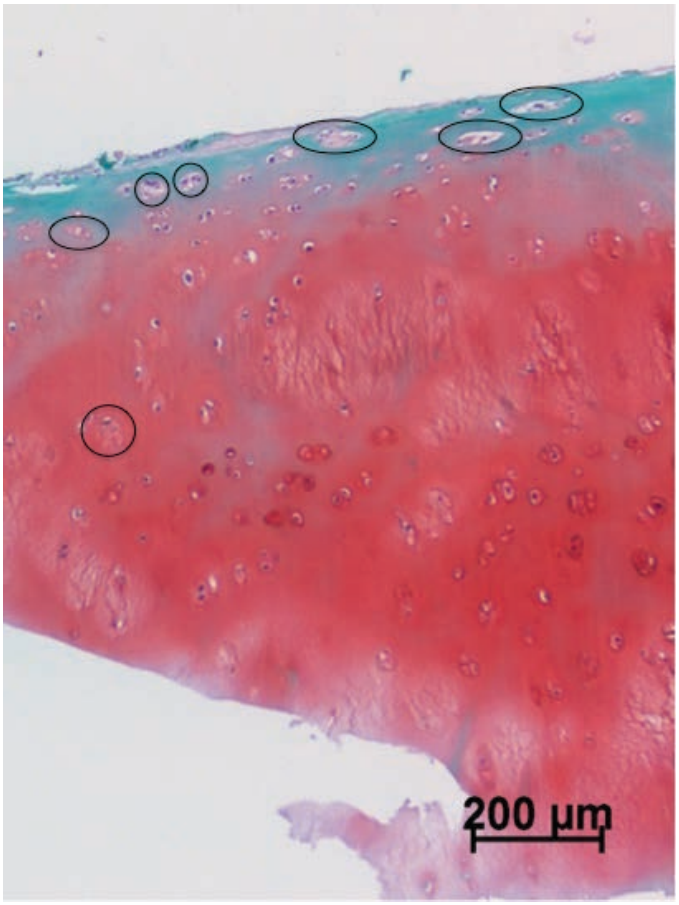


Figure A.8: Chondrocyte Clustering observed in a safranin-O stained cartilage explant. Prominent clusters are circled.

As previously indicated, normal chondrocytes are held in place due to their senescent state. However, in OA chondrocytes activate: proliferating and secreting matrix. This results in the chondrocytes moving and forming cell clusters. An example of chondrocyte clustering observed in OA cartilage explants can be seen in figure A.8. Counting the number of cell clusters is a method employed by researchers to convey the extent of OA progression. Clusters were only counted if they consisted of at least 3 chondrocytes. Figure A.9 demonstrates the limited differences between study groups. There appeared to be no difference in the number of chondrocyte clusters observed in OA, cartilage only or hADSC-treated groups after 15 days. However, hAMSCs in direct contact with cartilage demonstrated a trend towards reduced chondrocyte clustering and hAMSCs in indirect contact with cartilage demonstrated statistically reduced chondrocyte clustering compared to hADSC-treated groups.

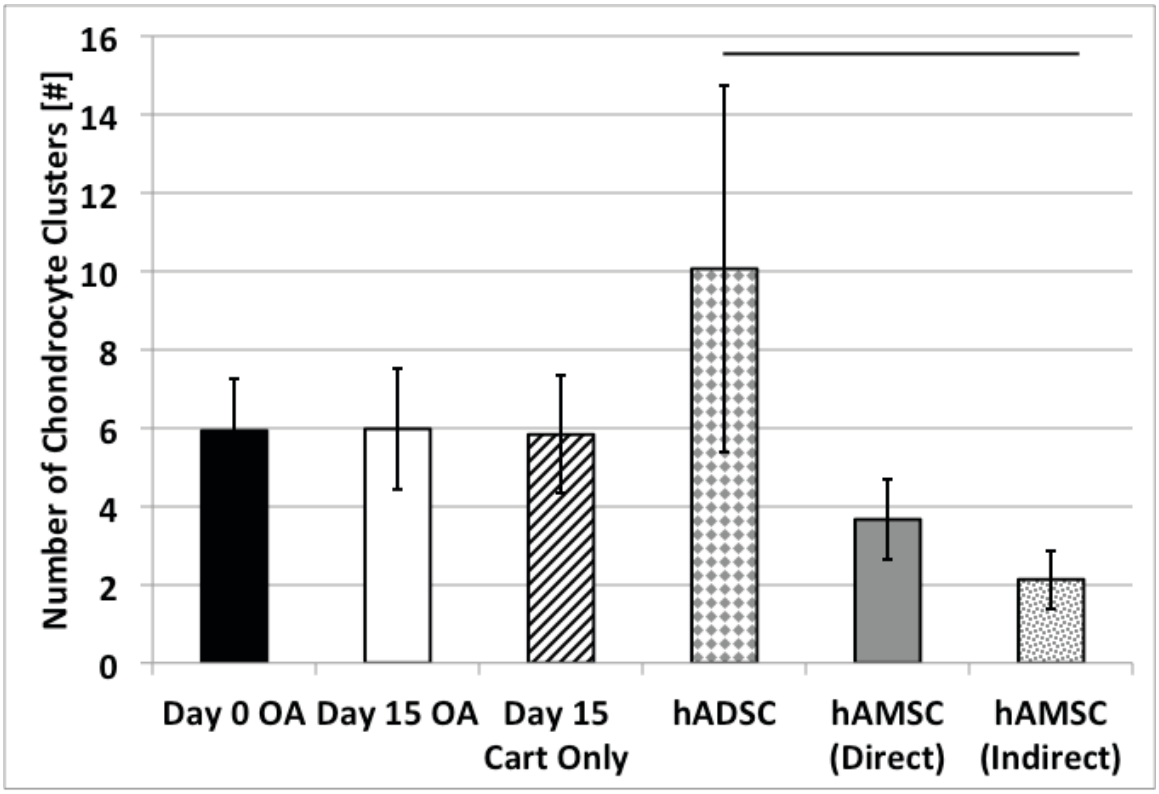


Figure A.9: Chondrocyte Clustering observed in OA explant co-culture over 15 days.

Figure A.10 shows a patient-matched set of safranin-o stained cartilage explants, demonstrating the trending decrease in clusters observed in hAMSC-treated groups.

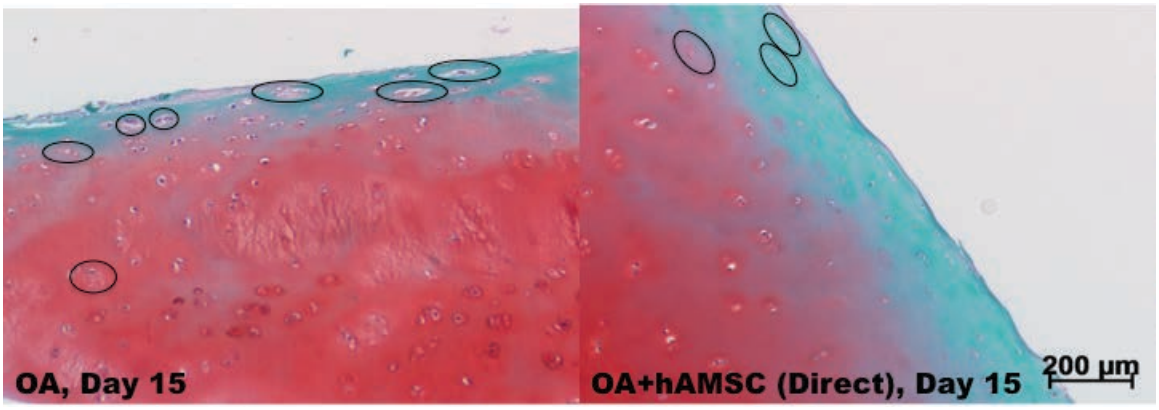


Figure A.10: Chondrocyte Clustering in Patient-Matched Cartilage Explants observed after 15 days

The depth of chondrocyte clustering is also considered to be reflective of the stage of OA; where clustering within the superficial-middle zones represents early stage OA and

clustering within the middle-deep zones represents late stage OA. Figure A.11 illustrates the relative depth of chondrocyte clusters observed within study groups.

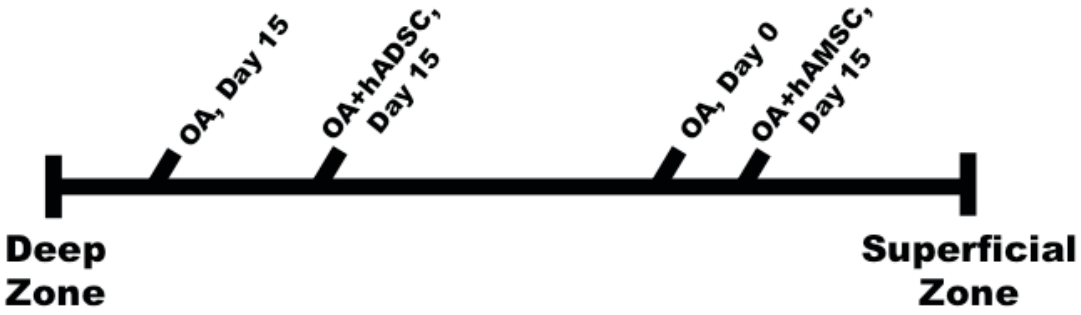


Figure A.11: Relative Depth of Chondrocyte Clustering observed after 15 days in explant co-culture.

Initial Feasibility Assessment for Guinea Pig Injection Study: Stem Cell Viability

In an attempt to ensure stem cell viability post submersion in HA and injection through a syringe, the viability of hAMSCs and hADSCs was tracked through this process in an initial feasibility study (n=3 per stem cell type). In order to gain meaningful insight into the affect of HA suspension and syringe injection, all stem cell culturing conditions and materials employed in this study were identical to those previously described in chapter VI (i.e. this feasibility study employed identical methods to those utilized in the actual guinea pig injections).

Methods: Passage 1 hAMSCs and hADSCs were plated under standard expansion culture conditions. At confluence, cells were passaged and initial viability of these passage 2 cells was established via Trypan Blue counting in a BioRad TC-20 cell counter. $1x10^6$ cells were submerged in individual aliquots of 100μL HA, loaded into a 21-gauge syringe and the total volume of HA+SC suspension was ejected. The HA+SC suspension was then counted to determine the effect of the HA carrier and injection process on cell viability.

Results: As illustrated in figure A.12, cell viability was not affected by submersion in HA followed by injection through the syringe. The average HA suspension and injection process lowered cell viability by <1.5%. No statistical differences were observed between initial and final viability counts or between stem cell types.

