The Effects of Aging and Chemotherapeutics on Mesenchymal Stem Cell Regenerative Properties

by

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Beane OS, Fonseca VS, Cooper LL, Koren G, Darling EM. (2014) Impact of Aging on the Regenerative Properties of Bone Marrow-, Muscle-, and Adipose-Derived Mesenchymal Stem/Stromal Cells. PLoS ONE 9(12): e115963. doi:10.1371/journal.pone.0115963 PMCID: PMC4277426

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CONFERENCE ABSTRACTS

Beane O, Darling L, Fonseca V, Darling E. September 2015. Dihydrofolate Reductase Levels Provide a Mechanism for Stem Cell Resistance to Chemotherapy. *Tissue Engineering and Regenerative Medicine International Society*. Boston, MA

Beane O, Cooper L, Koren G, Darling E. March 2014. Disparate Aging Effects Exist for Mesenchymal Stem Cells Derived from Different Tissues. *Orthopaedic Research Society*. New Orleans, LA.

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Chapter 1

Introduction

1.1 Motivation/Chapter Overview

Mesenchymal stem cells (MSCs) reside in various different tissues and exhibit the ability to differentiate along mesodermal lineages such as bone, fat, and cartilage. In addition to their multipotency, stem cells are immunosuppressant, reducing T-cell proliferation and cytokine production. Within their niches, stem cells are responsible for tissue maintenance and repair. As such, impairment of MSCs can lead to tissue damage. Several elements effect MSC function, including chemical and environmental factors. While research has focused on utilizing these cues to enhance stem cell function and regenerative properties, little is known about conditions that can impair MSCs. Moreover, how tissue source influences stem cell response to these chemical and environmental factors has not been studied extensively. The goal of this thesis was to examine adverse effects of aging and chemotherapeutics on MSC function and to reveal inherent differences among distinct cell types. This work is critical for identifying optimal MSC sources to be used for regenerative therapies.

Chapter 2 compares the use of pluripotent and mesenchymal stem cells for cartilage repair. This review covered recent literature investigating isolation procedures, surface marker profiles, and chondrogenic induction factors specific to various stem cell types. While several stem cell sources have been considered for cartilage regenerative therapies, an optimal type has not been identified since each has its strengths and weaknesses. This review sought to examine the variability and feasibility of stem cell use for this application by comparing current cell-specific methodologies.

Chapter 3 investigates disparate aging effects among MSCs derived from bone marrow, muscle, and adipose tissue. This study assessed differences in expansion and multipotent properties between cells isolated from young and old rabbit donors. Since MSCs are being considered as a treatment for diseases prevalent in older populations, the effects of aging on MSC function have been investigated to evaluate their potential use for autologous therapies. Several studies have successfully revealed adverse aging effects on a single cell source [1-3]. However, this response has not been compared among MSCs derived from different tissues. The goal of this study was to examine variability in age-induced impairment to identify an MSC type that is most resistant to adverse aging effects.

Chapter 4 examines the effects of several chemotherapeutic agents on normal cell response. This study compared differences in drug-induced impairment between ASCs and a non-stem cell fibroblast population. While chemotherapeutics are effective at killing cancerous cells, they also damage normal cells due to their non-specificity [4]. Studies have reported that normal cell types such as BMSCs and osteoblasts are impaired my chemotherapeutics, leading to long-term bone loss [5,6]. However little is known

about the drug sensitivity of other normal cell types that are responsible for tissue maintenance and repair, such as ASCs. The goal of this study was to characterize ASC response to several chemotherapeutic agents and reveal stem cell resistance or susceptibility to these treatments.

Chapter 5 investigates the effects of methotrexate (MTX) on mature vs stem cell function and the mechanism behind this response. This study examined the regenerative properties of MTX-treated BMSCs, ASCs, and osteoblasts. Moreover, knockdown and overexpression of dihydrofolate reductase (DHFR), the target of MTX, is assessed in treated cells. MTX is a commonly used chemotherapeutic agent that impairs both cancerous and normal cells by binding to and preventing the function of DHFR [7]. Treatment with this drug leads to long-term tissue damage by altering MSC differentiation potential and inhibiting mature cell proliferation [5,6]. However, whether these adverse effects vary among normal cell types is not known. Recent reports have shown that DHFR can contribute to MTX sensitivity, but the role of this protein in normal cell response has not been investigated thoroughly [8]. The goal of this study was to examine how cell type and DHFR expression influence normal cell response to treatment to identify mechanistic or regenerative approaches that prevent drug-induced damage.

The content of chapter 6 summarizes the main conclusions from each of the aforementioned studies. Future directions that will enable better understanding of this work's findings are also proposed.

1.2 Background

1.2.1 MSC Niches

Several MSC tissue sources have been identified since the initial discovery of these cells, including adipose tissue, muscle, and tendon [9]. For all of these sources, it is thought that MSCs reside within tissue-specific niches *in vivo* [9]. While research in this field is limited, studies that have investigated MSC niches have proposed that their location is perivascular, which could promote local regeneration as well as enable migration of MSCs to other tissues for repair [9]. Vascular localization of the stem cell niche is supported after observing positive expression of α- smooth muscle actin on several MSC types [10]. Moreover, BMSCs and dental pulp stem cells line blood vessels within their native tissues, while ASCs home to vasculature after they are reinjected into adipose tissue [11,12]. Niches are comprised of several components, including other resident, mature cells, extracellular matrix, and tissue-specific factors that influence MSC function through cadherins and integrins (Fig. 1.2.1, [9]) [13-15]. These constituents act together to prevent differentiation and exhaustion of MSCs in healthy tissues and initiate differentiation after injury to promote repair. If components of the MSC niche are impaired, this can result in tissue degradation [16]. Niche homeostasis is therefore essential for tissue health and maintenance, which has recently motivated researchers to better understand these microenvironments and how they influence MSC function. The perivascular location of MSCs suggests that cellular components of niches include resident cell types like pericytes and endothelial cells (ECs). It has been reported that pericytes play a role in blood flow regulation, vascular stabilization, and tissue maintenance [17]. Due to their multipotency, it has also been proposed that these cells are actually MSCs [9,18,19]. However, due to differences in surface marker profiles, this

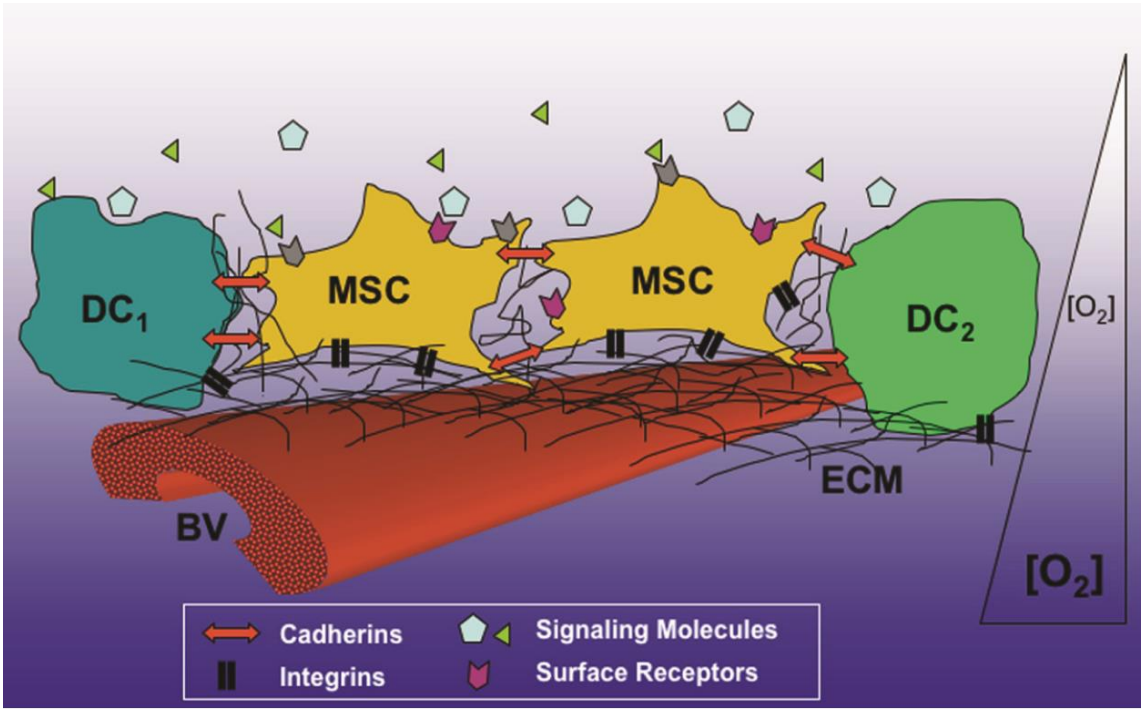

Figure. 1.2.1 MSC Niche. The perivascular MSC niche is comprised of many components, including differentiated cells ($DC_{1,2}$), signaling molecules, and ECM. Stem cells interact with these components through cadherins and integrins.

theory is has been debated [20]. ECs comprise the linings of blood vessels, helping to maintain their structure [21]. Within the MSC niche, it is likely that ECs and stem cells interact. Co-culture assays observed that combining ECs and ASCs promoted vascular assembly [22]. It has also been shown that ECs self-assemble with MSCs in 3D culture and that stem cell differentiation is affected by their presence [23].

While the signaling components within MSC niches are not well understood, several factors have been proposed to contribute to stem cell function within these microenvironments, including hypoxia and growth factors. Studies investigating the oxygen levels of bone marrow and adipose tissue observed regions with as low as 1-3% O_2 [24,25]. For BMSCs, it was also observed that cells in hypoxic culture conditions

proliferate faster and maintain their undifferentiated state for longer [26,27]. Fewer investigations have been conducted on ASCs. However, it has been reported that hypoxia improves the chondrogenic differentiation of these cells [28]. In addition to hypoxia, fibroblast growth factor-2 (FGF-2) has been considered to influence stem cell properties within their niches. Research has shown that FGF-2 increases ASC lifespan *in vitro* and could therefore be an important contributor to maintaining stem cell health within the niche [29]. Furthermore, FGF-2 stimulates muscle-derived stem cells to enter the cell cycle from a quiescent state, indicating the importance of this signaling cascade after injury [30]. Due to the positive effects of FGF-2 on stem cell function, it is often incorporated into expansion medium to mimic its role in MSC niches by maintaining stem cells in their undifferentiated state and improving proliferative capacity [31].

MSCs are also influenced by the ECM components that comprise their niches [32]. While ECM is important for structural purposes, it has more recently been recognized for its influence on cell behavior and role in development [33]. A recent investigation observed increased osteogenic differentiation of MSCs after being seeded on osteoblast-secreted ECM [34]. Moreover, collagen IV is an essential ECM component for stem cell quiescence in muscle and preserving stem cell self-renewal [35]. As would be expected, ECM and other components of stem cell niches vary based on tissue source. However, these dissimilarities are essential to maintain the unique properties of different stem cell types [32].

1.2.2 MSC Response to Environmental Stress

MSCs are exposed to both intracellular and extracellular stresses throughout their lifetime [36]. While these are often negligible within healthy tissues, factors such as

aging, trauma, and chemotherapy can lead to high levels of oxidative stress and DNA damage [36-39]. As such, researchers have been motivated to investigate MSC response to reactive oxygen species (ROS) production and double strand breaks (DSBs) to understand adverse effects and cellular DNA repair mechanisms that occur in these diseased environments.

ROS production at extreme amounts can lead to cell apoptosis or senescence due to a pro-oxidant/antioxidant imbalance [40,41]. For MSCs, high levels of hydrogen peroxide-induced ROS production trigger apoptosis through endoplasmic reticulum and mitochondrial pathways [42]. However, low levels cause MSC senescence [42,43]. ROS-induced senescence is regulated through activation of the p53 protein signaling cascade, which initiates entry of cells into growth arrest [44]. Upregulation of p21 in conjunction with p53 furthers cytostatic response [45]. While MSCs are susceptible to ROS-induced impairment, they are more resistant to oxidative stress than terminally differentiated cell types such as chondrocytes and fibroblasts [42,43]. MSC resistance has been proposed to result from p21, which returns to normal levels of expression in stem cells soon after hydrogen peroxide exposure [43]. Conversely, terminally differentiated cells exhibit elevated levels of p21 long after removal, which likely contributes to more severe, long-term effects. As a result, MSCs are capable of resisting oxidative stress more effectively.

DNA DSBs can lead to cell senescence, apoptosis or mutations, if repair is inaccurate [46]. Chemotherapy-induced DSB formation in MSCs activates ataxia telangiectasia mutated (ATM), which phosphorylates p53 and causes cell senescence through consequential telomere shortening [47]. However, relative to cancerous and terminally differentiated cells, MSCs are resistant to chemotherapy-induced apoptosis

[48,49]. MSC resistance to DSBs is reportedly regulated by expression of BCL-2, a family of proteins responsible for directing apoptosis [48,50]. In undifferentiated MSCs, Bcl-2 is downregulated and prevents cell death [48]. Conversely, protein expression increases during differentiation and sensitizes cells to apoptosis. Another protein responsible for MSC resistance to DNA damage is p73α, a member of the p53 family and inducer of apoptosis [51]. MSCs inherently express p73α at low levels. However, ectopic overexpression causes cells to undergo heightened apoptosis after drug exposure. Therefore, multiple mechanisms could contribute to MSC resistance to DNA DSBs.

1.2.3 Characterizing MSCs

Due to the clinical potential of MSCs, research in this field has grown dramatically, with new labs studying these cells every year. Unfortunately, increased interest in MSCs and their use *in vitro* has also led to ambiguities. Among labs, there are differences in isolation processes, expansion procedures, and characterization, which could suggest that resulting cell types are not similar enough for direct comparison across literature [52]. As such, researchers have aimed to determine properties that can be used to identify MSCs *in vitro* to instate some level of consistency among research groups and enhance understanding of these cells.

Two of the main criteria that are employed to classify MSCs are plastic adherence and differentiation potential (Fig. 1.2.2, [53]). The use of adherence as an MSC marker stems from early studies investigating bone marrow cell composition. Friedenstein and colleagues found that in addition to non-adherent haematopoietic stem cells (HSCs), bone marrow is also comprised of adherent stromal cells [54]. As a result, plastic adherence has been used to distinguish between MSCs and HSCs and is employed in isolation

procedures [55,56]. Plastic adherence is also exploited as a stem cell enrichment technique since it eliminates the contaminating, non-adherent fraction, which includes non-MSCs that are present in freshly isolated populations [57,58]. After identifying that adherent stromal cells exist in marrow, further characterization revealed that these cells are capable of multilineage differentiation, a hallmark of all stem cell types [59]. *In vivo*, stem cell differentiation promotes tissue maintenance, since it enables MSCs to replace mature cells that have undergone apoptosis [60]. MSC multipotency is measured *in vitro* by confirming differentiation along adipogenic, osteogenic, and chondrogenic lineages. This trilineage assay is not only used to confirm that populations are capable of differentiation, but it is also employed to refute that they are unipotent progenitor cells by showing that they can transform into cells from many different tissue sources. Quantifying lineage-specific metabolite production along these lineages is also important for understanding the differentiation capacity of isolated populations and their potential use in regenerative therapies.

Self-renewal is another property that is used to classify stem cell types like MSCs and refers to the ability of a cell to proliferate by clonal expansion and maintain its undifferentiated state [9]. Stem cell self-renewal is a significant property of MSCs since it perpetuates the stem cell pool within each niche and enables tissue repair after injury [61]. Self-renewal assays were first established for HSCs and involve re-transplantation of these populations into irradiated patients to assess the cells' ability to replenish pools of haematopoietic cells [62]. For MSCs, self-renewal investigations have only recently gained interest. While no standard assay has been established, one method that has been employed is examination of *in vitro* differentiation after multiple population doublings

[62-64]. However, this approach has received some criticism since it is less stringent than HSC assays. Another alternative self-renewal technique used for MSCs is the colony forming unit-fibroblast (CFU-F) assay, which tests the clonogenic capabilities of cells. CFU-F assays involve plating MSCs at low seeding densities and quantifying the number of colonies that form comprising more than 20-50 cells [65,66]. While differentiation is not always involved, it confirms the clonogenic capacity of MSCs, which is a component of self-renewal. Moreover, it is often used to quantify the number of stem cells present within populations. Genetic mechanisms that regulate this stem cell property have also

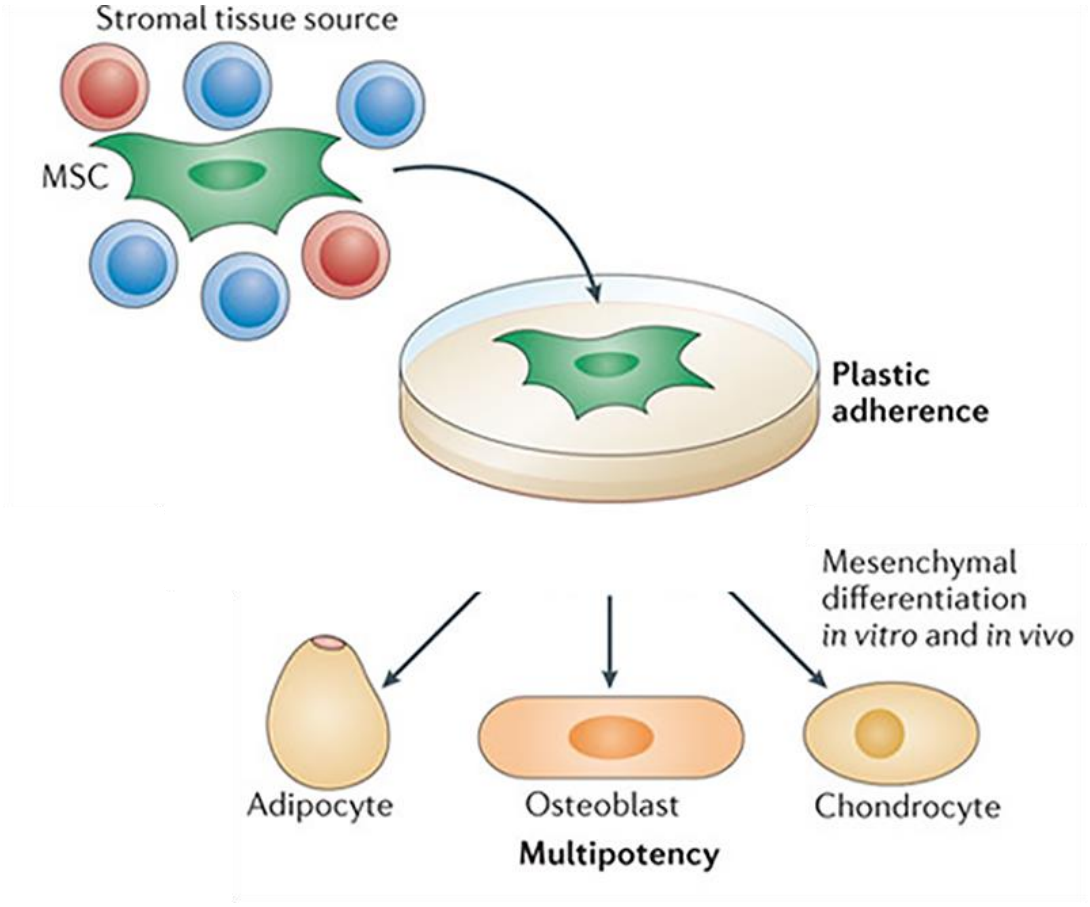

Figure. 1.2.2 MSC Characterization. Two of the main properties used to characterize and identify MSCs are plastic adherence and multipotency

been identified, including *oct-4* and *sox-2*, to enhance understanding of this function [67]. However, these genetic markers have not been studied extensively for MSCs and are therefore not a reliable method of confirming self-renewal capacity.

Surface marker profiles are another method used to characterize and identify MSCs. It has been proposed that these cells express CD105, CD73 and CD90, but lack CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR expression [52]. While surface markers have been employed extensively throughout literature with some degree of success, there are disadvantages associated with this technique, as well [68-70]. It has been reported that surface marker profiles of multipotent stromal cells change temporally during in vitro expansion and that they are inconsistent both across literature and among MSC types [71,72]. Furthermore, surface markers of MSCs often overlap with mature cells, suggesting that certain markers are not appropriate for accurate identification [73]. Due to the conflicting evidence about the reliability of surface marker characterization of MSCs, this approach is not always employed in stem cell studies.

1.2.4 MSC Multilineage Differentiation

In vitro differentiation of MSCs along adipogenic, osteogenic, and chondrogenic lineages are established techniques that, as previously mentioned, are used to not only confirm the stem cell status of cell populations, but to also provide insight into the regenerative capacity of cells. This process involves exposure of cells to induction factors that promote differentiation along each specific lineage. After a period of time in culture, cells are then examined for either genetic or metabolite markers to assess the extent of differentiation. Induced cultures are often compared with cultures that have not been exposed to lineage-specific induction factors in order to assess the degree of

differentiation. While this general procedure is employed for all forms of differentiation, induction factors and genetic markers are lineage-specific. Adipogenic differentiation has been investigated for a number of models and MSC types. Induction cocktails typically consist of varying concentrations of insulin, indomethacin, 3-isobutyl-1-methoxanthine (IBMX), and dexamethasone [74]. However, adipogenesis has also been induced successfully without indomethacin and/or IBMX. An alternative cocktail component that has been investigated is rosiglitazone, an agonist of the adipogenic regulator peroxisome proliferator-activated receptor (*PPARγ*). Rosiglitazone acts by binding to cells and sensitizing them to insulin, which enhances adipogenic differentiation [75]. Differentiation cocktails can vary depending on both the MSC tissue source and experimental model used. Cells are typically cultured in 2D and exposed to induction media for 2-3 weeks [76,77]. Though 3D cultures have also been used and can increase adipogenic capacity [78]. Adipogenic differentiation is assessed by several genetic or metabolic markers. Adipogenic genes such as lipoprotein lipase (*LPL*) or *PPARγ* are assessed by PCR at one week or earlier [79,80]. LPL is a lineage marker of adipocytes. Conversely, *PPARγ*, as mentioned previously, is considered a regulator of differentiation since increased expression leads to greater adipogenic potential, while decreased levels instead enhance osteogenic capacity [81,82]. Terminal adipogenic differentiation of MSCs is most commonly examined and is assessed by staining after 2-3 weeks of culture with Oil Red O (ORO), a dye specific for lipids (Fig. 1.2.3, [83]). To quantify the lipid amounts in induced and non-induced experimental groups, stained monolayers can be quantified by eluting the dye with isopropanol. The optical densities of eluents can then be determined using spectrophotometry. These values are examined

without normalization or are placed on a per cell basis to understand the contributions of individual cells. Alternatively, staining has been interpreted by manually counting the ORO positive and negative cells to obtain a percentage [2]. MATLAb has also been employed to quantify the size and number of lipids present in cultures, while ImageJ can be used to assess the percent of each experimental group covered in ORO [1,84].

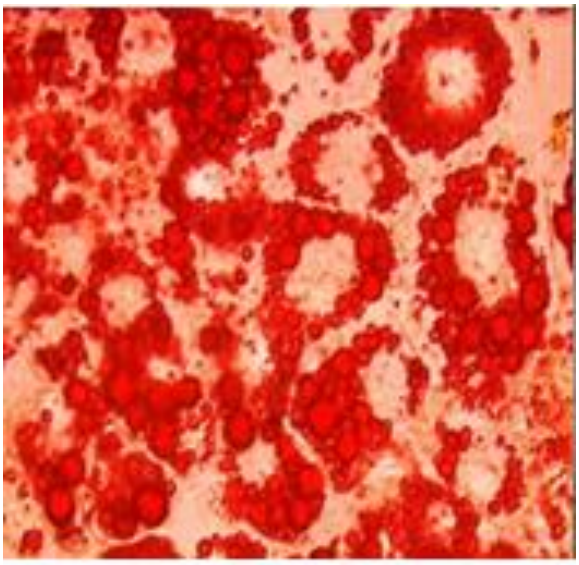

Figure 1.2.3 Oil Red O Staining. To examine lipid production in adipogenic cultures, ORO staining is used at the end of differentiation.

Osteogenic differentiation is typically induced using a cocktail comprised of β-glycerophosphate, ascorbate-2-phosphate, and dexamethasone, using various concentrations of each [85]. Certain cell populations, such as BMSCs, have enhanced osteogenic induction when members of the bone morphogenic protein (BMP) family are also added to the traditional cocktail [86]. It has also been reported that some cell types rely completely on BMPs and will not differentiate without their presence [87].

Another component that can be incorporated into osteogenic cocktails is Vitamin D_3, which has been used for ASCs [88,89]. Osteogenesis is typically induced in 2D for 3-4 weeks *in vitro* [90,91]. However, 3D cultures have been shown to significantly increase osteogenic genes relative to 2D cultures [92]. To evaluate differentiation at early time points, osteogenic genes such as alkaline phosphatase (*ALP*) and collagen type I alpha 1 (*COL1A1*) can be quantified at 4-5 days post-induction [90,93]. Conversely, ALP activity

can be quantified at ~1 week using commercially available ALP enzyme activity kits or visualized through ALP-specific stains [90,93,94]. ALP is a commonly used early marker of osteogenic differentiation since it is a regulator and initiator of calcified matrix production, the terminal differentiation marker for bone [95]. Calcified matrix deposition is assessed by staining with either alizarin red s (ARS) or von Kossa (Fig. 1.2.4, [96]). ARS can be evaluated by eluting the dye with cetylpyradinium chloride and quantifying the eluents with spectrophotometry [88]. Like with adipogenesis and ORO, values are then assessed or normalized to a per cell basis to understand contributions of individual cells. Conversely, ImageJ software is used to quantify the mineralization intensity of phase-contrast images after von Kossa staining [97].

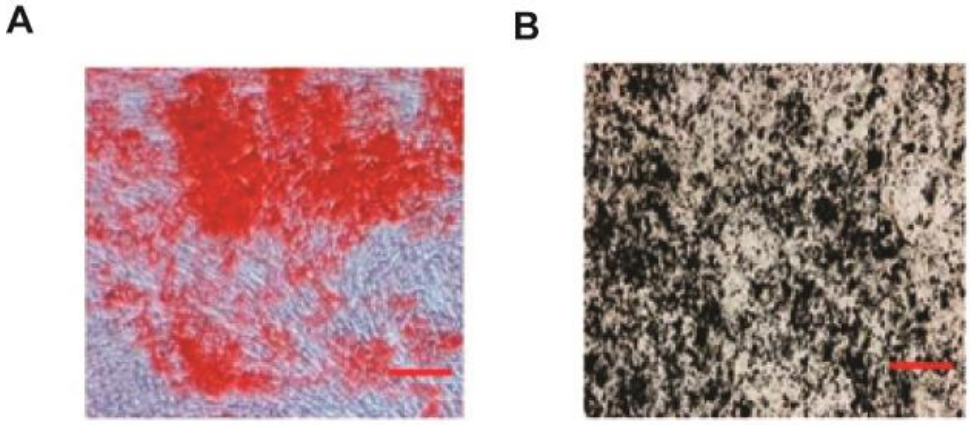

Figure. 1.2.4 Calcified Matrix Stains. (A) Alizarin red S or (B) von Kossa stains are commonly used to examine the calcified matrix production in osteogenic cultures. Scale bar=50 µm.

For chondrogenic differentiation, cocktails often consist of dexamethasone, ascorbate-2-phosphate, insulin, selenious acid, transferrin, sodium pyruvate, and transforming growth factor-β (TGF-β), which is the main inducer of chondrogenesis [98]. Several members of the TGF-β super family have successfully induced chondrogenic differentiation, including TGF-β1 and 3 and BMPs [88,99,100]. More than one member of the TGF-β superfamily can be combined to enhance differentiation further, such as

TGF-β3 and BMP2 [101]. Chondrogenic induction is most often conducted for 3 weeks in 3D culture to maintain the rounded morphology that is optimal for chondrocytes [102]. This is accomplished by forming cell-based pellets in untreated culture vessels, or by seeding cells in a biomaterial scaffold, such as alginate. There are several genes that can be assessed at ~2 weeks to evaluate early levels of chondrogenesis, including collagen type II alpha 1 (*COL2A1*) and aggrecan (*ACAN*), which are phenotypic markers of the chondrocytes [103]. Collagen type II and sulfated glycosaminoglycans (sGAGs) can then be evaluated at the end of differentiation to assess the amount of cartilage-specific matrix production. For collagen type II, pellets can be sectioned and stained using immunohistochemistry to qualitatively evaluate samples. Alternatively, pellets can be digested and sGAG amounts are quantified using a dimethylmethylene blue (DMMB) assay. This procedure involves binding of DMMB to sGAGs, which results in a change in the absorption spectrum and can be measured using spectrophotometry. Values can then be examined on a per sample basis, or normalized to DNA amount to examine contributions of individual cells [104].

1.3 Closing Remarks

The goal of this thesis was to examine differences among MSC types and explore factors that adversely affect stem cell function by building off of what is currently known and applied in the field. In chapter two, current literature was reviewed to expose differences among stem cell-specific induction factors for chondrogenesis. In chapter three, aging effects were compared among MSCs derived from bone marrow, muscle, and adipose tissue. Lastly, in chapters three and four, the effects of chemotherapeutic agents on MSC regenerative potential were explored along with the mechanisms behind

drug response. Understanding differences among MSC types and exposing factors that impair stem cell function is crucial for developing regenerative therapies that can be effectively used in the clinic.

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Chapter 2

Isolation, Characterization, and Differentiation of Stem Cells for Cartilage Regeneration

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Beane OS et al. *Ann Biomedical Eng*. 40 (2012) 2079-2097

2.1 Abstract

The goal of tissue engineering is to create a functional replacement for tissues damaged by injury or disease. In many cases, impaired tissues cannot provide viable cells, leading to the investigation of stem cells as a possible alternative. Cartilage, in particular, may benefit from the use of stem cells since the tissue has low cellularity and cannot effectively repair itself. To address this need, researchers are investigating the chondrogenic capabilities of several multipotent stem cell sources, including adult and extra-embryonic mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). Comparative studies indicate that each cell type has advantages and disadvantages, and while direct comparisons are difficult to make, published data suggest some sources may be more promising for cartilage regeneration than others. In this review, we identify current approaches for isolating and chondrogenically differentiating MSCs from bone marrow, fat, synovium, muscle, and

peripheral blood, as well as cells from extra-embyronic tissues, ESCs, and iPSCs. Additionally, we assess chondrogenic induction with growth factors, identifying standard cocktails used for each stem cell type. Cell-only (pellet) and scaffold-based studies are also included, as is a discussion of *in vivo* results.

2.2 Introduction

Stem cells are promising cell sources for many tissue engineering applications, including cartilage regeneration. Embryonic stem cells (ESCs), adult and extra-embryonic mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) have all been investigated as potential cell sources for cartilage applications (Fig. 2.2.1). The motivation for exploring the regenerative potential of these cells results from the lack of effective therapies currently available for patients suffering from joint diseases. One example is osteoarthritis, a pathology characterized by the degradation of hyaline cartilage.[79] Aside from total joint replacement, possible forms of treatment include microfracture, which allows for subchondral MSCs to populate the defect, and autologous chondrocyte implantation, which is a treatment using *ex-vivo* expanded chondrocytes and, potentially, stem cells. Unfortunately, both procedures can result in the formation of fibrocartilage, a mechanically inferior tissue to healthy hyaline cartilage. Tissue engineering approaches using primary chondrocytes are non-ideal since undamaged cartilage has to be destroyed to obtain the cells, and *in vitro* expansion is necessary to achieve sufficient cell numbers. This process also takes precious weeks, results in dedifferentiation, and raises the risk of contamination. Stem cells have become an attractive therapeutic alternative due to their relative abundance and multipotent capabilities, specifically their ability to undergo chondrogenesis.[148] An ideal stem cell

source has yet to be identified, as each has strengths and weaknesses. Studies have characterized these populations extensively, highlighting large variations in the different cell types, such as ease of isolation, differentiation potential, and surface marker expressions. Additional research has led to progress within all stem cell fields to optimize growth factor cocktails and delivery systems, although to varying degrees of success.

To induce stem cell chondrogenesis, many *in vitro* strategies have been explored,

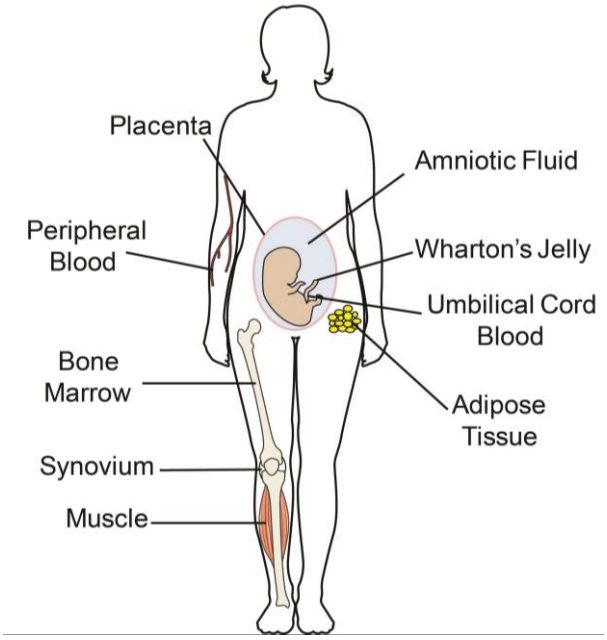

Figure 2.2.1 Stem cells can be isolated from multiple anatomical locations, encompassing adult and extra-embryonic tissues. The cell sources shown above have all been investigated for cartilage regeneration, although mesenchymal sources have been studied much more than other tissues.

including mechanical stimulation, the use of scaffolds or growth factors, or a combination of these techniques.[110] The most frequently used method of induction is treatment with chondrogenic medium in a pellet culture system.[27] Induction medium typically consists of insulin, transferrin, and selenous acid (ITS), dexamethasone, ascorbic acid, and sodium pyruvate, in addition to growth factors.[90, 148] Many growth factors have been considered for chondrogenic differentiation, as reviewed by Danisovic et al..[30] The most well-

characterized and implemented growth factors are part of the transforming growth factor-beta (TGF-β) superfamily, including TGF-β1, 2 and 3, as well as bone morphogenic proteins (BMPs). This review includes the reported optimal growth factors for chondrogenesis, identifying specific cocktails for each stem cell type. Following differentiation, chondrogenesis is confirmed by the presence of extracellular matrix, specifically type II collagen, proteoglycans, and glycosaminoglycans (GAGs), as reviewed by Vater et al.[155] Many methods are used to assess these components, the most common of which are stains specific to proteoglycans, such as toluidine blue, and stains that bind to GAGs or sulfated GAGs, such as alcian blue and safranin-O. An additional assay commonly used to measure GAG synthesis is 1,9-dimethyl methylene blue (DMMB), which can be used to provide quantitative data via spectrophotometry. We will use these reports of matrix synthesis to evaluate the relative effectiveness of stem cell type and culture environment for inducing the chondrocytic phenotype.

This review also seeks to highlight the differences inherent among human stem cell populations currently being investigated for cartilage applications, though for areas with limited human studies we will report results from animal models. Isolation procedures and surface marker expressions will be summarized for each cell type, as they are stem cell-specific. In addition, due to the extensive use of *in vitro* differentiation by means of pellet culture and 3D scaffolds, evaluation of studies successfully inducing chondrogenesis by these methods will be included. It has also been shown that *in vitro* chondrogenic ability is not always indicative of function *in vivo*, due to loss of the chondrocytic phenotype upon implantation.[114] As such, recent *in vivo* studies will be reviewed to stress the feasibility and variability of stem cell use. While previous articles

have assessed stem cells for cartilage tissue engineering, these typically focus on only the most common sources. Consideration of many stem cell types is necessary to evaluate the progress and potential of these populations. A compilation of our findings has been included in Table 2.2.1.