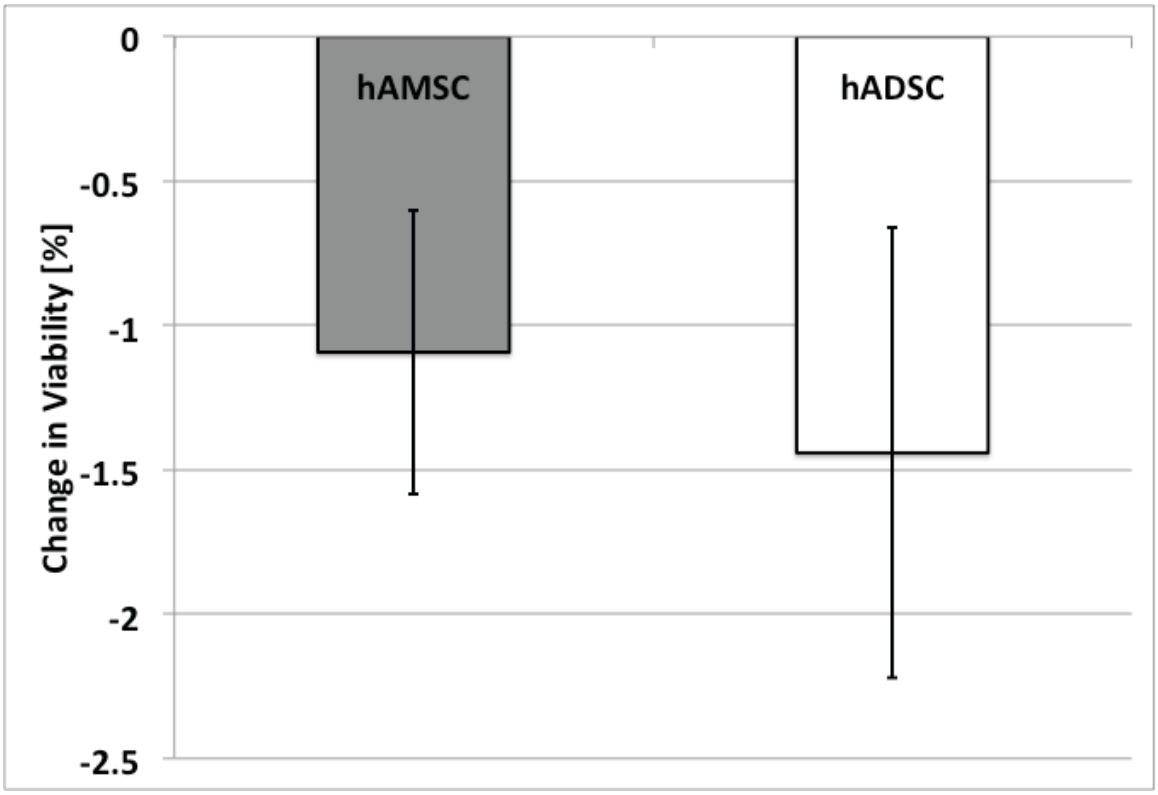


Figure A.12: Stem Cell Viability Assessment post submersion in the HA carrier, loading and injection through a syringe.

Initial Feasibility Assessment for Guinea Pig Injection Study: Stem Cell Injection

In an attempt to ensure the joint space could be successfully located, and the injections could be delivered into the intra-articular joint space, n=3 rats and n=6 guinea pigs were used in ultra-sound guided practice injections.

Methods: Ultrasound guidance was utilized similar to methods described by Vazquez et al.[261] Briefly, rat stifles were placed in flexion, the ultrasound probe was covered with gel and applied to the leg surface near the patella. A 21-gague syringe was loaded with PBS, and the needle was inserted posterior to the medial edge of the patellar ligament. After initial insertion, the leg was placed in extension to facilitate visualization of the intra-articular space. The needle was inserted until it was in direct contact with the femur, and the PBS was injected.

Results: As illustrated in figure A.13A, the intra-articular space was correctly identified. The needle was inserted into the intra-articular space and identified via ultra-sound (figure A.13B). The PBS was not injected until the needle was successfully guided to the femur surface (figure A.13C).

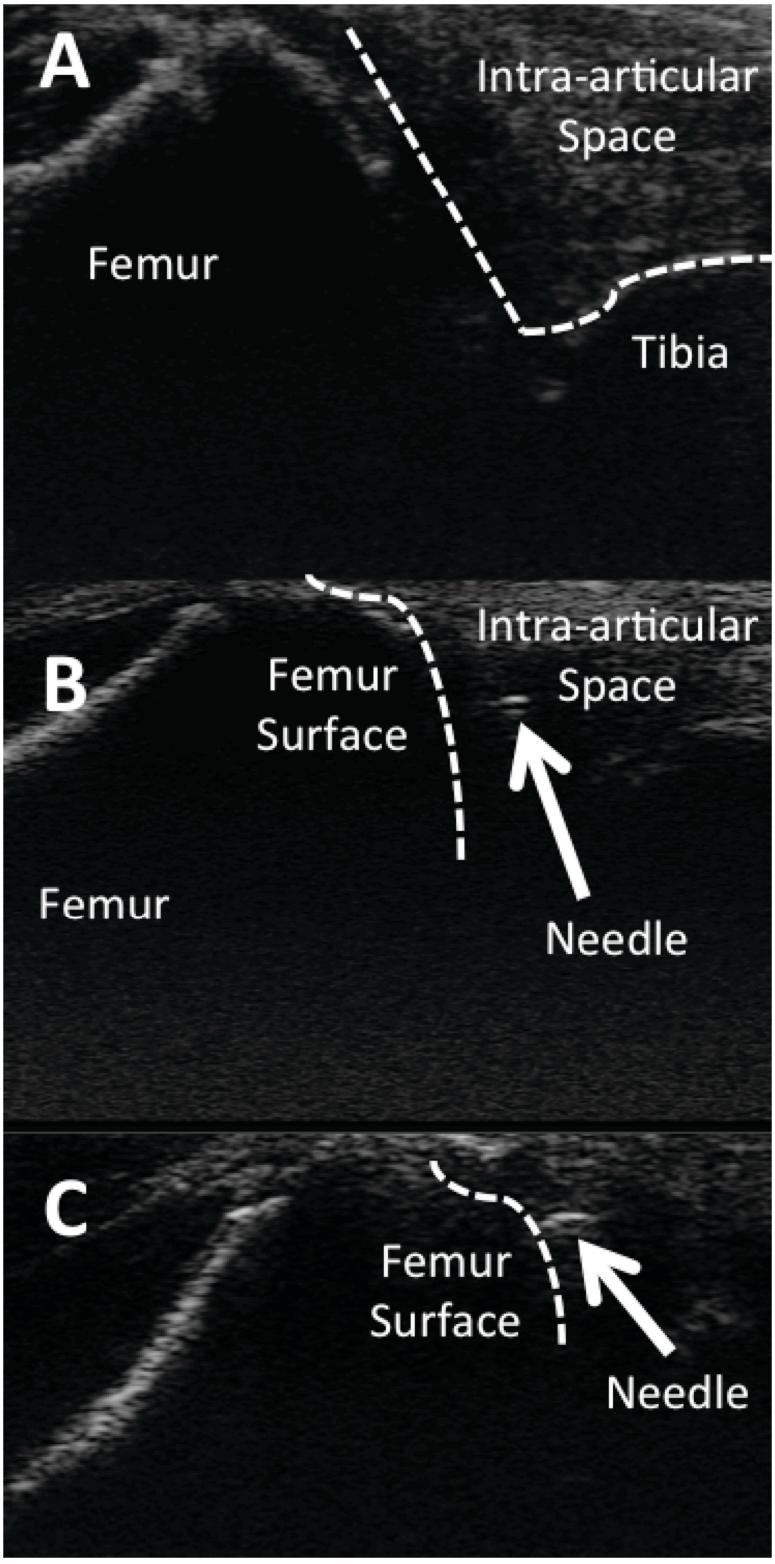


Figure A.13: Ultrasound Confirmation of Practice Injection showed that the intra-articular space could be identified (A), the needle could be inserted (B) and the PBS injection could be ejected when the needle was at the surface of the femur.

APPENDIX B

COMPREHENSIVE INFLAMATORY ASSESSMENT OF OA EXPLANT CULTURES

Initial probes into the inflammatory profile of the described OA explant culture system included enzyme-linked immunosorbent assay (ELISA) for primary OA mediators: IL-1β, TNF-α and MMP-13, were described in chapters IV and V. In order for more thorough model validation efforts as well as further mechanistic confirmation of the therapeutic efficacy of the described stem cell therapies, a comprehensive inflammatory cytokine array was completed on media samples from OA explant co-cultures (OA day 0 (n=13), OA day 15 (n=13), Macrophage depleted day 15 (n=5), OA+hADSC day 15 (n=4), OA+hAMSC in direct contact with cartilage day 15 (n=5), OA+hAMSC in indirect contact with cartilage day 15 (n=3)). This array provided quantitative measurements of 20 human chemokines and cytokines. 13 of these provide pro-inflammatory stimulation (IL-1α, IL-1β, TNF-α, RANTES, MCP-1, IL-8, IL-6, IL-2, IL-5, IL-12, MIP-1a, MIP-1b and GM-CSF), while 3 are known anti-inflammatory mediators (IL-4, IL-10, IL-13). An additional marker, VEGF, has been used as a marker of synovitis (as synovitis is characterized by enhanced angiogenesis within the synovium). In this appendix we describe the results of this array and highlight the implications of these results. For clarification, please see the below list of formal and alternative names for each mediator investigated.

Table B.1 Clarification of Terminology & Cytokine Source

Name on Cytokine Array	Abbreviation	Alternative Name(s)	OA Tissue Source of Cytokine [104,118,132,260,262,263]
Interleukin 1 alpha	IL-1α	Hematopoietin 1	Cartilage, Subchondral bone, Synovium
Interleukin 1 beta	IL-1β	Leukocytic pyrogen, Eukocytic endogenous mediator, Ononuclear cell factor, Lymphocyte activating factor	Cartilage, Subchondral bone, Synovium
Tumor Necrosis Factor alpha	TNF-α	Cachexin, or Cachectin	Cartilage, Subchondral bone, Synovium
Interleukin 8	IL-8	CXCL8	Synovium
Monocyte Chemoattractant Protein-1	MCP-1	Chemokine (C-C motif) ligand 2 (CCL2)	Synovium
Regulated on Activation, Normal T cell Expressed and Secreted	RANTES	Chemokine (C-C motif) ligand 5 (CCL5)	Subchondral bone, Synovium
Interleukin 6	IL-6	Interferon Beta 2	Cartilage, Subchondral bone, Synovium
Macrophage Inflammatory Protein-1a	MIP-1a	Chemokine (C-C motif) ligand 3 (CCL3)	Synovium
Macrophage Inflammatory Protein-1b	MIP-1b	Chemokine (C-C motif) ligand 4 (CCL4)	Synovium
Granulocyte-Macrophage Colony-Stimulating Factor	GM-CSF	Colony stimulating factor 2 (CSF2)	Synovium
Interleukin 10	IL-10	human Cytokine Synthesis Inhibitory Factor (CSIF)	NA
Interleukin 13	IL-13	NA	NA
Interleukin 4	IL-4	NA	NA