Table 2.2.1 Overview of stem cell characteristics.

Cell Type	**Method of Purification**	**Cartilage Synthesis Potential**	**Preferred Growth Factors**	**Quality of Regeneration *in vivo***
BMSC	FACS Plastic Adherence Density Gradient	Mass:0.25-9 GAG:0.8-122 Type II Collagen:0.04-50	TGFβ-3	Scaffolds are generally well-integrated and phenotypically similar to cartilage, though mechanically inferior.
ASC	FACS Plastic Adherence	Mass:0.1-1 GAG:0.3-120 Type II Collagen:0.02-3	TGFβ-1,3	Matrix secretion has been observed, as well as cartilage-like appearance. However, degree of integration has been inferior to that of BMSC scaffolds.
SDSC	FACS	Mass:1-4.5 GAG:2-45 Type II Collagen:1-2.25	TGFβ-3, BMP-2	Successful matrix secretion has been seen. Degree of integration has been both similar to BMSC, and inferior.
MDSC	Plastic Adherence	Mass:0.1-0.5	BMP-4	Appearance similar to native cartilage, though integration has been cited as inferior to BMSCs.
PBMSC	Plastic Adherence Density Gradient	-	TGFβ-1,3	Successful integration and regeneration has been reported.
WJSC	Plastic Adherence	-	TGFβ-1,3	-
UCBSC	Density Gradient	GAG:2.5	BMP-2	Cartilage-like matrix secretion and surface architecture have been cited.
AFSC	Plastic Adherence	GAG:3.5-4	TGFβ-1	Matrix secretion determined to be inferior to BMSCs.
PMSC	Plastic Adherence	Type II Collagen: 0.5-15	TGFβ-1	-
ESC	Mechanical Isolation	Mass: 0.6-3.75 GAG:0.5-42 Type II Collagen:2.5-100	TGFβ-1, BMP-7	Cartilage-like matrix secretion and surface architecture, as well as successful integration have been cited.
iPSC	-	-	TGFβ-1	Matrix secretion has been observed.

This table compiles general characteristics and procedures for all stem cell types concluded in this review. Quality of regenerated tissue was determined based on a rough comparison of pellet mass (mg) and quantified matrix secretion values, such as sulfated glycosaminoglycans (μg GAG/μg DNA) and type II collagen (normalized to GAPDH by PCR), . Other parameters were assessed based on literature cited in their respective sections. Stem cell types included are Bone Marrow Stem Cells (BMSC), Adipose-Derived Stem Cells (ASC), Synovial-Derived Stem Cells (SDSC), Mscle-Derived Stem Cells (MDSC), Peripheral Blood Mesenchymal Stem Cells (PBMSC), Wharton's Jelly Stem Cells (WJSC), Umbilical Cord Blood Stem Cells (UCBSC), Amniotic Fluid Stem Cells (AFSC), Placenta-Derived Mesenchymal Stem Cells (PMSC), Embryonic Stem Cells (ESC), and induced Pluripotent Stem Cells (iPSC).

2.3 Adult Stem Cell Sources

Adult stem cells are promising cell sources for cartilage repair due to their multipotency, lack of tumorogenicity, ease of isolation, and applicability to autologous transplantation procedures, which removes the risk of rejection associated with allogeneic sources.[62] Unfortunately, these cell types do have disadvantages.[111] When compared with alternative sources, such as extra embryonic stem cells or embryonic and embryonic-like stem cells, adult stem cells have limited self-renewal capacities. Additionally, as a person ages, these cells exhibit decreased proliferation rates and lessened differentiation potential and, in some cases, have similar regenerative characteristics as those associated with diseased patients.[64, 111]

2.3.1 Bone Marrow-Derived Stem Cells

Though their existence was discovered much earlier by Friedenstein et al., bone marrow-derived mesenchymal stem cells (BMSCs) were first characterized by Pittenger et al. in the late 1990s and remain the most commonly used stem cell for cartilage tissue engineering.[9, 17, 46, 118] The cells were identified by positive expression of STRO-1, CD105, CD44, CD71, CD90, CD106, CD120a, and CD124, and negative expression of hematopoietic markers, though later studies concluded negative expression of endothelial markers as well (Table 2.3.1).[4, 118] Additionally, cells possessed the ability to adhere to tissue culture plastic, as well as expand *in vitro*, and differentiate along mesenchymal lineages.[10] To obtain cells for assessment, Pittenger and colleagues employed a density centrifugation gradient, yielding approximately 0.001-0.01% of cells from the initial population. Low cell yield is still a persistent obstacle within the field, since it necessitates *in vitro* expansion to obtain sufficient cell numbers for therapies, a process

Table 2.3.1 Surface marker expression profiles of adult stem cells.

Markers	BMSC	ASC	SDSC	MDSC	PBMSC
CD10	+	+	+	+	–
CD13	+	+	+	+	
CD14	–	–	–*		–
CD20	–		–		
CD29	+	+			
CD31	–	–			–
CD34	–	+	–	+/–	+/–
CD44	+	+	+		+
CD45	–	–	–	–	
CD49a	+		+		
CD49d	–	+			
CD54	+	+			+
CD56	+*	–		+	
CD61	–	–			
CD62E	–	–	–		
CD71	+	+			
CD73	+	+	+		
CD80	–	–			
CD90	+	+	+	+	+
CD104	+	–			
CD105	+*	+	+*	+	+/–
CD106	+	+/–			+
CD117	–	+/–	–	+/–	+
CD133	+				+
CD144	+/–	–		+/–	
CD146	+	LOW			
CD166	+	+	+		+
CD271	+*	+*	+		
HLA-ABC	+	+			
HLA-DR	–	–			+
STRO-1	+*	+			–
SCA-1	+			+	
ALP	+	+	–		
NANOG	+	+			
OCT4	+	+			+
SOX2	+/–	+			
SSEA-4	+	+			

Markers are displayed in comparison with Bone Marrow Stem Cells (BMSC).[4, 29, 52, 118, 126, 151, 156] Other adult stem cell types include Adipose-Derived Stem Cells (ASC),[4, 52, 126, 147, 177] Synovial-Derived Stem Cells (SDSC),[4, 43, 102] Muscle-Derived Stem Cells (MDSC),[36, 67, 163] and Peripheral Blood Mesenchymal Stem Cells (PBMSC).[53, 58, 151] Bordered symbols indicate those that have been used for stem cell enrichment.
*Denotes markers found to enhance chondrogenesis.

that results in decreased differentiation potential.[24] Due to this setback, alternative methods have been pursued in an attempt to enhance cell yield efficiency and purification of cell populations.[24, 106] Some approaches include frequent medium changes, plastic adherence, cell sorting by surface markers, as well as exposure of cells to cytotoxic reagents during expansion.[24, 50, 56, 106] Many physical characteristics have also been used to identify BMSCs, including cell size and mechanical properties.[31, 96, 146, 172] To date, however, BMSC isolation from marrow, regularly harvested from the iliac crest, is still typically conducted by density gradient centrifugation.[105] Subsequently, to further purify the population, cells are plated on tissue culture plastic where non-adhered cells are later washed away.

The use of chondrogenic induction factors has been heavily investigated *in vitro*, as reviewed by Puetzer et al., leading to continuous modification of cocktails and culture environments.[119] One of the earliest studies attempting to optimize chondrogenic differentiation compared BMSC pellets exposed to TGF-β1, β2 or β3.[13] While chondrogenesis was induced under all conditions, after 21 days, it was discovered that TGF-β2 and β3 promoted the greatest GAG production, about twice that of pellets treated with TGF-β1. To enhance differentiation with TGF-β3, it was found that particular BMPs, most effectively BMP-2, could be incorporated.[133] Other uses for growth factors have also been considered, such as delaying impaired potency, a prominent issue after long-term expansion.[138] Sochaga et al. expanded BMSCs with fibroblast growth factor-2 (FGF-2) supplemented media and found that not only did this increase proliferation rates, but it also successfully delayed the loss of chondrogenesis. Furthermore, Cooke et al.

investigated the ability of co-culturing BMSCs with juvenile chondrocytes to enhance differentiation in pellet culture, without additional growth factors.[28] A two-fold increase in GAG levels was observed in comparison to pellets containing only BMSCs. This was a greater degree of induction than BMSC pellets exposed to TGF-β1.

Scaffolds of various materials have successfully induced chondrogenic differentiation of seeded BMSCs *in vitro*. Many biomaterials have been used to date, including collagen, a material known for its biocompatibility and ability to degrade.[121] Ng et al. investigated differences between type I collagen and type II collagen scaffolds.[111] After treatment of porcine BMSC-seeded constructs with TGF-β1, type I collagen scaffolds were superior to type II collagen in terms of GAGs per DNA production, 122.6 vs. 100.7 μg GAGs/μg DNA. Other groups have combined scaffold materials to test for enhanced potency. Zhou et al. incorporated hydroxyapatite (HA) into their collagen scaffold to better mimic osteochondral tissue.[176] After 4 weeks of treatment with TGF-β3, scaffolds with HA exhibited a two-fold increase of GAG production in comparison with those solely comprising of collagen. Additionally, encapsulation of cells by TGF-β3-incorporated fibrin hydrogels can also be used, as shown by Park et al.[114] GAG and relative type II collagen production revealed successful chondrogenic differentiation, 6 and 4.5 μg/μg DNA, respectively. Other materials include, but are not limited to, chitosan, alginate and polyglycolic acid (PGA).[135, 159, 165]

In the field of BMSC cartilage tissue engineering, there have been many encouraging studies using novel approaches that indicate these cells can treat cartilage pathologies *in vivo*. One study using a leporine (rabbit) model analyzed the use of BMSCs, among other stem cell types, to treat osteochondral defects.[92] Scaffolds were

comprised of demineralized bone matrix integrated with TGF-β1. After 12 weeks, BMSC-seeded scaffolds were well-integrated into the surrounding tissue and exhibited robust expression of type II collagen. An alternative study by Igarashi et al., conducted without growth factors, treated osteochondral defects in a canine model.[61] Their approach consisted of an *in situ* forming gel based on ultra-purified alginate containing autologous BMSCs. After 16 weeks of recovery, analysis of the treated area was reported as being smooth, firm, and glossy-white. Mechanical testing of the regenerated cartilage determined structural integrity was still inferior to that of native cartilage (9.2 vs. 12.2 MPa). Advances like these are what have enabled BMSC research in cartilage tissue engineering to reach clinical trials, where many are currently in progress.[1]

2.3.2 Adipose-Derived Stem Cells

Adipose-derived stem cells (ASCs) were first pursued by Zuk et al. in 2001 as an alternative to BMSCs in an attempt to overcome their limitations.[178] In contrast to bone marrow, not only is adipose tissue readily available, but it can be harvested using minimally invasive surgical procedures such as liposuction, and yields significantly more cells overall. Initial studies found that while ASCs exhibit the functional characteristics of stem cells, like multipotency and extended proliferation, they express some surface marker dissimilarities when compared to BMSCs. [13,37] Unlike BMSCs, ASCs show positive expression of CD49d and negative expression of CD106, although there is considerable overlap of other markers. To obtain these cells, protocols from the 1960's, originally designed for fat pads, are used and have since been modified for subcutaneous lipoaspirate, the standard source of ASCs. [16, 71] However, more recently, ASCs from alternative locations have been isolated using similar procedures, concluding that

variations in differentiation abilities exist among ASCs derived from epididymis, perinephrium, and subcutaneous adipose tissue.[71] Following extraction of fat tissue, the process of ASC isolation involves enzymatic digestion with type I collagenase, followed by subsequent centrifugation and washing steps.[16] The isolated cells generate a heterogeneous population including ASCs, fibroblasts, adipocytes, smooth muscle cells, and endothelial cells.[169] The negative effects of this cell contamination, including reduced proliferation and differentiation potential, continue to limit the use of ASCs for therapeutic applications.[122] Seeking to reduce these effects, protocols that aid in purification have been proposed, such as additional washing steps, density centrifugation, and sorting by surface markers.[5, 49, 122]

Many *in vitro* studies have been conducted to investigate the chondrogenic ability of ASCs. As such, cocktails have been refined to promote optimal differentiation. After reviewing current literature, there are few studies comparing cocktails directly, though many confirming that they have been used successfully, as reviewed by Puetzer et al.[119] Commonly used growth factors are TGF-β1, BMP-6, or a combined growth factor cocktail of TGF-β3 and BMP-6.[41] Additionally, TGF-β2 in conjunction with BMP-7 has proven to be a potent inducer of chondrogenesis.[77] Kim et al. assessed chondrogenic induction after treatment with numerous members of the TGF-β superfamily, including BMP-2, 6, 7 and TGF-β2. Individually, GAG amounts were at least twice those of control pellets for all factors. Combining BMP-2 and TGF-β2 resulted in a three-fold increase in GAG production when compared with controls, similar to BMSC pellets. Additionally, as seen with BMSCs, FGF-2 can be administered at low concentrations during expansion of ASCs to enhance proliferation rates and induce chondrogenesis.[22]

However, chondrogenic induction can be hindered by FGF-2 when combined with BMP-6 and TGF-β1.[54] It has also been shown that chondrocytes can enhance chondrogenesis of ASCs by means of soluble factors, or direct interaction through co-culture.[88] Lee et al. assessed differentiation of ASCs in co-culture with chondrocytes, after exposure to chondrocytic conditioned medium, or exposure to a cocktail of TGFβ-2 and BMP-7. The co-culture condition resulted in a 37% increase in GAG production as compared with controls, while ASC pellets exposed to conditioned medium resulted in a 25% increase. However, the growth factor condition induced the greatest degree of chondrogenesis, increasing GAG levels by 50% over controls.

Many materials have been employed as scaffolds to obtain chondrogenic differentiation of ASCs. While early groups studied the ability of agarose, and gelatin to function as a scaffold material for ASC chondrogenesis, modification of these materials has since occurred.[10] Nieto-Aguilar et al. combined fibrin and agarose to produce a novel hydrogel for such applications. Seeded scaffolds were cultured in chondrogenic induction medium supplemented with TGF-β1 for extended periods. Though early assessment confirmed no differentiation had occurred, after 20 days, 98.7% and 86.7% of cells were positive for expression of GAGs and type II collagen, respectively. Recently, mechanically stimulated chitosan/gelatin scaffolds induced potent chondrogenesis of insulin-like growth factor-1 (IGF-1) induced ASCs.[91] Collagen and proteoglycan levels were increased roughly three-fold from controls when assessing scaffolds not exposed to mechanical stimulation. An even greater increase, four-fold, was seen when comparing mechanically stimulated scaffolds with controls. Poly(lactic-co-glycolic acid) (PLGA) has also been used as a scaffold material.[116] Park et al. recently observed chondrogenic

differentiation of leporine ASCs in PLGA scaffolds incorporating dexamethasone and TGF-β1. Though incorporation of TGF-β1 yielded higher GAGs per DNA production than PLGA scaffolds without growth factors (0.15 vs. 0.25 µg GAGs/µg DNA), seeded scaffolds incorporating only dexamethasone produced the most GAGs per DNA (~0.275 µg GAGs/µg DNA).

In addition to cartilage assessment *in vitro*, past studies have demonstrated that ASCs are capable of producing cartilage *in vivo* using numerous approaches. Jung et al. examined the influence of fibrin glue on the *in vivo* chondrogenesis of ASCs when implanted subcutaneously in a nude murine model. [70] Prior to implantation, the stem cells were differentiated *in vitro* by media supplemented with TGF-β1. Post-operation, constructs were qualitatively analyzed at 12 weeks, with the resulting tissue appearing to be structurally sound and glossy. Mehlhorn et al. assessed chondrogenesis of ASCs seeded on PLGA scaffolds.[101] Differentiation was induced *in vitro* by means of TGF-β1 supplemented medium after cells had migrated into the polymer matrix. Analysis of implants after 8 weeks revealed the presence of type II collagen and proteoglycans in scaffolds, suggesting successful differentiation and maintained chondrogenic phenotype. TGF-β1 was also investigated during an *in vivo* study to assess treatment of leporine defects with various MSC sources.[92] The growth factor was incorporated into demineralized bone matrix scaffolds, and subsequently seeded with MSCs. While ASC-treated defects were superior to controls, defects treated with BMSCs were observed to have greater surface architecture and integration.

2.3.3 Synovial Membrane-Derived Stem Cells

De Bari et al. first characterized synovial membrane-derived mesenchymal stem cells (SDSCs) in 2001 by examining their multipotency, proliferation rates, and surface marker expressions.[32] Many commonalities with BMSCs were found, though later studies determined SDSCs to express CD49d, whereas BMSCs do not.[32, 102] After collagenase digestion and subsequent expansion, which is the isolation procedure predominantly used today, analysis by De Bari et al. revealed many advantageous characteristics of these cells for tissue engineering applications.[32] SDSCs possessed considerable proliferative abilities, as seen by maintained growth rates over 30 population doublings, and multi-lineage differentiation capabilities. Since this early study, many groups have determined and confirmed superior chondrogenic and proliferative abilities compared to other MSC types, further supporting the importance of SDSC research.[42, 128] Like with many adult stem cell types, isolating SDSCs yields a heterogeneous population, of which fibroblasts and macrophages are persistent contaminants.[43, 69] To overcome this concern, many studies have investigated enrichment through surface marker expression. For this approach, cells are either sorted for negative expression of CD14 shortly after cell isolation or are expanded prior to sorting to exclude macrophages.[43]

In terms of *in vitro* differentiation, beginning with De Bari et al., TGF-β1 was shown to enhance chondrogenesis, as evidenced by GAG production.[32] Later, *in vitro* differentiation was optimized after treatment with several cytokines, including TGF-β3 and BMP-2.[137] Induction with TGF-β3, alone, resulted in pellets averaging 0.8 mm in diameter, larger than controls averaging roughly 0.5 mm in diameter. With the addition of BMP-2, average pellet diameter strikingly increased to roughly 1.7 mm. Significant GAG

and type II collagen production was also noted. Alternatively, BMP-2 has been combined with TGF-β1 to induce differentiation of SDSCs. To discern the influence of passage on chondrogenesis, Han et al. differentiated cells from P0-P8, determining the maximum potential at P1, yielding roughly 37 μg GAGs/μg DNA.[51] Additionally, chondrogenic differentiation of porcine-derived cells by treatment with TGF-β1 has been reported.[14]

Few recent studies have investigated the chondrogenic differentiation of SDSC-seeded scaffolds. Qi et al. recently determined chondrogenesis of CD105+ murine SDSCs integrated into chitosan-alginate scaffolds.[120] Following a two-week culture period in medium containing TGF-β3 and BMP-2, expression of type II collagen was observed, as well as GAG production, averaging 5.5 μg GAGs/μg DNA. SDSCs have also been seeded in polyethylene glycol diacrylate-based hydrogels and phosphoester-polyethylene glycol-based hydrogels.[42] While both successfully induced chondrogenic differentiation, the latter proved to be superior, as shown by type II collagen and proteoglycan production.

In vivo studies have further confirmed the chondrogenic abilities of these cells. Koga et al. treated cartilage defects in a leporine model after suspending SDSCs in a collagen gel.[81] Defects exhibited profuse matrix production after 4 weeks, increasing after 12 weeks. This repair was similar to BMSC-treated defects. Li et al. investigated SDSC treatment of defects in a leporine model, as well, with demineralized bone matrix-seeded scaffolds, incorporating TGF-β1.[92] The results from this group were not as successful as Koga et al., as BMSC-treated defects were superior in integration as well as cartilage production. Studies have also been conducted specifically to enhance integration of SDSCs in leporine knee osteochondral defects.[136] It was found that not only did cells

adhere faster with the addition of magnesium, but cartilage production of SDSCs was expedited, resulting in greater matrix production.

2.3.4 Muscle-Derived Stem Cells

The discovery of muscle-derived stem cells (MDSCs) in mammals, isolated using a freeze/thaw procedure, occurred in the mid-1990s.[117] Though this study confirmed potency of these cells, it was not until years later that their surface markers were extensively characterized.[67] Seeking to expedite the isolation procedure, Young et al. determined that MDSCs express CD10, CD13, CD56, and Sca-1, while lacking CD45, similar to BMSCs.[171] As reviewed by Wu et al., additional surface marker expressions include CD34, desmin, MyoD, CD144, and c-Kit, though papers have also contradicted this positive expression.[163] Additional markers, distinct from BMSCs, have yet to be determined. Because of their unique surface marker expression within muscle-derived populations, fluorescence-activated cell sorting (FACS) is occasionally used to select for MDSCs, though can be unreliable due to overlapping expression with non-MDSCs.[20] However, the most common isolation method is the modified preplate technique. Preplating attempts to isolate MDSC populations based on their adherence rates to tissue culture plastic or collagen-coated flasks, whereby stem cells are the slowly adhering fraction, obtained 4-5 days post-harvest.[47] Cells are initially released from biopsies by means of enzymatic digestion.[103] A recent study aiming to purify MDSC populations further discovered that Percoll density gradient centrifugation should also be considered as a viable option.[20]

In vitro chondrogenesis of MDSCs has been accomplished in a few studies, generally incorporating BMP-4. Kuroda et al. isolated cells from murine muscle biopsies

and transduced them with BMP-4.[85] Colonies were then formed and subsequently differentiated with and without the addition of TGF-β1. In control media, cultures of BMP-4-transduced cells had more type II collagen-producing colonies than those of non-transduced cells. Additionally, exposure to TGF-β1 did not enhance this effect. Similar to the Kuroda et al., another group transduced murine MDSCs with BMP-4, and then co-pelleted these cells with either vascular endothelial growth factor (VEGF)-blocked or VEGF-producing MDSCs and subsequently treated with TGF-β3.[84] It was found that pelleting with VEGF-blocked MDSCs enhanced differentiation two-fold when compared to pelleting with VEGF-producing cells or pellets consisting solely of unmodified MDSCs. Recently, Ye et al. isolated murine MDSCs for chondrogenic assessment.[166] Following induction of chondrogenesis with TGF-β1 for 2 weeks, a 25-fold increase in type II collagen gene expression was observed over controls. Alternatively, Lu et al. confirmed that TGF-β1 induced chondrogenesis in human MDSCs.[93]

Few groups have recently explored scaffolds for chondrogenic differentiation of MDSCs. Alginate beads have been used successfully, while treating MDSCs with TGF-β1.[7] Following 21 days, staining with alcian blue and assaying for aggrecan revealed presence of chondrogenic markers. A study by Nawata et al. incorporated BMP-2 into type I collagen scaffolds.[109] Toluidine blue staining and type II collagen expression confirmed chondrogenesis.

Many of the previously described MDSC studies investigated the chondrogenic potential of MDSCs *in vivo*, in addition to *in vitro*. Kuroda et al. determined the ability of differentiated MDSCs to treat osteochondral defects in a murine model when mixed with fibrin glue.[85] After 24 weeks, the cells were well-integrated into the surrounding tissue,

and regenerated cartilage looked white, glossy, and smooth. Similar repair was seen by Kubo et al. after treating osteochondral defects with VEGF-blocked cells, in addition to fibrin-embedded BMP-4-transduced cells.[84] In a gender study by Matsumoto et al., cells derived from both males and females were transduced with BMP-4 and mixed with fibrin glue before treating osteochondral defects in nude rats. Unlike defects treated with male MDSCs, those treated with female-derived MDSCs did not heal effectively.[98] A more recent study by Li et al. compared *in vivo* chondrogenesis of cells derived from various source tissues.[92] Leporine knee defects were treated with demineralized bone matrix scaffolds seeded with undifferentiated MSCs. BMSCs proved to be superior to MDSCs on many levels including integration and surface architecture.

2.3.5 Peripheral Blood Stem Cells

The field of peripheral blood mesenchymal stem cells (PBMSCs), an idea initially proposed in 2000 by Zvaifler et al., is relatively new, as their existence is still being investigated due to contradicting reports in early studies.[58, 127, 179] These non-hematopoietic cells isolated from peripheral blood have been determined to lack STRO-1, unique from BMSCs, and can be isolated from blood samples using Ficoll or Percoll gradients and subsequent plating to further isolate by means of plastic adherence.[52, 53, 58, 151] While this method successfully isolates PBMSCs, it results in extremely low yields (0.0002% of initial population), thus, groups have made many modifications to address this issue.[94] FACS has been explored to isolate cells based on expression of various surface markers, specifically positive expression of CD133 or negative expression of CD14.[58, 151] Fibrin microbeads have also been used successfully, due to their ability to bind to matrix-dependent cells.[73] In this study, treating patients with granulocyte colony-

stimulating factor increased cell yield dramatically, although yield was still only 0.5% of the initial population.

Chondrogenesis of these cells has been achieved *in vitro* using a variety of methods, many similar to those used for BMSCs, though without the incorporation of scaffolds. It was determined that PBMSCs in 2-D culture induced along the chondrogenic lineage by TGF-β3 express type II collagen precursors, though contradicting results of this finding exist.[25, 123] Chong et al. also analyzed the effect of TGF-β3 on the differentiation of pelleted human PBMSCs.[26] Average GAG concentrations of PBMSCs pellets were found to be similar to those of BMSCs, roughly 3 and 3.25 µg GAGs/µg DNA, respectively. Unfortunately, both of these levels were still lower than those of chondrocyte pellets, 4 µg GAGs/µg DNA. Tondreau et al. induced chondrogenesis of PBMSCs by treatment with TGF-β1. Following 2-3 weeks of culture, the presence of GAGs indicated successful differentiation. Though few groups have assessed the function of these cells *in vivo*, an encouraging study was recently conducted by Saw et al.[130] Patients with chondral defects first underwent subchondral drilling. PBMSCs were harvested one week after the procedure, and then re-injected intraarticularly with hyaluronic acid once a week for 5 weeks. Long-term, post-operative assessment concluded regeneration of hyaline cartilage at the treatment site. Patients reported minimal adverse side effects apart from discomfort.

2.4 Extra-Embryonic Stem Cell Sources

Cells from extra-embryonic sources have recently been proposed for the purposes of regenerative medicine. These cells are isolated from tissue discarded after birth and are characterized as being in a more developmentally primitive state[62]. This translates into

higher proliferation rates and, potentially, increased multi-lineage differentiation capabilities. Unfortunately, extra embryonic stem cells must be cryogenically stored prior to autologous use, which can be associated with high costs and potentially result in deleterious effects.

2.4.1 Wharton's Jelly Stem Cells

Though isolation of cells was first achieved in 1991, characterization of MSCs derived from Wharton's jelly, the mucoid connective tissue surrounding the umbilical vein, was first conducted by Wang et al. in 2004.[99, 157] These cells isolated from Wharton's jelly, also termed umbilical cord matrix, were determined to express surface markers similar to BMSCs, though a more recent study reported lack of CD18 expression (Table 2.4.1).[44, 86] In their initial study, Wang and colleagues investigated not only surface marker expression, but also the differentiation potential of Wharton's jelly stem cells (WJSCs) along the chondrogenic lineage.[157] Pelleted cells were exposed to medium supplemented with TGF-β1 for 3 weeks and then assessed by immunohistochemical analysis. Staining illustrated rich deposits of type II collagen in both control and experimental pellets. Alternatively, Baksh et al. analyzed the differentiation of TGF-β3-treated pellets.[12] Compared with controls, GAG levels of WJSC pellets were 4 times as high. Additional studies have been conducted incorporating the use of scaffolds. Fong et al. fabricated polycaprolactone (PCL)/type I collagen nanofibrous scaffolds prior to seeding WJSCs and observed significant production of cartilage matrix molecules.[45]

Though these studies suggest the therapeutic potential of WJSCs for hyaline cartilage applications, more promising results were observed for successful fibrocartilage

Table 2.4.1 Surface marker expression profiles for extra-embryonic stem cells.

Markers	BMSC	WJSC	UCBSC	AFSC	PMSC
CD9	+	+			
CD10	+	+		+	
CD11b	–	–			–
CD13	+	+			
CD14	–	–	–	–	–
CD18	+	–			
CD19	–		–		–
CD29	+	+	+	+	
CD31	–	–		–	
CD34	–	–	–	–	–
CD44	+	+	+	+	+
CD45	–	–	–	–	–
CD49b	+		+		
CD49d	–		+		
CD49e	+			+	
CD51	+	+	+		
CD54	+			+	
CD58	–		+	+	
CD73	+	+		+	+
CD90	+	+	–	+	+
CD105	+	+	+	+	+
CD117	–			+	–
CD133	+		–	–	+
CD144	+/–	–			
CD166	+	+		+	+
CD349	+				+
HLA-ABC	+	+/–	+	+	+
HLA-DR	–	–	–	–	–
STRO-1	+				–
NANOG	+	LOW		+	
OCT4	+	+	+	+	
SSEA-4	+		+	+	–

Markers are displayed in comparison with Bone Marrow Stem Cells (BMSC).[4, 15, 29, 52, 118, 126, 151, 156] Extra-embryonic cells include Wharton's Jelly Stem Cells (WJSC),[44, 59, 86, 112, 157] Umbilical Cord Blood Stem Cells (UCBSC),[89, 100] Amniotic Fluid Stem Cells (AFSC),[4, 8, 112] and Placenta-Derived Mesenchymal Stem Cells (PMSC).[52, 134] Bordered symbols indicate those that have been used for stem cell enrichment.

fabrication, indicated by type I collagen production.[11, 158] An early study by Bailey et al. explored the potential of PGA scaffolds seeded with WJSCs to generate temporomandibular joint condylar cartilage.[11] Four weeks after seeding scaffolds with TGF-β1-differentiated cells, quantification of GAG content revealed higher levels in WJSC scaffolds than those seeded with chondrocytes, approximately 3 μg and 1 μg, respectively. Additionally, WJSC scaffolds exhibited a significant amount of type I collagen, more than was seen in chondrocyte pellets, whereas type II collagen production was nominal in both. These results inspired further investigation by the same group to determine optimal seeding density.[158] Concentrations higher than 25 million cells/mL successfully sustained GAG production while simultaneously enhancing the mechanical integrity of the construct.

2.4.2 Umbilical Cord Blood Stem Cells

Confirmed homing capacity of MSCs and circulatory properties of hematopoietic stem cells inspired Erices et al. to investigate whether umbilical cord blood could also serve as a stem cell source, though the presence of mesenchymal-like cells had previously been reported.[40, 167] Expression of common mesenchymal stem cell surface markers was seen, and again confirmed in a study directly comparing those of BMSCs and umbilical cord blood mesenchymal stem cells, but expression of CD49d has been observed in umbilical cord blood stem cells.[89, 100, 156] Further assessment of surface markers by Chang et al. determined the existence of two MSC subpopulations within umbilical cord blood, one being positive for expression of CD90 and the other being negative.[19] Similar chondrogenic and osteogenic abilities were observed between populations. Conversely, adipogenic differentiation potential was greater in the population expressing CD90.

While these studies have successfully obtained MSCs from umbilical cord blood for analysis, low or even undetectable levels of MSCs in cord blood continue to hinder their clinical use.[40, 64, 174] Research within the field has continued, however, due to the availability of the tissue and their superior proliferative capacity.[74] *In vitro* chondrogenesis has been investigated and achieved using various growth cocktails. In the presence of TGF-β1, pelleted umbilical cord blood stem cells are capable of producing twice as much type II collagen as control pellets, similar to ASCs, though BMSCs under the same conditions produce three times as much as their controls.[124] Differentiation with TGF-β3 and BMP-2 was also analyzed by Zhang et al.[174] The pellet mass obtained from three different donors was quantified following differentiation with TGF-β3 and BMP-2, TGF-β3 alone, and BMP-2 alone. It was noted consistently for all donors that BMP-2 promoted the largest pellets. However, this also led to production of hypertrophic chondrocytes. Collagen scaffolds were also seeded with differentiated umbilical cord blood stem cells and then implanted subcutaneously in a murine model. Post-operative assessment three weeks later confirmed matrix section, as well as type II collagen production. The constructs were visually white, stiff, and glistening, features resembling cartilage. Most recently, De Mara et al. compared the chondrogenic induction efficiencies of BMP-2 and BMP-6 on umbilical cord blood stem cells.[34] Type II collagen expression was superior in cell monolayers exposed to BMP-2, as shown by western blotting. No comments on manifestation of hypertrophic chondrocytes were made.

2.4.3 Amniotic Fluid Stem Cells

The earliest report of amniotic fluid-derived stem cells (AFSCs) is by In ’t Anker in 2003, though amniotic fluid cells expressing OCT-4 were publicized just prior and

were further characterized by De Coppi et al. a few years later.[33, 65] Cells were isolated from second trimester fluid after centrifugation and subsequent culturing. Flow cytometry revealed positive expression of MSC markers, however, positive expression of CD117 and negative expression of CD10 were also determined, unique from BMSCs.[8, 33, 112, 153] The ability of AFSCs to undergo chondrogenesis was studied in depth by Kolambkar et al. in 2007 by comparing the effects of TGF-β1, TGF-β3, and BMP-2.[82] The combination of TGF-β3 and BMP-2, and TGF-β3 alone, produced the lowest increase in GAG production, 1.9- and 2.2-fold greater than controls, whereas TGF-β1 alone produced a 2.7-fold increase. Staining for type II collagen was substantial in pellets exposed to TGF-β1 or β3. However, minimal staining was found after treatment with the combination of TGF-β3 and BMP-2. Tsai et al. also differentiated AFSCs using TGF-β3 and confirmed proteoglycan production after 3 weeks.[152] Because AFSC research is still preliminary, aside from the few studies investigating *in vitro* chondrogenesis, cartilage repair applications have not been investigated thoroughly.[162] An *in vivo* study has been conducted by Park et al., encapsulating cells with TGF-β3-containing fibrin gels.[113] While *in vitro* analysis revealed substantial GAG levels, on par with BMSCs, post-implantation assessment determined GAG production of BMSCs to be superior to AFSCs *in vivo*, roughly 7 and 4 μg GAGs/μg DNA.

2.4.4 Placenta-Derived Mesenchymal Stem Cells

In 2003, Zhang et al. first identified MSCs in term placenta.[175] Soon after, further characterization was performed by In ‘t Anker et al., when they investigated the specific membranes of the placenta, as the amnion and chorion are of fetal origin, and the decidua portions are of maternal origin.[64] Cells were isolated simply by mincing the membranes

and then filtering the resulting suspension. Their surface markers have been characterized to include many BMSC markers, with the exception of negative expression of STRO-1 and SSEA-4.[134] Since that time, though many encouraging studies have been conducted in the field of placenta-derived mesenchymal stem cells (PMSCs), very few targeted cartilage applications.[57] Groups have studied the amnion and the chorion, determining characteristic differences between the two sources, as well as their ability to differentiate. Portman-Lanz et al. determined that chondrogenesis of chorion-derived cells with TGF-β1 was superior to amnion-derived cells. Conversely, Cavallo et al. discovered that with the addition of 10% serum, chorion-derived cells do not undergo chondrogenesis. However, type II collagen gene expression of amnion-derived cells was determined to increase by roughly 15-fold.[18] Alternatively, amnion-derived cells have been differentiated successfully after exposure to TGF-β3, as seen by type II collagen production.[6]

2.5 Embryonic and Embryonic-Like Stem Cell Sources

Embryonic and embryonic-like stem cells, such as induced pluripotent stem cells (iPSCs), have been considered for regenerative medicine applications due to their pluripotency and capacity to continuously self-renew.[111] Use of ESCs requires immunosuppressants, but iPSCs can be transformed from patient-specific cells into embryonic-like cells such that autologous transplantation is possible.[39] However, tumorogenicity is an unwavering concern for both cell types, in addition to transmission of zoonotic factors introduced during the expansion process, since murine feeder layers are essential for maintaining proliferation rates and pluripotency.[39, 125, 148] The therapeutic

versatility of ESCs and iPSCs may outweigh these concerns in the long run for tissues that cannot be repaired using other cell types.

2.5.1 Embryonic Stem Cells

The field of human ESC research stems from their discovery by Thomson et al., who successfully isolated lines of human ESCs and determined their pluripotent characteristics.[144] Characteristically, ESCs express SSEA3, SSEA4, TRA-1-60, TRA-1-8, OCT4, SOX2 and NANOG as reviewed by Draper et al. and De Miguel et al. (Table 2.5.1).[35, 38] Steps to isolate these cells from an *in vitro* fertilization-produced embryo include culturing until it reaches the blastocyst stage, whereby cell masses can then be isolated.[144] Currently, mechanical isolation, by which the blastocyst is plated and trophectoderm cells are subsequently removed, is used to obtain these cells without the

Table 2.5.1 Surface marker expression profiles for embryonic and embryonic-like stem cells.

Markers	BMSC	ESC	iPSC
CD9	+	+	
Alkaline Phosphatase	+	+	
NANOG	+	+	+
OCT4	+	+	+
SOX2	+/–	+	+
SSEA-4	+	+	

Markers are displayed in comparison with Bone Marrow Stem Cells (BMSC).[4, 15, 118, 126, 151] Embryonic and embryonic-like cells include Embryonic Stem Cells (ESC)[35, 38] and induced Pluripotent Stem Cells (iPSC).[173]

use of zoonotic components, a feature of other techniques such as immunosurgery that involve use of animal enzymes.[140] Expansion of ESCs on tissue culture plastic, unlike adult stem cells, adversely leads to rapid differentiation. Early preventative measures, including the addition of a mouse embryonic fibroblast feeder layer or conditioned media, were proven to be effective, though risk of zoonotic pathogens were a concern for clinical applications.[125] Alternatives, such as use of human-derived cells, were proven to be a safer method.[139]

Due to their tumorigenic tendencies upon implantation, well-controlled ESC differentiation along the chondrogenic lineage *in vitro* is essential.[148] While recent findings using ESCs for cartilage engineering are inferior to BMSC, ASC, and SDSC results, elucidation of optimal chondrogenic conditions may make ESCs a viable option in the future (Fig. 2.6.1). Many approaches incorporating growth factors have been assessed, most commonly employing the use of embryoid body (EB) systems, a method highly dependent on the stage of development and frequently leading to heterogeneous differentiation.[48, 148] A few groups have attempted to promote chondrogenesis without EB formation. An initial study conducted by Yang et al. compared stem cells directly differentiated, without EB formation, by TGF-β1.[164] Under both conditions, chondrogenesis did not occur. In contrast, Gong et al. successfully induced chondrogenesis with TGF-β1 using a high-density adherent micromass culture system.[48] However, this induction was accomplished by supplementing media with BMP-2, as well. Moreover, in the same study, it was shown that BMP-2 alone was capable of stimulating chondrogenic differentiation. Type II collagen production of BMP-2-treated cells was seven times that of control pellets. Chondrogenesis has also been induced by

BMP-2, using EB formation, after first treating with TGF-β3.[80] However, it was found that treatment with TGF-β1 and IGF-1 after TGF-β3 was more effective, as seen by a two-fold increase in type II collagen production. Alternatively, using a gelatin-coated flask, Nakagawa et al. induced chondrogenesis of ESCs cultured with BMP-7, and even more so when combined with TGF-β1.[107] Analysis determined generation of high levels of GAGs per DNA, reaching roughly 21 μg GAGs/μg DNA with BMP-7 alone, and 41 μg GAGs/μg DNA when treated with BMP-7 combined with TGF-β1. As normalized to GAPDH levels, RT-PCR also revealed a seven-fold increase in mRNA expression of type II collagen.

Incorporating scaffolds into the differentiation process has been conducted with much success. Tigli et al. assessed ESC differentiation after seeding onto either chitosan or silk-fibroin scaffolds incorporated with BMP-6.[131] Following 4 weeks of differentiation, histological and RT-PCR analysis identified chitosan to be inferior in terms of chondrogenesis. Upon investigating the effect of modulus on chondrogenic differentiation, Nam et al. cited the ability of low modulus PCL scaffolds to induce chondrogenesis, as seen by upregulated type II collagen and aggrecan expression.[108] Conversely, neither type I collagen nor type II collagen scaffolds successfully induced differentiation of ESCs.[111] While constructs were also treated with TGFβ-1, type II collagen and GAG production were not observed.

Successful demonstration of ESC chondrogenesis *in vivo* has been conducted numerous times. Hwang et al. obtained chondrogenic-committed cells using an ESC-chondrocyte co-culture system and subsequently seeded PEG-RGD scaffolds prior to subcutaneous implantation in a murine model.[60] Analysis of implanted hydrogels after 24

weeks confirmed an increase in pellet weight and long-term chondrogenic commitment. A more recent study reported encouraging results when treating osteochondral defects in a murine model using ESCs embedded in a hyaluronic acid-based hydrogel.[149] Prior to implantation, scaffolds were treated *in vitro* with various cocktails, whereby BMP-7 and TGF-β1 were deemed superior. Assessment after 12 weeks revealed exceptional integration of the scaffold, and successful treatment of the defect, as seen by regenerated, hyaline-like cartilage.

2.5.2 Induced Pluripotent Stem Cells

In the late 1990s, Wilmut et al. transferred cell nuclei from an embryonic cell line to adult lamb somatic cells, thereby successfully producing a viable offspring.[161] This study confirmed the potential of an adult cell to be reprogrammed into an embryonic cell. These results inspired Takahashi, a decade later, to investigate the factors that cause this phenomenon.[142] After assessing genes known to maintain pluripotency of embryonic stem cells, such as OCT3/4, c-Myc, SOX2, and KLF4, they successfully generated pluripotent cells from murine embryonic and adult fibroblasts, termed induced pluripotent stem cells (iPSCs). Soon after, they, along with Yu et al., reproduced these results in adult human fibroblasts.[141, 173] However, Yu et al. were the first to report pluripotency in cells reprogrammed to express OCT4, SOX2, NANOG and LIN28.[173] These results supported the potential of these cells to be used for regenerative medicine applications, and confirmed similar surface marker expression to ESCs. Unfortunately, this method was burdened by a low success rate, prompting others to explore alternative routes, such as using microRNAs and creating new vectors.[39] One of the largest obstacles that still remains with iPSCs is the tumorogenic tendencies linked to the factors

employed, further stressing the need for alternative techniques. Few groups have induced chondrogenesis of iPSCs, though Wei et al. cited chondrogenic differentiation of induced osteoarthritic chondrocytes following *in vitro* and *in vivo* assessment.[160] *In vitro*, cells were transfected with TGF-β1 and embedded in alginate with chondrocytes. RT-PCR determined type II collagen and GAG levels to be comparable to chondrocytes and two-fold greater than iPSCs cultured without chondrocytes. Following two weeks of *in vitro* differentiation, constructs were implanted subcutaneously in a murine model. Post-operative assessment 6 weeks later confirmed chondrogenesis had been achieved, as seen by type II collagen production. Teramura et al. differentiated murine iPSCs along the chondrogenic lineage by treating cells in a micromass culture with STEMPRO® chondrogenic medium.[143] Following 2 weeks of treatment, type II collagen expression was increased 20-fold from non-induced iPSC cultures. While these groups have cited successful chondrogenic differentiation, the relative novelty of the field restricts assessment of their potential, though future exploration remains promising.

2.6 Conclusion

Since their discovery, stem cells have been considered propitious candidates for many tissue engineering applications, especially cartilage regeneration. Currently, there is a great deal of research supporting this favoritism. However, further investigation is necessary to improve upon current methods of differentiation and, ultimately, regeneration. This literature review confirms great variability in results from stem cell studies (Fig. 2.6.1). Individual findings are influenced by how the cells were handled, including isolation procedures, surface marker analyses, and differentiation assessments, as well as the use of multiple growth factors to stimulate chondrogenesis (Table 2.6.1).

Some differences can be attributed to the influence of distinct, stem cell niches prior to isolation. While MSCs from various tissues have been shown to play similar roles, such as repair and immunosuppression, the inherent differences among microenvironments

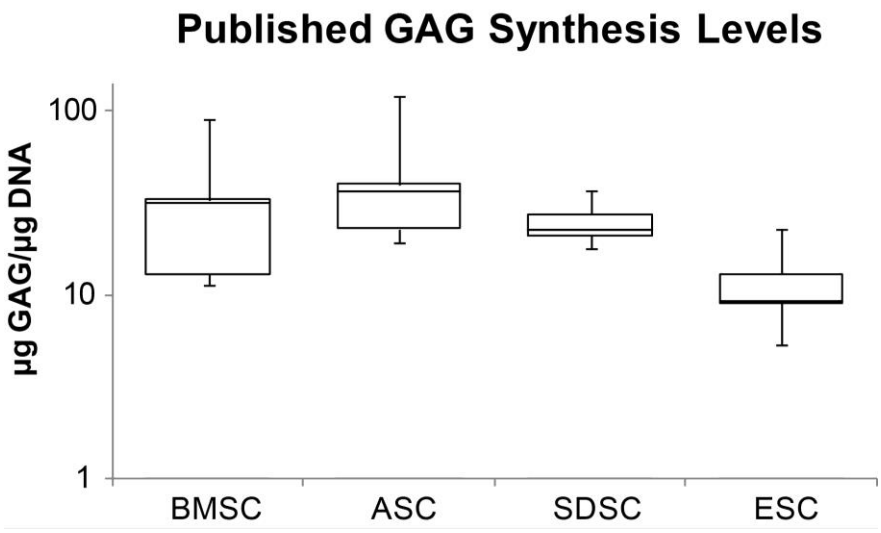

Figure 2.6.1 Comparison of reported GAG synthesis amounts per DNA after chondrogenic induction of human-derived cells using box-and-whisker plots. Results were calculated from recent literature by averaging the largest GAG value reported from each study. Cell types and their respective citations include Bone Marrow Stem Cells (BMSC, n=16),[2, 3, 37, 55, 72, 75, 76, 78, 113, 115, 135, 154, 159, 165, 170, 176] Adipose-Derived Stem Cells (ASC, n=11),[2, 21, 23, 37, 54, 63, 68, 76, 77, 113, 168] Synovial Membrane-Derived Stem Cells (SDSC, n=5),[51, 82, 90, 97, 104] and Embryonic Stem Cells (ESC, n=6).[87, 107, 145, 149, 150, 164]

could influence the properties of resident stem cells.[83] MSCs (e.g., BMSCs and ASCs) are not all the same cell type, as demonstrated by the widely differing responses observed when treated with the same growth factors. MSCs are not stem cells by the classical definition, due to their finite proliferative capacities. Instead, they can be more accurately described as multipotent, progenitor cells. This description is supported by a study showing that cells from adipose tissue differentiate through different molecular mechanisms than those from skin, while still reaching the same desired cell type.[66]

Table 2.6.1Standard and alternative growth factors used for the induction of chondrogenic differentiation.

Stem Cell Type	Standard Growth Factors	Alternative Growth Factors
BMSC	TGF-β3[13, 119, 176] TGF-β1[11, 13, 111, 119, 138, 176]	TGF-β3, BMP-6[132, 133] TGF-β3, BMP-4[133] TGF-β3, BMP-2[133] TGF-β2[13, 77] BMP-7[115]
ASC	TGF-β1[10, 22, 95, 101, 116, 178] TGF-β3, BMP-6[54] TGF-β3[54, 113] BMP-6[77]	TGF-β2, BMP-7[77, 88] TGF-β2, BMP-6[77] TGF-β2, BMP-2[77] TGF-β1, BMP-6[95] TGF-β1, BMP-2[168] TGF-β2[76, 77] BMP-7[77] BMP-2[77]
SDSC	TGF-β3, BMP-2[120, 137] TGF-β1[14, 32, 42, 104]	TGF-β1, BMP-2[51] TGF-β3, BMP-7[136] TGF-β3[42, 97]
ESC	TGF-β1[60, 87, 107, 145, 164] BMP-2[48, 108, 149, 150]	TGF-β1, BMP-2[149] TGF-β1, BMP-7[48, 107, 149] BMP-7[107, 149]

Description of growth factors commonly used, as well as alternative options, for chondrogenic differentiation of stem cells derived from human and animal sources as reported in recent literature. Information relates to Bone Marrow Stem Cells (BMSC), Adipose-Derived Stem Cells (ASC), Synovial-Derived Stem Cells (SDSC), and Embryonic Stem Cells (ESC).

Results depict contrasting stem cell properties, potentially caused by microenvironments or idiosyncrasies of these cells aligning to their roles in the tissue. The goal of this review was to provide a comprehensive summary of stem cell sources for chondrogenesis while addressing these characteristic differences. However, new tissues are continuously being investigated as promising cell depots. Likewise, previously established stem cell sources, such as skin, are showing promise in the area of cartilage regeneration.[129]

Many modifications should be considered in order to enable standardization and reliable comparisons within the field. This is a difficult proposition, however, since investigators have distinct goals for their projects, and allowing direct comparisons to other stem cell sources may not be one of them. With respect to 3D environments for

chondrogenesis, employing basic pellet cultures, rather than scaffolds, could provide standardization that allows greater insight into chondrogenic abilities of each cell type. This would eliminate interactions between cells and scaffold materials, permitting assessment of baseline differentiation. Additionally, consistency among reported parameters should be improved upon. Though mechanical properties are considered supremely important for determining tissue functionality, type II collagen and sGAG quantification should be reported as well, potentially using a quantitative, enzyme-linked immunosorbent assay (ELISA) or DMMB assay, respectively. These can be further normalized by quantifying data on a per cell basis, rather that total amounts, which can vary based on number of cells and size of the pellet. True control samples, where stem cells are not induced, would also enhance standardization, enabling examination of fold-increases that can be used for cross-study comparisons. Furthermore, wet weight and dry weight should be considered, since the hydration of cartilage tissue is an informative parameter. Though current studies comparing stem cells have provided great insight, treatment with universal cocktails hinders the ability to identify optimal stem cell sources for cartilage repair, as direct comparisons are difficult to make. Additionally, many *in vivo* studies fail to assess regeneration using previously determined optimal conditions, specifically growth factors. This suggests that greater effort to adapt *in vitro* results to *in vivo* studies could improve upon cartilage regeneration, potentially revealing an optimal stem cell source for future therapies.

2.7 Acknowledgments

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Chapter 3

Impact of Aging on the Regenerative Properties of Bone Marrow-, Muscle-, and Adipose-Derived Mesenchymal Stem/Stromal Cells

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3.1 Abstract

Mesenchymal stem/stromal cells (MSCs) are promising cell sources for regenerative therapies due to their multipotency and ready availability, but their application can be complicated by patient-specific factors like age or illness. MSCs have been investigated for the treatment of many musculoskeletal disorders, including osteoarthritis and osteoporosis. Due to the prevalence of these diseases in older populations, researchers have studied how aging affects MSC properties and have found that proliferation and differentiation potential are impaired. However, these effects have never been compared among MSCs isolated from multiple tissue sources in the same, healthy donor. Revealing differences in how MSCs are affected by age could help

identify an optimal cell source for musculoskeletal therapies targeting older patients. MSCs were isolated from young and old, rabbit bone marrow, muscle, and adipose tissue. Cell yield and viability were quantified after isolation procedures, and expansion properties were assessed using assays for proliferation, senescence, and colony formation. Multipotency was also examined using lineage-specific stains and spectrophotometry of metabolites. Results were compared between age groups and among MSC sources. Results showed that MSCs are differentially influenced by aging, with bone marrow-derived stem cells having impaired proliferation, senescence, and chondrogenic response, whereas muscle-derived stem cells and adipose-derived stem cells exhibited no negative effects. While age reduced overall cell yield and adipogenic potential of all MSC populations, osteogenesis and clonogenicity remained unchanged. These findings indicate the importance of age as a factor when designing cell-based therapies for older patients.

3.2 Introduction

Mesenchymal stem/stromal cells (MSCs) hold promise in regenerative therapies due to their multipotency and availability. MSCs are being considered for the treatment of a wide range of pathologies, and researchers are especially interested in their potential to treat musculoskeletal disorders such as osteoarthritis, osteoporosis, and osteonecrosis [1-3]. Bone marrow is the most commonly investigated source tissue for these applications, although cells from other tissues like muscle and fat have also been used effectively [4-7]. Recent reports have determined that bone marrow-derived mesenchymal stem cells (BMSCs) can slow the degradation of articular cartilage or even regenerate it in osteoarthritic animal models [8,9]. Similarly, muscle-derived stem cells

(MDSCs) and adipose-derived stem cells (ASCs) have been used successfully in treating bone defects *in vivo* [10,11].

The prevalence of the aforementioned musculoskeletal diseases in older populations has motivated researchers to investigate the impact of aging on MSC properties to evaluate their functionality for autologous treatments. Though conflicting results exist, past findings have indicated that the regenerative potential of MSCs deteriorates with age, which suggests a possible limitation in their use. In individual studies, old BMSCs and ASCs have diminished osteogenesis compared with their younger counterparts [12,13]. Furthermore, aged MDSC proliferation is slowed compared with young MDSC rates [14]. Similar adverse aging effects have been reported for periosteal progenitor cells and even hematopoietic stem cells [15,16]. While these past studies are beneficial for understanding how aging might affect a *single* MSC type, their experimental designs do not allow for a direct, comparative analysis of MSC types in a donor from multiple source tissues. Since MSC properties are variable among donors [17] and experimental approaches are often inconsistent, it is difficult to relate trends across literature to make a reliable and accurate conclusion. The effects of aging have not been compared among MSCs derived from different tissue sources of healthy donors, an important consideration since cell origin has also been shown to influence properties such as differentiation and proliferation [18]. Exploring whether tissue source disparately influences MSC susceptibility to adverse aging effects could identify a cell type that is minimally impaired, making this cell source an optimal candidate for use in regenerative therapies for older patients.

This study aimed to investigate the effects of aging on BMSCs, MDSCs, and ASCs derived in matched groups from young and old donor animals. We hypothesized that not all cell sources would see similar impairment of therapeutic characteristics with age. MSCs were isolated from rabbit donors, and their initial viabilities and yields were quantified to evaluate cell susceptibility to isolation procedures and determine the availability of cells in different tissue sources and age groups. Proliferation rates, cell senescence, and clonogenicity were quantified and compared among populations to examine the impact of aging on MSC expansion properties. Lastly, multilineage differentiation potential was investigated by quantifying the production of lineage-specific metabolites for adipogenesis, osteogenesis, and chondrogenesis.

3.3 Materials and Methods

3.3.1 MSC Isolation and Culture

BMSCs, MDSCs, and ASCs were isolated from young (4-6 months) and old (4-5 years), female, New Zealand white rabbits (RSI Farms, Mocksville, NC, USA) as previously described (N = 5 for each age group) [5,19-21]. Brief summaries of these isolation procedures are provided below. To limit the effect of donor variability and strengthen comparisons among MSC sources, all three tissues were isolated from the same donor for each of the ten animals. The influence of isolating tissues from the same set of donors was investigated for all parameters listed below and included in the interpretation of the results. All animal work was performed in accordance with the local guidelines of the institutions and only after approval by the Institutional Animal Care and Use Committee (Permit numbers: 0015-10 and 0218-12) in accordance with the Guide

for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

BMSCs were obtained from the long bones of individual rabbits following established protocols, with minor modifications [20-22]. Connective tissue was removed from femurs, which were then cut with bone scissors to expose the marrow cavity. Marrow was collected by centrifuging long bones at 400 g for 1 minute. Pellets were resuspended in BMSC culture medium (DMEM/F-12, 15% FBS, 1% antibiotic/antimycotic (Hyclone)), centrifuged, and then incubated in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM K_2CO_3, and 0.1 mM EDTA) at room temperature for 10 minutes. The remaining cells were passed through a 70 µm strainer, centrifuged once more, and plated in BMSC culture medium (passage 0). Cells were counted before plating to determine viability and cell yield. To expand BMSCs, cells were maintained at 37°C, 5% CO_2, and upon reaching 80% confluence, the cells were trypsinized and replated (passage 1). At passage 2, BMSCs were cryopreserved in freezing solution consisting of 80% FBS, 10% culture medium, and 10% dimethyl sulfoxide. Post-thawing, BMSCs were expanded once more (passage 3) before being used for experiments. This procedure (expansion to passage 3, post-thawing) was followed for all MSC types in this study.

MDSCs were isolated from hindlimb skeletal muscle using a modified preplate technique [5]. Briefly, muscle was washed with phosphate-buffered saline (PBS), and connective and fat tissues were removed. The remaining muscle tissue was minced and digested at 37°C in 0.2% type XI collagenase for one hour, then in 2.4 U/mL dispase for 45 minutes, and lastly, in 0.1% trypsin for 30 minutes. The resulting cell pellet was

resuspended in MDSC culture medium (high glucose DMEM (DMEM/HG), 10% FBS, 10% horse serum (Hyclone), 0.5% chick embryo extract (United States Biological), and 1% antibiotic/antimycotic) and passed through a 70 μm strainer. Cells were then sequentially drawn through an 18G, 23G, and 27G needle, counted to determine cell viability and yield, and plated in collagen type I-coated flasks (Sigma). After two hours, the cell suspension was replated in new collagen-coated flasks. This process was repeated every 24 hours for an additional 3 days to isolate the slowly adhering cell fraction containing MDSCs. It should be noted that viability and cell yield were quantified immediately following cell isolation to be consistent with BMSC and ASC data sets. Actual MDSC yields within the harvest population could not be calculated since the cells were isolated only after the extended preplating procedure. To a certain extent, this is also true of BMSC and ASC harvests, which result in heterogeneous populations containing both stem and non-stem cells. Once preplating was completed, MDSCs were expanded in monolayer and cryopreserved at passage 2 as described above.

ASCs were isolated from inguinal fat pads using previously established methods [19]. Briefly, adipose tissue was washed thoroughly with PBS and then digested at 37°C for one hour in an equal volume of 0.1% type I collagenase. The sample was centrifuged and resuspended in ASC stromal medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic). After washing two more times with stromal medium, the cells were incubated in erythrocyte lysis buffer as described for BMSCs, passed through a 70 μm strainer, counted to quantify viability and yield, and plated in tissue culture-treated flasks. The following day, stromal medium was replaced with ASC culture medium consisting of DMEM/F-12, 10% FBS (Zen-Bio), 1% antibiotic/antimycotic, 0.25 ng/mL

transforming growth factor-β1 (TGF-β1), 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) [19]. ASCs were expanded in monolayer to passage 2 and cryopreserved as described above.

3.3.2 Population Doubling Time (PDT)

To examine the effects of aging on MSC proliferation, PDTs for each experimental group were quantified. Briefly, 2,500 cells were seeded in 6-well plates with their respective culture media (n = 24 for each experimental group). Cells from three wells per group were typsinized and counted every 1-2 days using a hemocytomer. Cell count data were plotted for each MSC population, and PDTs were calculated from the log phase of each growth curve [23].

3.3.3 Colony Forming Unit (CFU) Assay

To assess how aging affects the clonogenicity of MSC populations, cells from each experimental group were plated at low seeding densities and allowed to form colonies. Based on previous empirical findings, 500 MDSCs, 1,000 ASCs, and 2,000 BMSCs were plated in 100 mm dishes in their respective culture media (n = 5). Following two weeks of growth, plates were fixed and stained with 0.5% crystal violet in methanol for 15 minutes. A dissection scope was used to count colonies comprising > 50 cells and CFU formation was quantified by dividing the number of colonies by the number of cells originally plated. The CFU area was also determined by imaging plates with a Nikon D5000 digital camera (Nikon Corporation, Tokyo, Japan) and then processing with ImageJ software (National Institutes of Health).

3.3.4 Senescence-Associated β-Galactosidase (SABG) Assay

MSC senescence was examined by staining for SABG [24,25]. MSCs were plated at 5,000 cells/cm^2 in 24 well plates (n = 3) and grown until ~70% confluent. Monolayers were fixed with 0.5% glutaraldehyde and washed thoroughly with PBS. Plates were then stained with X-gal solution (1 mg/ml X-gal, 5 mM $K_4Fe(CN)_6 \cdot H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$ in PBS, pH 6) for 24 hours at 37°C. To visualize all cells, nuclei were labeled with 4′,6-diamino-2-phenylindole (DAPI, Thermo Fisher Scientific), and the number of cells positive for SABG activity versus total cells was quantified by brightfield and fluorescence imaging, respectively. For each well, at least 300 cells from 3-6 images were counted to obtain percentages.

3.3.5 Multilineage Differentiation

Osteogenic Differentiation

Osteogenesis was assessed by quantifying alkaline phosphatase activity and calcified matrix deposition in response to chemical induction. MSCs were plated at 8,000 cells/well in 96-well plates with their respective culture media. Upon reaching confluence, monolayers were exposed to osteogenic (DMEM-HG, 50 ng/mL BMP-2 (a kind gift from Dr. Nic Leipzig), 10 mM β-glycerophosphate, 0.15 mM ascorbate-2-phosphate (Sigma), 1% antibiotic/antimycotic) or control (DMEM-HG, 10% FBS, 1% antibiotic/antimycotic) medium (n = 8 osteogenic, 8 control) [26]. Media were changed every 2-3 days, and after 1 week of induction, half of the wells (n = 4 osteogenic, 4 control) were assessed for alkaline phosphatase (ALP) activity using a BioVision ALP

activity kit according to the manufacturer's protocol (Mountain View, CA). After a total of 3 weeks of induction, the remaining wells were fixed with 3.7% paraformaldehyde (Thermo Fisher Scientific), and alizarin red S (ARS, Sigma) stain was applied to visualize calcified matrix deposition for each sample. Following qualitative imaging, spectrophotometry was used to measure the optical density of eluted samples at 540 nm [27]. To compare osteogenesis between age groups and among tissue sources, the differentiation response of samples was quantified by normalizing ALP and ARS values of induced wells to their respective controls. To allow for analysis on a per-cell basis, nuclei stained with either Hoechst (Invitrogen) or DAPI were visually counted for each ALP and ARS sample prior to biochemical processing. Osteogenesis assays were only completed for MDSCs and BMSCs because ASC monolayers consistently became excessively contractile and balled-up during induction, preventing accurate assessment of matrix deposition and cell numbers.

Adipogenic Differentiation

Adipogenesis was assessed by quantifying intracellular lipid accumulation in response to chemical induction. MSCs were plated at 8,000 cells/well in 96-well plates with their respective culture media. Upon reaching confluence, monolayers were exposed to adipogenic (DMEM-HG, 10% FBS, 1.7 µM insulin, 1 µM dexamethasone, 0.2 mM indomethacin, 0.5 mM isobutyl-1-methylxanthine (Sigma), and 1% antibiotic/antimycotic) or control medium (n = 4 adipogenic, 4 control) [28]. Media were changed every 2-3 days for 2 weeks before fixing samples with 3.7% paraformaldehyde. Oil red O (ORO, Sigma) stain was then used to visualize intracellular lipid production.

After qualitative imaging, ORO dye was eluted, and optical densities were measured at 500 nm using spectrophotometry [27]. To compare adipogenesis among MSC types, the differentiation response was quantified by normalizing optical density values of induced wells to their respective controls. Like with osteogenesis, DAPI-stained nuclei were counted for each sample to allow for analyses on a per-cell basis.

Chondrogenic Differentiation

Chondrogenesis was assessed by quantifying sulfated glycosaminoglycan (sGAG) synthesis in response to chemical induction. Cell pellets were formed by placing MSCs in V-bottom, 96-well plates at 50,000 cells/well and centrifuging for 5 minutes at 400 g. Culture medium was then replaced with either chondrogenic (DMEM-HG, 10 ng/ml TGF-β1, 0.15 mM ascorbate-2-phosphate, 100 nM dexamethasone, 1% ITS+ Premix (BD Biosciences), and 1% antibiotic/antimycotic) or control medium (chondrogenic medium without TGF-β1) (n = 4 chondrogenic, 4 control) [19,27,29,30]. Media were changed every 2-3 days for 3 weeks. For assessment, microtissues were digested with papain (Sigma), and sGAG amounts were quantified using the dimethylmethylene blue (DMMB) assay at pH 1.5 and measuring samples at 525 nm [27,31]. To normalize sGAG amounts to approximate cell numbers, DNA content of samples was quantified using the PicoGreen assay (Invitrogen), with fluorescence measured at 480 nm excitation, 520 nm emission. Since some control samples produced no sGAGs, the differentiation response was quantified by normalizing induced values to the average of non-induced controls within each group.

3.3.6 Statistical Analysis

Two-way ANOVA was used to detect differences across tissue sources and ages for initial cell yield and viability, proliferation, senescence, clonogenicity, and differentiation. Significance levels for individual comparisons were determined using a Tukey post-hoc test ($p < 0.05$ for significance). Non-normal data sets were transformed prior to statistical analysis. Statistical comparisons of relative, metabolite production across tissues were not conducted since "optimal" differentiation cocktails for each individual MSC source could not be assured, making it inappropriate to directly compare across MSCs for this set of data. The differentiation frequency for each lineage was determined by quantifying the positive differentiation response between control and induced samples for each experimental group using a Student's t-test.

3.4 Results

3.4.1 Cell yields and viabilities following isolation

Cell yields and viabilities for freshly isolated young and old cell populations were quantified to examine how age influences cell availability within tissue sources as well as survival after isolation. For all tissue sources, old cell yields were significantly smaller than young by 15-55%, based on age as a factor ($p < 0.05$, Fig. 3.4.1A). Cell yields ranged from 0.1-210 x 10^6 cells/g, with BMSC yields being significantly greater than MDSC and ASC yields by at least 200-fold ($p < 0.001$). As mentioned previously, yields reflect total cell numbers obtained

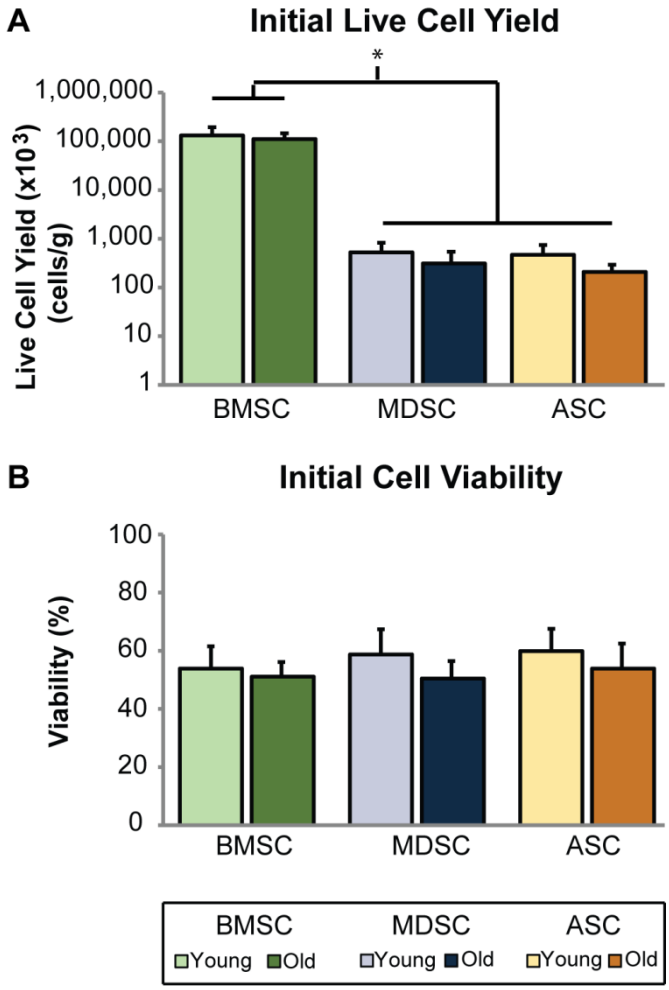

Figure 3.4.1 MSC yield and viability following isolation. Age adversely affected initial cell yield and viability of MSCs. After cell isolation, initial cell yield and viability were quantified. (A) Live cell counts were normalized to harvested tissue mass, with results indicating that cell yield was significantly reduced based on age as a factor ($p < 0.05$). However, individual comparisons within tissue source showed no significant differences between age groups. BMSC yields were significantly larger than MDSC and ASC yields ($p < 0.001$). (B) While viability was also reduced across MSC types, this trend did not quite reach significance ($p = 0.051$). Error bars depict standard deviations.

from the tissue and not only MSCs. Although average, old cell viabilities for all tissue sources were 5-15% lower than young viabilities, this trend did not reach significance ($p = 0.051$, Fig. 3.4.1B). Overall cell viabilities were comparable among all three tissue sources, ranging from 42-67%, slightly lower than other reported findings, potentially due to differences in isolation procedures or animal model used [32-38].

3.4.2 Expansion properties of young and old MSCs

To understand how MSC expansion properties are affected by age, PDT, SABG expression, and clonogenicity were examined for young and old cells. Quantifying the proliferation of MSCs during exponential growth revealed that BMSC PDT was impaired with donor age (36% slower, $p < 0.005$), while PDTs for MDSCs and ASCs were not ($p = 0.60$, Fig. 3.4.2A). Comparisons across tissue sources revealed that BMSC population doubling times were significantly longer than MDSCs and ASCs on average by at least 130% ($p < 0.001$).

Quantitative and qualitative assessment of X-gal staining showed that while SABG activity increased noticeably with donor age for BMSCs (46% higher), this trend did not reach statistical significance ($p = 0.095$, Fig. 3.4.2B, C). Conversely, no apparent differences were observed with age for MDSCs or ASCs ($p = 0.88$). Comparisons of senescence across tissue sources showed that BMSC SABG activity was at least 140% higher than in MDSC and ASC activity ($p < 0.001$).