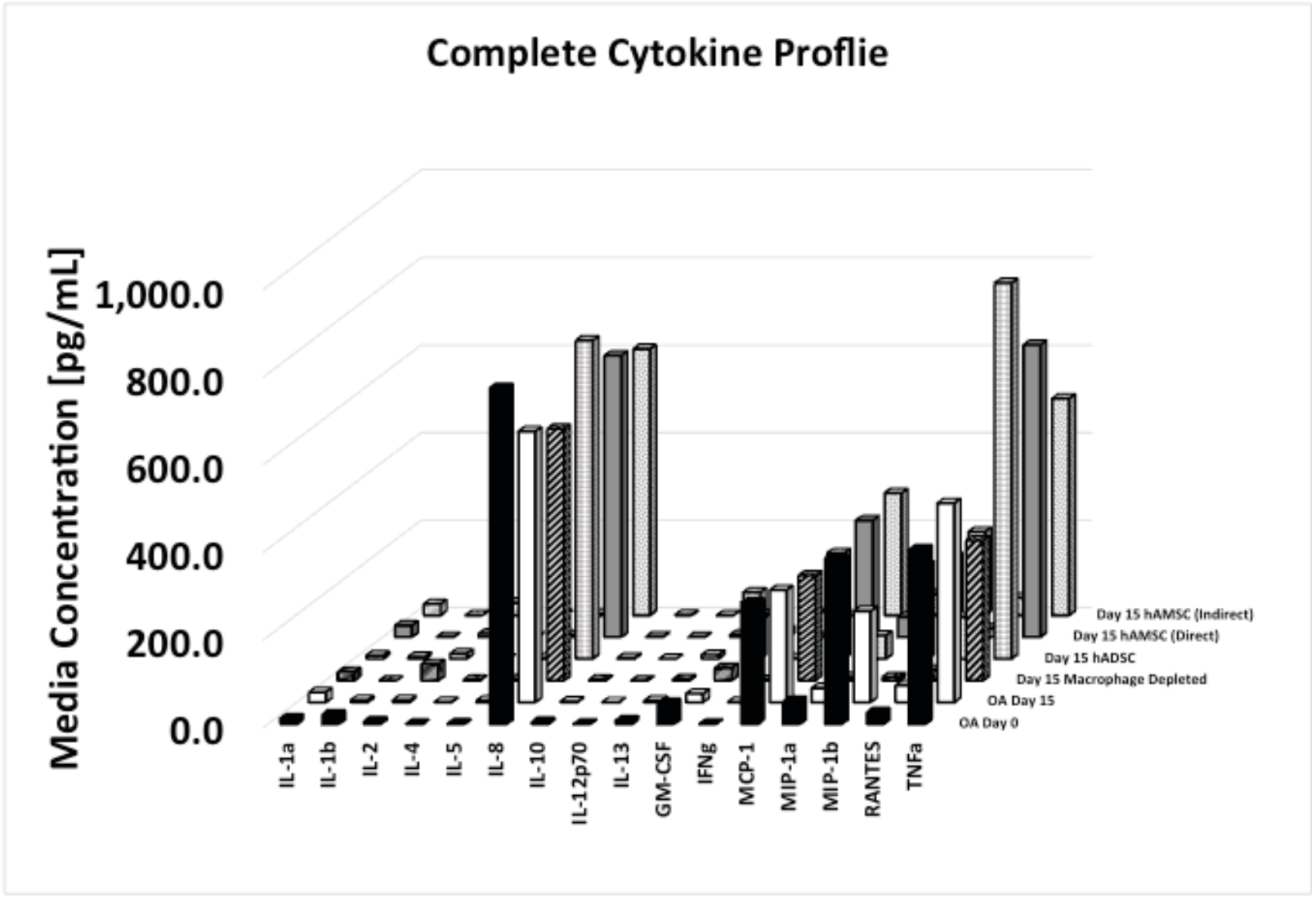


Figure B.1: Summary of Mediators Investigated.

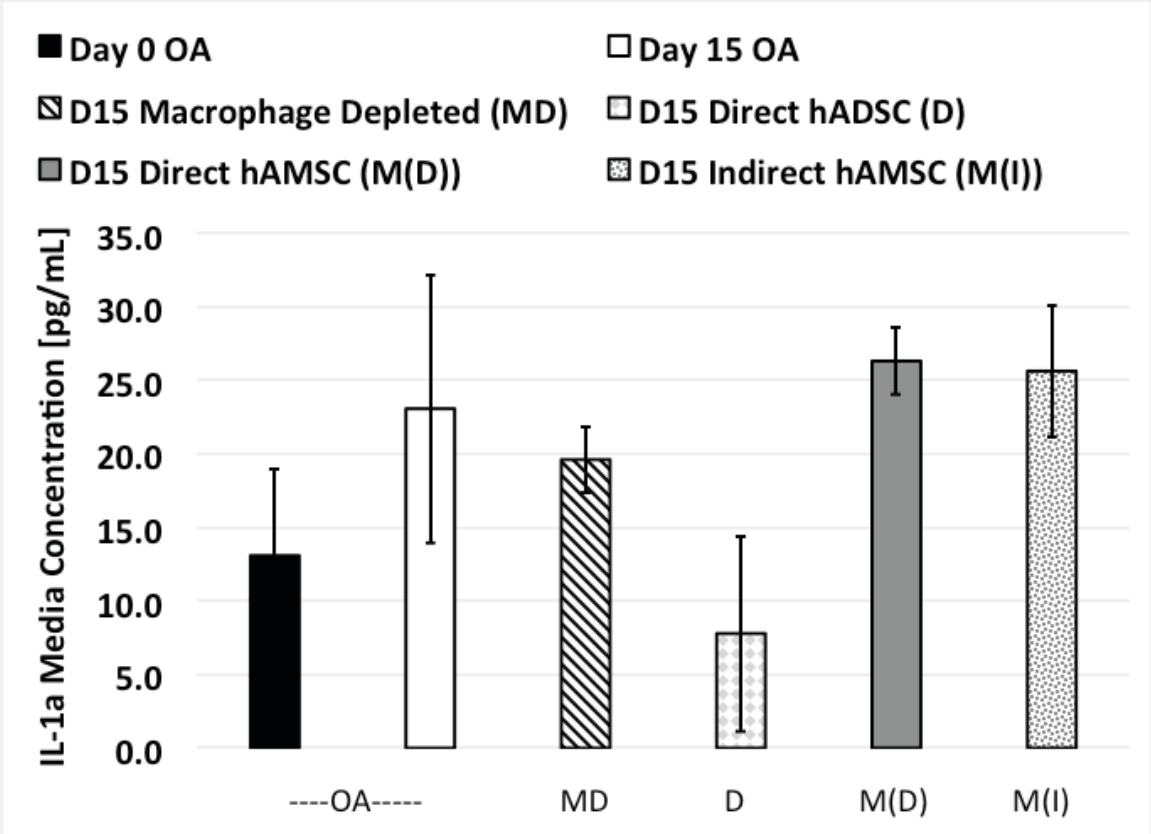


Figure B.2: Summary of IL-1α concentrations from each OA explant group examined.

IL-1α is typically secreted in early stage OA. IL-1a has been used as a prognostic indicator of early OA, as its serum detection successfully differentiated early stage OA patients from controls.[262] Like IL-1β, IL-1α is a potent pro-inflammatory mediator, which stimulates the secretion of numerous downstream pro-inflammatory mediators.[252] It would be expected that due to the removal of macrophages and increased anti-inflammatory activity, IL-α would decrease in macrophage depleted and stem cell treated groups, respectively. However, no statistical differences are noted.

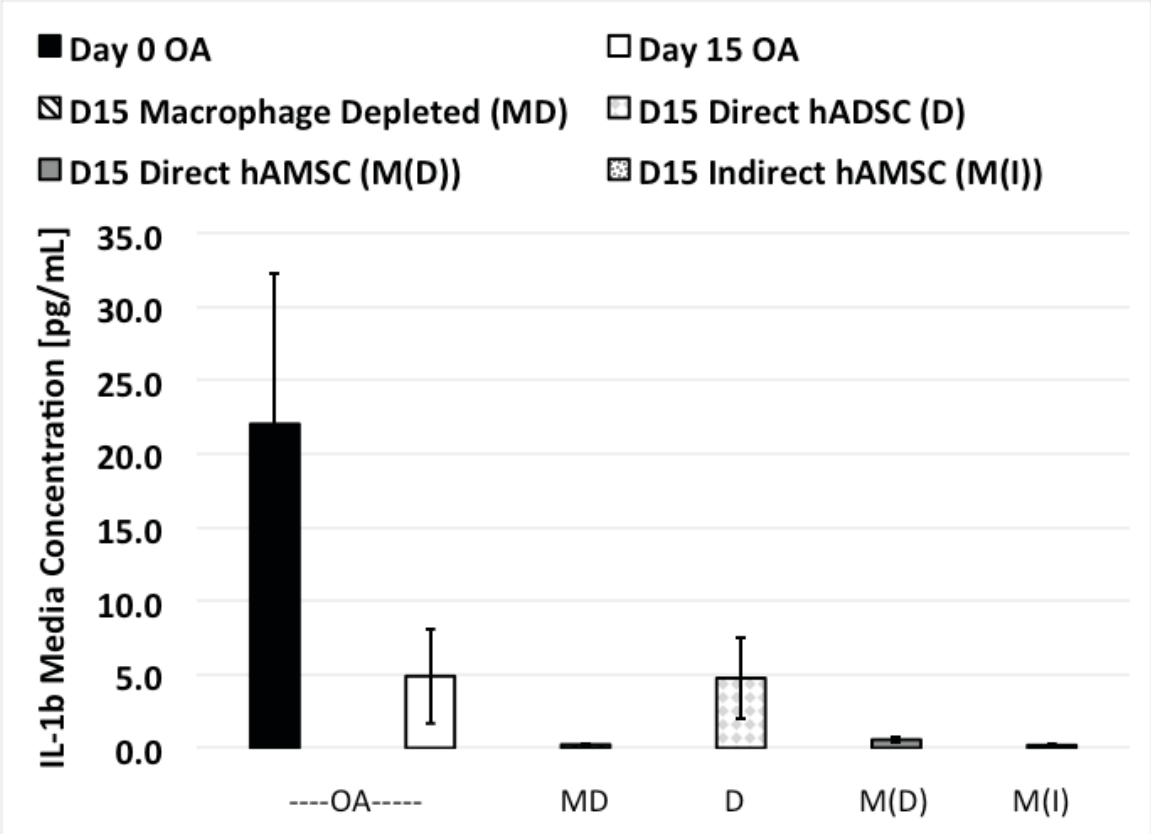


Figure B.3: Summary of IL-1β concentrations from each OA explant group examined.

IL-1β is typically secreted in late stage OA. IL-1β stimulates the production of cartilage degrading enzymes MMP-1, MMP-3 and MMP-13, cytokine IL-6 and chemokines IL-8, MCP-1 and RANTES.[118] As this cytokine has been previously discussed in detail, this discussion will focus specifically on the reported results. Consistent with the ELISA data described in chapters IV and V, IL-1β decreased in macrophage depleted and stem cell treated cultures. Additionally, trends towards decreased Il-1β expression in amnion groups were observed ($p<0.055$ compared to OA day 0; $p<0.061$ compared to hADSC treated cultures). At all points in culture, our results fell within physiological ranges, further demonstrating this OA co-culture system provides a more natural model of Il-1β involvement in OA pathogenesis.[136,147,149] Others

have noted extremely low concentrations of IL-1β in ex vivo cultures.[252–254] This has been attributed to the extreme lability associated with this cytokine, and researchers have cautioned conclusions based solely on IL-1β biochemical analysis.[173,255] However, our other cytokine results coupled with our previous analyses described in chapters IV and V reinforce the validity of such conclusions.

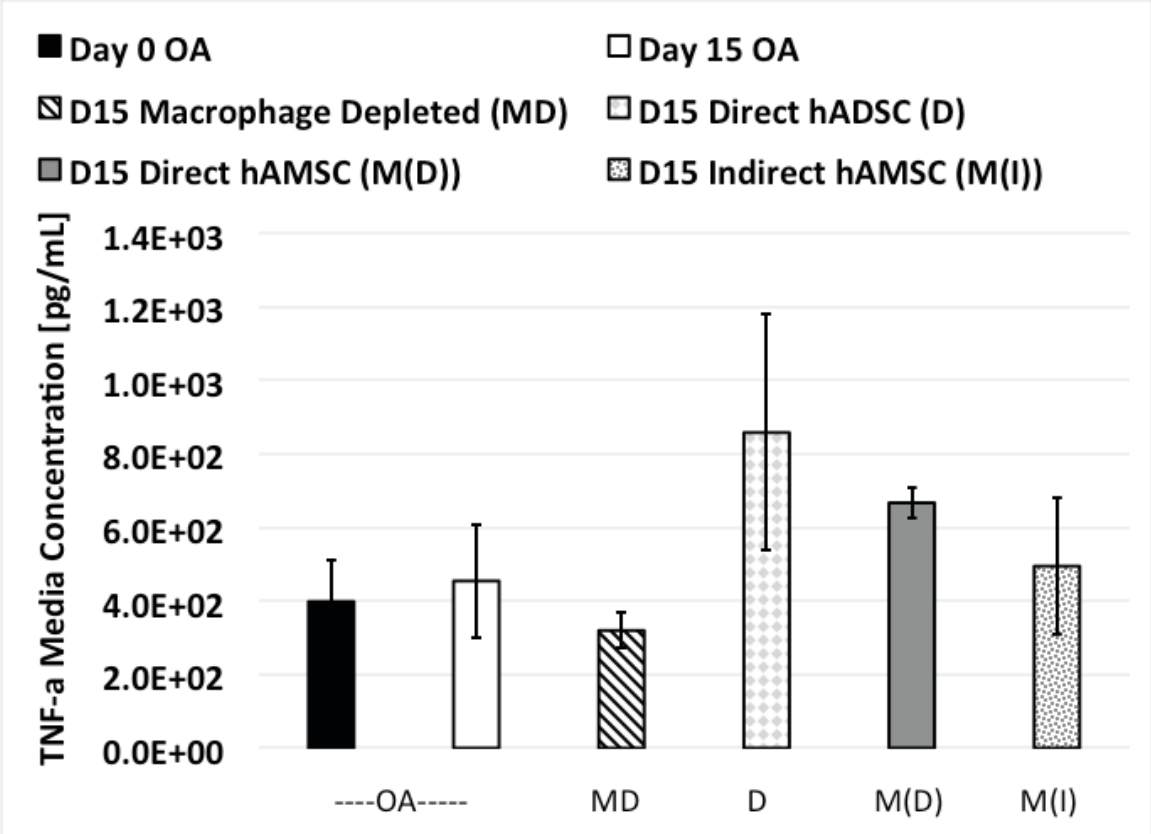


Figure B.4: Summary of TNF-α concentrations from each OA explant group examined.