Clonal expansion of young and old MSC samples showed that CFU formation ranged from 0.4-40%, and were comparable between age groups for all MSC populations ($p > 0.25$, Fig. 3.4.3A, B). Comparisons among tissue sources indicated that MDSC CFU

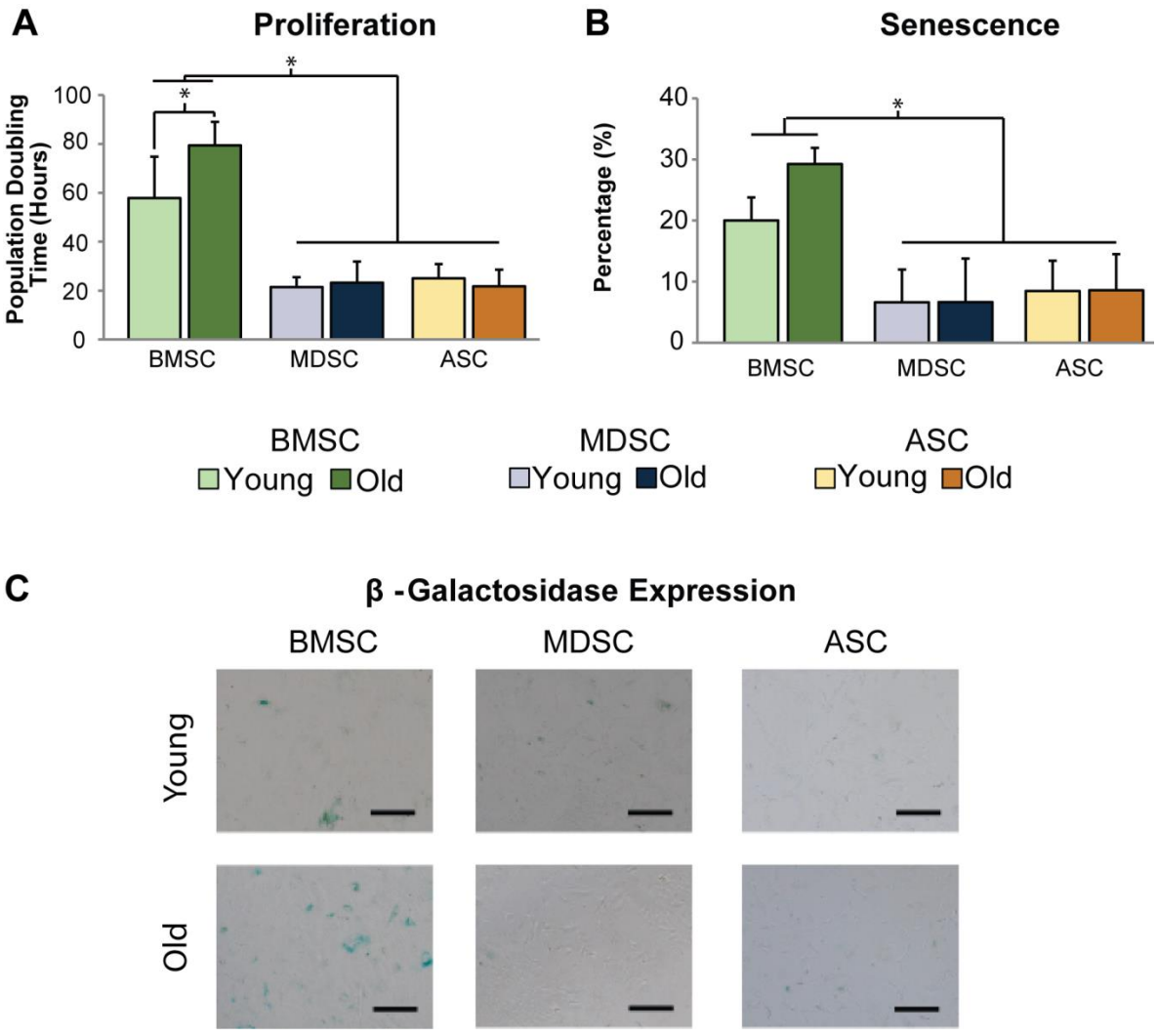

Figure 3.4.2. Cell expansion properties. Characterization of MSC expansion properties revealed differences in age-related changes across MSC populations. (A) PDTs of old BMSCs were significantly longer than young BMSC PDTs ($p < 0.005$). However, no differences were observed between age groups for MDSCs or ASCs. Additionally, BMSC PDTs were significantly longer than MDSCs and ASCs ($p < 0.001$). (B) Quantitative and (C) qualitative assessment of β-galactosidase staining showed that senescence was greater in old BMSC populations than young, although this trend did not reach significance ($p = 0.095$). MDSC and ASC senescence was not affected by age. Statistical analysis also determined that senescence was significantly greater in BMSC populations than MDSCs and ASCs ($p < 0.001$). Error bars depict standard deviations. (Scale bars = 100 μm)

efficiencies were 7-fold higher than BMSC efficiencies ($p < 0.005$). ASC CFU efficiencies were not significantly different from BMSC ($p = 0.22$) or MDSC efficiencies ($p = 0.051$). Average CFU area ranged from 5.3-7.4 mm (Fig. 3.4.3A). However, we were unable to detect differences between the age groups for any MSC population ($p > 0.50$) or tissue sources ($p = 0.12$).

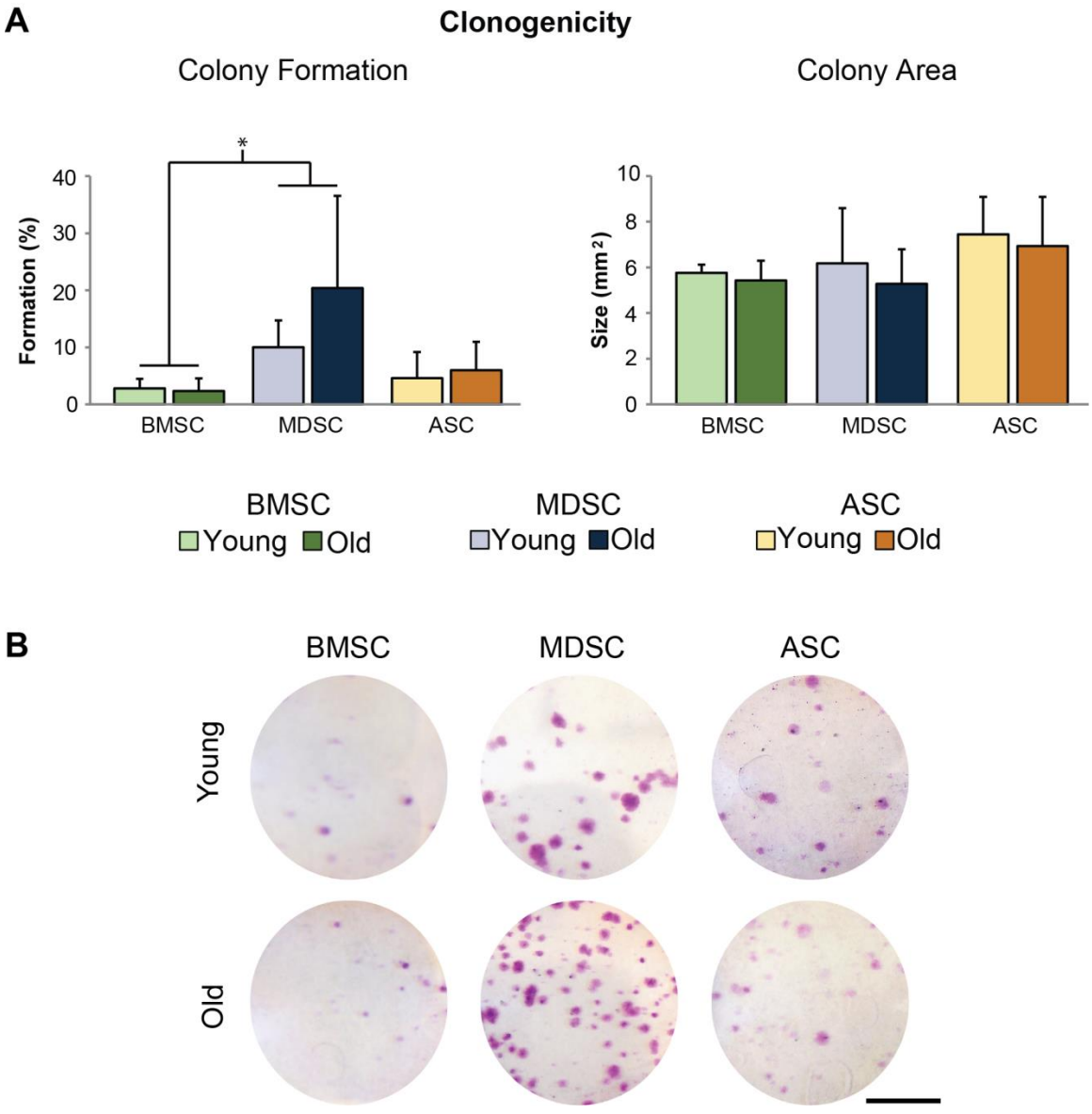

Figure 3.4.3. Clonogenicity of MSC populations. Quantification of colony formation and area indicated that age had no universal effect on MSCs for either parameter. However, MDSC colony formation was significantly higher than BMSC formation ($p < 0.005$). Error bars depict standard deviations. (Scale bars = 25 mm)

3.4.3 Multilineage differentiation potential of young and old MSCs

Differentiation of young and old MSCs was evaluated based on lineage-specific metabolite production for the osteogenic, adipogenic, and chondrogenic lineages. Histological staining of osteogenic samples with ARS after 21 days of induction showed no differences in calcified matrix coverage between young and old cells within BMSC and MDSC groups (Fig. 3.4.4). Quantification of ALP activity on day 7 and calcified

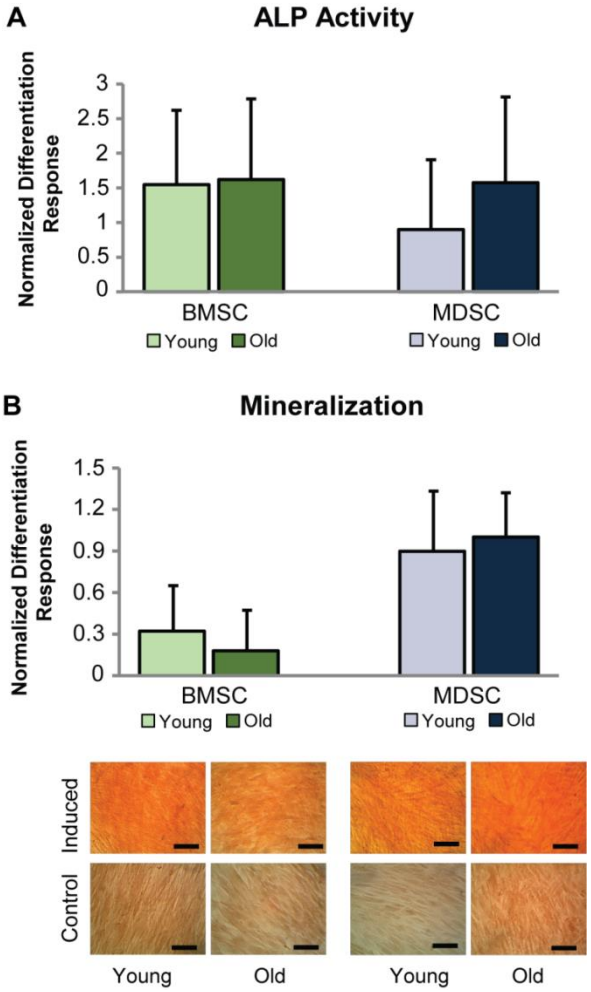

Figure 3.4.4 MSC osteogenic differentiation. Age did not adversely affect BMSC or MDSC osteogenesis. (A) Control-normalized differentiation response of ALP activity after 7 days of induction was comparable between age groups for BMSCs and MDSCs. (B) Calcified matrix production was examined quantitatively and qualitatively with ARS stain after 21 days of induction. Results were similar between age groups for BMSCs and MDSCs. ASC osteogenic differentiation was not characterized since cells became contractile upon induction and balled up, preventing accurate assessment. Error bars depict standard deviations. (Scale bars = 100 μm)

matrix deposition on day 21 showed that the normalized differentiation response was similar for young and old cells within BMSC and MDSC groups ($p = 0.41$, Fig. 3.4.4A, B). ALP and ARS raw values were also examined on a per-sample and per-cell basis, along with raw values for adipogenic and chondrogenic markers (Table 3.4.1). As mentioned previously, ASCs were not included in the osteogenic assessment due to complications caused by their excessively contractile phenotype.

Qualitative assessment of ORO staining revealed that chemically induced, old BMSC and MDSC populations produced less lipids than their respective young cell populations, whereas in control medium, no differences existed between young and old groups (Fig. 3.4.5). Interestingly, old ASCs in control medium exhibited more extensive lipid staining than young ASCs, but this difference was not observed for the induced condition. Quantifying ORO staining of young and old adipogenic cultures confirmed that this differentiation response was reduced with age for all MSC populations, based on age as a factor ($p < 0.05$, Fig. 3.4.5). The most severe loss in adipogenic potential was observed for ASCs. Although lipid accumulated in old, induced ASC cultures, the normalized induction response was minimal since control samples also produced large amounts of lipids. Conversely, young ASCs differentiated as expected, with induced samples having more lipids than controls. Comparing between young and old cells revealed that the normalized differentiation response of old cells was only 6% that of young. Moreover, it was determined that raw adipogenic values of old ASCs were not significantly increased from controls ($p = 0.27$, Table 3.4.1) but were increased for young ASCs ($p < 0.01$). For old BMSCs and MDSCs, the normalized differentiation response

Table 3.4.1Detailed Differentiation Response of Young and Old MSCs

			BMSC		MDSC		ASC	
Marker	**Values**	**Differentiation Condition**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**
ALP Activity[a] (mU)	Raw ($x10^{-3}$)	Induced	116 ± 65	55 ± 50	45 ± 40	32 ± 26	-	-
		Control	44 ± 28	25 ± 27	24 ± 21	11 ± 7		
	Cell Numbers ($x10^{3}$)	Induced	22 ± 13	20 ± 11	47 ± 13	39 ± 13	-	-
		Control	20 ± 10	12 ± 4	29 ± 9	26 ± 8		
	Normalized ($x10^{-6}$ per cell)	Induced	6.1 ± 5.0	2.6 ± 1.3	0.8 ± 0.5	0.9 ± 0.7	-	-
		Control	2.8 ± 22	2.1 ± 2.0	0.8 ± 0.7	0.5 ± 0.3		
	Differentiation Frequency		4/5	4/5	2/5	3/5	-	-
Calcified Matrix[a] (OD)	Raw ($x10^{-3}$)	Induced	133 ± 41	103 ± 34	247 ± 116	194 ± 96	-	-
		Control	104 ± 28	87 ± 16	130 ± 51	94 ± 34		
	Cell Numbers ($x10^{3}$)	Induced	23 ± 13	18 ± 9	42 ± 10	32 ± 6	-	-
		Control	22 ± 12	13 ± 6	26 ± 10	24 ± 8		
	Normalized ($x10^{-6}$ per cell)	Induced	8.1 ± 4.3	7.1 ± 3.7	5.7 ± 2.1	6.0 ± 2.3	-	-
		Control	6.5 ± 4.7	8.9 ± 5.5	5.2 ± 1.6	3.9 ± 0.5		
	Differentiation Frequency		3/5	2/5	5/5	5/5	-	-
Lipid[b] (OD)	Raw ($x10^{-3}$)	Induced	143 ± 50	100 ± 60	85 ± 16	91 ± 80	124 ± 28	107 ± 40
		Control	47 ± 10	44 ± 25	32 ± 19	43 ± 26	47 ± 10	129 ± 74
	Cell Numbers ($x10^{3}$)	Induced	24 ± 13	16 ± 10	48 ± 5	41 ± 10	33 ± 10	26 ± 8
		Control	23 ± 10	14 ± 8	47 ± 6	37 ± 11	29 ± 10	21 ± 7
	Normalized ($x10^{-6}$ per cell)	Induced	7.5 ± 3.0	7.9 ± 4.3	1.8 ± 0.5	2.0 ± 1.4	5.5 ± 4.5	5.7 ± 5.4
		Control	2.8 ± 1.4	5.2 ± 5.7	0.7 ± 0.5	1.0 ± 0.4	2.3 ± 2.5	6.9 ± 5.1
	Differentiation Frequency		5/5	3/5	5/5	4/5	4/5	1/5
sGAG[c] (ug)	Raw	Induced	33.0 ± 36.4	3.3 ± 2.0	1.8 ± 1.2	3.7 ± 2.6	4.5 ± 1.4	3.9 ± 1.4
		Control	3.6 ± 2.8	2.0 ± 1.6	1.1 ± 1.4	3.1 ± 1.8	3.2 ± 2.1	2.7 ± 1.5
	DNA (ng)	Induced	127 ± 86	45 ± 12	42 ± 20	35 ± 9	39 ± 25	36 ± 20
		Control	171 ± 97	86 ± 62	96 ± 54	77 ± 28	80 ± 20	70 ± 23
	Normalized ($x10^{-3}$ per ng DNA)	Induced	224 ± 124	75 ± 43	68 ± 56	20 ± 14	195 ± 137	140 ± 80
		Control	24 ± 16	46 ± 42	27 ± 43	10 ± 4	40 ± 27	50 ± 36
	Differentiation Frequency		5/5	2/5	2/5	2/5	4/5	4/5

Mean ± standard deviation is reported. No data were reported for ASC osteogenesis due to their contractile phenotype during differentiation. Differentiation frequency indicates number of donors successfully differentiating based on induced and control metabolite production. [a] Indicative of osteogenesis; [b] Indicative of adipogenesis; [c] Indicative of chondrogenesis

was 75% and 44% of young cells, respectively, but raw adipogenic values were still significantly increased compared to controls ($p < 0.05$, Table 3.4.1).

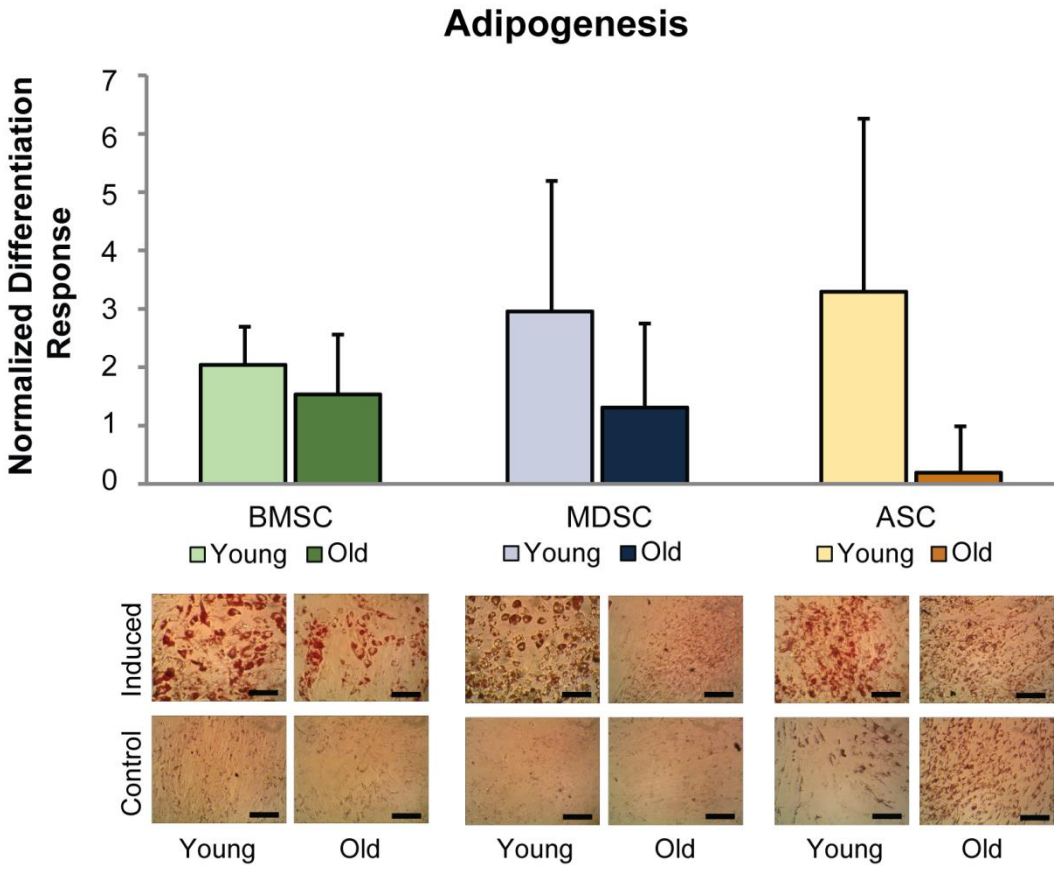

Figure 3.4.5. MSC adipogenic differentiation. Old age impaired the adipogenic potential of BMSCs, MDSCs, and ASCs. Quantification of eluted ORO stain revealed significant decreases in the overall, control-normalized differentiation response based on age as a factor ($p < 0.05$). However, individual comparisons within tissue sources showed no significant differences between age groups. Qualitatively, staining showed that lipid production of induced samples was reduced for old BMSCs and MDSCs compared with their young counterparts. No readily apparent, age differences were observed for these cells in control conditions. Conversely, lipid production in old ASCs was noticeably greater than young ASCs in control conditions, while induced samples were similar. Error bars depict standard deviations. (Scale bars = 100 μm)

Chondrogenesis assessment showed that increases in sGAG production were significantly reduced with age for BMSCs ($p > 0.001$) but not MDSCs or ASCs ($p > 0.33$, Fig. 3.4.6). The normalized differentiation response of old BMSCs was only 7% of young BMSCs. Although BMSC chondrogenesis was reduced significantly with age, similar amounts of normalized and raw metabolite production were observed among all

old MSC populations (Table 3.4.1). Quantifying the chondrogenic differentiation frequency of each MSC population showed that fewer old BMSC samples differentiated than young, while frequencies were comparable between age groups for MDSCs and ASCs (Table 3.4.1).

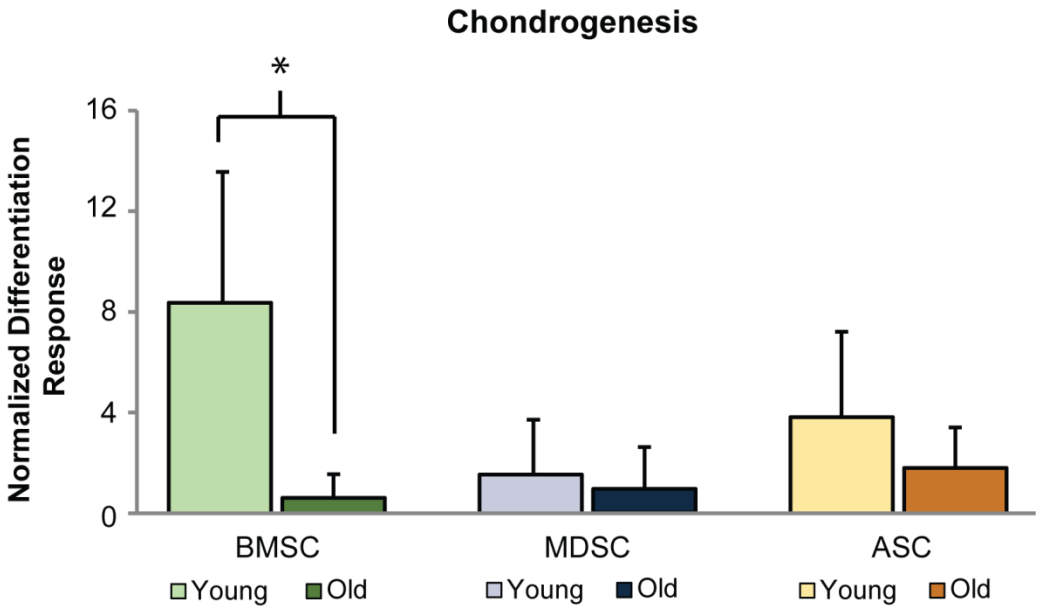

Figure 3.4.6. MSC chondrogenic differentiation. Chondrogenesis was reduced with age for BMSCs, but not for MDSCs or ASCs. Quantification of sGAG production showed that the control-normalized differentiation response of old BMSCs was significantly lower than young BMSCs ($p < 0.001$). Conversely, no significant differences were observed between age groups for MDSCs or ASCs. Error bars depict standard deviations.

3.5 Discussion

The results of this study indicate that donor age disparately affects tissue-specific MSC regenerative properties such as cellular proliferation, senescence, and differentiation potential. All MSC types showed lower cell yields and impaired adipogenesis with age. Old BMSCs exhibited slower PDTs, increased senescence, and inferior chondrogenesis, whereas old MDSCs and ASCs did not. Beyond these findings, very few differences were observed between the regenerative properties of young and old

MSC cell types. For example, age had no effect on CFU formation or area measurements for any MSC population. Investigating the effects of age on MSC properties is a research area of high relevance due to the potential of MSCs for treating musculoskeletal disorders that are prevalent in older populations, including osteoarthritis and osteoporosis. Past studies conducted on individual MSC types have found that proliferation and differentiation potential are typically impaired with donor age [13,14,39-45], though this is not always the case. Few studies have investigated whether MSCs derived from different tissue sources are similarly susceptible to the aging process [46], and none have investigated this using healthy, donor-matched MSC comparisons. We hypothesized that since cell source has previously been shown to influence the abilities of MSCs to resist toxic environments like oxidative stress [47], specific tissues may also influence resident MSC resistance to adverse aging effects. Comparing how different tissue-derived MSCs are affected by aging can potentially identify an MSC type that best maintains its therapeutic potential in older populations, providing a preferred cell source for clinical procedures with these patients.

Differences in aging effects among MSC populations were observed for measured expansion properties. Results showing that age significantly affected PDT for BMSCs but not for MDSCs or ASCs suggest that BMSCs lose their proliferative properties earlier than MSCs from other tissues. Our data are consistent with previous studies investigating ASCs, BMSCs, or both in other experimental models, which found that aging slowed PDT in BMSCs but not ASCs [39,40,46,48-50]. Few studies have examined the effects of age on MDSCs, but none have compared aging of MDSCs with other MSC populations [51]. Our data are the first to indicate that MDSCs are less susceptible to aging than

BMSCs with respect to PDT. MDSCs and ASCs were also shown to be more resistant than BMSCs to age-related senescence, although this trend did not reach significance. An increase in senescence with age has been reported for MSCs from other species besides rabbit, including non-donor matched, human and rhesus macaque studies [46,50]. An increase in senescent cells could account for the longer PDT measured for old BMSCs since fewer cells would be proliferating. The expansion properties of MSCs are important for their clinical use due to the large number of cells necessary for therapies. Longer PDTs and elevated senescence increase the amount of time that old BMSCs must be expanded for *in vitro*. Longer monolayer expansion times could potentially impair old BMSC differentiation potential when compared with young BMSCs [52,53]. Use of MDSCs or ASCs may be preferable to BMSCs for regenerative therapies since their expansion properties are maintained with age.

Results showed that age had measurable, disparate effects on the *in vitro* differentiation response of BMSCs, MDSCs, and ASCs. This was primarily true for chondrogenesis, where there was a dramatic loss in BMSC chondrogenic potential with age but no effect for MDSCs or ASCs. While changes to BMSC chondrogenesis have been investigated previously, we found no studies that examined these effects for MDSCs or ASCs. Osteoarthritis is a prevalent pathology in older populations, and because there are currently no effective, long-term therapies, many MSC types are being considered as a treatment option. Since our results suggest that MDSCs and ASCs maintain their chondrogenic potential with age better than BMSCs, they may serve as promising candidates for cell-based cartilage therapies.

Lipid accumulation data revealed that age reduces the adipogenic differentiation response of BMSCs, MDSCs, and ASCs, which could be highly relevant for reconstructive and cosmetic surgery procedures. The most noticeable reduction in adipogenesis was for ASCs, where induced values were not significantly increased from controls. While these results suggest that old ASCs do not significantly respond to induction medium, large amounts of lipid were still produced in both induced and control samples. Therefore, old ASCs may still be viable for adipogenic regenerative therapies but limited since differentiation cannot be as well controlled through induction factors. Findings showed that old MDSCs and BMSCs still respond to adipogenic differentiation cocktails, but their application may be restricted since their differentiation response is reduced from that of young cells.

No aging effects were observed for BMSC or MDSC osteogenesis. ALP activity, an early osteogenic marker, and calcified matrix deposition, a late osteogenic marker, were similar for young and old BMSCs and MDSCs. These data suggest that the osteogenic differentiation potential of BMSCs and MDSCs is not affected by age and that both MSC sources could be considered for bone-related applications. Our findings related to BMSC osteogenic potential are supported by other groups, emphasizing the importance of these cells for treating bone damage in elderly populations [54,55]. However, other factors such as decreased BMSC numbers and slowed proliferation rates should also be considered. MDSC osteogenic potential has not been thoroughly investigated, and these are the first data to determine that MDSC osteogenesis is maintained with age. The ability of both young and old MDSCs to undergo osteogenesis supports their use in regenerative treatments.

Age reduced overall cell yield for populations isolated from bone marrow, muscle, and adipose tissue, consistent with previous reports [13,56]. And while not statistically significant, initial cell viability was also reduced with age for all MSC populations. The effects of aging on harvested cell viability are rarely reported, but this is a critically important parameter for determining how many usable cells are actually available for a treatment. Having a reduction in cell yield and viability is concerning since it suggests that many older tissue sources might not provide a large abundance of cells for regenerative medicine applications. The reduction in initial viability with age might indicate why there was a decrease in initial cell yield in older populations. If more cells are undergoing apoptosis or necrosis in old tissues, or are too fragile to withstand isolation procedures, fewer cells are available for therapeutic applications.

While our results show that age reduces the differentiation response of some MSC populations, this reduction does not necessarily indicate that they have less regenerative potential than "unimpaired" MSCs. For example, BMSC chondrogenesis was reduced with age while MDSC and ASC chondrogenesis was maintained. However, when comparing total sGAG/DNA values, chondrogenesis for old BMSCs was still much more robust than for old MDSCs, even after undergoing age-related reductions in differentiation potential. For many applications, the absolute amount of matrix production is what matters, regardless of how much it has decreased from peak levels. Ultimately, *in vivo* studies will be necessary to compare actual therapeutic potentials for each MSC type, taking into account total matrix production as well as other important characteristics such as cell yield, viability, and proliferation. While the reported differentiation potential, expansion properties, and cell yield data are consistent with current literature, some

discrepancies do exist. Dissimilarities in these trends could be due to many factors, including donor variability, or differences in animal model systems, age groups, and culture conditions. Contrasting isolation methods could also contribute to disparities. One notable result that differed from other studies is that we observed no reduction in BMSC osteogenesis with age. Our observations could be influenced by the age range assessed. It is possible that the osteogenic potential of rabbit BMSCs is impaired at a younger age than even the "young" group examined in this study. A dramatic decrease in osteogenesis at early ages has been reported previously [50,57]. Examining neonatal rabbit BMSC osteogenesis would confirm whether differentiation potential is reduced prior to 4-6 months of age. Other discrepancies could also be explained by the different model systems used in various studies. For example, the significant reduction in rabbit ASC and BMSC adipogenesis we observed is consistent with multiple, mouse MSC studies [44,58]. However, other groups studying human BMSCs or ASCs reported no age-related reduction in adipogenesis [49,59], suggesting that some results may be specific to smaller animal models. Discrepancies could also be attributed to donor-to-donor variability and differences in methodology, since results have been shown to vary widely within a single species [6,46,56,58]. The number of contradicting reports suggests that comparisons across studies of single MSC types is not reliable and stresses the need to directly compare aging effects among MSC sources within individual experiments, as was done in this study. Isolating MSCs from a single donor is likewise important to help minimize the effects of species and donor-to-donor variability.

The cell populations examined in this study are heterogeneous in their composition, consisting of stem and progenitor cells as well as fully differentiated cell

types. While antigen-based purification was not used, empirical results for multipotency and clonogenicity suggest that MSCs comprised a large proportion of these populations. Furthermore, surface marker profiles can be inconsistent across literature [60-62], and "purified" MSCs are still inherently heterogeneous, with variable regenerative potential [29]. Many studies have investigated MSCs without purification [49,58,59], and evidence exists that separating these cells into antigen-specific populations may even impair their differentiation response compared to the unsorted cells [63]. Clinical applications reflect this in the use of whole bone marrow versus purified BMSCs in many transplantation procedures [64], making the assessment of non-purified MSC populations equally as important as purified populations.

The current study used a rabbit animal model to allow for isolation of multiple MSC types in sufficient numbers from young and old donors. This experimental design is not feasible with smaller animals, particularly since fat tissue, and the ASCs residing in it, does not exist in sufficient amounts in rat and mouse pups. Investigating young and old age groups is complicated for longer-lived, large animal models, leaving rabbits as the most viable option. While the observed results may not be completely conserved across other experimental models, rabbits have been used extensively and reliably in multiple MSC studies focusing on regeneration of age-related musculoskeletal disorders [8,21,65]. This suggests that the findings and conclusions presented in this work should be applicable to future studies regardless of model system. Furthermore, rabbits were an appropriate model to isolate bone marrow, muscle, and adipose tissue from the same donors, a procedure that would be much less feasible in humans. For most parameters and MSC types, donor-dependent trends were not present (S3.9.1). In other words, a specific

donor could not be categorized as “strongly” or “weakly” regenerative, with all MSCs performing similarly well or poor in comparison with other donors. Rather, the relative ranking of MSC performance *within* a donor was variable, lending support to having an experimental design that incorporates matched-donor MSCs. For the current study, comparisons among the MSC types are more reliable than if each cell type came from a separate donor animal, which would simply exacerbate the effects of donor-to-donor variability.

3.6 Conclusion

Understanding the effects of age on MSC properties is important due to their potential use in treating musculoskeletal disorders that are prevalent in older populations. Furthermore, comparing the effects of aging on different MSC populations could help optimize treatments by identifying an MSC source that is not adversely affected by age. This study compared the regenerative properties of young and old BMSCs, MDSCs, and ASCs. We determined that aging effects on MSCs from different source tissues were dissimilar. In particular, old BMSCs suffered from reduced chondrogenic potential and impaired expansion properties, while MDSCs and ASCs did not. Additionally, the adipogenic potential of all MSC types was adversely affected by aging. Although aging adversely affected the properties of some MSCs more than others, total metabolite production was still comparable across all three MSC types. Keeping in mind the aforementioned caveats regarding stem cell-specific induction cocktails, these results suggest that old BMSCs, MDSCS, and ASCs are equally promising candidates for therapies targeting older populations. Further investigation into the mechanism responsible for differential changes between young and old cells among MSC sources is

warranted and, along with the presented findings, can be used to inform future studies focused on designing musculoskeletal treatments for the world's rapidly aging population.

3.7 Acknowledgements

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3.9 Supplemental Figures

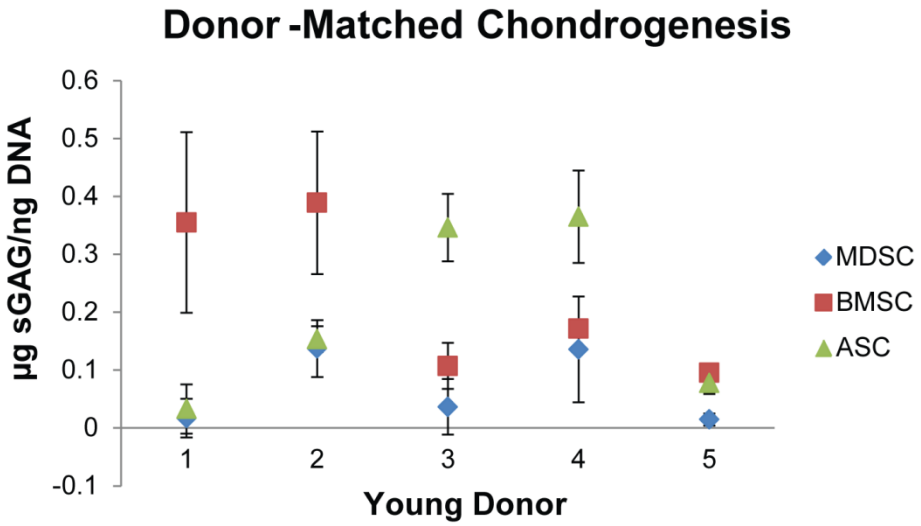

Figure S3.9.1. Influence of using matched-donors. Isolating MSC populations from the same donor rabbits enabled us to examine patterns in cell properties both within and across donors. A graph of chondrogenesis reveals that trends among BMSCs, MDSCs, and ASCs are inconsistent within each donor. No evidence supported the existence of uniformly "strong" or "weak" donors, with some MSC types performing well for one donor but not for others. Donor-to-donor variability was still high, but by isolating all three cell types from single animals, the impact of this variability was lessened, increasing overall confidence in the study's conclusions. Error bars depict standard deviations.

Chapter 4

Adipose-Derived Stem Cells Retain their Regenerative Potential after Methotrexate Treatment

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Beane OS et al. *Exp Cell Res.* 327 (2014) 222-232

4.1 Abstract

In musculoskeletal tissues like bone, chemotherapy can impair progenitor cell differentiation and proliferation, resulting in decreased bone growth and mineralization throughout a patient's lifetime. In the current study, we investigated the effects of chemotherapeutics on adipose-derived stem cell (ASC) function to determine whether this cell source could be a candidate for repairing, or even preventing, chemotherapy-induced tissue damage. Dose-dependent proliferation rates of ASCs and normal human fibroblasts (NHFs) were quantified after treatment with cytarabine (CY), etoposide (ETO), methotrexate (MTX), and vincristine (VIN) using a fluorescence-based assay. The influence of MTX on the multipotency of ASCs and freshly isolated stromal vascular

fraction (SVF) cells was also evaluated using lineage-specific stains and spectrophotometry. ASC and NHF proliferation were equally inhibited by exposure to CY and ETO; however, when treated with MTX and VIN, ASCs exhibited greater resistance. This was especially apparent for MTX-treated samples, with ASC proliferation showing no inhibition for clinically relevant MTX doses ranging from 0.1 to 50 μM. Additional experiments revealed that the differentiation potential of ASCs was not affected by MTX treatment and that upregulation of dihydrofolate reductase possibly contributed to this response. Moreover, SVF cells, which include ASCs, exhibited similar resistance to MTX impairment, with respect to cellular proliferation, clonogenicity, and differentiation capability. Therefore, we have shown that the regenerative properties of ASCs resist the cytotoxicity of MTX, identifying these cells as a potential key for repairing musculoskeletal damage in patients undergoing chemotherapy.

4.2 Introduction

The influence of chemotherapy on cellular function is of increasing interest to the stem cell field. While the effects of chemotherapeutics on bone marrow stem cells (BMSCs) and hematopoietic stem cells have been studied for a number of years (Gardner, et al., 1993; J. Li, et al., 2004), researchers have only recently begun investigating the effects of these drugs on adipose-derived stem cells (ASCs) (Liang, et al., 2011; Zimmerlin, et al., 2011). Advancements in drug formulations have resulted in increased patient survival, but unfortunately many of these chemotherapeutics also have severe side effects, such as long-term musculoskeletal damage (Schriock, et al., 1991). Reports suggest that these impairments result from the slowed proliferation, restricted differentiation, and apoptosis of progenitor cells, such as BMSCs (Georgiou, et al., 2012).

Investigating the influence of chemotherapeutics on other stem cell types, like ASCs, will help identify whether these adverse side effects are universal or whether susceptibility is specific to BMSCs. Furthermore, the possibility of identifying alternative mesenchymal stem cell sources resistant to the cytotoxic effects of these drugs would provide a means to treat, or even prevent, tissue damage. The regenerative properties of ASCs make them an attractive cell population for these therapies. Moreover, they have been shown to withstand other toxic environments like oxidative stress, suggesting ASCs might also resist functional damage induced by chemotherapeutics (Ertas, et al., 2012).

Only limited investigations have been made into the area of mesenchymal stem cell response to chemotherapeutics (Liang, et al., 2011; Qi, et al., 2012). The effects of these treatments on ASC proliferation and differentiation potential have not previously been investigated thoroughly. Differences in mechanism of action suggest that, like BMSCs, ASCs will vary in their susceptibility to chemotherapeutic agents (J. Li, et al., 2004). Therefore, it is necessary to evaluate many commonly used drugs in order to understand the impact of each individual treatment on ASC regenerative properties. Comparing chemotherapeutic effects on ASCs versus a normal, non-stem, somatic cell type provides a simple way to identify resistance or susceptibility to a given drug since neither cell type is directly targeted.

The goal of this study was to evaluate the effects of methotrexate (MTX), vincristine (VIN), cytarabine (CY), and etoposide (ETO) on ASC regenerative properties. Chemotherapeutic agents were chosen based on their diverse mechanisms of action and their application towards a wide range of cancers. MTX functions by binding to and inhibiting dihydrofolate reductase (DHFR), an essential protein for DNA synthesis

(Cronstein, 1997). Conversely, VIN prevents microtubule formation, CY inhibits polymerase function, and ETO targets topoisomerase II (George, et al., 1965; D. D. Ross, et al., 1990; W. Ross, et al., 1984). After treatment with individual chemotherapeutics *in vitro*, ASC growth was compared with untreated ASCs and treated/untreated normal human fibroblasts (NHFs). These comparisons enabled us to identify significant deviation from normal growth trends and evaluate the resistance of ASCs to chemotherapeutics relative to untargeted, non-stem somatic cells. We also assessed how multilineage differentiation potential was affected by drugs that did not inhibit ASC proliferation, and investigated the possible mechanism behind this immunity. These results provide important insight towards understanding how ASC regenerative properties are affected by commonly used chemotherapeutics and identify mechanisms of chemotherapeutic resistance that could be useful in designing regenerative therapies for cancer patients.

4.3 Materials and Methods

4.3.1 Cell culture

ASCs, originally isolated from the subcutaneous fat of healthy human, non-diabetic, non-smoking female donors, aged 18-60 years (N=7), were purchased from Zen-Bio, Inc. (superlot #36; Research Triangle Park, NC). ASCs were grown in expansion medium consisting of DMEM/F-12 (HyClone), 10% FBS (Zen-Bio), 1% antibiotic/antimycotic (HyClone), 0.25 ng/mL transforming growth factor-β1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) (B. T. Estes, et al., 2008). ASCs were maintained in humidified incubators at 37°C, 5% CO_2 and

passaged at 80% confluence with 0.25% trypsin-EDTA (HyClone). Experiments used ASCs at passage 4-6 (P4-6).

NHFs derived from neonatal human foreskins (a gift from Dr. Jeffrey Morgan) were expanded in high glucose DMEM (DMEM-HG, HyClone), 10% FBS (HyClone), and 1% penicillin/streptomycin (HyClone) (Youssef, et al., 2011). Experiments used NHFs at P6-9.

4.3.2 Isolation and culture of stromal vascular fraction (SVF) cells

All procedures were approved by the internal review board (IRB) at Rhode Island Hospital. Human lipoaspirate was obtained from female donors, aged 20-56 (N=3). SVF cells were isolated using established protocols (B. T. Estes, et al., 2010). Briefly, liposuction waste tissue was washed thoroughly with phosphate-buffered saline (PBS) and digested for one hour at 37°C in an equal volume of 0.1% type I collagenase (Worthington Biochemical Corporation). Samples were centrifuged at 300 g for 5 minutes, and the pellet was resuspended in stromal medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic) to neutralized the enzyme. Cells were washed twice more and incubated for 10 minutes at room temperature in an erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM K_2CO_3, and 0.1 mM EDTA) to remove red blood cells. The remaining cell population was centrifuged and resuspended in stromal medium, then filtered sequentially through 100 µM and 70 µM strainers before cell counting. All samples were stored in liquid nitrogen using freezing medium containing 80% FBS, 10% dimethyl sulfoxide, and 10% expansion medium. Prior to experimentation, cells were quickly

thawed and plated in expansion medium at 2,000 cells/cm^2. SVF cells were treated with chemotherapeutics at P0. Clonogenicity and differentiation were assessed at P1.

4.3.3 Chemotherapeutic agents

The following chemotherapeutics (and concentrations) were used in our experiments: MTX (0.1-50 µM, MP Biomedicals), VIN (0.01-1 µM, Cayman Chemicals, Ann Arbor, Michigan), CY (1-100 µM, Accela ChemBio, Shanghai, China), and ETO (0.1-5 µM, MP Biomedicals). The concentration range for each drug was chosen based on clinically relevant doses reported in the plasma of patients receiving chemotherapy (J. Li, et al., 2004). While treatment times ranging from 48-72 hours have been used in the past (Georgiou, et al., 2012; J. Li, et al., 2004; Qi, et al., 2012), a duration of 24 hours was used in the current study based on pilot work showing a dramatic response of ASCs and NHFs to the listed chemotherapeutics within that time frame.

4.3.4 Proliferation assay

To examine the effects of chemotherapeutics on cell proliferation, ASCs and NHFs were quantified on days 6-10 after treatment with various chemotherapeutic agents. Cells were plated at 2,000 cells/cm^2 in 96-well plates, a cell density chosen since it was high enough to detect fluorescence and low enough to enable the greatest amount of proliferation prior to reaching confluence. After adhering overnight, cells were treated with MTX, VIN, CY, or ETO for 24 hours. Following treatment, samples were rinsed with PBS, and fresh expansion media were added to ASC and NHF wells. Cells were cultured for an additional 10 days, quantifying cell counts on days 6-10 using a

spectrophotometer-based assay developed and fully validated in our lab (Fig. S4.9.1, 4.9.2). In brief, cell nuclei were stained with 5 μg/mL Hoechst 33342 dye (Invitrogen) for 30 minutes, rinsed three times with warm PBS, and assessed by spectrofluorometry using adsorption and emission wavelengths of 350 nm and 461 nm, respectively. Cell numbers were quantified based on sample fluorescence using a reference curve. To assist in comparing data across sample sets, values were presented as a percentage of control counts determined on day 10 for each individual chemotherapeutic.

4.3.5 Cell viability

To assess how 24 hour MTX treatment (2.5 μM) affects cell viability, ASCs and NHFs were monitored using a live/dead assay. Cells were stained on the day of MTX treatment removal (day 0) as well as the following ten days by incubating cultures in 2 μM calcein AM (AnaSpec Inc, Fremont, CA), 4.5 μM propidium iodide (EMD Millipore), and 1 μg/mL Hoechst for 30 minutes. Cellular viability was quantified by taking 16-24 images/well (N=3 wells) at 10x and counting stained cells (N>200).

4.3.6 Cell division

To determine if MTX inhibits cell division, Ki-67 immunostaining was performed on untreated and 2.5 μM MTX-treated ASCs and NHFs on days 0-10. Samples were fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilized with 0.25% Triton X (Thermo Fisher Scientific) for 15 minutes. Cells were blocked in 3% bovine serum albumin (Thermo Fisher Scientific) for 1 hour, stained with a Ki-67 antibody (1:200, EMD Millipore) for 30 minutes, and then stained with an Alexa Fluor 488-

conjugated secondary antibody (Life Technologies) for 30 minutes. Samples were incubated in 5 μg/mL 4',6-diamino-2-phenylindole (DAPI, Thermo Fisher Scientific) to visualize all cell nuclei. To determine Ki-67 expression, 16-24 images/well (N=3 wells) were taken at 10x and stained cells (N>200) were counted.

4.3.7 Senescence-associated β-galactosidase activity

To investigate how MTX treatment affects cell senescence, β-galactosidase staining in ASCs and NHFs was quantified after treatment with 2.5 μM MTX on days 0-10. Cells were fixed with 0.5% glutaraldehyde for 5 minutes and then washed with PBS. Plates were then incubated in X-gal solution (1 mg/ml X-gal (Genesee Scientific Corp, San Diego, CA) , 5 mM $K_4Fe(CN)_6 \cdot H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$ in PBS, pH 6) overnight at 37°C. Cell nuclei were stained with DAPI as described above. The number of X-gal-positive cells versus total cells was quantified by brightfield and fluorescence imaging, respectively. To determine the percentage of senescent cells, 10 images/well (N=3 wells) were taken at 10x and stained cells (N>200) were counted.

4.3.8 Multilineage differentiation

Adipogenic and osteogenic differentiation

To examine whether MTX affected differentiation potential, ASCs were treated with MTX as described previously and expanded until cultures were 80% confluent. For freshly isolated SVF cells, samples were thawed and plated at 2,000 cells/cm^2 in T-25 flasks. After adhering overnight, cells were treated with 2.5 μM MTX for 24 hours. Once

treatment was removed, cells were cultured for an addition 6 days, until reaching 80% confluence. Once at 80% confluence, ASCs and SVF cells were uplifted and plated at 8,000 cells/well in 96-well plates with expansion medium. Upon reaching 100% confluence, ASCs were exposed to 200 μL of adipogenic, osteogenic, or control (stromal) medium (Guilak, et al., 2006; Zheng, et al., 2006). Adipogenic medium contained DMEM/F-12, 10% FBS, 1 μM dexamethasone, 10 μM insulin, 0.5 μM isobutyl-1-methylxanthine, 200 μM indomethacin (Sigma), and 1% antibiotic/antimycotic. Osteogenic medium contained DMEM-HG, 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.15 mM ascorbate-2-phosphate, 10 nM 1,25-$(OH)_2$ vitamin D_3 (Sigma), and 1% antibiotic/antimycotic. Media were changed every 2-3 days for two (adipogenesis) or three (osteogenesis) weeks before fixing cell monolayers with 3.7% paraformaldehyde (Thermo Fisher Scientific). Oil Red O (ORO, Sigma) staining was used to assess lipid production in adipogenic and control cultures. Alizarin Red S (ARS, Sigma) staining was used to assess calcified matrix deposition in osteogenic and control cultures. After imaging stained wells, ORO and ARS dyes were eluted, and optical densities were measured with spectrophotometry at 500 and 540 nm, respectively. Optical densities were normalized to cell number by counting nuclei stained with DAPI (Carpenter, et al., 2006). Assessment of SVF cell osteogenesis was only conducted for one donor, since cells from the other donors became highly contractile during the differentiation procedure, balling up and preventing comparative matrix deposition measurements.

Chondrogenic differentiation

Untreated and MTX-treated ASCs and SVF cells were placed in V-bottomed, 96-well plates at 50,000 cells/well and centrifuged at 400g for 5 minutes to form pellets. Expansion medium was replaced with 200 µL of chondrogenic or control media. Chondrogenic medium contained DMEM-HG, 10% FBS, 10 ng/ml TGF-β1, 0.15 mM ascorbate-2-phosphate, 100 nM dexamethasone, 1% ITS+ Premix (BD Biosciences), and 1% antibiotic/antimycotic (Gonzalez-Cruz, et al., 2012). Media were changed every 2-3 days, and after 3 weeks of culture, pellets were digested with papain (Sigma). Sulfated glycosaminoglycan (sGAG) amounts were quantified using previously established protocols (Awad, et al., 2003; Guilak, et al., 2006). Briefly, 200 µL of DMMB dye (Polysciences, Inc., pH 1.5) was added to 50 µL of pellet digests in 96-well plates and optical densities were measured at 525 nm. sGAG values were normalized to DNA amounts, determined using the PicoGreen assay (Invitrogen), measuring fluorescence at 480 nm excitation and 520 nm emission.

4.3.9 Colony forming unit-fibroblast assessment

The effect of MTX on the clonogenicity of heterogeneous SVF cell populations was examined using the colony forming unit-fibroblast (CFU) assay. Untreated and MTX-treated SVF cells were plated at 1,000 cells/100 mm dish (BD Biosciences) in stromal medium. Cells were cultured for 2 weeks, fixed with methanol, and stained with 0.5% Crystal Violet (Thermo Fisher Scientific). Colonies containing at least 50 cells were counted using a dissection microscope.

4.3.10 Western blotting

To investigate the mechanism of action behind ASC MTX resistance, dihydrofolate reductase (DHFR) protein expression was assessed by western blot. DHFR is the target of MTX, and upregulation during MTX treatment has been noted for other resistant cell types (Goldman and Matherly, 1985). Untreated and MTX-treated ASCs, NHFs, and SVF cells were lysed on ice for 30 minutes in RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with HALT protease and phosphatase inhibitors (Pierce). Protein concentrations were determined using a BCA Assay (Pierce). Equal amounts of protein were separated on SDS-PAGE gels (Bio-Rad), transferred onto PVDF membranes (Millipore) and confirmed with Ponceau Red stain. Membranes were blocked with Blotto (50 mM Tris (pH 7.5), 185 mM NaCl, 0.05% Tween 20, 3% (w/v) nonfat dry milk) and incubated with DHFR (1:500, Abcam) or β-tubulin (1:2000, Developmental Studies Hybridoma Bank) antibodies overnight. Membranes were washed for one hour in Blotto and then stained with peroxidase-conjugated secondary antibodies (Sigma, eBioscience Inc., San Diego, CA). Membranes were washed for 30 minutes in Blotto without milk and then visualized by enhanced chemiluminescence (EMD Millipore).

4.3.11 Statistical analysis

Three-way ANOVA (cell type, concentration, day) was used to assess proliferation data. Two-way ANOVA was used to assess differentiation data of ASCs (treatment, differentiation condition), SVF CFU and proliferation data (donor, treatment), as well as SVF osteogenesis (treatment, differentiation condition). Three-way ANOVA

was used to assess SVF adipogenesis and chondrogenesis (donor, treatment, differentiation condition), as well as viability, cell division, and senescence (cell type, treatment, day). Following each ANOVA, the significance level of individual comparisons was determined using a Tukey post-hoc test.

4.4 Results

4.4.1 Cell growth after chemotherapeutic treatment

To compare how ASC and NHF proliferation is influenced by chemotherapeutics, cells were treated with individual drugs and counted on days 6-10. ASC proliferation was not inhibited by MTX treatment, whereas NHF proliferation was. No significant reductions in ASC counts were determined for any time point or concentration of MTX (Fig. 4.4.1A). Interestingly, cell counts of ASCs after 2.5 or 50 μM MTX treatments were 30-40% higher than non-treated controls on day 10 and days 8-10, respectively ($p < 0.05$), although results after 50 μM MTX treatment were variable. Conversely, NHF cell counts were reduced from control values at all time points by 80-95% after 2.5 or 50 μM MTX ($p < 0.001$). ASCs and NHFs treated with 0.1 μM MTX were not significantly different from their respective control values. These effects did not change when ASCs were treated and cultured in NHF medium or when NHFs were treated and cultured in ASC medium (Fig. S4.9.3). Similar to MTX, ASC proliferation was less inhibited by VIN ($p < 0.001$, Fig. 4.4.1B). Treatment with 0.01 μM reduced ASC counts from controls by 55-77% ($p < 0.001$), and higher doses of 0.125 or 1 μM inhibited ASC growth by 75-79% ($p < 0.001$). Conversely, all concentrations of VIN reduced NHF counts from controls by 79-90% ($p < 0.001$). Treatment with CY caused similar inhibition of ASC

and NHF growth (Fig. 4.4.1C). ASC and NHF counts were significantly reduced from control counts by 55-95% after treatment with concentrations ranging from 1 to 100 µM ($p < 0.001$). As with CY, ETO treatment resulted in similar growth inhibition of ASCs and NHFs (Fig. 4.4.1D). Counts were significantly reduced from control values following treatment with ETO concentrations ranging from 0.1-5 µM ($p < 0.05$). Specifically, 0.1 µM ETO lowered cell counts of ASCs and NHFs by a maximum of only 16% ($p< 0.05$), while 1.25 and 5 µM ETO reduced counts by 70-75% ($p < 0.001$).

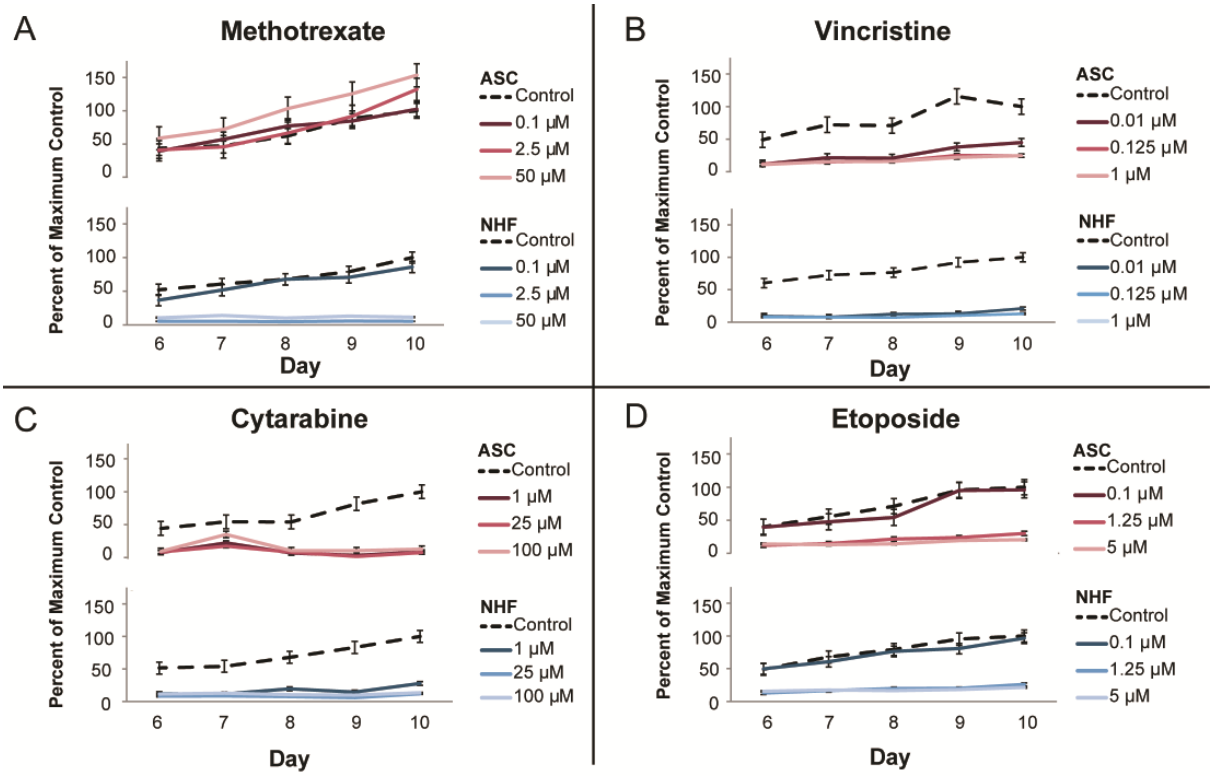

Figure 4.4.1 ASC (red lines) and NHF (blue lines) proliferation were differentially inhibited by chemotherapeutic agents. ASCs and NHFs were quantified 6-10 days after a 24 hour treatment with different concentrations of (A) methotrexate, (B) vincristine, (C) cytarabine, (D) or etoposide. Cell counts are presented as the percent of ASC or NHF untreated control counts on day 10. ASC growth was not inhibited by any concentration of methotrexate, while NHFs were impaired after 2.5 and 50 µM treatments. Similarly, ASC proliferation was more resistant to vincristine exposure than NHFs. However, both cell types were equally impaired by cytarabine and etoposide. Error bars show standard error.

4.4.2 MTX effects on cell expansion properties

To understand the effects of 2.5 μM MTX on ASC and NHF expansion properties, viability, cell division, and senescence were examined on days 0-10 after treatment. Results from the live/dead assay revealed that MTX had disparate effects on ASCs versus NHFs (Fig 4.4.2A). Statistical analysis indicated no significant differences existed between untreated and MTX-treated ASCs at any time point ($p = 0.682$). Conversely, the viability of MTX-treated NHFs was significantly reduced from untreated NHFs by 10-30% on days 2-10, consistently decreasing over time and reaching viabilities as low as 62% on day 10 ($p < 0.001$). MTX-treated NHF viability was also 9-37% lower than MTX-treated and untreated ASC viability on days 2-10 ($p<0.001$). Results also showed that untreated NHF viability was 9-12% lower than untreated ASCs ($p < 0.001$), although this viability percentage of ~90% was relatively constant for days 4-10.

Immunostaining of Ki-67 indicated that MTX treatment reduced cell division in NHF samples but had no effect in ASCs (Fig. 4.4.2B). Untreated and MTX-treated ASCs showed no statistically significant differences at any time point ($p =0.457$). Conversely, Ki-67 expression in MTX-treated NHFs was significantly reduced compared to untreated NHFs by 25-99% on days 2-10, reaching percent expressions as low as 0.25% on day 10 ($p < 0.001$), which suggests no active cell division at all. Furthermore, MTX-treated NHF Ki-67 expression was 30-99% lower than untreated and MTX-treated ASC populations on days 2-10 ($p<0.001$). Comparisons between untreated ASCs and NHFs indicated that NHF Ki-67 expression was 5-65% lower than ASC expression on days 2-10 ($p < 0.001$), which is to be expected since NHFs are larger in size than ASCs and reach confluence sooner in control conditions.

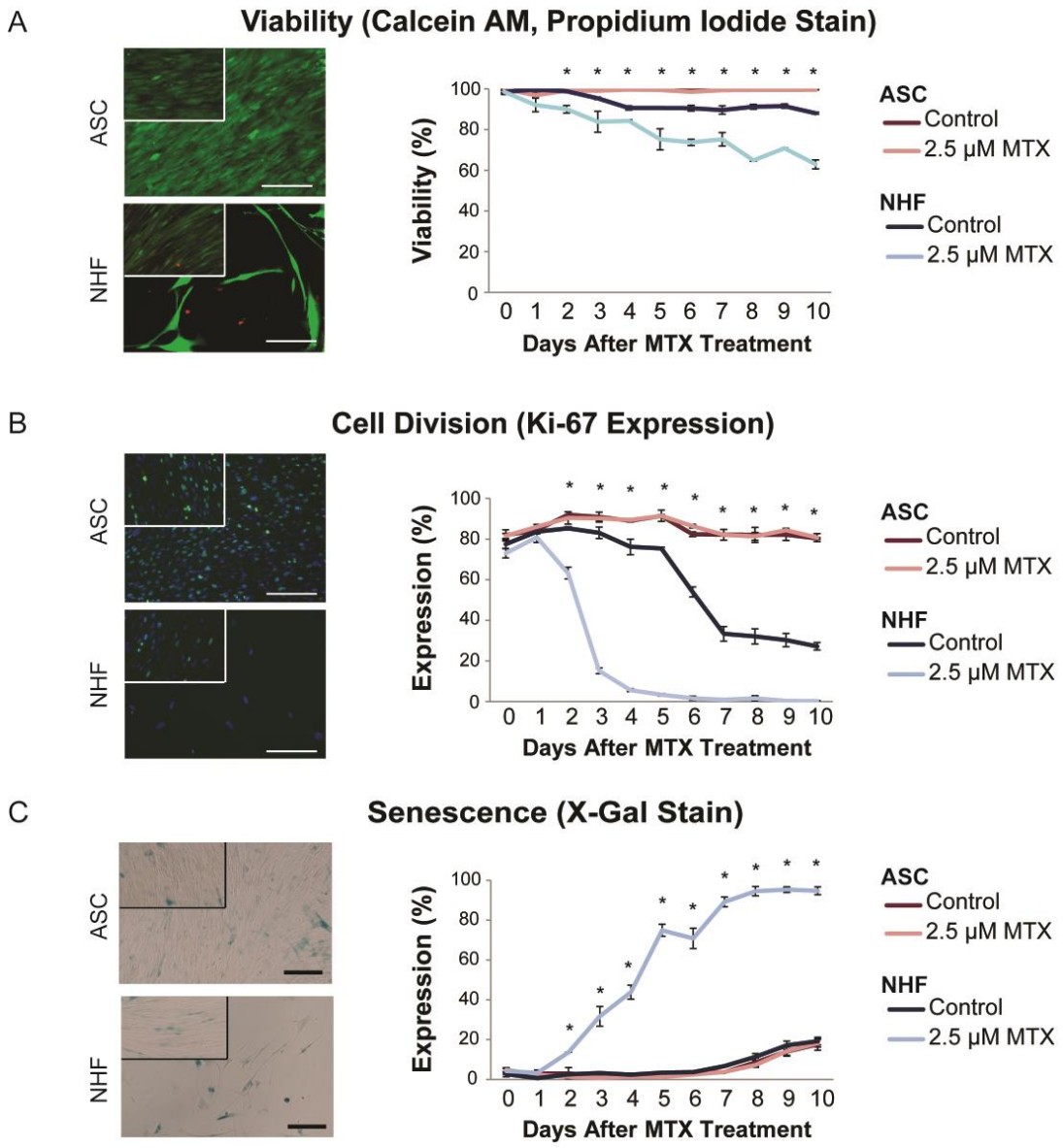

Figure 4.4.2 Methotrexate effects on cell viability, cell division, and senescence revealed clear differences between ASCs and NHFs. (A) Live/dead staining indicated that ASC viability remained at 100% following 2.5 µM MTX treatment while NHF viability steadily dropped over time, Representative images of live (green) and dead (red) cells at day 10 indicate differences in ASC and NHF samples following MTX treatment (insets show controls). B) Ki-67 immunostaining confirmed that MTX had no effect on ASC division. Conversely, cell division in NHFs was significantly reduced compared to controls. Representative images of Ki-67 (green) and DAPI (blue) staining on day 10 suggest prevalent Ki-67 expression in ASCs, with minimal expression in NHFs. C) X-gal staining determined that MTX did not affect ASC senescence. However, MTX-treated NHF samples had significantly more senescent cells than untreated controls, with nearly all cells senescing by day 8. Images of senescent (blue) cells on day 10 indicate a greater percentage of senescence in NHFs than ASCs. (Scale bar indicates 200 µm, *$p < 0.001$ between untreated and MTX-treated NHFs).

Quantifying β-galactosidase expression of ASCs and NHFs indicated that MTX did not influence ASC senescence but did increase NHF senescence. Untreated and MTX-treated ASCs showed no statistical differences in senescence at any time point (Fig. 4.4.2C). For NHFs, senescence of MTX-treated cells increased 5-22-fold over untreated NHFs on days 2-10, reaching 95% positive staining for β-galactosidase by day 8 ($p < 0.001$). Moreover, senescence of MTX-treated NHFs was 9-55-fold greater than untreated and MTX-treated ASCs ($p<0.001$). No differences between untreated ASCs or NHFs were observed, although senescence of both cell types increased at later time points as cultures became confluent.

4.4.3 MTX effects on ASC differentiation potential

To determine whether ASC differentiation potential is resistant to MTX, commercially available, enriched populations of ASCs were treated with 2.5 μM MTX and then expanded before replating for differentiation. Results from the adipogenic, osteogenic, and chondrogenic assays indicated that the multipotency of ASCs was not inhibited by 2.5 μM MTX treatment. Histological stains of lipid and calcified matrix revealed comparable amounts of metabolite production between untreated and MTX-treated ASCs (Fig. 4.4.3A). Both untreated and MTX-treated adipogenic populations produced significantly more lipid than their respective non-induced controls ($p < 0.001$). While MTX treatment resulted in a slight increase in lipid production for adipogenic (11%) and non-induced (29%) samples over non-MTX-treated samples (Fig. 4.4.3B, $p < 0.05$), the average increases in lipid production over non-induced controls were comparable for untreated and MTX-treated populations (0.5-fold and 0.65-fold increase,

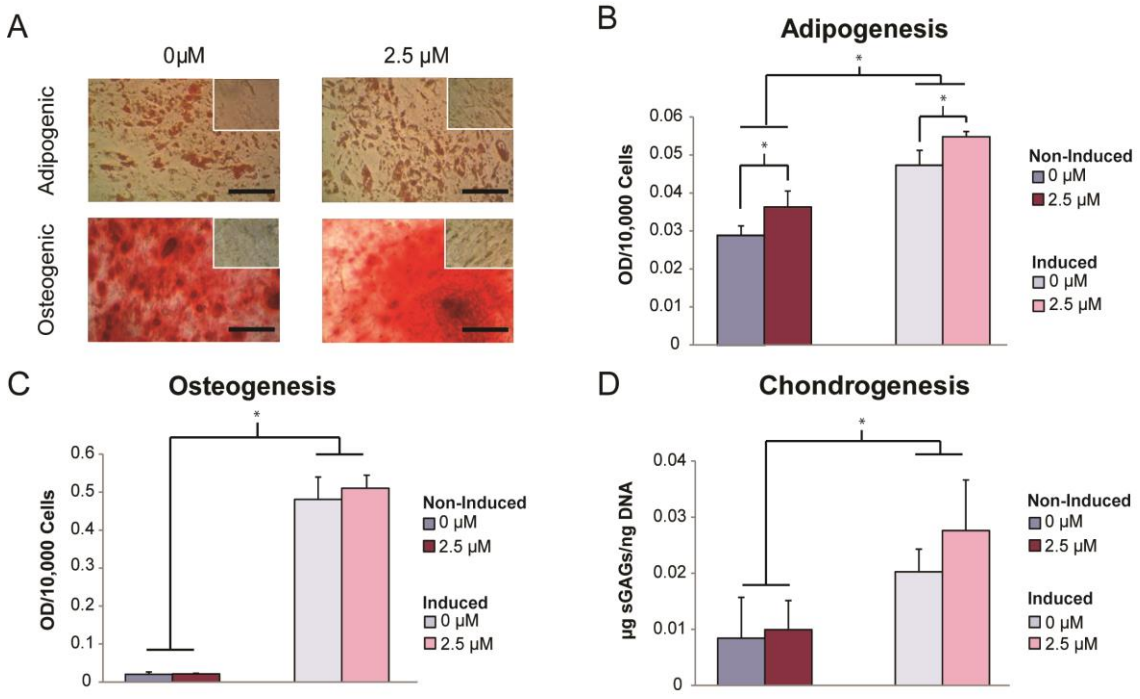

Figure 4.4.3 Treatment with 2.5 μM MTX had no negative effect on ASC multilineage differentiation potential. (A) Oil Red O and Alizarin Red S stains confirmed comparable lipid and calcified matrix production for induced and non-induced (insets), untreated and methotrexate-treated ASCs. Optical densities of eluted, metabolite-specific stains were quantified to determine differences in (B) adipogenic, (C) osteogenic, and (D) chondrogenic differentiation potential. While MTX caused statistically significant increases in lipid production for induced and non-induced samples over untreated wells, these differences were minor. No differences were observed between untreated and methotrexate-treated osteogenic or chondrogenic differentiation potentials. Error bars show standard deviation. (Scale bar indicates 50 μm, *$p < 0.05$)

respectively). Analysis of osteogenesis and chondrogenesis showed that increases in metabolite production over non-induced controls were comparable for untreated and MTX-treated ASCs in osteogenic (22.5-23.5-fold increase, $p < 0.001$) and chondrogenic (1.4-1.8- fold increase, $p < 0.05$) culture environments. Calcified matrix and sGAG production amounts were also similar for untreated and MTX-treated samples (Fig. 4.4.3C, D).

4.4.4 MTX effects on SVF properties

While properties of expanded ASCs were shown to be largely unaffected by MTX, it was unknown whether freshly isolated cells would also resist inhibitory effects of the drug. Therefore, we investigated the influence of MTX on freshly isolated, heterogeneous SVF populations obtained from 3 different donors. After expanding and counting SVF cells treated with 2.5 µM MTX, results showed that that the drug actually increased proliferation of all donor populations, with MTX-treated samples having 17-50% more cells than untreated samples (Fig. 4.4.4A, $p < 0.05$). Moreover, colony counts for all

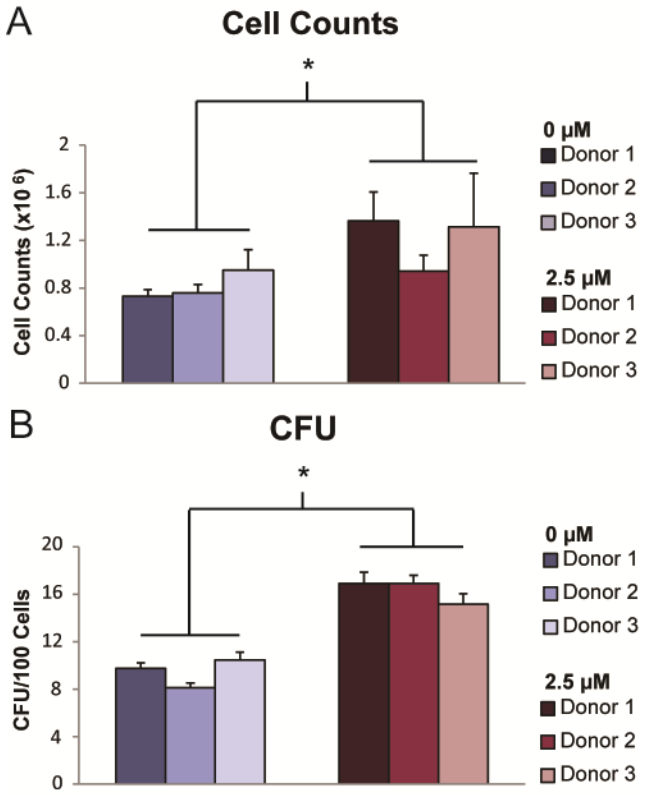

Figure 4.4.4 MTX treatment increased the proliferation and clonogenicity characteristics of SVF cell populations. (A) After monolayer expansion of untreated and MTX-treated SVF populations, cell counts of MTX-treated populations were higher than untreated populations. (B) Likewise, colony counts of treated SVF populations were significantly higher than untreated populations when assessed via a CFU assay. Error bars show standard deviation. (*$p < 0.05$)

MTX-treated donor populations were 50-100% higher than untreated populations (Fig. 4.4.4B, $p < 0.001$). Despite these other changes, MTX treatment did not affect the differentiation potential of SVF cells. Metabolite production for all lineages was significantly higher in induced samples than non-induced controls for both untreated and MTX-treated groups. Histological images illustrated that similar amounts of lipids and calcified matrix were produced by untreated and MTX-treated samples (Fig. 4.4.5A).