TNF-α stimulates the production of cartilage degrading enzymes MMP-1, MMP-3 and MMP-13, cytokine IL-6 and chemokines IL-8, MCP-1 and RANTES.[118] As this cytokine has been previously discussed in detail, this discussion will focus specifically on the reported results. No statistical differences are noted. Notably, the ELISA data described in chapter V showed a more distinctive trend of TNF-α reduction in amnion treated groups. This reduction concomitant with the observed reduction in IL-1β by was hypothesized to be a mechanism behind the enhanced mitigation observed in hAMSC treated groups, as it has been demonstrated that reductions in both are necessary for OA mitigation.[150] This discrepancy in TNF-α results can likely be attributed to differences in sample sizes as well as in the detection method employed.

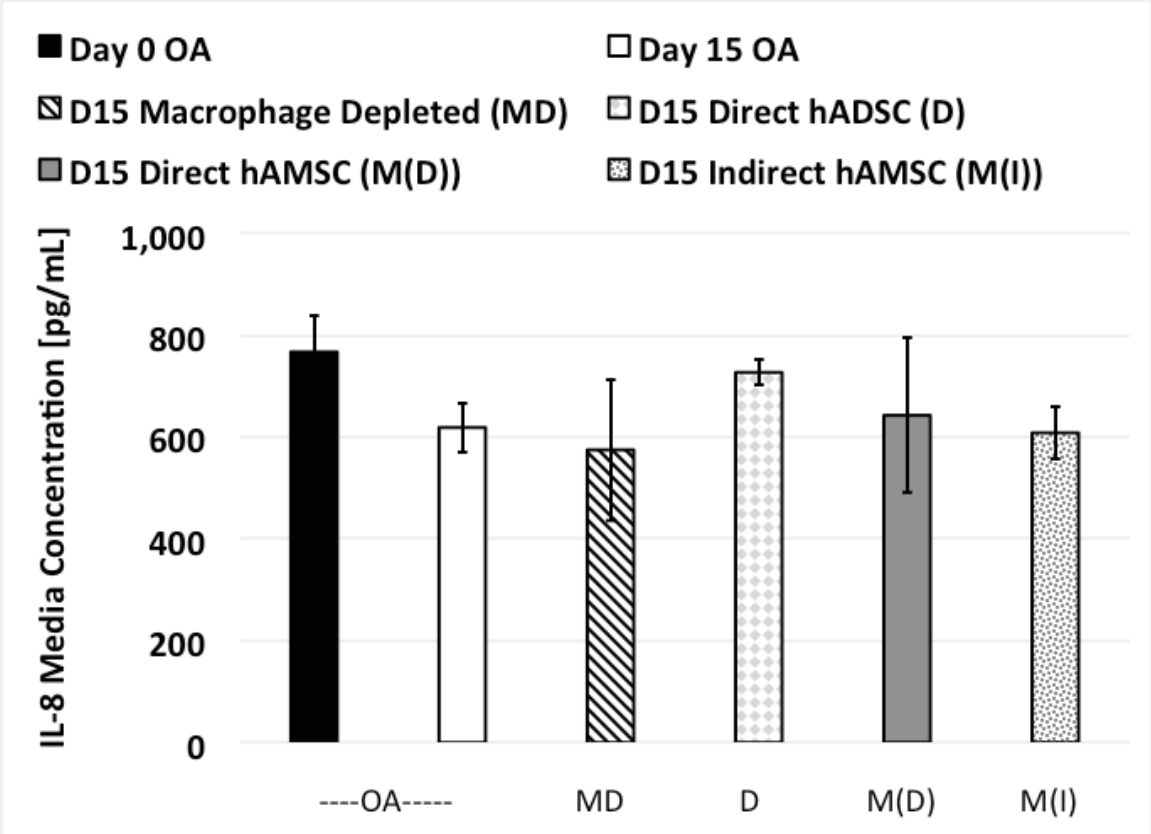


Figure B.5: Summary of IL-8 concentrations from each OA explant group examined.

IL-8 is a chemokine responsible for monocyte/macrophage recruitment. IL-8 induces proteoglycan loss through the up regulation of MMPs as well as IL-6 production.[118] It would be hypothesized that macrophage depleted and stem cell treated cultures would express less IL-8 compared to other culture groups. However, no statistical differences are noted.

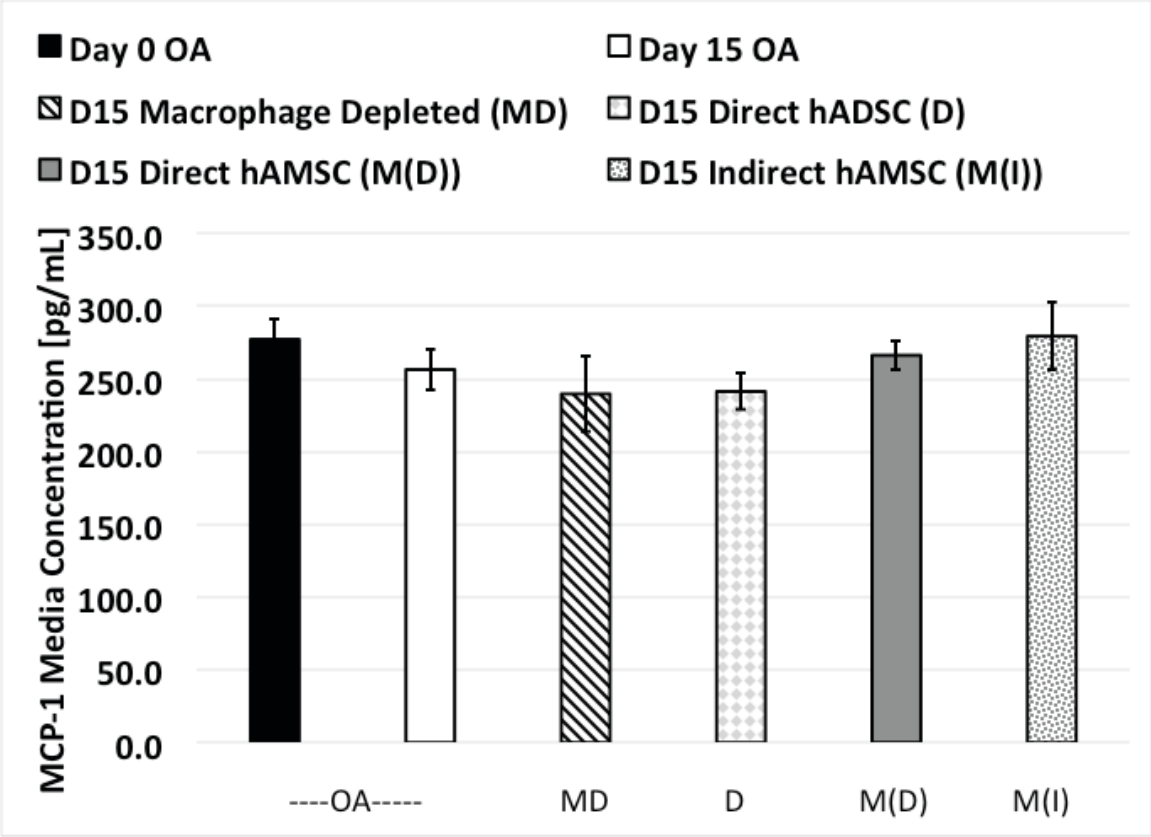


Figure B.6: Summary of MCP-1 concentrations from each OA explant group examined.

MCP-1 plays a primary role in the recruitment and infiltration of monocytes/macrophages into target tissues.[264] MCP-1 is specifically associated with chemotaxis of monocyte, memory T lymphocyte and natural killer cells.[264] MCP-1 is secreted by a variety of cells, but it is largely associated as a macrophage secretion, and is thus used as a marker of pro-inflammatory activity. MCP-1 induces proteoglycan loss through the up regulation of MMPs as well as IL-6 production.[118] It would be hypothesized that macrophage depleted and stem cell treated cultures would express less MCP-1 compared to other culture groups. However, no statistical differences are noted.

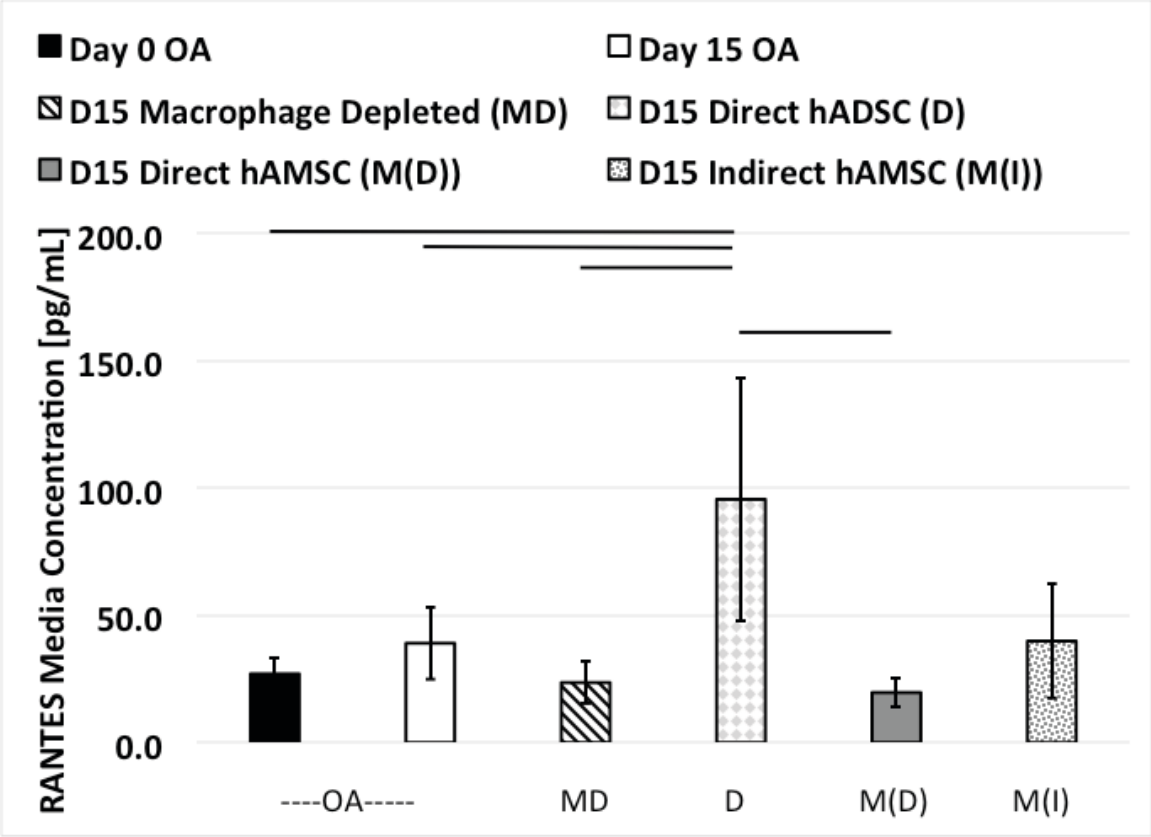


Figure B.7: Summary of RANTES concentrations from each OA explant group examined.