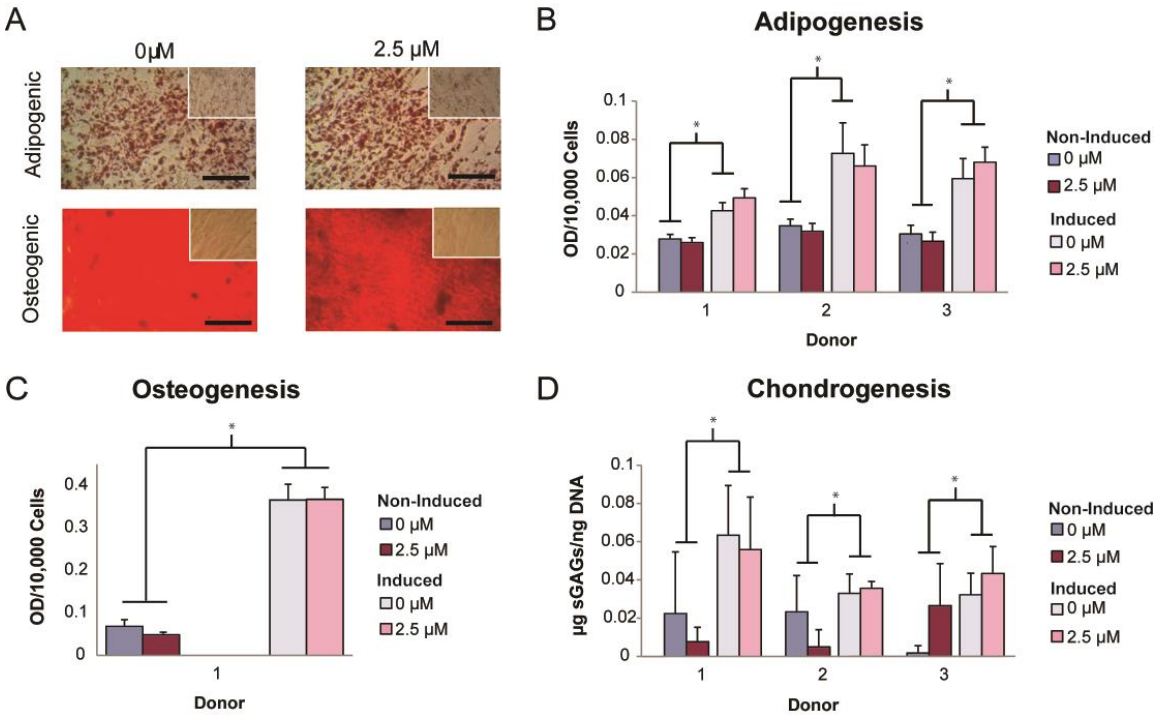

Figure 4.4.5. Treatment with 2.5 μM MTX had no negative effect on SVF cell multilineage differentiation potential. (A) Oil Red O and Alizarin Red S staining confirmed comparable lipid and calcified matrix production for induced and non-induced (insets), untreated and methotrexate-treated SVF cells (representative images for one donor). Optical densities of eluted, metabolite-specific stains were quantified to determine differences in (B) adipogenic, (C) osteogenic, and (D) chondrogenic differentiation potential of three, unique, SVF cell populations. No differences were observed between untreated and methotrexate-treated adipogenic, osteogenic, or chondrogenic differentiation potentials. Osteogenic assessment was only conducted on donor 1, as donors 2 and 3 became overly contractile and could not maintain a monolayer throughout three weeks of matrix deposition. Error bars show standard deviation. (Scale bar indicates 50 μm, *$p < 0.05$)

These findings were supported by quantitative data showing that lipid, calcified matrix, and sGAG levels were comparable within both the induced and non-induced control sample conditions (Fig. 4.4.5B-D, $p < 0.05$). As mentioned in the methods, osteogenesis was only included for donor 1, since SVF cells from donors 2 and 3 could not maintain a monolayer morphology throughout the 3-week induction period, preventing accurate comparisons across donors.

4.4.5 DHFR protein expression after MTX treatment

Previous studies suggested that DHFR protein upregulation may be an MTX-specific mechanism of resistance in multiple cell types. Therefore, we conducted western blot experiments to explore the role of DHFR in the MTX resistance exhibited by freshly isolated (SVF cells) and monolayer expanded ASCs. Protein expression was compared between untreated and 2.5 μM MTX-treated SVF cells, ASCs and NHFs. DHFR expression was upregulated differentially between SVF cells, ASCs, and NHFs (Fig. 4.5.1A). Quantification of protein levels via densitometry determined that average DHFR upregulation in SVF cells and ASCs following MTX treatment was at least 11-fold greater than that observed in NHFs (Fig. 4.5.1B). Furthermore, SVF upregulation was 2-fold greater than ASC upregulation, on average.

4.5 Discussion

The results of this study indicate that ASCs are resistant to high-dose MTX treatment, whereas clinically relevant concentrations of VIN, CY, and ETO inhibit cellular growth. The effects of chemotherapy on mesenchymal stem cells, such as ASCs,

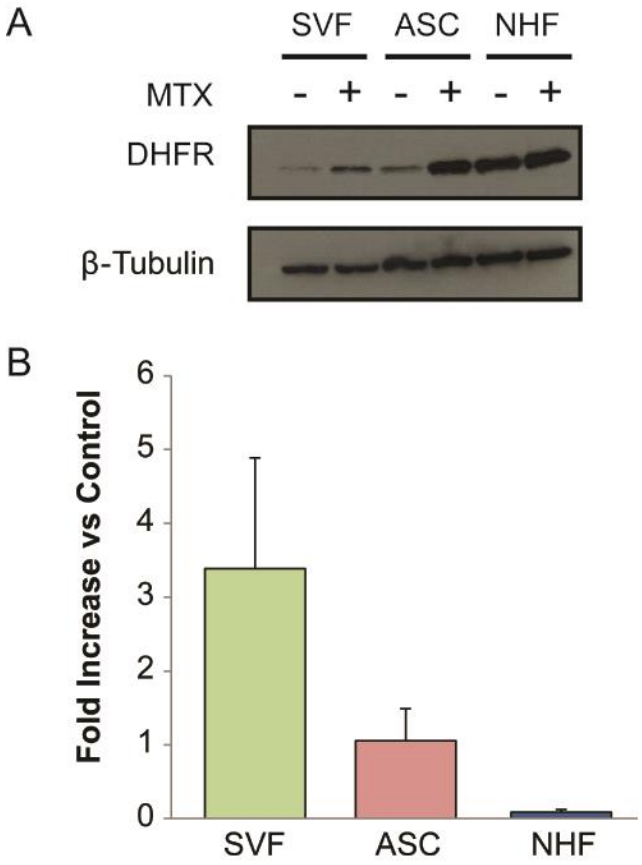

Figure 4.5.1 DHFR upregulation in SVF cells and ASCs was greater than NHFs after MTX treatment. (A) Western blot was used to compare protein expression of DHFR in untreated and 2.5 μM MTX-treated SVF cells, ASCs, and NHFs. (B) Quantification of band intensity revealed that SVF cells and ASCs upregulate DHFR 11-fold more than NHFs in the presence of MTX. Error bars show standard deviation.

is an important area of investigation since these cells play a critical role in tissue maintenance and tissue regeneration (Liang, et al., 2011). However, differences in mechanism of action suggest that not all chemotherapeutics will uniformly affect ASC function. Therefore, examining the influence of individual agents on ASC regenerative properties is necessary to determine drug-specific resistance or sensitivity for these cells. The goal of this study was to assess the response of ASCs versus an untargeted, somatic, non-stem cell population (NHFs) following *in vitro* exposure to common chemotherapeutics. We sought to identify resistance or susceptibility of ASCs to the

tested drugs and improve upon our current understanding of chemotherapy effects. Furthermore, we aimed to investigate a potential mechanism behind any drug resistance to elucidate the phenomena observed in our results. Initial experiments used monolayer-expanded ASCs, which are more homogeneous than freshly isolated cells, to examine the effects of chemotherapeutics on regenerative properties. To investigate whether these effects were conserved for a more complex cell population, subsequent experiments used heterogeneous, SVF cells to examine the proliferation and differentiation capabilities of drug-treated samples.

To determine the effects of MTX, VIN, CY, and ETO on ASC and NHF proliferation, cells were counted on days 6-10 following treatment with specified drug concentrations. Most interestingly, we observed that ASC growth was not inhibited by MTX at any concentration (0.1-50 μM). Conversely, NHF growth was inhibited after treatment with as low as 2.5 μM, which is within the clinically relevant range (Kearney, et al., 1979; J. Li, et al., 2004). While the current study showed no dose dependent impairment for ASCs exposed to MTX, Qi et al. observed decreases in ASC proliferation when treating with 550 μM MTX for 48 hours, suggesting that longer exposure at much higher drug concentrations can negatively affect ASC growth (Qi, et al., 2012). The other chemotherapeutics investigated in this study, VIN, CY, and ETO, all inhibited ASC proliferation, although variability existed among drug type and concentrations. ASCs and NHFs responded comparably to CY and ETO, suggesting similar susceptibility to these drugs, which prevent DNA synthesis via inactivation of polymerase and inhibition of topoisomerase II, respectively (D. D. Ross, et al., 1990; W. Ross, et al., 1984). However, cellular response to VIN was not as uniform. While most drug concentrations resulted in

greatly decreased proliferation, these decreases were less for ASCs than NHFs. Therefore, ASCs may be better equipped to rectify inhibition of microtubule formation, the mechanism of action for VIN (George, et al., 1965). This is supported by a study by Liang et al. that found ASCs could recover after exposure to 0.1 μM VIN (Liang, et al., 2011). Discrepancies between these findings and our own, which showed no recovery after exposure to 0.125 μM VIN, could be due to the slightly higher VIN-treatment concentration or other differences in the medium composition, such as serum fraction. It remains to be examined whether the superior resistance of ASCs over NHFs is conserved at even lower concentrations of VIN. However, those results may not be of great translational interest since ASCs and NHF growth was inhibited at clinically relevant VIN concentrations (0.1 μM) (J. Li, et al., 2004). The variability among ASC response to MTX, VIN, CY, and ETO suggests that ASCs are not impervious to all chemotherapeutics. In particular, high concentrations of VIN, CY, and ETO reduced cell counts by 70-95%. The clinically relevant range of doses used in this study illustrate the impact chemotherapeutics can have on the function of ASCs and other somatic cells such as NHFs. ASC senescence during chemotherapy could impair their ability to maintain tissue integrity, leading to degradation akin to the effects of chemotherapy on BMSCs and osteoblasts in bone. However, additional experiments are necessary to determine whether our *in vitro* results are indicative of the *in vivo* response. Cumulative effects of chemotherapeutics and the influence of the surrounding microenvironments during long-term *in vivo* treatment may result in different findings, as has been shown previously to occur with BMSCs (Georgiou, et al., 2012).

While we have discussed the impact of clinically relevant chemotherapeutic concentrations on ASC and NHF proliferation, it is important to note that these concentrations are based off of plasma levels and not necessarily the concentrations within adipose tissue. Reports on methotrexate and vincristine indicate that drug concentrations are 3-10-fold lower in adipose tissue than plasma (Anderson, et al., 1970; Behan, et al., 2010). These data suggest that the concentrations used in this study are higher than those found within the ASC niche during treatment. However, since it has been suggested that ASCs reside within the adipose vasculature (Tang, et al., 2008), ASCs may be exposed to concentrations closer to those in plasma. Therefore, the range of doses used in our study, which includes levels above and below physiologically relevant plasma concentrations, is representative of concentrations that ASCs would be exposed to within adipose tissue. Of particular relevance to our findings related to methotrexate, we showed that even at high concentrations relative to physiological dosing, ASCs exhibited a remarkable resistance to the negative effects of the drug. To further understand the effects of MTX on ASC and NHF expansion, cells were treated with 2.5 μM MTX and assessed for viability, cell division (Ki-67), and senescence (X-gal) on the day that treatment was removed (day 0) as well as the subsequent 10 days. Results confirmed that MTX had no effect on ASC viability, cell division, or senescence. Conversely, MTX-treated NHF viability and cell division were significantly reduced from untreated levels, and the fraction of senescent cells was significantly increased. These data confirm that ASC expansion properties are resistant to 2.5 μM MTX *in vitro,* and reveal why MTX-treated ASC counts were not lower than untreated ASC counts in our proliferation assay. The cell death and growth arrest observed for NHFs is consistent

with previous studies and indicates why low NHF counts existed after MTX treatment. Published findings suggest that exposure to MTX can cause either cell death or senescence, although this response is dependent on MTX treatment concentration and time. For example, Hattangadi, et al. determined that MTX caused 40% of MCF-7 breast tumor cells to apoptose and a greater fraction of cells to undergo growth arrest (Hattangadi, et al., 2004). While untreated ASC and NHF division was reduced and senescence was increased over time, this can be attributed to the cells reaching confluence (Fagman, et al., 2003; Severino, et al., 2000).

In addition to proliferation and growth characteristics, we investigated the effects of MTX on cellular differentiation potential. We compared the adipogenic, osteogenic, and chondrogenic differentiation outcomes of untreated and MTX-treated, homogeneous ASC populations. Analysis of normalized, metabolite levels revealed MTX caused no change in osteogenic or chondrogenic differentiation, while slightly increasing adipogenic response. These results suggest that ASC differentiation potential is not impaired by 24 hour exposure to 2.5 µM MTX treatment *in vitro*. Combined with our proliferation findings, ASCs appear to be a promising stem cell source that is less sensitive to the negative effects of this commonly used chemotherapeutic agent compared to other somatic cell types. This is important since previously published studies have shown that BMSCs are susceptible to MTX. *In vitro* treatment of BMSCs with 0.1 µM MTX for 48 hours resulted in increased adipogenesis but had no effect on osteogenesis. When replicated *in vivo*, treatment still increased adipogenesis but showed significant inhibition of osteogenesis (Georgiou, et al., 2012). The authors proposed that MTX-impaired BMSC function contributes to bone disorders arising after MTX treatment

(Georgiou, et al., 2012). To determine superior MTX resistance of ASCs over BMSCs, experiments directly comparing these cell types must be conducted.

Given the abilities of ASCs to maintain their differentiation potential after short-term treatment with concentrations 25-fold higher than clinically relevant doses (Kearney, et al., 1979) they could be an attractive source for regenerative therapies for patients undergoing MTX treatment. Acute lymphoblastic leukemia, the most common form of childhood cancer, is one disease primarily treated with this drug. Currently, young patients undergoing chemotherapy face the risk of never recovering MTX-induced, bone mineral density loss, leaving them at higher risk for osteoporosis and bone fractures throughout their lifetime (Mandel, et al., 2004). However, it is possible that treatment with a resistant stem cell population, like ASCs, could ameliorate these defects by regenerating tissue damage after MTX exposure or even preventing bone loss altogether if introduced prophylactically. To verify that ASCs are a promising cell source for these regenerative therapies, additional investigations into the extent of ASC MTX-resistance is necessary. Studies should examine the effects of longer treatments and whether ASC resistance to MTX extends to the *in vivo* environment.

While enriched, homogeneous ASC populations were resistant to the effects of MTX, it was unknown whether cells comprising freshly isolated, heterogeneous SVF populations would also withstand MTX cytotoxicity. SVF cells from lipoaspirate are heterogeneous, consisting of ASCs, adipocytes, smooth muscle cells, endothelial cells, and fibroblasts (Yoshimura, et al., 2006). ASC purification approaches use a variety of strategies, the most effective of which is often serial passaging. Rapid enrichment of *freshly isolated* ASCs is problematic since there are no universally accepted surface

markers for mesenchymal stem cells (Lv, et al., 2014). If cells are to be isolated from a patient and re-implanted with no extensive, monolayer expansion steps, then SVF cells are an important population to examine. Moreover, a secondary aim of this study was to determine whether the regenerative properties of SVF samples could be enhanced by MTX exposure based on the hypothesis that resident ASCs would proliferate while non-stem cells would not, effectively enriching the sample for our cell type of interest. Freshly isolated cells were treated with MTX and then analyzed for their clonogenicity and differentiation potential. Quantifying the number of colonies formed by untreated and treated SVF cells revealed that MTX increased colony counts by 50-100%. Since ASC quantity and CFUs correlate, our results indicate that MTX treatment effectively enriched ASCs within the heterogeneous populations. This enrichment should equate to a more robust differentiation response following chemical induction. However, analysis of adipogenic, osteogenic, and chondrogenic differentiation potential for untreated and MTX-treated SVF samples showed that lineage-specific metabolite production did not increase as a consequence of treatment. We hypothesize that ASC proportions in these heterogeneous cell populations, while higher, was not increased enough to see an effect on the metabolite production of the overall SVF samples. Interestingly, these findings suggest that the various cell types in SVF combine to exhibit a similar, resistant response to MTX as the more homogeneous, monolayer-expanded ASCs.

Experimental results also showed that ASC resistance to MTX is not passage-dependent or donor-dependent. Groups have reported that monolayer expansion can influence many characteristics of ASCs including surface marker expression, proliferation, and differentiation potential (Mitchell, et al., 2006; Schellenberg, et al.,

2011; Zhao, et al., 2012). However, increased CFU counts and uninhibited differentiation potential of MTX-treated, freshly isolated SVF cells suggests that monolayer expansion does not cause MTX resistance of ASCs. This response was consistent for cells from multiple, human donors. While differentiation potential has been determined to be donor dependent (Guilak, et al., 2006), ASC MTX resistance is hypothesized to be an inherent function of this specific cell type, not influenced by differences in donor physiology.

Data showed that in response to drug treatment ASCs and SVF cells upregulated DHFR protein, the target of MTX and hypothesized mechanism behind ASC resistance (Cronstein, 1997). DHFR is involved in DNA biosynthesis and is essential for proliferation. During treatment, MTX binds available DHFR, inhibiting its function and leading to cell senescence or apoptosis. Therefore, DHFR upregulation due to an increase in gene expression infers cellular resistance, as this provides excess, unbound DHFR for continuation of necessary bioprocesses (Goldman and Matherly, 1985). Quantification of protein amounts for SVF cells, ASCs, and NHFs revealed that the increase in DHFR expression from baseline levels is at least 11-fold greater in ASCs and SVF cells than MTX-sensitive NHF populations. These results suggest that DHFR upregulation in the presence of MTX likely contributes to ASC resistance, though studies examining DHFR gene expression should be conducted to further confirm this.

Western blot experiments also revealed that SVF cell DHFR upregulation was 2-fold greater, on average, than upregulation of purified ASCs, despite SVF populations containing a greater fraction of terminally differentiated cells than ASC populations. Two reasons for the disparate responses of SVF and ASC DHFR upregulation have been hypothesized. First, ASCs are exposed to culture medium supplemented with growth

factors, which have been suggested to improve chondrogenesis, while potentially reducing ASC "stemness" (R. T. Estes, et al., 2006). Loss of "stemness" may prevent ASCs from upregulating DHFR as much as the ASCs present in SVF populations. An additional contributing factor could be that freshly isolated SVF populations are more prolific than expanded ASCs. Studies have shown that non-passaged SVF cells express surface markers associated with proliferation that are lost during expansion (Suga, et al., 2009). As such, while both expanded and freshly isolated ASCs are able to upregulate DHFR and resist adverse MTX effects, the metabolic and prolific properties of freshly isolated ASCs could enable them to upregulate DHFR more than passaged cells.

It is possible that DHFR upregulation is not exclusively responsible for ASC resistance but may contribute in conjunction with complementary mechanisms. Other studies have reported that MTX resistance could be a result of low reduced folate carrier expression, overexpression of breast cancer resistance protein, or upregulation of multidrug resistance-associated protein 3 (Cowman and Foote, 1990; Guo, et al., 1999; Wall and Rubnitz, 2003). Superior DNA repair abilities of ASCs may also contribute to resistance, since accumulation of single- and double-strand breaks causes apoptosis during chemotherapy (J. C. Li and Kaminskas, 1984). Furthermore, differences in growth rates could contribute to the superior chemotherapeutic resistance of ASCs over NHFs. Chemotherapeutic agents are designed specifically to target rapidly dividing cells, while slowly dividing cells are not as susceptible to damage. Since ASCs have a longer doubling time than NHFs, 36 versus 24 hours, respectively, it is possible that this difference renders ASCs more resistant to these treatments than NHFs. Further study is

warranted, since inducing targeted, MTX resistance via chemical treatment would be a more straight-forward, therapeutic approach than cellular transplantation.

A possible consequence of ASC upregulation of DHFR is increased proliferation rates. Results showed that ASC growth after MTX treatment determined that ≥ 2.5 µM MTX increased cell numbers 30-40% from control levels. This trend was conserved for SVF populations, as well, with cell counts of 2.5 µM MTX-treated populations being consistently higher than counts of untreated populations. DHFR overexpression has been implicated previously in increased proliferation, with cells in zebra fish embryos proliferating faster when DHFR is overexpressed (Sun, et al., 2011). While DHFR expression in NHFs was also elevated in the presence of MTX, these cells were still impaired by drug treatment. This is not unexpected since DHFR has previously been noted to increase protein expression in MTX-treated osteosarcoma cells, while mRNA expression decreased (Yoon, et al., 2010). Therefore, it is possible that DHFR protein upregulation was an initial response of the cells to the treatment but could not be sustained. NHF cell senescence after treatment suggests that expression is eventually lost, due to the reliance of cell proliferation on DHFR. Examining DHFR levels at a later time point could confirm this hypothesis.

4.6 Conclusion

The effects of chemotherapeutic agents on ASC proliferation and differentiation potential are imperative to understanding the musculoskeletal consequences of high-dose treatments and potentially identifying preventative treatments for patients receiving chemotherapy. This study revealed differential resistance and susceptibility of ASCs to commonly used chemotherapeutics. We have identified multiple drugs that impair ASC

proliferation, revealing concerns for patients with respect to mesenchymal stem cell function. However, we also determined that ASC regenerative properties were unaffected by MTX, a chemotherapeutic known to cause long-term musculoskeletal injury. This study's findings support further investigation into the applicability of ASCs for regenerative therapies targeting patients undergoing MTX treatment.

4.7 Acknowledgements

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4.9 Supplemental Figures

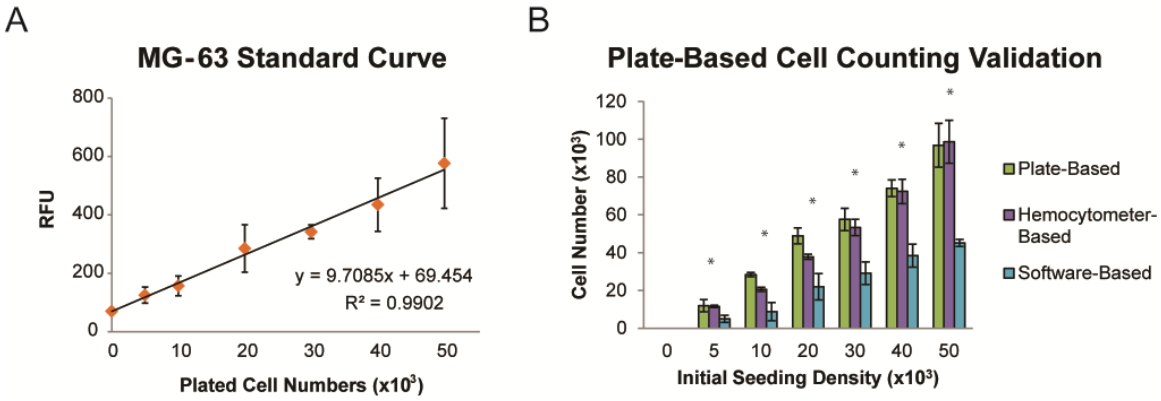

Figure S4.9.1 A novel, plate-based cell counting method was validated for reproducibility and accuracy in comparison to hemocytometer and software-based approaches. A) A standard curve was determined for MG-63 cells (cell line used in preliminary studies) correlating fluorescent units with cell counts. Known cell numbers were plated in 96-well plates at values ranging from 5,000-50,000 cells. Following overnight adherence, cells were stained with Hoechst and fluorescence was quantified using spectrophotometry. Values were plotted to obtain an equation correlating fluorescence values with cell counts. B) Our plate-based counting technique was compared with established methods to confirm consistency. Known cell amounts were plated in 96-well plates and allowed to proliferate. After 24 hours, cells were counted using plate-based (fluorescence of Hoechst-stained nuclei), hemocytometer-based (manual counting), and software-based (imaging and quantifying DAPI-stained nuclei) approaches. Statistical analysis confirmed that no significant differences existed between plate and hemocytometer-based approaches. Conversely, the software-based technique yielded counts significantly lower than plate and hemocytometer-based methods, due to difficulties identifying overlapping nuclei as individual cells. These data confirm that our plate-based approach is comparable to the commonly used hemocytometer-based counting technique. Furthermore, the plate-based approach is potentially more accurate than the software-based approach, a less conventional, but validated method. (*$p < 0.001$ between software-based and plate/hemocytometer-based counts)

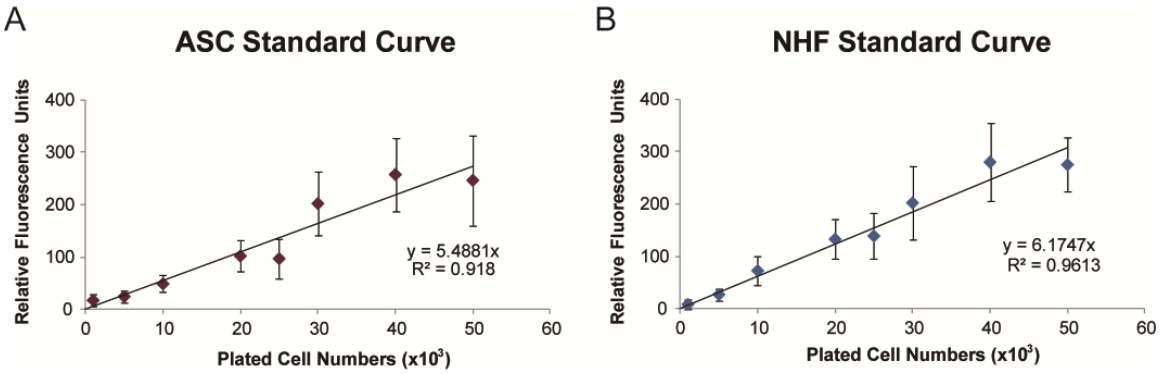

Figure S4.9.2.To assist with cell counting, a simple fluorescence-based assay was developed in our lab. Standard curves for (A) ASCs and (B) NHFs were generated by plating known numbers of each cell type (ranging from 0-50,000 cells) in 96-well plates. The following day, nuclei were stained with Hoechst dye, and spectrofluorometry was used to quantify relative fluorescence units. Data were plotted to obtain an equation correlating relative fluorescence units with cell counts. This assay was extensively validated for each cell type and served as a reliable method for quantifying proliferation. While relative fluorescence units could also be used to assess proliferation, converting to cell numbers provided for more intuitive descriptions.

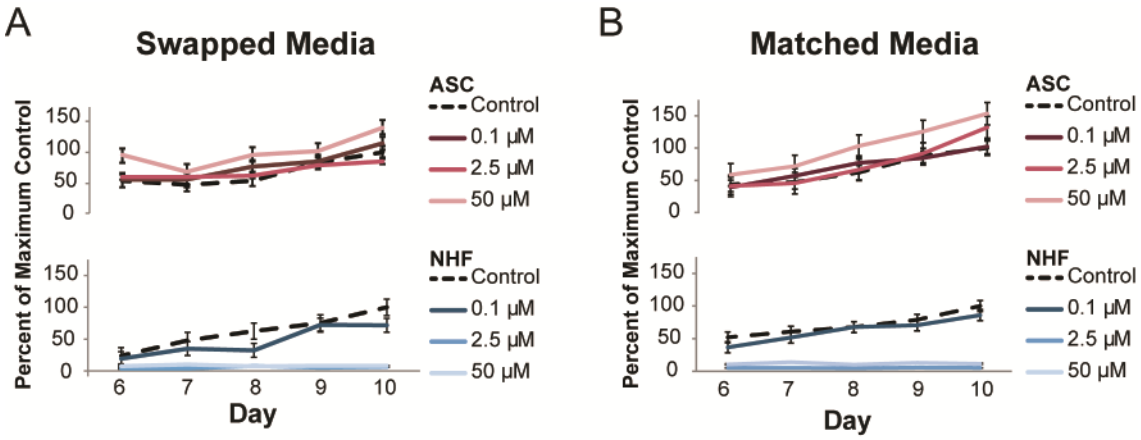

Figure S4.9.3 To confirm that adipose-derived stem cell and normal human fibroblast media were not contributing to the effects of methotrexate, cells were treated with the drug in swapped media. (A) ASCs cultured and treated in NHF medium were resistant to methotrexate treatment. NHFs cultured and treated in ASC medium were inhibited by 2.5 and 50 µM methotrexate. (B) Swapped media results were consistent with findings for cells treated in matched media conditions (from Fig. 4.4.1A).

Chapter 5

Stemness and Dihydrofolate Reductase Influence Non-Cancerous Cell Response to Methotrexate

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5.1 Abstract

Methotrexate (MTX) is a commonly used chemotherapeutic agent that kills cancer cells through binding of dihydrofolate reductase (DHFR). Due to its non-specificity, MTX also impairs normal cell function and causes long-term damage to healthy tissue. These consequences have been investigated extensively in bone-derived cells due to their sensitivity to the drug. While DHFR likely plays a role in normal cell response to MTX, research in this area is limited. Moreover, how MTX sensitivity differs among cell types responsible for maintaining tissues is unknown. The goal of this study was to investigate the role of DHFR and subsequent nucleotide synthesis in normal cell response to MTX. We also sought to compare adverse drug effects among normal cell

types to identify sensitive populations and resistant cell sources for regenerative procedures targeting patients undergoing chemotherapy. DHFR overexpression or exogenous nucleoside delivery rescued cells from adverse MTX effects. Conversely, DHFR knockdown impaired MTX-treated adipose-derived stem cell (ASC) osteogenesis. ASC and bone marrow stem cell proliferation was more resistant to MTX than that of terminally differentiated osteoblasts. However, stem cells became susceptible to the drug after beginning osteogenic differentiation These results suggest that the ability of stem cells to survive and maintain their surrounding tissues likely depends on whether they are in a "stem" state when exposed to MTX. Therapeutic strategies that delay the differentiation of stem cells until clearance of the drug may produce more favorable outcomes in the long-term health of treated tissues.

5.2 Introduction

Chemotherapeutics are an effective treatment for many forms of cancer due to their targeting of rapidly dividing cells [1-4]. However, because of the non-specificity of these drugs, normal, non-cancerous cells also exhibit sensitivity to treatments [5]. Chemotherapy-induced impairment of normal cells results in long-term damage, which can include tissue loss or dysfunction [6,7]. Methotrexate (MTX) is a commonly used chemotherapeutic agent known to harm both normal and cancerous cells through binding and inhibition of dihydrofolate reductase (DHFR) [8]. DHFR is an essential protein responsible for reduction of folate and dihydrofolate to tetrahydrofolate, which is necessary for nucleotide production and subsequent DNA biosynthesis [9]. MTX has been employed to combat prevalent cancers, such as acute lymphoblastic leukemia and breast cancer [10,11]. The drug is also used long-term at low concentrations for treatment

of rheumatoid arthritis and has recently been considered as a potential therapy to reduce obesity-related inflammation of adipose tissue [12,13]. While this drug is widely effective in the clinic, previous studies have found that MTX can cause impaired function of bone-derived cells, including osteoblasts (OBs) and bone marrow stem cells (BMSCs) [14-18]. High dose treatment can lead to a reduction in OB and BMSC calcified matrix deposition and proliferation, which ultimately results in long-term bone loss and renders patients at risk for bone defects [6,19,20]. Folinic acid or nucleosides can be delivered to patients undergoing MTX therapy to remedy the resulting nucleotide deficiencies and ameliorate toxicity [21,22]. However, research in this field is limited. Due to the link between cell impairment and long-term tissue damage, it is important to identify adverse effects of chemotherapeutics on cells that are responsible for tissue maintenance and function. Furthermore, since many normal cell types have exhibited sensitivity to chemotherapeutics, it is also necessary to explore mechanistic or regenerative rescue approaches that can minimize adverse effects to normal cells and treat long-term tissue loss.

Adipose-derived stem cells (ASCs) are a regenerative cell population located in adipose tissue [23]. Due to their multilineage differentiation potential, these cells are being considered for use in a broad spectrum of cell-based therapies [24]. Within their niche, ASCs help regulate tissue metabolism and are responsible for tissue maintenance by replacing adipocytes during cell turnover and promoting angiogenesis [24-27]. Their clinical promise and significant regulatory role *in vivo* makes ASCs an important cell type to understand more completely. Unfortunately, not much is known about their response to harmful agents like MTX, which is an important consideration given the

prevalence of MTX treatments prescribed in the clinic. Our group has previously shown that ASCs are relatively resistant to MTX when compared with a normal, non-stem cell fibroblast population [28]. We also determined that ASCs upregulate DHFR more than fibroblasts during MTX treatment, potentially identifying a resistance mechanism that could be implemented in normal cells to prevent unwanted impairment. However, the role of DHFR in ASC MTX-resistance is still not completely understood. Furthermore, little is known about how ASC MTX response compares with other normal cell types shown to be MTX-sensitive, like OBs and BMSCs [29]. Comparing the MTX response of ASCs with other cell types could reveal the extent of ASC MTX-resistance and potentially identify it as a regenerative cell population capable of treating tissue loss after chemotherapy.

This study aimed to investigate how modulating DHFR in normal and stem cell types influences MTX response *in vitro*. We hypothesized that DHFR overexpression or exogenous nucleoside delivery would increase resistance of MTX-sensitive cell types, like normal human fibroblasts (NHFs) and osteoblasts (OBs), while DHFR knockdown would lead to drug susceptibility in MTX-resistant ASCs. To examine the role of DHFR and nucleotide synthesis in MTX cell response, NHFs were transfected with DHFR overexpression plasmids. Alternatively, glycine, adenosine, and thymidine (GAT) were delivered to normal, somatic cell types to explore the rescue capacity of this approach. To understand more about ASC MTX resistance, proliferation and differentiation potential were assessed after DHFR knockdown. Moreover, drug response of non-transfected ASCs was compared with that of bone marrow-derived stem cells (BMSCs) and OBs to evaluate differences in drug sensitivity among these cell types.

5.3 Materials and Methods

5.3.1 Cell Types and Culture

Four different cell types were used in this study: ASCs, NHFs, BMSCs, and OBs. All cells were isolated from human donors and used at low passage number. In most cases, a single donor was used, so interpretation was limited to phenomenological findings and the investigation of molecular mechanisms.

ASCs were isolated from human lipoaspirate following an established protocol [30] with minor modifications, as described previously [28]. Waste tissue was obtained from one, female donor (age 56) following procedures approved by the internal review board (IRB) at Rhode Island Hospital. ASCs were grown in expansion medium comprised of DMEM/F-12 (HyClone, GE Healthcare), 10% FBS (Zen-Bio), 1% antibiotic/antimycotic (HyClone, GE Healthcare), 0.25 ng/mL transforming growth factor-β1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) [31]. Experiments used ASCs at passage 5.

NHFs isolated from neonatal human foreskins (a gift from Dr. Jeffrey Morgan) were expanded in high glucose DMEM (DMEM-HG, HyClone, GE Healthcare), 10% FBS (HyClone, GE Healthcare), and 1% penicillin/streptomycin (HyClone) [32]. NHFs were maintained in humidified incubators at 37°C, 5% CO_2 and passaged at 80% confluence with 0.25% trypsin-EDTA (HyClone, GE Healthcare). Experiments used NHFs at passage 9.

BMSCs derived from one, female donor (age 20, a gift from Dr. Anita Shukla, purchased from Lonza, lot 0000305526) were cultured in α-MEM (HyClone, GE

Healthcare), 15% FBS (HyClone, GE Healthcare), and 1% penicillin/streptoymycin [33]. Experiments used BMSCs at passage 5.

OBs derived from human calvarial bone (a gift from Dr. Dioscaris Garcia, purchased from ScienCell (Carlsbad, CA)) were were grown in Osteoblast Medium (ScienCell) according to the manufacturer's instructions. Experiments used OBs at passage 5.

5.3.2 NHF Plasmid Transfection and Fluorescence-Activated Cell Sorting (FACS)

To assess the effects of DHFR overexpression on NHF MTX response, cells were transfected with DHFR-GFP or GFP control plasmids. Briefly, NHFs were uplifted, centrifuged, and resuspended at 10 x 10^6 cells/mL in 4°C DMEM-HG. Cells were transfected with either GFP or DHFR-GFP fusion expression plasmids (12.5 ug DNA/mL) via electroporation using an Amaxa Nucleofector 2b device according to product literature (program T-016; Lonza). After recovering in complete medium for 10 min, cells were plated at ~70,000 cells/cm^2 in 6-well plates and incubated at 37°C overnight to allow for protein expression. Due to low transfection efficiencies (~10-15%), fluorescence-activated cell sorting (FACS) was used to collect pure populations. Briefly, transfected cells were uplifted 24 h post-electroporation, resuspended at ~0.5 x 10^6 cells/mL in Hank's Buffered Salt Solution (Hyclone, GE Healthcare), and filtered through a 40 μm cell strainer. Cells were sorted using an Influx high-speed cell sorter (BD Biosciences, San Jose, CA) using a 488 nm laser and a 530/30 bandpass filter. Non-transfected cell populations were used as a control to gate forward and side scatter profiles as well as to define fluorescence intensity. Cells with fluorescent signal at least one order of magnitude over background noise was considered positive and were

collected. Positively transfected NHFs were immediately plated at 8,000 cells/cm^2 in 96 well plates, and after adhering overnight, cultures were treated with 0-0.2 µM MTX for 24 h. Cells were then lysed for protein extraction or were washed with 1x PBS and expanded further for proliferation studies.

5.3.3 ASC siRNA Delivery

The role of DHFR in ASC MTX response was examined by silencing DHFR gene expression prior to drug exposure. ASCs were plated in 96 well plates at 5,300 cells/cm^2 and allowed to adhere for 24 h. To achieve knockdown, cells were transfected for 24 h with 0.3 uL/well RNAiMAX (Life Technologies) and either 50 nM control (ON-TARGET plus Non-targeting Pool D-001810-10-05, Dharmacon) or *DHFR* (ON-TARGET plus Human *DHFR* siRNA-SMART pool L-008799-00-0010, Dharmacon) siRNA in antibiotic/antimycotic-free medium. A subset of cells was lysed to extract mRNA and protein 48 h post-transfection, and the remaining cells were treated for 48 h with 0-50 µM MTX. Cultures were washed with 1x PBS following MTX-removal, and fresh expansion or induction media were added, as described below.

5.3.4 Exogenous Nucleoside/Glycine-Adenosine-Thymidine (GAT) Treatment

NHFs

Temporal effects of GAT delivery on MTX-treated NHF proliferation were evaluated to optimize rescue regimen. NHFs were plated at 2,000 cells/cm^2 and allowed to adhere overnight. For temporal GAT studies, cells were treated with 0.2 µM MTX for 24 h. During or after MTX treatment, cultures were exposed to one of four different GAT delivery regimens (Fig. 5.3.1): no GAT (medium was supplemented with DMEM-HG

vehicle control), A GAT (GAT was added for the final 18 h of MTX treatment), B GAT (A GAT, plus cultures were supplemented immediately following MTX-removal for 48 h), and C GAT (GAT was added immediately following MTX-removal for 48 h). GAT was added to reach final concentrations of 0.134 mM G (Fisher Scientific), 7.5 µM A (Acros Organics), and 8.26 µM T (Acros Organics) [34].

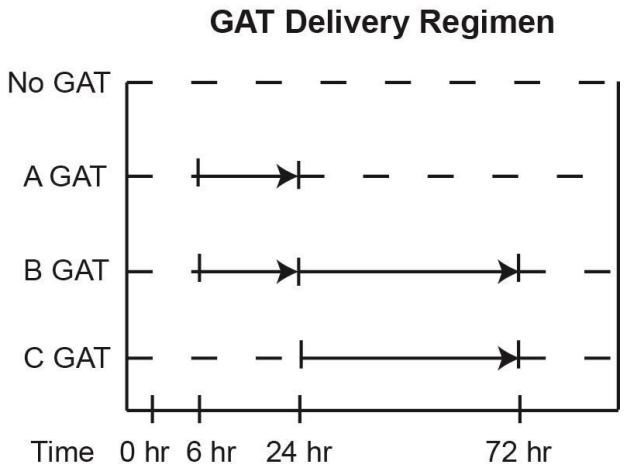

Figure 5.3.1 GAT delivery regimen schematic. GAT was delivered to MTX-treated NHF samples following four different regimens.

To assess the effects of GAT concentration on NHF MTX rescue, only the B GAT regimen was used. Cells were treated with MTX concentrations ranging from 0.1 to 10 µM MTX for 24 hours. Medium was supplemented with either vehicle control, low-dose GAT (LD GAT; 0.067 mM G, 3.75 µM A, 4.13 µM T) or high-dose GAT (HD GAT; 0.67 mM G, 37.5 µM A, 41.3 µM T). MTX was removed, and wells were washed with 1x PBS. Fresh expansion medium was then added with the final GAT dose.

ASCs, BMSCs, and OBs

GAT rescue effects on MTX-treated ASCs, BMSCs, and OBs were assessed using HD GAT, following the B GAT regimen. Cells were plated at 2,000 cells/cm^2 in 96

well plates. Cells were grown for 2-4 days to ensure entrance into log phase and then treated with 10 μM MTX for 48 h. Media were supplemented with a vehicle control or HD GAT, following the B GAT regimen, as described previously. Upon MTX removal and 1x PBS wash, the final 48 h GAT dose was added to wells along with either cell-specific expansion media for proliferation studies or induction media for differentiation studies, as described below.

5.3.5 Western Blot

Protein levels were assessed via western blot for DHFR overexpression and knockdown in NHFs and ASCs, respectively, following previously established protocols [28]. Equal amounts of protein were separated on pre-cast SDS-PAGE gels (Bio-Rad) and transferred onto PVDF membranes (Millipore) before probing for DHFR (1:500, Abcam) or β-Tubulin (1:1000, Developmental Studies Hybridoma Bank, Iowa City, IA). Blots were then developed using WesternSure PREMIUM Chemiluminescent Substrate (LI-COR, Lincoln, NE) and scanned using a C-Digit Blot Scanner (LI-COR). Densitometry was used to semi-quantitatively assess protein levels using Image J (NIH).

5.3.6 mRNA Extraction and qPCR

Effects of siRNA delivery on mRNA expression were examined by qPCR. mRNA was extracted 48 hours after cells were transfected with either control or DHFR siRNA using a QuickRNA Mini Prep Kit (Zymo Research, Irvine, CA) following manufacturer instructions. Reverse transcription was then conducted using a SuperScript III First Strand cDNA Synthesis Kit (Life Technologies, Grand Island, NY). Taqman Gene Expression Assay human primers for *DHFR* (Hs00758822_s1) and a reference gene, *GAPDH* (Hs03929097_g1) were used for amplification. Real-time fluorescence signals

were then measured on a CFX Connect Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA), and relative expression was determined using the comparative C_T method [35].

5.3.7 Proliferation

Cell proliferation was used to indicate the relative health of the experimental groups. To quantify proliferation, cells were stained 2 days post-MTX removal with 5 µg/mL Hoechst 33342 dye (Invitrogen, Life Technologies) for 15 minutes. Stained nuclei were imaged using a Cytation 3 reader (BioTek Instruments, Winooski, VT) and were quantified with Gen5 data analysis software (BioTek Instruments).

5.3.8 Multilineage Differentiation

Adipogenic Differentiation

To evaluate the effects of MTX and GAT rescue on differentiation, cells were induced along adipogenic (ASCs, and BMSCs) or osteogenic (ASCs, BMSCs, and OBs) lineages after treatment removal. For adipogenesis, ASCs (siRNA transfected and non-transfected) and BMSCs were exposed to induction media [DMEM/F-12 (ASCs) or DMEM-HG (BMSCs), 10% FBS, 1 µM dexamethasone, 10 µM insulin, 0.5 µM isobutyl-1-methylxanthine, 200 µM indomethacin (Sigma), and 1% antibiotic/antimycotic] or control media [DMEM/F-12 (ASCs) or α-MEM (BMSCs), 10% FBS, 1% antibiotic/antimycotic] immediately following MTX removal [36]. After two weeks of culture, samples were fixed with 3.7% paraformaldehyde (Thermo Fisher Scientific). Oil red O (ORO, Sigma) staining was used to visualize intracellular lipid droplets in

adipogenic and control cultures. A custom MATLAB program was employed to quantify large lipid diameters and counts (> 4 μm diameter), as previously described [37].

Osteogenic Differentiation

For osteogenesis, ASCs and BMSCs were exposed to induction media consisting of DMEM-HG (ASCs) or α-MEM (BMSCs), 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.15 mM ascorbate-2-phosphate, 10 nM 1,25-$(OH)_2$ vitamin D_3 (Sigma), and 1% antibiotic/antimycotic or control media consisting of DMEM/F-12 (ASCs) or α-MEM (BMSCs), 10% FBS, 1% antibiotic/antimycotic [38]. For OB "osteogenesis," cells were exposed to induction medium consisting of α-MEM, 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, 0.15 mM ascorbate-2-phosphate, and 1% antibiotic/antimycotic or control medium, as described for BMSCs [39]. ASCs, BMSCs, and OBs were cultured for 1 or 3 weeks before assessing either alkaline phosphatase (ALP) activity or calcified matrix production, respectively. After one week of induction, ALP activity was quantified using a Biovision ALP Activity Assay following manufacturer instructions [28]. After three weeks of culture, cells were fixed, and calcified matrix deposition was visualized using alizarin red S stain (ARS, Sigma). After imaging stained samples, ARS was eluted, and optical density was measured with spectrophotometry at 540 nm. Osteogenic data are presented on a per sample basis. However, variations in trends between per sample and per cell normalization existed for some assays. An interpretation of these differences is included in supplemental information (Supplemental Fig. S5.8.1). Experimental cell counts were determined by counting nuclei stained with 4′,6-diamino-2-phenylindole (DAPI, Thermo Fisher Scientific), as described for proliferation.

5.3.9 Statistical Analysis

Two-way ANOVA was used to detect differences in proliferation among experimental groups for each individual cell type. For differentiation, two-way ANOVA was used to distinguish differences among experimental groups of induced conditions. Significance levels for individual comparisons were determined using a Tukey post-hoc test ($p < 0.05$ for significance). To control for non-experimental variability inherent in the differentiation assays, all data were normalized to the average value of individual runs for each experiment. Non-normal data sets were transformed prior to statistical analysis. The effects of DHFR knockdown on non-MTX-treated ASCs were analyzed using a Student's t-test.

5.4 Results

5.4.1 NHF DHFR Overexpression

NHFs were transfected with GFP control or DHFR-GFP plasmids to investigate whether increasing DHFR expression would give NHFs resistance to MTX. Transfection of NHFs with DHFR-GFP plasmids dramatically increased DHFR protein expression when compared with endogenous levels of GFP-transfected control cells, as revealed by western blot. Moreover, treatment with 0.1 μM MTX significantly increased both endogeneous and plasmid DHFR expression by 110-120% (Fig. 5.4.1A, B, $p < 0.005$). Quantifying NHFs after treatment with 0.1 or 0.2 μM MTX revealed that DHFR-GFP transfection led to a 30-40% increase in cell counts over control, GFP-transfected cells (Fig. 5.4.1C, $p < 0.001$). This overexpression resulted in no significant differences being observed between 0.1 μM MTX treated and non-treated DHFR-GFP-expressing NHFs.

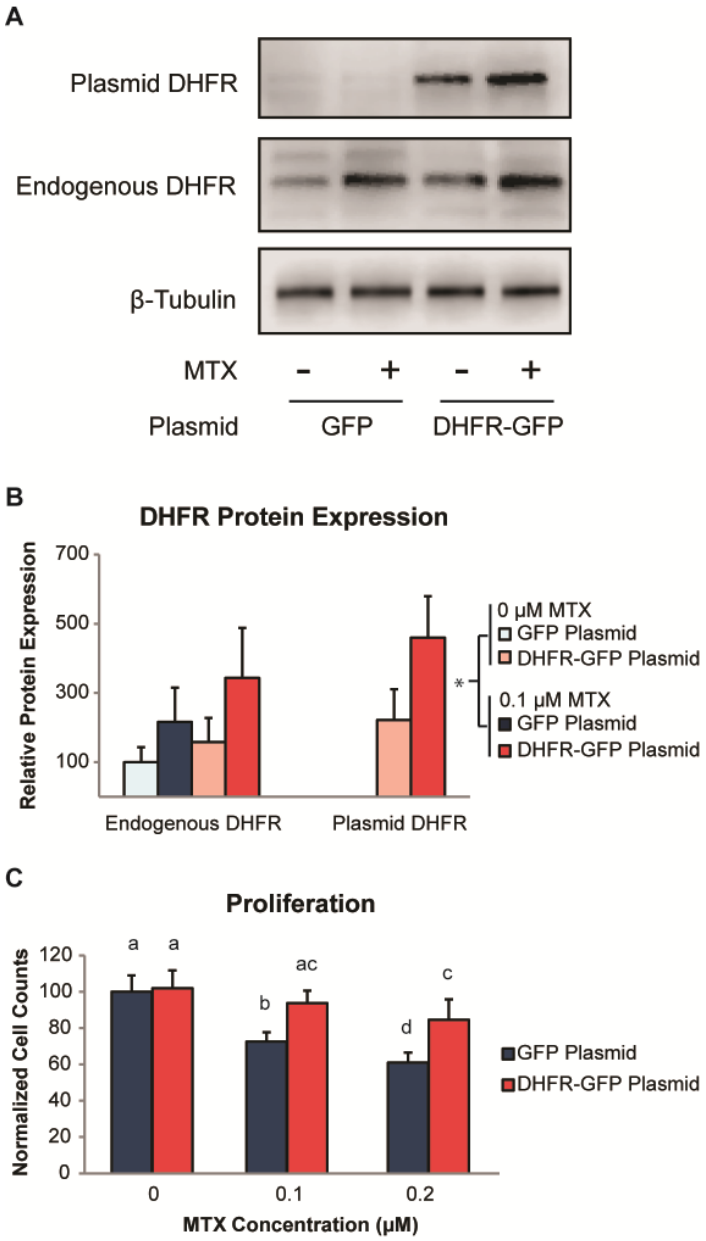

Figure 5.4.1 Effects of MTX on GFP and DHFR-GFP-transfected NHFs. (A) Protein expression for GFP and DHFR-GFP-transfected NHFs after treatment with 0.1 μM MTX. (B) Quantification of band intensity revealed that DHFR expression was significantly increased based on MTX as a factor ($p < 0.005$), but was not significant for individual comparisons. (C) Cell counts of DHFR-GFP-transfected NHFs were significantly higher than GFP-transfected cells after MTX treatment ($p < 0.001$). While 0.1 μM MTX-treated GFP-DHFR NHF counts were similar to untreated controls, 0.2 μM MTX-treated counts were significantly reduced ($p < 0.005$). Quantified protein expression is presented as a percent of untreated, endogenous DHFR expression in GFP-transfected cells. Cell counts are presented as a percent of untreated, GFP-transfected control counts. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

However, treatment with 0.2 µM MTX still impaired cell proliferation, leading to a 20% reduction compared to untreated controls ($p < 0.005$).

5.4.2 NHF GAT Rescue

To investigate the temporal effects of exogenous GAT delivery on NHF MTX rescue, cells treated with 0.2 µM MTX were exposed to a variety of GAT regimens (Fig. 5.3.1). Comparing cell counts of untreated and MTX-treated groups revealed that MTX significantly reduced NHF counts by 30-70% in No GAT, A GAT, and C GAT conditions (Fig. 5.4.2A, $p < 0.001$). However, no significant differences were observed

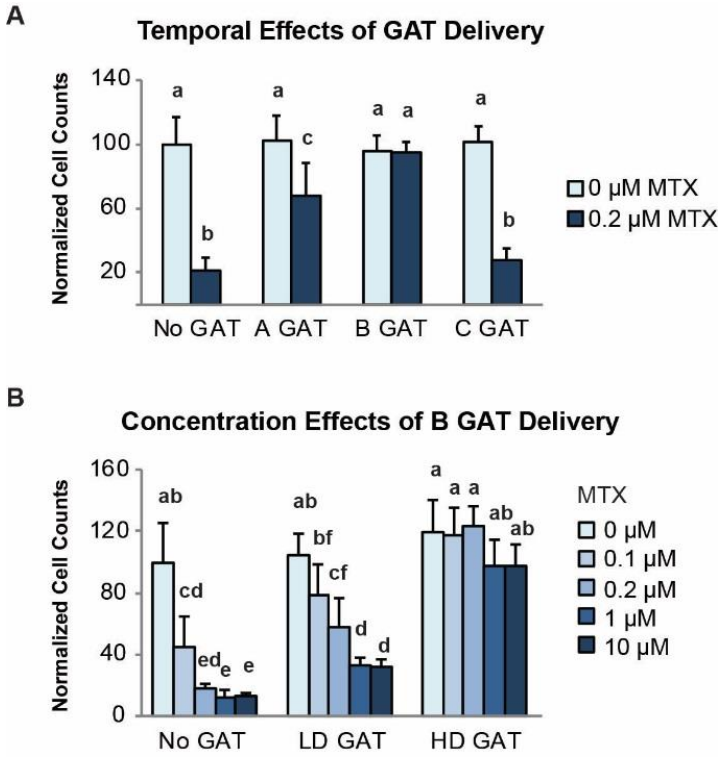

Figure 5.4.2 Temporal and concentration effects of nucleoside/GAT delivery for rescue of MTX-treated NHFs. (A) GAT delivery regimens rescued cell numbers to varying degrees, with B GAT providing the most complete recovery. (B) Successful GAT rescue depended on both the concentration of MTX and concentration of GAT. Too little GAT resulted in incomplete rescue of cell numbers, although some recovery could be observed for lower MTX concentrations. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

between untreated and MTX-treated counts in B GAT conditions. To assess how GAT concentration influences NHF MTX rescue, MTX-treated cells (0.1-10 μM) were exposed to either LD or HD GAT following the B GAT regimen (Fig. 5.4.2B). Within the No GAT condition, treatment with ≥ 0.1 μM MTX significantly reduced cell counts from untreated controls by 60-90% ($p < 0.001$). For LD GAT wells, the 0.1 μM MTX treatment did not significantly reduce cell counts from untreated controls. However, treatment with 0.2-10 μM MTX reduced NHF counts by 30-90% ($p < 0.001$). Conversely, for HD GAT conditions, no significant decreases were observed between untreated and MTX-treated cell counts at any concentration.

5.4.3 ASC DHFR Knockdown Effects

To examine how DHFR knockdown influences ASC MTX-resistance, ASCs were transfected with either control or DHFR siRNA, and their proliferative and differentiation potential was assessed following exposure to MTX. Western blot revealed that DHFR expression of transfected cells, 48 hours post-transfection, was reduced by 90%, consistent with knockdown of *DHFR* mRNA levels (Fig. 5.4.3A, B, C). ASC proliferation after MTX treatment (0.1-50 μM) showed that neither MTX exposure nor DHFR knockdown had an effect on cell numbers (Fig. 5.4.3D). Adipogenesis was similarly unaffected, with large lipid counts and diameters in adipogenic samples being comparable between control and DHFR knockdown conditions, with no observable effects of MTX (Fig. 5.4.4A, B). Conversely, ASC osteogenesis showed measurable changes in experimental groups. Treatment of ASCs with ≥ 0.1 μM MTX significantly reduced

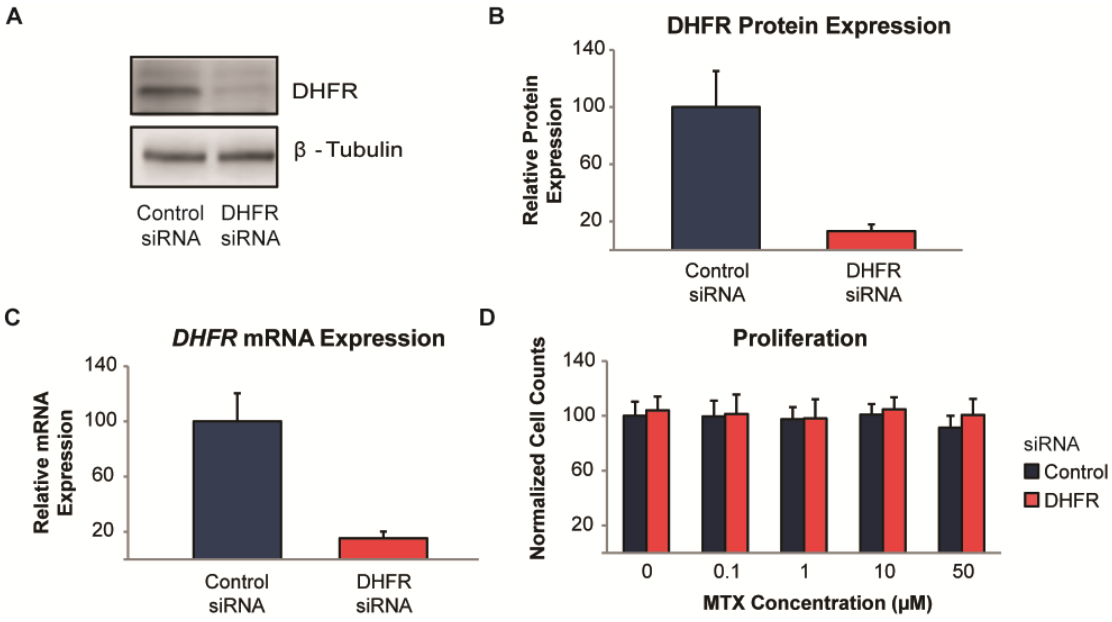

Figure 5.4.3 Effect of DHFR knockdown on MTX-treated ASC proliferation. (A) Protein blot of control and DHFR siRNA-treated ASCs. (B) Quantification of band intensity showed an 85% reduction in DHFR protein levels after knockdown. (C) qPCR confirmed the effectiveness of DHFR siRNA transfection, also showing a 85% reduction in mRNA expression. (D) DHFR knockdown had no effect on ASC proliferation, regardless of MTX concentration. Protein and mRNA expression are presented as a percent of control siRNA samples. Cell counts are presented as a percent of untreated counts in control siRNA samples. Error bars depict standard deviation.

ALP activity by 10-60% for both control and DHFR knockdown conditions (Fig. 5.4.4C, $p < 0.05$). Moreover, ALP activity in both untreated and MTX-treated samples was significantly reduced in DHFR knockdown conditions from 3-20% ($p < 0.01$).

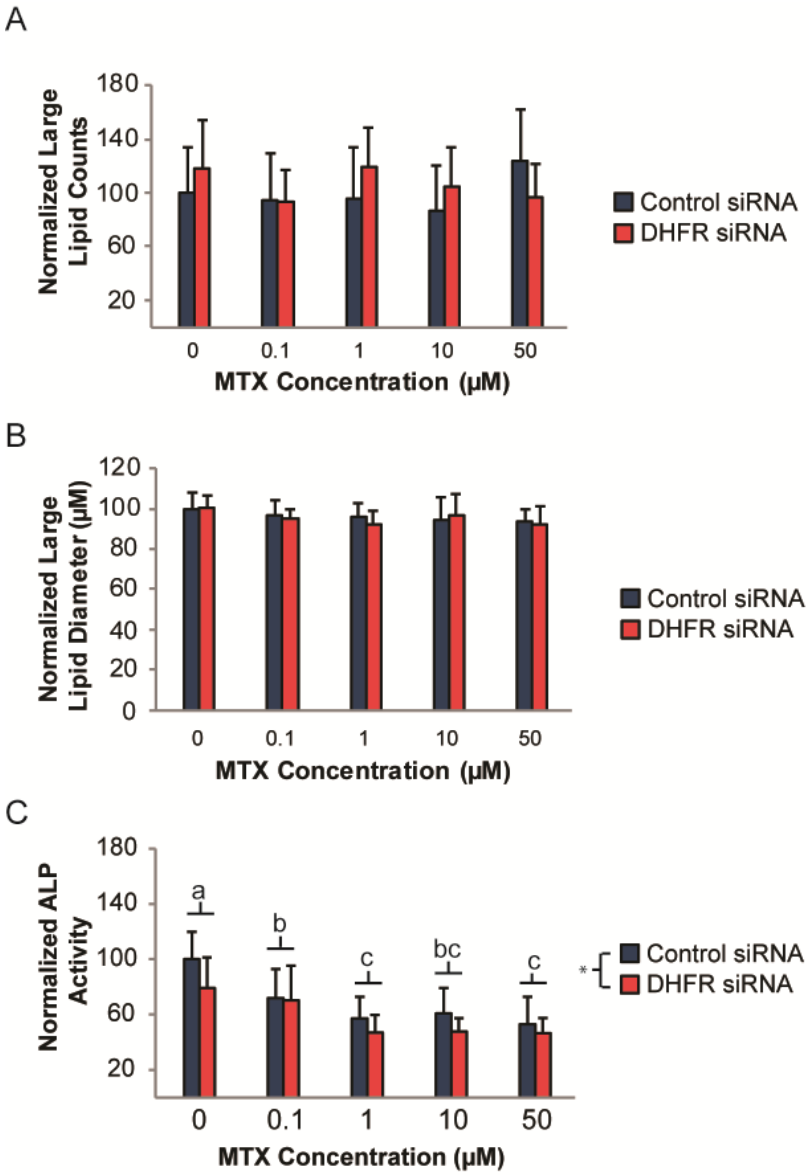

Figure 5.4.4 Effects of DHFR knockdown on MTX-treated, ASC differentiation. Quantification of large (A) lipid count and (B) diameter revealed no differences between siRNA types or among MTX concentrations for adipogenic differentiation. (C) For osteogenic differentiation, treatment with ≥ 0.1 μM MTX and knockdown of DHFR both reduced ALP activity of ASCs ($p < 0.05$ and 0.01, respectively). No individual comparisons showed statistically significant differences. Values are presented as a percent of untreated levels within control siRNA conditions. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

5.4.4 MTX Effects on ASC, BMSC, and OB Proliferation

To evaluate differences in MTX response and to assess the rescue potential of GAT delivery, proliferation of MTX-treated ASCs, BMSCs, and OBs was examined. Without GAT delivery, MTX treatment had no effect on ASCs or BMSCs but significantly reduced OB cell counts by 30% (Fig. 5.4.5, $p < 0.001$). When GAT was delivered, MTX had no significant effect on OB proliferation. However, counts were significantly reduced from untreated/No GAT counts by 30% ($p < 0.001$). Based on GAT as a factor, ASC counts were reduced significantly from No GAT conditions by 6%-15% in untreated and MTX-treated groups ($p < 0.05$). Conversely, no significant differences were observed for BMSCs.

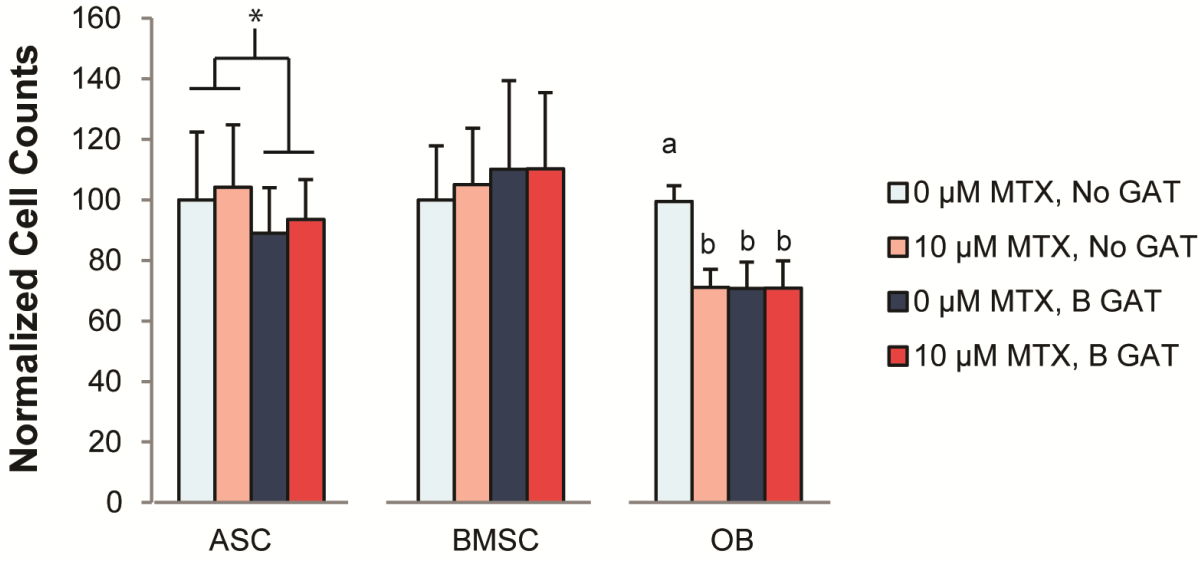

Figure 5.4.5 Proliferation of MTX-treated ASCs, BMSCs, and OBs. For ASCs, MTX had no effect on proliferation, but overall cell counts were significantly reduced based on GAT as a factor ($p < 0.05$). No effect was observed for individual comparisons. Likewise, BMSCs showed no effect due to GAT or MTX. OB proliferation was adversely affected by MTX treatment and GAT delivery ($p < 0.001$), but these effects were not additive. Cell counts are presented as a percent of untreated/No GAT control counts. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

5.4.5 MTX Effects on ASC, BMSC, and OB Differentiation

To examine how MTX treatment and GAT delivery influenced ASC, BMSC, and OB differentiation potential, MTX-treated cells were induced along adipogenic (ASCs, BMSCs) or osteogenic (ASCs, BMSCs, OBs) lineages. Imaging of adipogenic cultures showed similar ORO staining among induced conditions within ASC and BMSC sample groups (Fig. 5.4.6A, B). Quantification of ORO revealed that large lipid counts in ASCs were comparable between untreated and MTX-treated conditions (Fig. 5.4.6C). However,

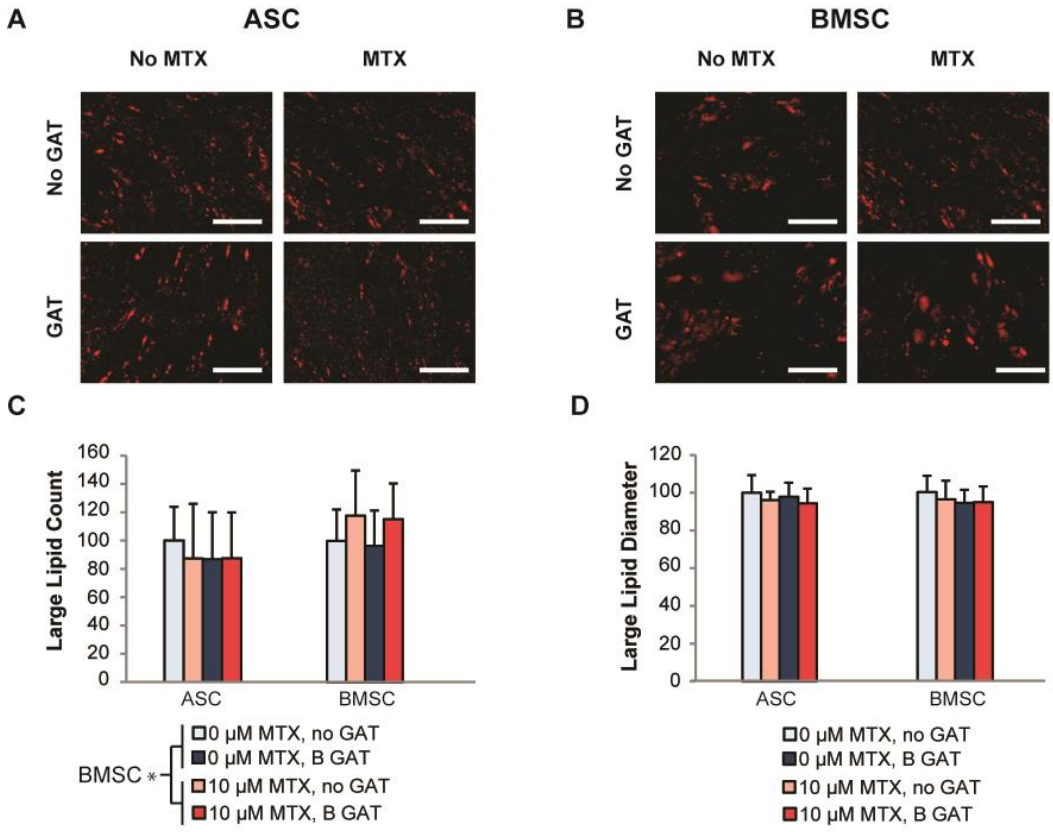

Figure 5.4.6 Adipogenic potential of MTX-treated ASCs and BMSCs. (A, B) Imaging of oil red O staining revealed similar amounts of lipid production among groups for ASCs and BMSCs. (C) Quantification of large lipid counts showed both MTX and GAT had no effect on ASC adipogenesis. Conversely, MTX treatment significantly increased large lipid counts of BMSC samples for both No GAT and GAT conditions ($p < 0.05$) but was not significant for individual comparisons. (D) MTX treatment did not affect large lipid diameter in ASC or BMSC cultures. Large lipid count and diameter are presented as a percent of ASC or BMSC untreated/No GAT control values, respectively. Error bars depict standard deviation. (Scale bar indicates 200 μm).