RANTES is largely (but not exclusively) a macrophage secreted product that induces proteoglycan loss through the up regulation of MMPs as well as IL-6 production.[118] RANTES is up-regulated by other potent pro-inflammatory mediators, including IL-1β and TNF-α.[265] RANTES is readily detected in normal adult joint tissues; however, it has been readily detected in OA patient joint tissues.[265] MCP-1, RANTES and MIP-1b are all chemotactic for monocytes, but they bind with specific (different) receptors.[264] This could be one explanation why the observed trends between these three cytokines are different. Notably, hAMSC-treated cultures in direct contact with cartilage exhibit statistically reduced RANTES expression compared to hADSC-treated cultures. Therefore, it could be hypothesized that hAMSC-treated cultures would also demonstrate the down-regulation of primary OA pro-inflammatory mediator IL-6.

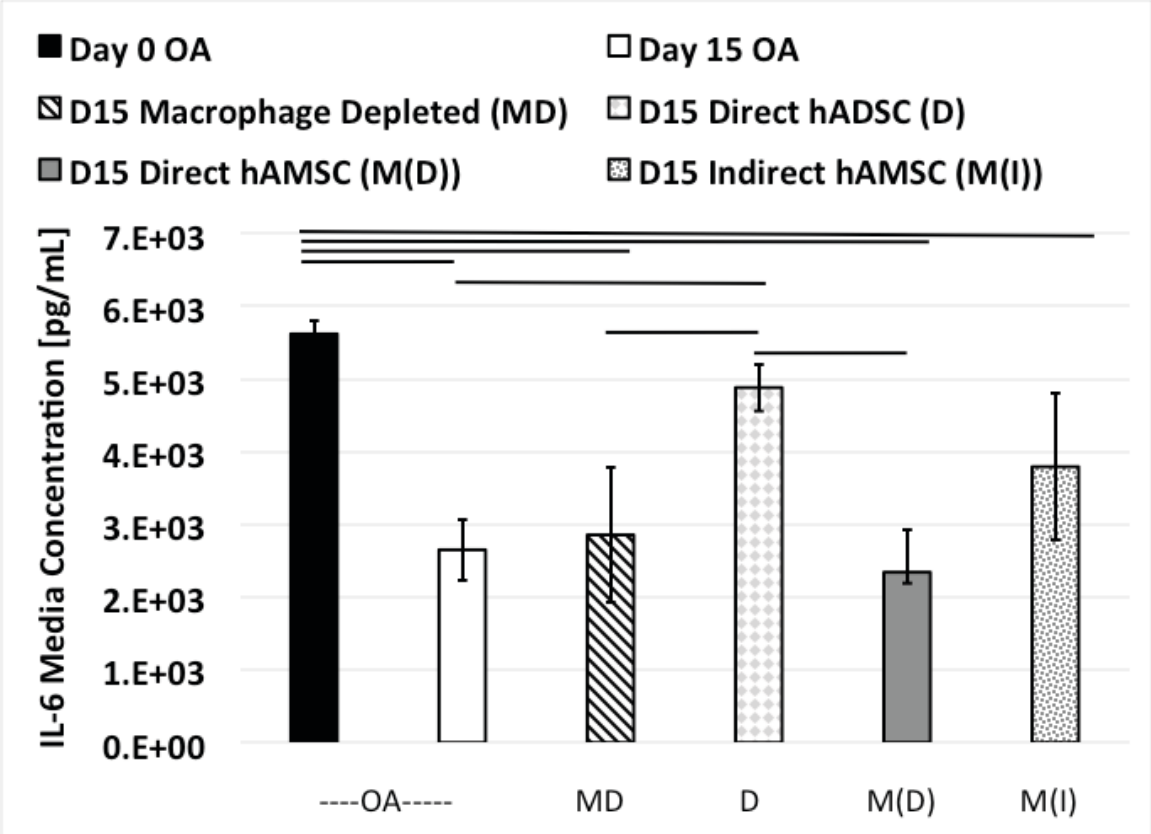


Figure B.8: Summary of IL-6 concentrations from each OA explant group examined.

IL-6 is a prominent cytokine in the pathogenesis of osteoarthritis.[118] IL-6 is known to be elevated within the synovial fluid of OA patients.[118] IL-6 secretion results in the reduction in type II collagen from cartilage via the up regulation of MMP-1 and MMP-13.[118] IL-6 secretion is dependent on IL-1, TNF-α, IL-8, MCP-1 and RANTES production (i.e. IL-6 is a downstream mediator). Since the previously described data demonstrates trending and/or statistical reductions in IL-1β, TNF-α and RANETS expression in hAMSC-treated groups (compared to hADSC-treated groups), it was hypothesized that a reduction in IL-6 would also be observed. Our results confirm this hypothesis for hAMSCs in direct contact with the cartilage.

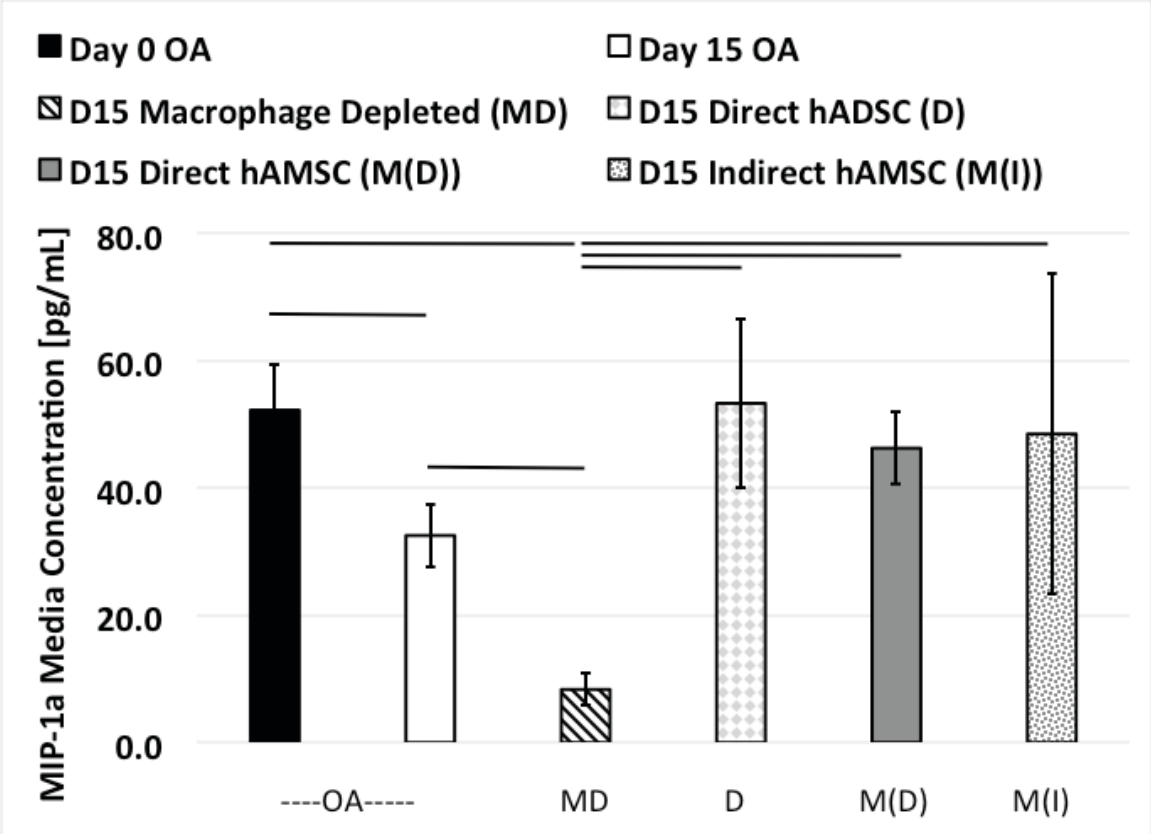


Figure B.8: Summary of MIP-1a concentrations from each OA explant group examined.

MIP-1a is a chemoattractant factor for monocytes/macrophages, and it has been implicated specifically in osteoarthritis pathology within human joints.[266] MIP-1a has been used as a prognostic indicator of early OA, as its serum detection successfully differentiated early stage OA patients from controls.[262] Additionally, MIP-1a has been correlated with IL-8 concentrations.[262] Apart from the macrophage-depleted group, our MIP-1a results show similar trends to those of IL-8. As MIP-1a is a primarily macrophage secreted product, it is logical that its secretion would be reduced under conditions of macrophage depletion.

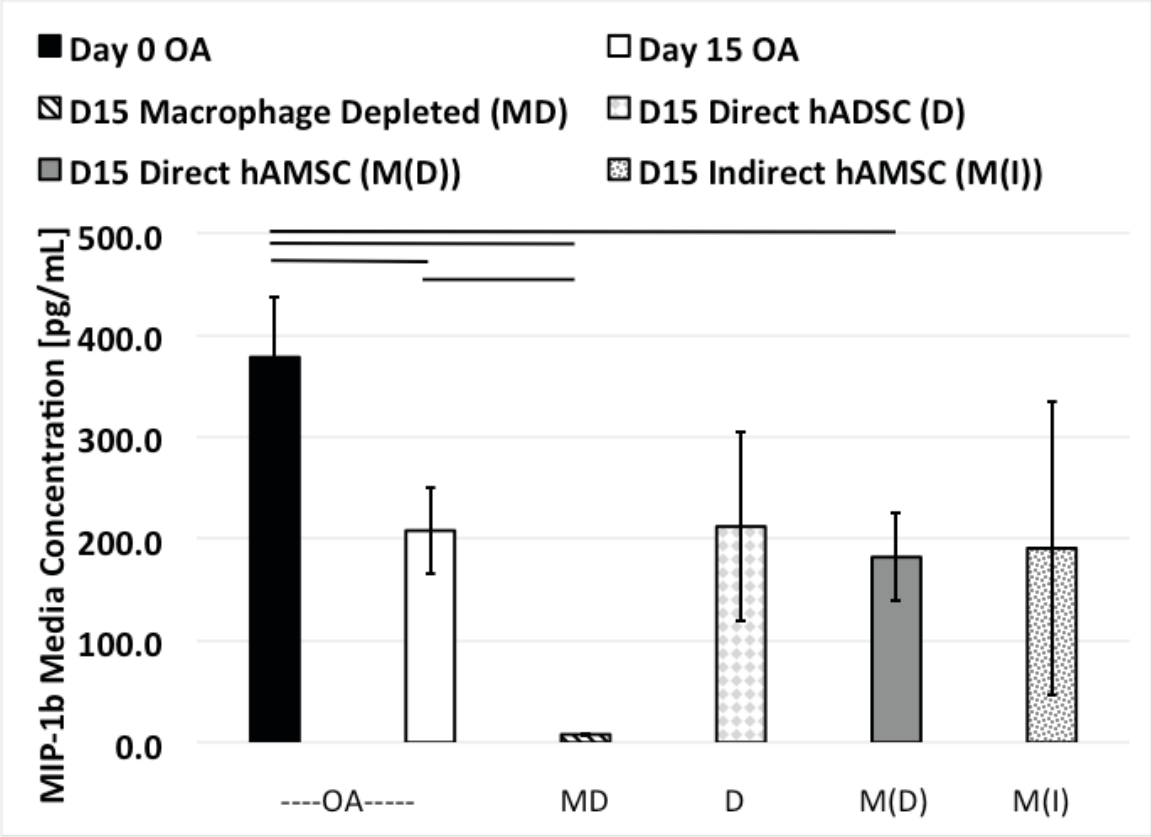


Figure B.9: Summary of MIP-1b concentrations from each OA explant group examined.

MIP-1b is a chemoattractant factor for monocytes/macrophages, and it has been implicated specifically in osteoarthritis pathology within human joints.[266] In fact, MIP-1b is the only reported cytokine with elevated levels in OA patients compared to rheumatoid arthritis patients.[266] Elevated MIP-1b expression has been correlated with worsened joints (as evaluated through clinical scoring systems) and local expression of pro-inflammatory mediators.[262] The statistically reduced expression of MIP-1b in the macrophage-depleted group could explain and/or be explained by the reduction in other pro-inflammatory mediators observed (see figure B.17 for more information on the inter-relationships of this cytokine).

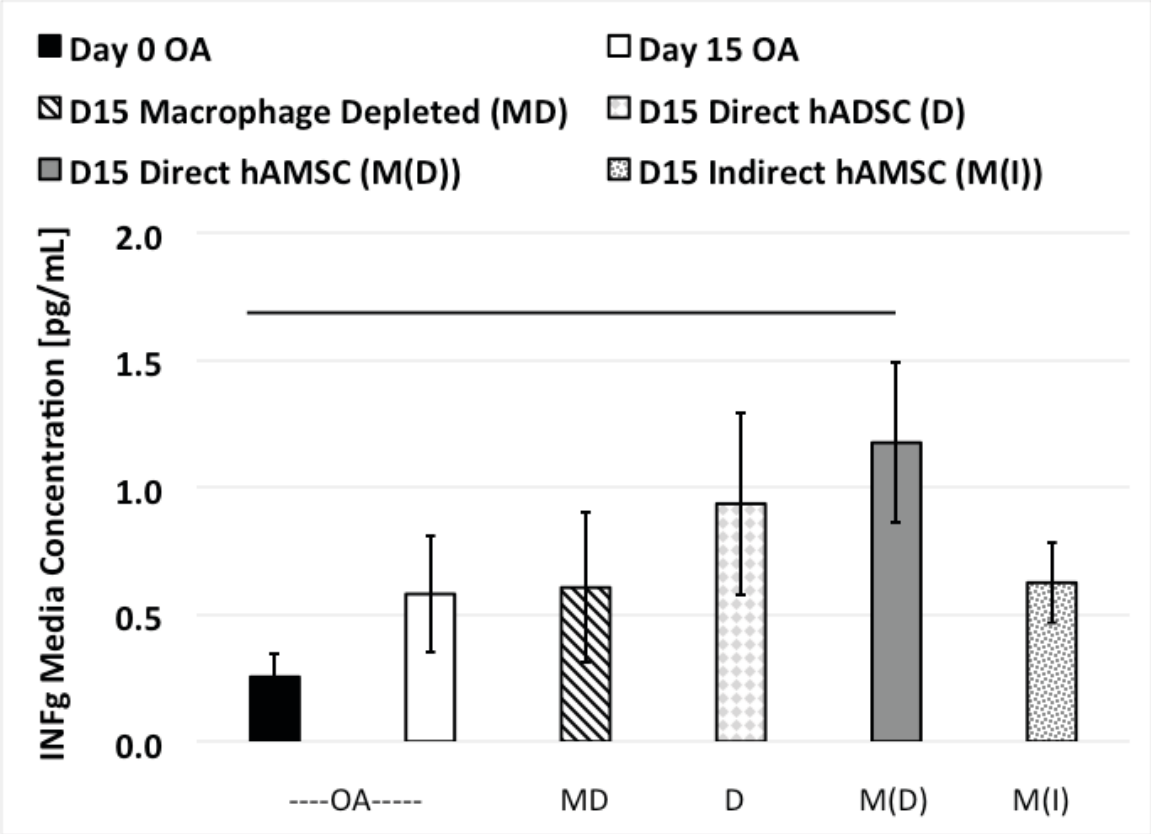


Figure B.10: Summary of INFg concentrations from each OA explant group examined.

INF-g promotes the differentiation of and is secreted by M1 macrophages.[256] INF-g, VEGF and/or GM-CSF stimulation results in the up-regulation of monokines, including, IL-1β, IL6 and MCP-1.[262] Interestingly, INFg expression appears to increase in stem cell treated cultures. This is counter to our hypotheses and the previously described IHC M1/M2 macrophage counts described in chapter V. However, as previously indicated, biochemical mediator quantification is extremely labile.[118,255,265] Many markers are involved in numerous complex biochemical cascades. Therefore, there may be an additional mechanism that is currently not accounted for in our discussions. Conclusions drawn from this cytokine data alone should be interpreted cautiously.

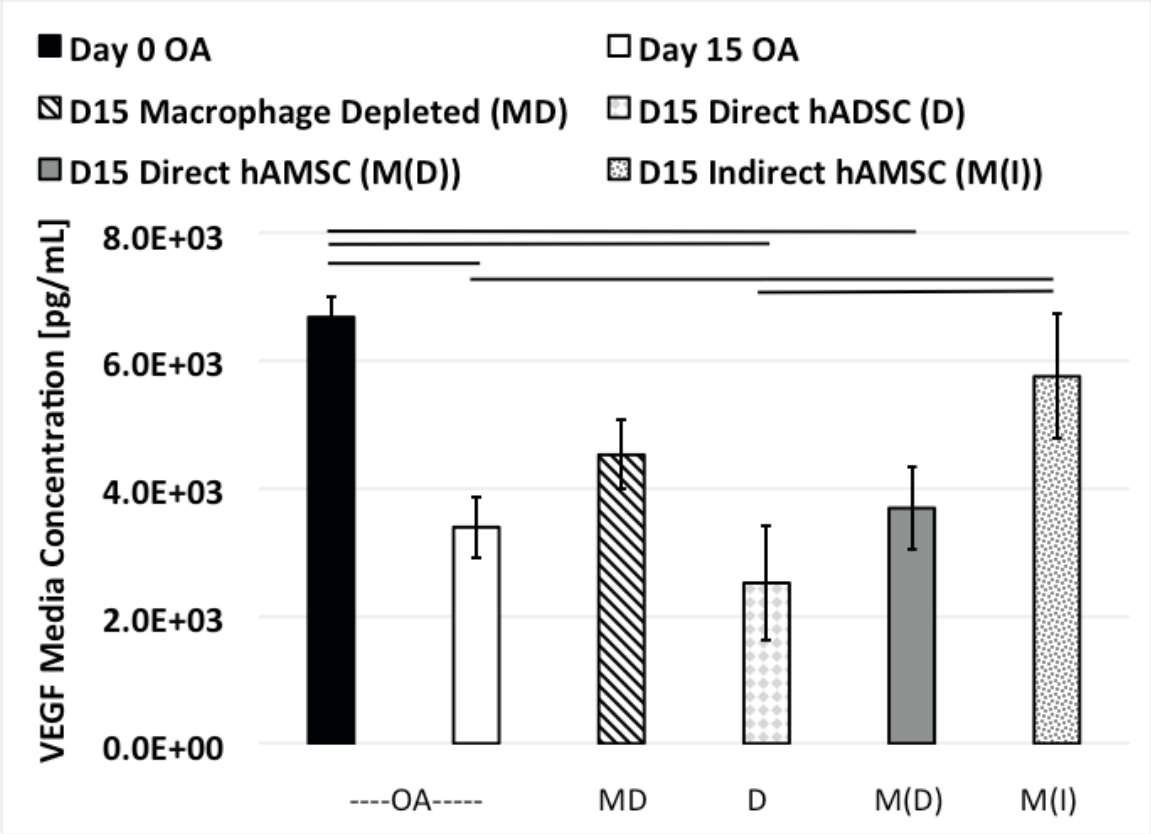


Figure B.10: Summary of VEGF concentrations from each OA explant group examined.

VEGF has a complicated and not clearly defined role in OA pathogenesis. Classically, VEGF is a fibroblastic secretion promoting angiongenesis.[132,256] Thus, VEGF has been associated with blood vessel growth in wound healing as an M2 macrophage secreted product.[256] However, as indicated previously, INF-g, VEGF and/or GM-CSF stimulation results in the up-regulation of monokines, including, IL-1β, IL6 and MCP-1.[262,267] Our data does not seem to provide clarity into which mechanism is likely involved in our explant co-culture system.

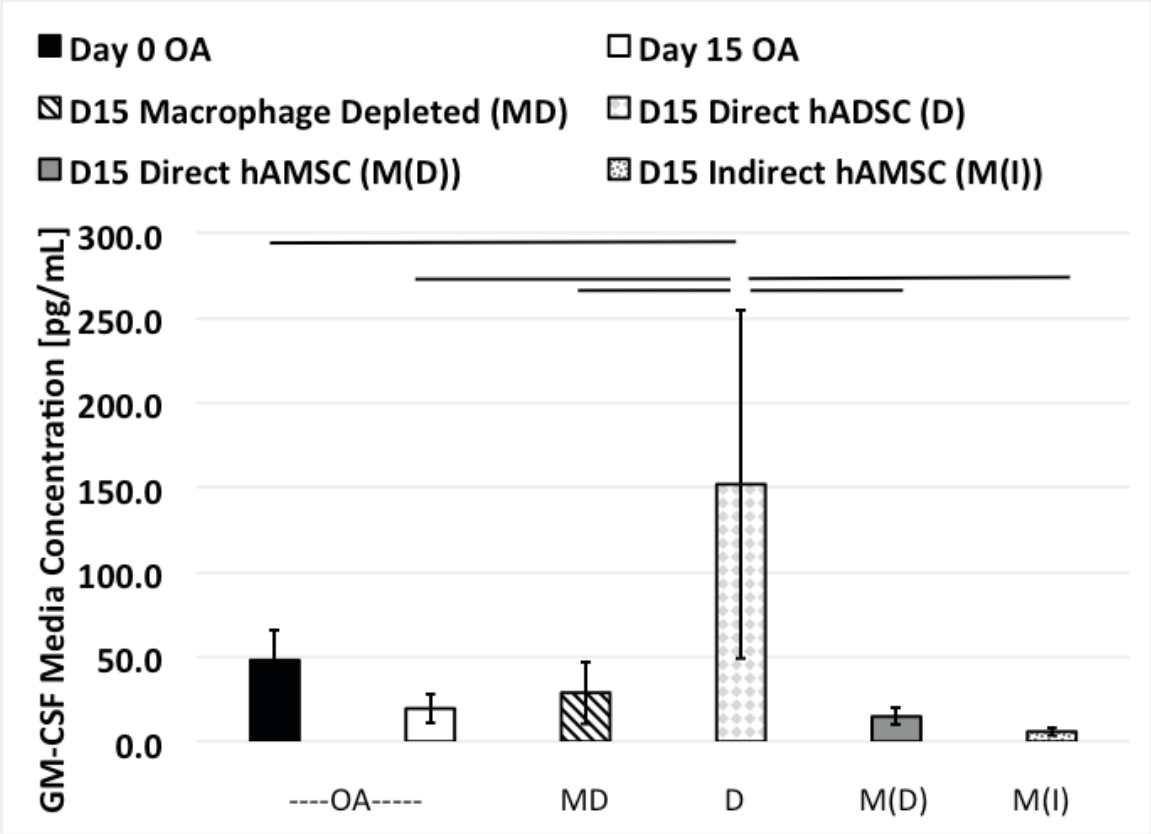


Figure B.11: Summary of GM-CSF concentrations from each OA explant group examined.

GM-CSF is only involved in the development of macrophages under inflammatory conditions (i.e. diseases such as OA) as opposed to normal, homeostatic conditions.[257] GM-CSF is associated with the differentiation and maintenance of M1 macrophages.[257] As indicated previously, INF-g, VEGF and/or GM-CSF stimulation results in the up-regulation of monokines, including, IL-1β, IL6 and MCP-1.[262,268] In light of the IHC results described in chapters IV and V, we would have expected levels of GM-CSF to be highest in OA and hADSC treated cultures. It is surprising to see these levels so low in OA cultures. However the relationship between high GM-CSF expression in hADSC treated cultures with low expression in hAMSC treated cultures seems to corroborate the previously described IHC data.

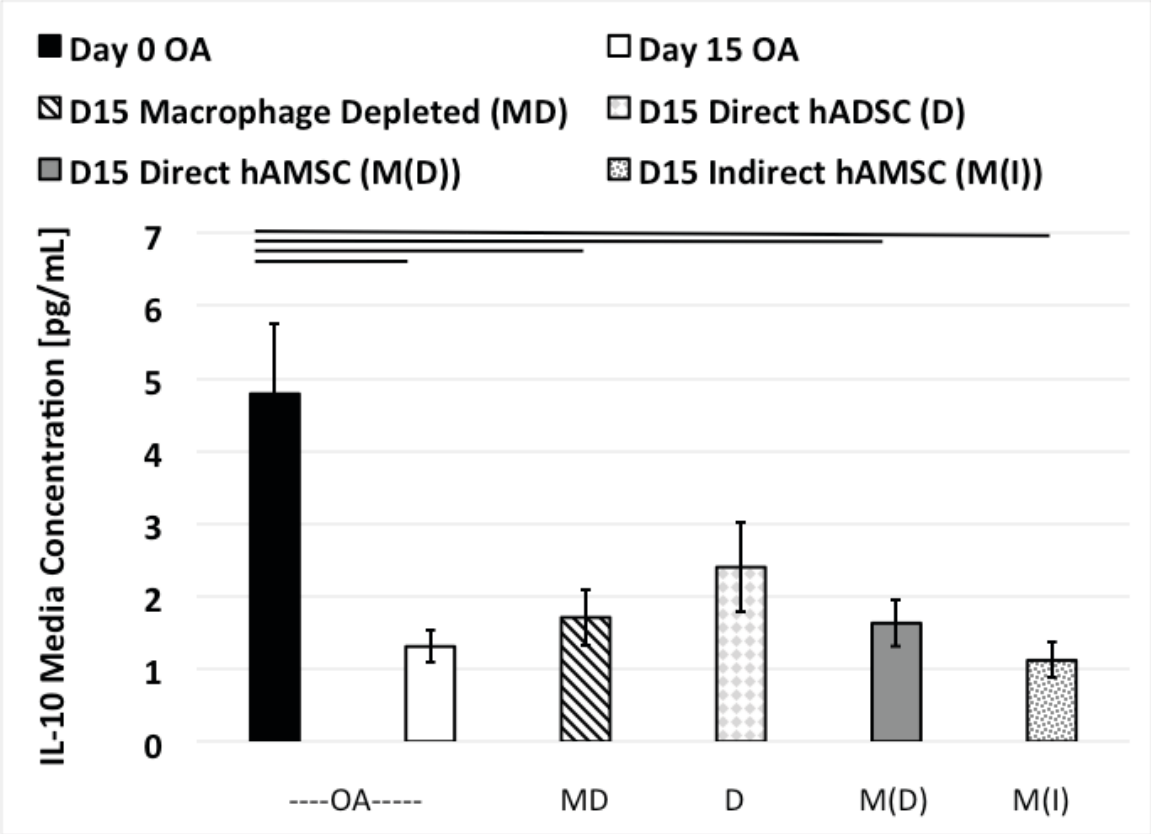


Figure B.12: Summary of IL-10 concentrations from each OA explant group examined.

IL-10 is the first of three anti-inflammatory mediators measured through our analyses (although some prefer to classify IL-10 as a regulatory, as opposed to anti-inflammatory, cytokine[269]). IL-10, IL-13 and IL-4 induce an M2 phenotype within macrophages.[256,257] IL-10 suppresses TNF-α, as it directly inhibits localization of nuclear NF-κB.[267,270] However, IL-10 less reliably suppresses IL-1 synthesis. As previously indicated, the suppression of both IL-1β and TNF-α are necessary for inflammatory modulation.[150] This could be one mechanistic explanation for the beneficial therapeutic effects observed with combination IL-4/IL-10 therapies[270] as well as reports that IL-10 alone is not an effective immunomodulator.[268] IL-10 has also been shown to increase IL-1Ra synthesis (IL-1Ra is a competitive inhibitor of IL-1β).[270]

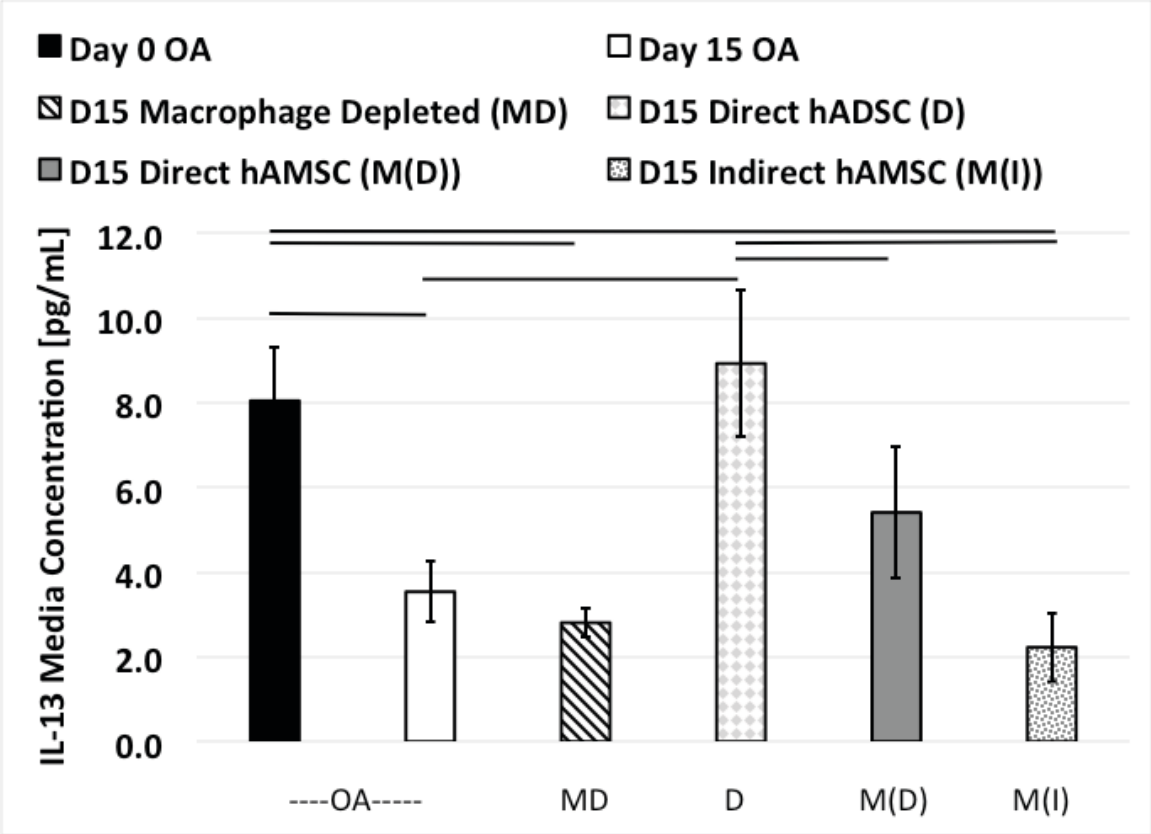


Figure B.13: Summary of IL-13 concentrations from each OA explant group examined.

IL-13, IL-10 and IL-4 induce an M2 phenotype within macrophages.[256,257] IL-13 and IL-4 have demonstrated the ability to mitigate cartilage degradation in vitro.[258] While IL-4 and IL-10 have direct effects on chondroprotection through the down regulation of ADAMTSs and MMPs, respectively, IL-13 produces an indirect anti-inflammatory effect through the regulation of PGE_2.[259] There is limited data providing insight into the relative efficacy of IL-4 and IL-13 therapeutic strategies. However, this mechanistic insight seems to offer a potential explanation for the heightened and more rapid mitigation of OA observed in our data. This is described further in figure B.17.

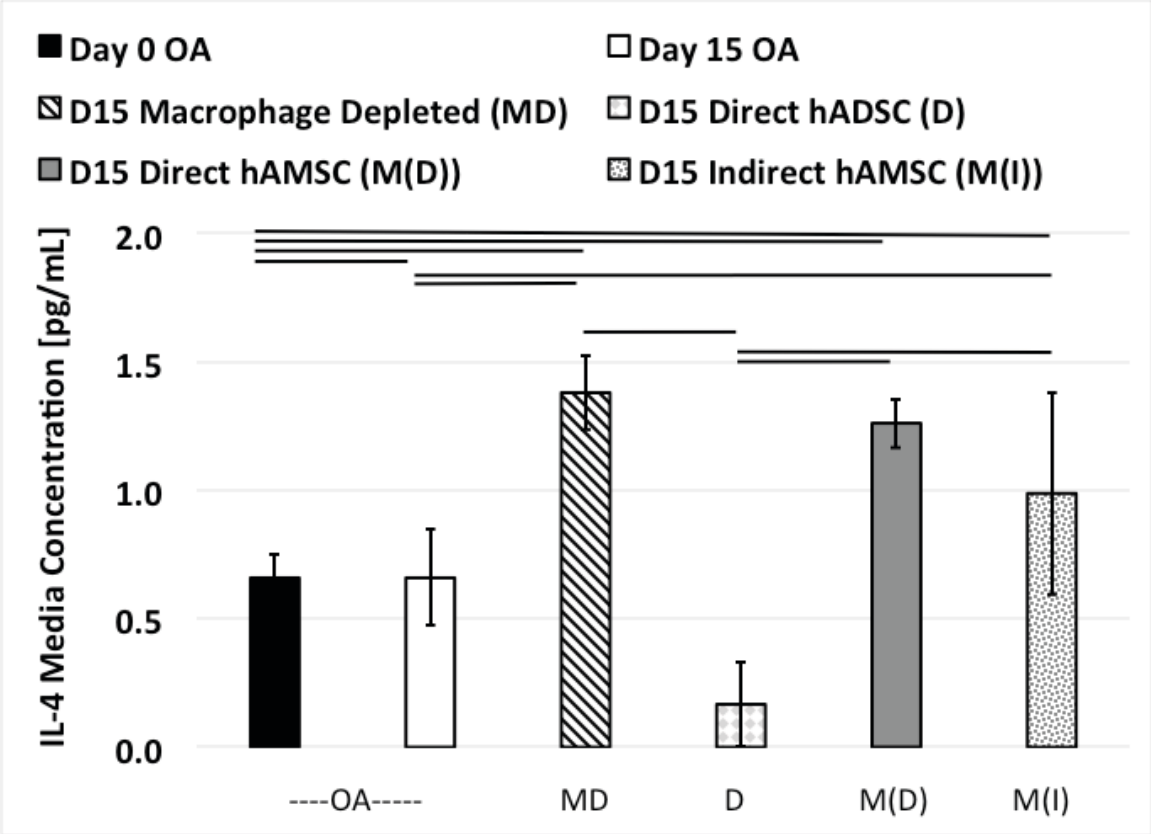


Figure B.14: Summary of IL-4 concentrations from each OA explant group examined.

IL-4, IL-10 and IL-13 induce an M2 phenotype within macrophages.[256,257] IL-4 suppresses IL-1β and TNF-α expression.[258,267,268] This suppression effect can be enhanced through concomitant expression of IL-10. IL-10 alone is not an effective suppressor of pro-inflammatory cytokines and does not result in significant chondroprotection.[267] However, IL-4 alone offers both immunomodulatory and chondroprotective effects.[267] hAMSC treatment significantly increases IL-4 expression (compared to OA cultures and hADSC-treated cultures). This provides mechanistic insight into a key up-stream anti-inflammatory mediator that could explain the previously results described results in chapter V. This is described further in figure B.17.

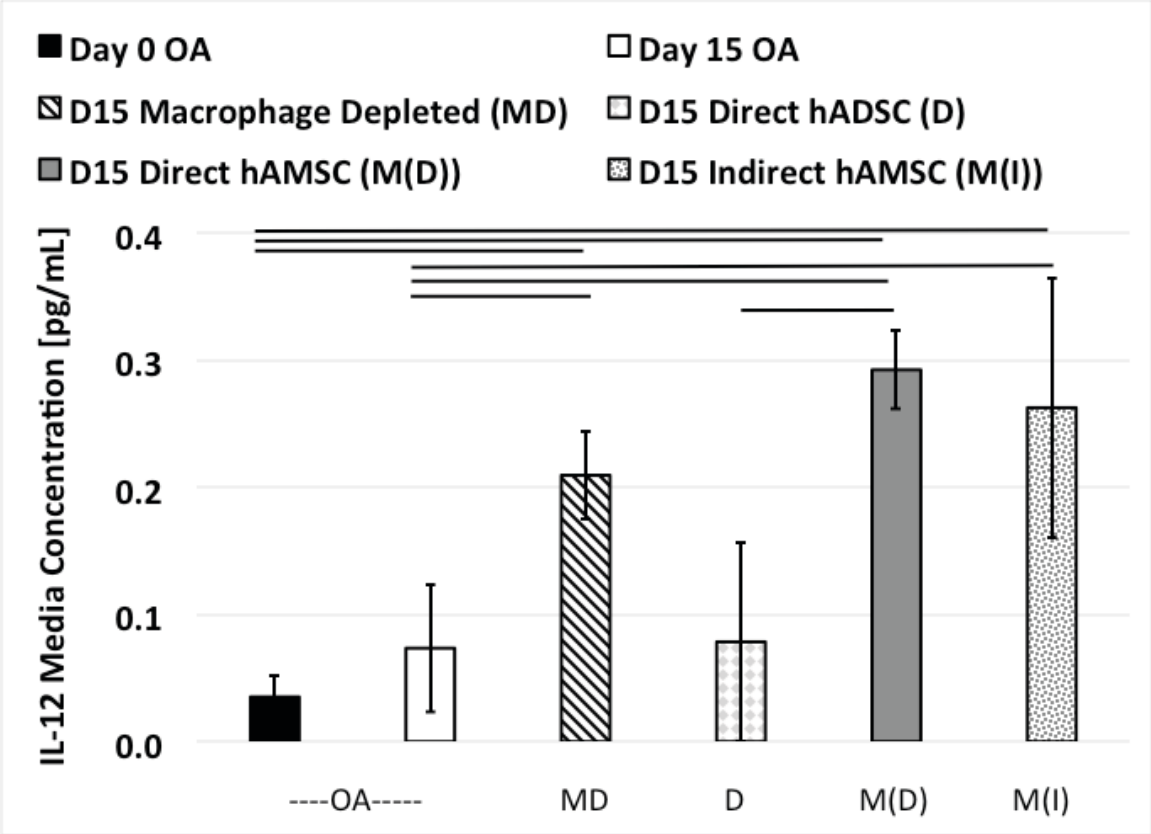


Figure B.15: Summary of IL-12 concentrations from each OA explant group examined.

IL-12 is classically considered to maintain and regulate inflammation within the synovium.[271] IL-12 is a macrophage secretion associated with the recruitment of helper T cells (Th_1).[132] In this activation pathway, IL-12 functions to protect the body against intracellular pathogens through the up-regulation of INFg and IL-2.[272] However, the trends in our INFg and IL-2 results do not seem to support this activation pathway of IL-12. More recently, evidence supports a less significant role for IL-12 in pro-inflammatory responses, as IL-23 (not IL-12) is necessary for inflammatory reactions.[272] For example, IL-23 and not IL-12 was required for the development of collagen-induced arthritis in mice.[272] Though there is not a consensus within the scientific community, IL-12 is now hypothesized to play a protective role in immunity.[272] IL-12 has also been shown to have an inhibitory effect on IL-17 (a marker of rejection and diseases characterized by chronic

inflammation).[272] Therefore, the elevated levels of IL-12 observed in macrophage depleted and amnion stem cell treated groups may represent an under investigated mechanism of immune-regulation.

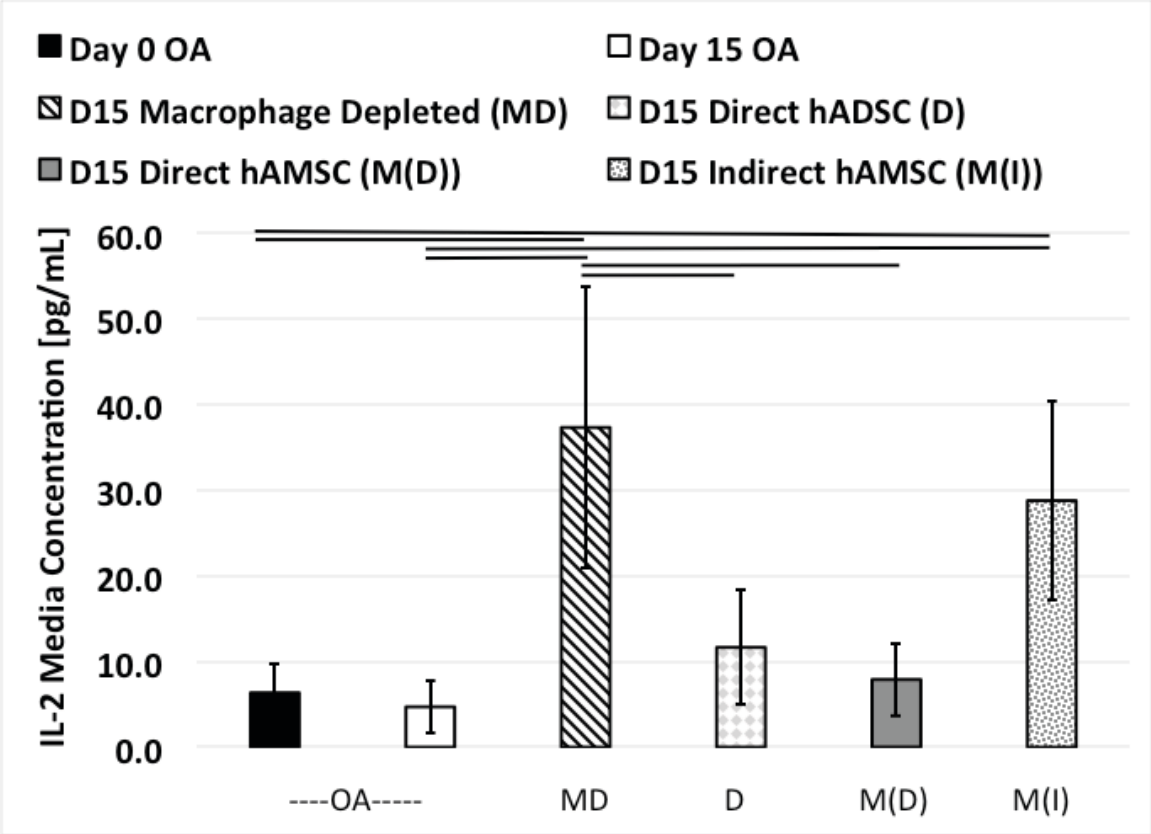


Figure B.16: Summary of IL-2 concentrations from each OA explant group examined.

IL-2 is associated with the recruitment of helper T cells (Th_1) and promotion of inflammation via the stimulation of IL-17.[132,272] Interestingly, our IL-2 results do not follow the stimulatory patterns expected with the classical activation of IL-12. The low levels of expression observed in most groups support the known *auxiliary* role of T-cells in OA. However, the heightened concentrations observed in the macrophage depleted and indirect hAMSC treated cultures are unexpected results.

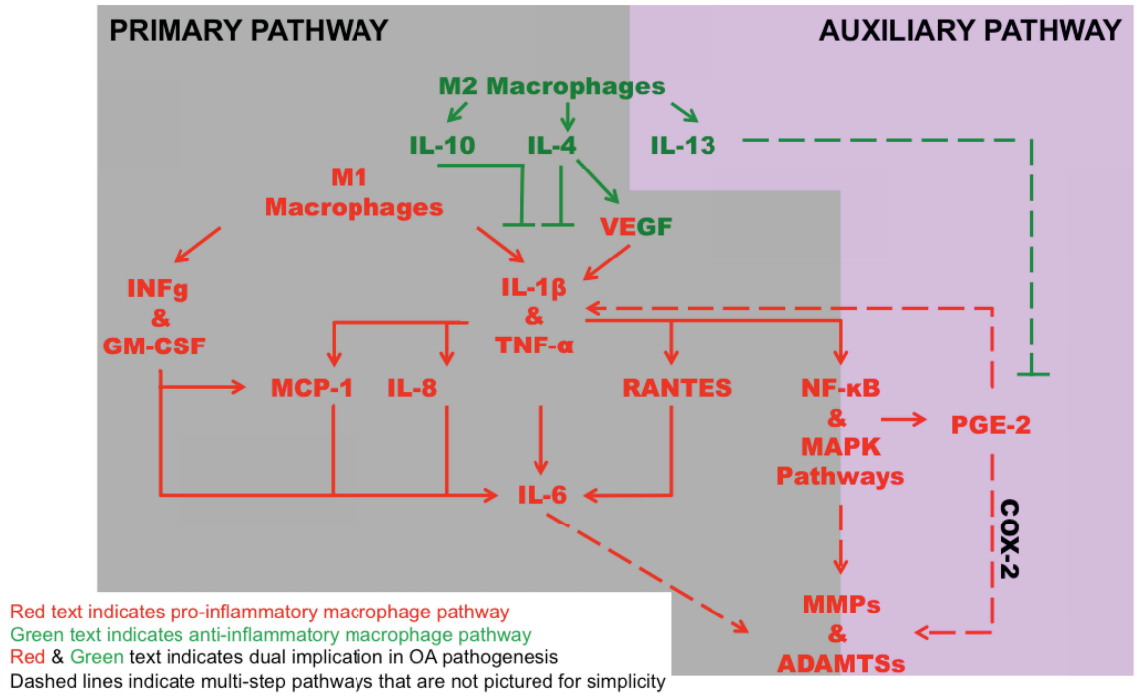


Figure B.17: Simplified Summary of Examined Cytokine Relationships. Red text indicates pro-inflammatory (M1) macrophage pathway. Green text indicates anti-inflammatory (M2) macrophage pathway. Mixed colors indicate a multi-functional (pro and anti inflammatory) role in OA. Dashed lines indicate multi-step pathways that are not pictured for simplicity. Note: PGE_2 does not directly stimulate IL-1 and TNF production; it increases expression of surface TLRs therefore increasing the binding activity of IL-1β and TNFα. PGE primarily acts via the COX-2 pathway.

The observed heightened expression of IL-13 by hADSC treated cultures may imply an insufficient or delayed anti-inflammatory effect due to its indirect role in chondroprotection. Primary PGE_2 stimulates ADAMTS and MMP production via the COX-2 pathway, an auxiliary pathway in OA progression.[273,274] PGE_2 has secondarily been described as up-regulating the production of TLRs on cell surfaces, ultimately increasing the production of IL-1β and TNF-α.[273] IL-4 has a more direct inhibitory role, acting on the primary NF-κB, MAPK and JAK/STAT pathways of OA progression.[258,267,268] The up-regulation of IL-4 by hAMSC-treated cultures may explain the rapid and more extensive mitigation of disease progression observed.

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