BMSC large lipid counts were increased by 13-20% after MTX treatment ($p < 0.05$). Measurement of large lipid diameter showed that both ASC and BMSC values were similar between their respective untreated and MTX-treated conditions (Fig. 5.4.6D). Examination of osteogenic cultures revealed that without GAT, MTX treatment reduced ASC and OB ALP activity by 30% (Fig. 5.4.7A, $p < 0.01$). No significant differences

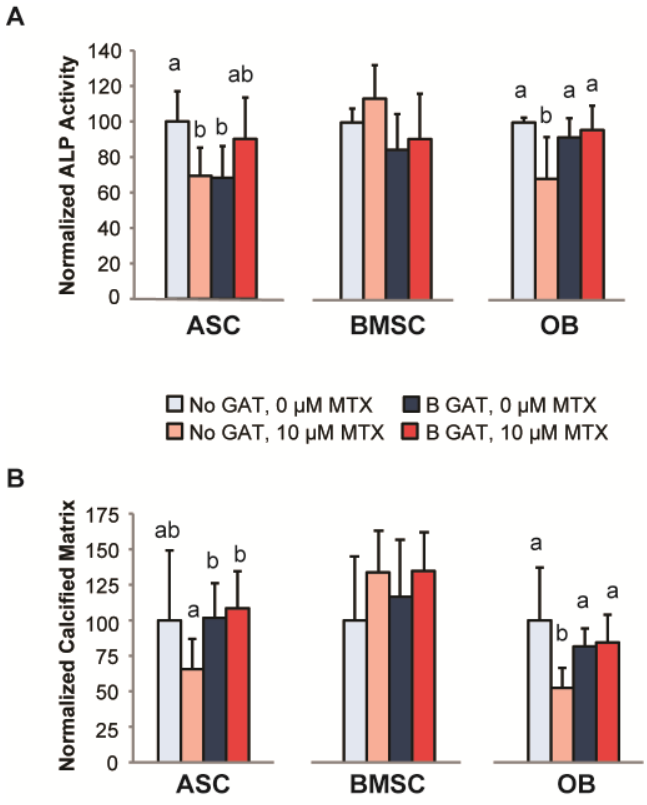

Figure 5.4.7 Osteogenic capacity of MTX-treated ASCs, BMSCs, and OBs. (A) MTX treatment significantly reduced the ALP activity of ASCs and OBs ($p < 0.01$) in No GAT conditions but did not affect BMSCs. No significant differences were observed between untreated/No GAT and MTX-treated/GAT conditions for any cell type, indicating complete rescue. (B) MTX significantly reduced the calcified matrix production of OBs ($p < 0.005$) but had no significant effect on ASCs or BMSCs. As for ALP activity, no differences were observed between untreated/No GAT and MTX-treated/GAT conditions for any cell type. Values are presented as a percentage of ASC, BMSC, or OB untreated/No GAT control levels, respectively. Error bars represent standard deviation. Groups with different letters are significantly different ($p < 0.05$).

were observed in BMSC samples. In the absence of MTX, GAT delivery adversely affected ALP activity in ASCs. Evaluation of ARS staining showed that MTX treatment reduced calcified matrix production of OBs by 50% in No GAT conditions (Fig. 5.4.7B, $p < 0.005$). A decrease in calcified matrix, although not significant, was also observed for ASCs. Similarly, no significant MTX effects were observed for BMSC calcified matrix production. For all cell types, MTX-treated/GAT conditions showed ALP and ARS levels comparable to the untreated/No GAT conditions, indicating a rescue effect.

5.5 Discussion

The results of this study indicate that DHFR, and subsequent nucleotide synthesis, is central to the MTX response of normal, healthy cells but less critical for mesenchymal stem cells. DHFR overexpression or exogenous nucleoside/GAT delivery rescued sensitive, somatic cells from adverse MTX effects. Furthermore, differences were observed for mature versus stem cell response to MTX, with undifferentiated ASCs and BMSCs exhibiting greater resistance than terminally differentiated OBs. These data suggest that sensitivity to MTX is influenced by DHFR expression in conjunction with cell type.

Proliferation experiments of MTX-treated NHFs indicated that DHFR-transfection reduced the adverse effects of MTX. These data suggest that NHF proliferation is susceptible to MTX effects due to insufficient DHFR expression. Arnoldo and colleagues observed similar results when they overexpressed DHFR in HEK cells, indicating that low DHFR expression renders multiple cell types sensitive to the drug [40]. Furthermore, it has been reported that DHFR overexpression is a MTX-resistance mechanism of leukemia cells, confirming that cell response to MTX is often DHFR-

related [41]. While DHFR-transfected cells were resistant to 0.1 μM MTX, treatment with 0.2 μM MTX significantly reduced cell counts from untreated controls. These results indicate that while overexpression of DHFR is capable of rescuing NHFs at low MTX concentrations, there is not enough plasmid-generated DHFR present to rescue cells at higher MTX concentrations. As such, rescue with DHFR overexpression is limited and would be difficult to implement for clinical applications. While a more efficient promoter in the plasmid might address this issue, lack of safe and efficacious *in vivo* transfection approaches also restrict the practical use of this approach to protect non-cancerous, somatic cells [42].

Due to the limitations observed with plasmid-based MTX rescue, exogenous GAT delivery was investigated as a more clinically relevant option. Previous reports have indicated that exposure to exogenous purines and pyrimidines can remedy the deficiencies that result from MTX treatment and can thusly rescue cells from adverse effects [34,43]. Temporal GAT studies with NHFs found that exposure to GAT for the final 18 h of MTX treatment (A GAT), or exposure for the 48 h following MTX removal (C GAT) were inadequate rescue regimens. However, when combined (B GAT), GAT successfully prevented MTX impairment of NHF proliferation. This illustrates that adverse MTX effects are irreversible after 24 h, as has been reported previously, and that damage continues to occur after MTX is removed [44]. Cellular damage after drug removal is likely due to formation of MTX polyglutamates, which occurs by the addition of glutamates to MTX [45]. These MTX polyglutamate chains can be retained by cells for days following treatment due to their length and continue to cause damage [46]. Additional GAT experiments in the current study revealed that increasing GAT amounts

were necessary to rescue NHFs at higher MTX concentrations. These results are consistent with previous literature and indicate that low amounts of GAT do not adequately remedy the large purine and pyrimidine deficiencies that occur at higher MTX concentrations [47]. As such, we have shown that GAT rescue must be tuned to the level of MTX administered. While high doses of GAT did not adversely affect non-MTX-treated NHFs, proliferation and differentiation studies revealed that excess GAT was detrimental to ASC and OB properties. GAT damage is likely due to a surplus of thymidine, which can lead to accumulation of thymidine triphosphate and restrict DNA synthesis [48-50]. Therefore, even in the presence of MTX, excess GAT can impair cells, with susceptibility varying by cell type. To minimize unintended damage, GAT may be most suitable for protecting cells exposed to low MTX concentrations, since less harmful levels of GAT could be delivered and still prevent nucleotide deficiency.

Unlike NHFs, undifferentiated ASCs exhibited resistance to MTX; however, cell numbers and metabolite production decreased when ASCs were placed in an osteogenic environment. Osteogenic induction assays revealed that treatment with MTX decreased ASC ALP activity, with or without knockdown, at concentrations as low as 0.1 μM. ALP activity was also reduced by DHFR knockdown in both untreated and MTX-treated, differentiating ASCs, suggesting that expression levels of this protein can influence osteogenesis. Multiple reports have shown that osteogenic capacity of bone-derived cells is impaired by MTX treatment and leads to long-term effects, such as osteoporosis and bone defects [14,15]. However, to our knowledge, no studies have looked at how DHFR knockdown alone influences osteogenic capacity and/or differentiation, which is relevant since MTX binds and inhibits this protein. Our data showing the importance of high

DHFR expression on osteogenesis indicate a potential reason as to why this tissue lineage is especially susceptible to MTX effects. Moreover, our results support further investigation into targeting DHFR expression of normal, osteoblastic cells as a method of preventing bone loss in patients undergoing chemotherapy.

Only a subset of ASC characteristics was observed to be dependent on DHFR and sensitive to MTX. While ASC osteogenesis was influenced by protein levels, ASC adipogenesis and proliferation were not. Thusly, DHFR expression is not the sole regulator of MTX resistance for ASC proliferation or adipogenesis. The data also suggest that ASCs do not depend on high levels of DHFR to divide or undergo adipogenic differentiation, which may indicate an additional mechanism of MTX resistance. To our knowledge, this is the first study to investigate the role of DHFR in ASC function. These results are important for understanding how necessary bioprocesses are regulated within ASCs. Moreover, they provide encouraging insight for adipogenic reconstructive procedures targeting patients undergoing MTX therapy.

Since ASCs exhibited resistance to MTX until undergoing osteogenesis, stemness could serve as an alternative mechanism of resistance. BMSC MTX response further supports this hypothesis, being that no adverse drug effects were observed until adipogenic induction. A previous report investigating cancer stem cells also considered this theory, since it was observed that acquisition of stemness can lead to drug resistance [51]. Our data suggest that resistance to chemotherapeutics might not just be limited to cancer “stem” cells but might extend to healthy stem cell types as well. Investigation into the expression of stemness factors common between these abnormal and normal cells might reveal targets capable of reducing cancer stem cell drug resistance. These results

are also important for developing regenerative therapies to treat bone loss in patients undergoing chemotherapy. While ASCs and BMSCs may be potential cell sources for treatment of drug-induced tissue damage, their regenerative properties could be limited if MTX is still present within the microenvironment when they start differentiating.

Comparing the MTX response of ASCs, BMSCs, and terminally differentiated OBs further supported a potential role for stemness in MTX resistance. Proliferation assays showed that MTX treatment significantly reduced OB cell counts, while ASCs and BMSCs were unaffected, which is consistent with previous reports [20,29,52]. These data indicate that OBs are more susceptible to adverse MTX effects than stem cells with regards to proliferation, potentially due to their terminally differentiated state. In addition to stemness, an explanation for these differences could lie in their variable proliferation rates, with OBs exhibiting faster population doubling times than ASCs and BMSCs (Table 5.5.1). Since chemotherapeutic agents like MTX target rapidly dividing cells, OBs may be inherently more susceptible to the drug than cells that divide more slowly [4]. Alternatively, OB MTX sensitivity could be a result of their reliance on DHFR for cell division. Knockdown experiments in the current study showed that DHFR expression influenced ASC osteogenesis. It is possible that proliferation of mature osteoblasts might also be regulated in part by DHFR.

While proliferation assays indicated stem cells possessed a superior resistance to MTX, osteogenic differentiation revealed that this resistance was not absolute. Results showed that MTX treatment impaired osteogenesis to varying degrees in ASCs and OBs, while BMSCs exhibited no change, which is consistent with previous literature

Table 5.5.1 Population Doubling Times of Normal Cells

Cell Type	Population Doubling Time (h)
ASC	**21**
BMSC	**51**
NHF	**24**
OB	**13**

[14,15,53]. Interestingly, adverse MTX effects could be ameliorated by GAT delivery. These data indicate that DHFR, and subsequent nucleotide synthesis, likely contributes to ASC and OB osteogenesis but does not influence BMSCs. Understanding differences in drug sensitivity and how mesenchymal stem cells (MSCs) regulate differentiation is important for optimizing regenerative therapies to treat drug-induced damage. Furthermore, our data suggest that impaired OB function may contribute more to MTX-induced bone loss than any effect on BMSCs. It should be noted that on a per cell basis, MTX had no adverse effects on ASC osteogenesis (Fig. S5.8.1). These results are due to a reduction in cell numbers following treatment and suggest that the osteogenic capacity of surviving stem cells is retained. Cells that do not survive are hypothesized to be differentiating rapidly, increasing their susceptibility to remaining MTX polyglutamates. It is possible that more cells would resist adverse MTX effects if differentiation was delayed after treatment. However, this is not always possible in clinical applications of the drug.

Looking further into the effects of MTX on cellular differentiation potential revealed that the drug had no effect on ASC adipogenesis but significantly increased BMSC large lipid counts. These data suggest that there are differences in ASC and BMSC MTX sensitivity and that DHFR may play a larger role in adipogenesis of BMSCs

than ASCs. Georgio and colleagues have also reported that MTX increases the adipogenic potential of BMSCs in a rat model [14]. They later found that this response is due to a reduction in β-catenin expression, a regulator of adipogenic and osteogenic differentiation [55]. Reports on ASCs have also shown that knockdown of β-catenin expression increases adipogenic potential. However, it is possible that while MTX reduces β-catenin expression in BMSCs, it does not affect expression in ASCs. Further investigation into the interaction between DHFR and β-catenin in these two cell types is necessary to confirm this hypothesis. Since low β-catenin expression has also been shown to reduce osteogenic potential in MSCs, it would be interesting to see if this protein, in conjunction with DHFR, contributes to the MTX-induced osteogenic reduction observed for ASCs [55]. Since ASC adipogenesis is not adversely affected by MTX when compared with BMSCs, adipose tissue might not be as susceptible as bone to MTX-induced tissue loss.

Although the 24-48 h MTX treatment regimens used in this study effectively revealed adverse consequences of the drug, longer treatments should also be considered. Our findings revealed that BMSC proliferation was resistant to a 48 h MTX treatment. However, previous reports have cited that treatments lasting 28-66 days, mimicking long-term rheumatoid arthritis therapy, do impair BMSC division [19,56]. Therefore, it is important to consider that use of longer MTX treatments could have yielded different results. An additional, influential factor is how the *in vivo* environment affects cellular drug sensitivity. While *in vitro* studies are imperative for understanding the mechanistic effects of MTX on cell function, it has been reported that discrepancies can exist between *in vitro* and *in vivo* drug effects. For example, Georgiou and colleagues found that *in*

vivo, BMSC osteogenic potential is reduced by MTX but remains unchanged *in vitro* [14]. The authors concluded that the microenvironment plays a role in stem cell susceptibility to MTX. As such, cells might have been more susceptible to adverse drug effects, had they been treated within their niches.

The current study demonstrated that both DHFR and cell type can influence MTX response. Findings revealed that increased DHFR expression or exogenous nucleoside delivery could rescue non-stem cell types from adverse MTX effects, while DHFR knockdown sensitized ASCs to the drug only during osteogenesis. Understanding mechanisms behind normal cell MTX response is crucial for identifying targets for potential rescue techniques. Results also showed that OBs are more susceptible to adverse MTX effects than ASCs and BMSCs, which helps with understanding the reason for drug-induced tissue damage but raises further concerns for patients undergoing chemotherapy. The resistant characteristics of mesenchymal stem cells provide some hope, particularly if they can be used to prevent or regenerate healthy tissue loss during chemotherapeutic treatment.

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5.8 Supplemental Figures

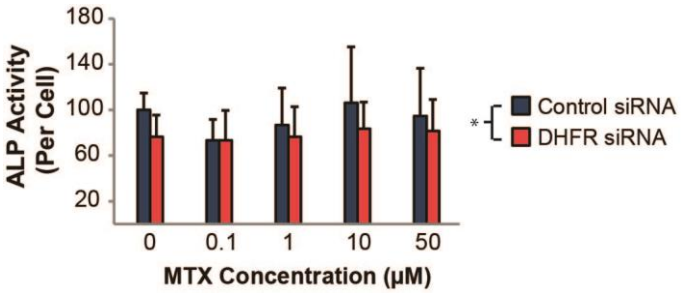

Figure S5.8.1 Effects of DHFR knockdown on ASC osteogenesis. On a per cell basis, MTX had no significant effect on ASC ALP activity. However, DHFR siRNA reduced ALP activity overall compared to control siRNA ($p < 0.05$), but no individual comparisons showed significance. ALP activity is presented as a percent of untreated values within control siRNA conditions. Error bars depict standard deviation. Groups with different letters are significantly different ($p < 0.05$).

Chapter 6

Conclusion and Future Work

MSC function is influenced by factors such as donor age, chemotherapeutic exposure, and cell type. Chapter two reviewed published studies and identified inherent differences among stem cell types being considered for cartilage repair. The original work in chapters three through five revealed the sensitivity of different MSC types to both age-induced impairment and chemotherapy. This work also explored regenerative and mechanistic approaches to prevent normal cell damage during treatment.

6.1 Chapter Two

Chapter two discussed current methods employed to isolate, characterize and differentiate stem cells for cartilage repair. Purification and optimal induction components were found to be highly dependent on stem cell type. Furthermore, 3D culture techniques and tissue functionality measures varied across literature, making comparisons difficult. To address this concern, standardizing the approaches used to assess chondrogenic potential should be considered. The use of 3D cell pellets instead of scaffolds and reporting type II collagen and sGAG amounts on a per cell basis would

enable more reliable comparisons across studies and potentially identify an optimal cell source for cartilage regeneration.

6.2 Chapter Three

Chapter three compared the regenerative properties of young and old MSCs derived from muscle, bone, and adipose tissue. Adverse aging effects were found to be variable among MSC types. BMSC proliferative capacity was more susceptible to age-induced impairment than MDSCs and ASCs. Since older patients are more susceptible to bone loss, these results could indicate that a reduction in BMSC proliferation capacity contributes to this side effect [1]. While muscle loss also increases with age, MDSCs did not suffer reduced proliferation rates [2]. Therefore, decreased myogenic differentiation potential could contribute more to age-induced muscle atrophy than lack of proliferative capacity. Examining the effects of age on myogenic differentiation of these cells would help confirm this theory.

Further investigation revealed that the adipogenic differentiation response of all MSC types was adversely affected by age. In old ASCs, increased spontaneous lipid production in non-induced conditions contributed to this trend, since control lipid levels were comparable to induced samples. Unlike bone and muscle, adipose tissue volume increases with age [3]. This work suggests that spontaneous lipid production of ASCs could contribute to the increased fat volume observed in older patients rather than an increase in signals that induce differentiation.

Results also revealed that while chondrogenic potential was reduced with age for BMSCs and not ASCs or MDSCs, total metabolite production was still comparable

among all old populations. This suggests that muscle, bone, and adipose tissue are equally promising MSC sources for regenerative therapies targeting older demographics. However, using optimal, cell type-specific induction cocktails should be investigated since this could yield different results and identify one cell source with greater chondrogenic capacity. These experiments would especially be of interest for optimizing osteoarthritis regenerative treatments.

6.3 Chapter Four

Chapter four investigated the effects of chemotherapy on ASC and terminally differentiated fibroblast properties. Results showed that ASC and NHF proliferation was similarly impaired by several chemotherapeutic agents, revealing concerns for patients undergoing treatment. Adipose-derived cell senescence can increase insulin resistance, a reported side effect of chemotherapy that occurs due to increased p53 expression and pro-inflammatory cytokine secretion [4,5]. This work suggests that ASC impairment could also contribute to this condition, since these cells are susceptible to adverse side effects of treatments. Investigating p53 expression and monitoring cytokine release post-chemotherapy in ASCs could implicate their involvement with this pathogenesis and help develop therapies to prevent insulin resistance in patients.

Additional results showed that ASCs were more resistant than NHFs to MTX and exhibited proliferative capacity comparable to untreated controls. Furthermore, ASCs retained their multilineage differentiation potential after treatment. These data indicate that ASCs are a potential source for regenerative therapies that target MTX-induced tissue loss. To test the potential of this regenerative therapy, i*n* vivo studies should be conducted to examine how ASCs respond to MTX in tissue microenvironments, since

these interactions can intensify adverse effects. Alternative studies could investigate the response of cells to MTX in 3D tissue culture conditions to understand how the morphology and cell-cell interaction of ASCs influence their response. As part of these studies, drug effects could be compared with a cancer cell type to narrow down relevant concentrations that damage cancerous cells, but not normal cells.

Further investigation revealed that DHFR was upregulated more in MTX-treated ASCs than NHFs, which could suggest a potential mechanism of resistance. However, several proteins have been shown to contribute to MTX resistance, including low reduced folate carrier expression, multidrug resistance protein 1-5, and breast cancer resistance protein [6-11]. Investigating the expression levels of these proteins would potentially provide an alternative mechanism of resistance or identify a protein that works in conjunction with DHFR to elicit this response.

6.4 Chapter Five

Chapter five examined the influence of DHFR and cell type on normal cell MTX response. Overexpression of DHFR and endogenous nucleoside delivery rescued sensitive, normal cells from adverse MTX effects. Additionally, DHFR knockdown increased MTX sensitivity of ASC osteogenesis. These data suggest that DHFR influences normal cell response to MTX and could be a target for rescue techniques. Furthermore, these results are the first to link DHFR expression to ASC osteogenic potential, potentially revealing why bone is especially susceptible to adverse MTX effects. To better understand the role of DHFR in osteogenesis, additional experiments could examine how protein expression changes throughout differentiation. Unlike osteogenesis, ASC proliferation and adipogenesis were unaffected by MTX treatment or

knockdown. This work indicates that DHFR does not play a role in the resistance of these properties. These results are encouraging for adipogenic regenerative therapies during MTX treatment, such as those used to treat Parry Romberg syndrome which results in craniofacial tissue loss [12].

Additional findings showed greater MTX resistance of undifferentiated MSCs compared to differentiating stem cells and OBs. These results suggest that stemness is a potential mechanism of resistance and raise concerns about the use of MSCs for regenerative therapies targeting drug-induced tissue loss. Testing the recovery potential of treated cells, as was done in chapter four with ASCs, would enable optimization of MSC regenerative therapies that could be used after chemotherapy instead of during. Superior MTX resistance of undifferentiated vs differentiated cell types could also suggest variability in DHFR expression. Results from chapter four showed that baseline DHFR levels were higher in MTX-sensitive NHFs than resistant stem cells, which could indicate greater reliance of differentiated cells on this protein to function. Additional work could include investigating whether this trend is also consistent for MTX-resistant, undifferentiated BMSCs versus sensitive OBs. If MSCs consistently express DHFR at low levels, this marker could be used to remove terminally differentiated cells that highly express DHFR in heterogeneous populations.

The ability of exogenous GAT delivery to rescue cells from MTX was also examined in chapter five. The results of these experiments revealed that the B GAT regimen prevented adverse side effects of MTX on normal cells, although this response was cell type- and property-dependent. These results indicate that GAT could be used at lower concentrations to minimize adverse drug effects on normal cells and potentially

prevent long-term tissue damage during chemotherapy. The regimens tested solely looked at GAT delivery 6 and 24 hr into MTX treatment. Additional studies could include looking at the rescue abilities of GAT delivery at 12 and 18 h to see how long nucleoside treatment can be delayed and still minimize effects. These experiments could be run in parallel with cancer cells to determine a time point when cancer cells can no longer be rescued, while normal cells still are.

6.5 Closing Remarks

The work in this thesis revealed that aging and chemotherapy influence MSC regenerative properties. Furthermore, the response to these and other environmental cues was shown to be cell type-dependent. Given the dramatic effects of the aforementioned conditions on MSCs, this work emphasizes the importance of niche homeostasis and supports further research into other pathological environments such as obesity and diabetes. Since MSC response to aging and chemotherapy differed based on tissue source, research into other adverse conditions should also consider many cell types to understand individual effects. Identifying factors that alter MSC function is necessary to understand consequential long-term tissue loss and optimize regenerative therapies.

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Appendices

Appendix A

7.1 Optimization of Human Adipose-Derived Stem Cell Adipogenic Induction Medium

Introduction

Mesenchymal stem cell (MSC) adipogenesis has been successfully induced using a range of media cocktails. The most commonly incorporated components are indomethacin, insulin, isobutylmethylxanthine (IBMX), and dexamethasone. Depending on the cell type and tissue source, the concentrations and combinations of these factors can vary. As such, it is necessary to optimize adipogenic differentiation for each MSC population of interest.

Two approaches are commonly used to differentiate MSCs. The first uses only one differentiation cocktail for the entirety of induction. These cocktails typically contain most or all of the aforementioned components. The second approach uses two cocktails, termed induction and maintenance medium. Induction medium often contains all of the aforementioned components, while maintenance medium is solely supplemented with insulin [1,2]. Differentiation using this technique requires initial culture in induction medium for 2-3 days then maintenance medium is swapped in. Cells are then cultured in maintenance medium for the remainder of differentiation. Conversely, induction medium can be swapped back after 3 days in maintenance medium. This cycle is then repeated several times for up to 2 weeks to stimulate lipid production. The goal of this study was

to optimize human adipose-derived stem cell (ASC) adipogenic differentiation using various media compositions and each of these techniques.

Methods

Adipogenic Differentiation

Adipogenesis was investigated using p5 human ASCs (superlot #36; Research Triangle Park, NC), originally isolated from the subcutaneous fat of healthy human, non-diabetic, non-smoking female donors, aged 18-60 years (N=7). Cells were plated in expansion medium (DMEM/F-12 (HyClone), 10% FBS (Zen-Bio), 1% antibiotic/antimycotic (HyClone), 0.25 ng/mL transforming growth factor-β1, 5 ng/mL epidermal growth factor, and 1 ng/mL fibroblast growth factor (R&D Systems) at 8,000 cells per well in 96 well plates. After reaching confluence, monolayers were exposed to various induction cocktails (Table 7.7.1) or control medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic). For medium 4, induction medium was replaced with maintenance medium (DMEM/F-12, 10% FBS, 1.7 µM insulin) after 48 hours for the remainder of differentiation. After 2 weeks, samples were fixed with 3.7% paraformaldehyde. Oil Red O (ORO, Sigma) staining was used to assess lipid production in adipogenic and control cultures. After imaging stained wells, ORO dye was eluted, and optical densities were measured with spectrophotometry at 500 nm. Experimental values were normalized to control levels.

Component	Media[Source]			
	1[3]	2[4]	3[5]	4[2]
Base Medium	DMEM/F-12	DMEM-HG	DMEM/F-12	DMEM/F-12
FBS	3%	10%	10%	10%
Insulin	1.7 µM	1.7 µM	10 µM	1.7 µM
Dexamethasone	1 µM	1 µM	1 µM	1 µM
IBMX	500 µM	0.5 µM	0.5 µM	500 µM
Indomethacin	200 µM	-	200 µM	-

Table 7.1.1 Adipogenic Medium Cocktails. A summary of the various induction cocktails used to assess human ASC adipogenic differentiation.

Statistical Analysis

A student's t-test was used to make individual comparisons between control and induced conditions for each adipogenic media type.

Results

Multiple cocktails were tested to assess their ability to induce ASCs along the adipogenic lineage. Medium 2 increased normalized absorbance values by 170% when compared with controls (Fig. 7.1.1, $p < 0.05$). Media 1 and 3 also significantly increased absorbance values by 90% and 140%, respectively ($p < 0.05$). No significant differences were observed between control and medium 4.

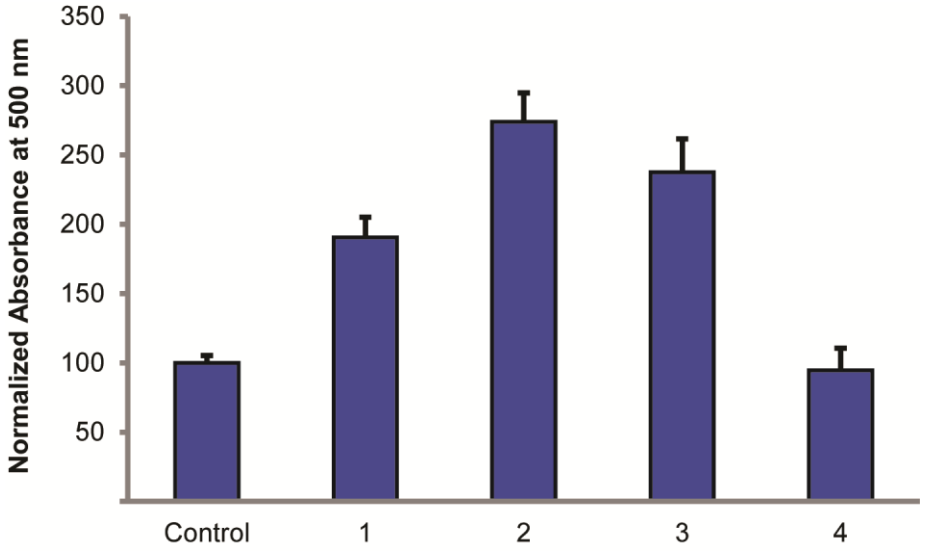

Figure 7.1.1 ASC Adipogenesis. Quantification of eluted ORO dye revealed significant increases in ASC lipid production over control values for induction media 1-3 ($p < 0.05$). Conversely, no significant differences were observed between control and medium 4 values.

Discussion/Conclusion

ASC adipogenic differentiation is dependent upon the induction medium composition they are exposed to. While several media successfully induced adipogenesis for ASCs (media 1-3), medium 4 did not. Failure of medium 4 to induce adipogenesis could be a consequence of the induction/maintenance approach used for this cocktail. Previous studies have cycle several times between induction and maintenance media, while this study did not [1]. Among the media that did increase lipid production, medium 2 induced the largest response. The potency of medium 2 could be due to the insulin concentration in this cocktail, which was larger than the others tested. Identifying an optimal adipogenic induction medium for ASCs enables further research into examining the regenerative potential of these cells.

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Appendix B

7.2 Identification of Multipotent, Rabbit Muscle-Derived Stem Cells Using the Preplate Technique

Introduction

MDSCs have been characterized from a number of experimental models including human, mouse, and rabbit [1-3]. Like with most MSCs, isolation procedures result in heterogeneous populations, including stem cells and other residence cell types. To enrich for MDSCs after isolation, researchers often employ the "preplate" method to obtain the slowly adhering stem cell population. This process involves transferring freshly isolated, non-adherent cell suspensions to new, tissue culture-treated flasks every 2-24 hours. After 3-4 days, the slowly adhering fraction of MDSCs will attach and expand.

While a number of groups have successfully obtained multipotent stem cell populations using pre-plating, there is variability among techniques. Most groups denote MDSCs as the population obtained 96 h after isolation (preplate 6, pp6), while others have found MDSCs to be those isolated after 72 hours (pp5) [4,5]. The aim of this study was to identify the adherent fraction of rabbit-derived muscle cells that contains multipotent MDSCs. Muscle-derived cell populations were isolated from rabbits and then pp5 and 6 were induced along adipogenic, osteogenic, and chondrogenic lineages to examine multipotency.

Materials and Methods

MDSC Isolation

MDSCs were isolated from hindlimb skeletal muscle of 5month old, male, New Zealand white rabbits (RSI Farms, Mocksville, NC, USA) using a modified preplate technique [6]. Briefly, muscle was washed with phosphate-buffered saline (PBS), and connective and fat tissues were removed. The remaining muscle tissue was minced and digested at 37°C in 0.2% type XI collagenase for one hour, then in 2.4 U/mL dispase for 45 minutes, and lastly, in 0.1% trypsin for 30 minutes. The resulting cell pellet was resuspended in MDSC culture medium (high glucose DMEM (DMEM/HG), 10% FBS, 10% horse serum (Hyclone), 0.5% chick embryo extract (United States Biological), and 1% antibiotic/antimycotic) and passed through a 70 µm strainer. Cells were then sequentially drawn through an 18G, 23G, and 27G needle, counted to determine cell viability and yield, and plated in collagen type I-coated flasks (Sigma). After two hours, the cell suspension was replated in new collagen-coated flasks. This process was repeated every 24 hours for an additional 3 days for pp5 (n = 3 donors) and 4 days for pp6 (n = 2 donors) to isolate the slowly adhering cell fractions. Fewer populations were assessed for pp6 since cell yields wre typically very low for this group. Once preplating was completed, MDSCs were expanded in monolayer and cryopreserved at passage 2 as described above. All experiments were then conducted using passage 3 cells.

Adipogenic Differentiation

To induce adipogenesis, cells were plated at 8,000 cells/well in 96 well plates. After reaching confluence, wells were exposed to either adipogenic medium (DMEM/F-12, 3% FBS, 1.7 µM insulin, 1 µM dexamethasone, 500 µM isobutylmethoxanthine, 200

μM indomethacin (Sigma), 1% antibiotic/antimycotic) or control medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic) following a previously established protocol (n = 6 for each experimental condition) [7]. After 3 weeks, cells were fixed with 3.7% paraformaldehyde and stained with ORO. To quantify lipid amounts, ORO dye was eluted, and optical densities were measured at 500 nm using spectrophotometry [8]. OD's were placed on a per-cell basis by visually counting DAPI-stained nuclei. To compare adipogenesis among preplate populations, induced values were normalized to non-induced controls.

Osteogenic Differentiation

To assess the osteogenic capacity of MDSC preplate populations, cells were plated at 8,000 cells per well in a 96 well plate. After reaching confluence, monolayers were cultured in either osteogenic induction medium (DMEM/F-12, 10 mM β-glycerophosphate, 0.120 mM ascorbate-2-phosphate, 15 nM dexamethasone, 10 nM 1,15-$(OH)_2$ vitamin D_3 (Sigma), 1% antibiotic/antimycotic) or control medium, as described for adipogenesis, using a previously established protocol with minor modifications (n = 6 for each experimental group) [7]. After 3 weeks of culture, monolayers were fixed with 3.7% paraformaldehyde and stained with alizarin red s to examine calcified matrix production. Following qualitative imaging, spectrophotometry was used to measure the optical density of eluted samples at 540 nm [8]. Optical densities were then normalized on a per-cell basis and to non-induced controls as described for adipogenesis.

Chondrogenic Differentiation

The chondrogenic potential of MDSCs was examined by plating 50,000 cells in a v-shaped, 96-well plate. Cells were given either chondrogenic induction medium

(DMEM-HG, 10% FBS, 10 ng/ml TGF-β1 (R&D Systems), 0.15 mM ascorbate-2-phosphate, 100 nM dexamethasone, 1% ITS+ Premix (BD Biosciences), and 1% antibiotic/antimycotic) or control medium as, described for adipogenesis, using previously established protocol with minor modifications [7]. After 3 weeks in culture, pellets were digested with papain, and sulfated glycosaminoglycan (sGAG) amounts were quantified using a DMMB assay (Accurate Chem. And Sci. Corp.). The PicoGreen assay (Invitrogen) was used to normalize sGAGs to cell amounts. Since some control pellets did not have any sGAGs, induced values were not normalized to their respective controls.

Statistical Analysis

To determine if differentiation had occurred, a student's t-test was used to analyze significant differences between control and induced lineage-specific metabolite production.

Results

To identify the multipotent stem cell population of slowly adhering muscle-derived cells, pp5 and 6 multilineage differentiation was compared after isolation from multiple rabbit donors. Assessment of lipid production revealed that average adipogenic values were 9 – 40-fold higher than controls for all donors and both preplate types (Fig. 7.2.1A, $p < 0.05$). For osteogenesis, calcified matrix production was 1.5-3-fold higher in osteogenic samples than controls for all pp5 rabbit donors (Fig. 7.2.1B, $p < 0.05$). Conversely, only rbt1 pp6 donor had significantly higher osteogenic values than controls ($p < 0.05$). Quantification of pp5 cell sGAG amounts revealed significant increases in

metabolite production over controls for all rabbit donors ($p < 0.05$). However, only rbt 3 pp6 donor had significantly higher chondrogenic values than controls ($p < 0.05$).

Discussion/Conclusion

While all pp5 cells exhibited trilineage differentiation, neither rbt 1 nor rbt 3 pp6 muscle-derived cells differentiated along all three lineages. These data indicate that the multipotent population of rabbit-derived cells resides in the pp5 slowly adhering fraction. Differences between these results and previous literature reporting multipotency in pp6 cells could be due to donor and experimental model variability. Identifying the multipotent MDSC population in muscle-derived cells enables further investigation into the regenerative properties of these cells.

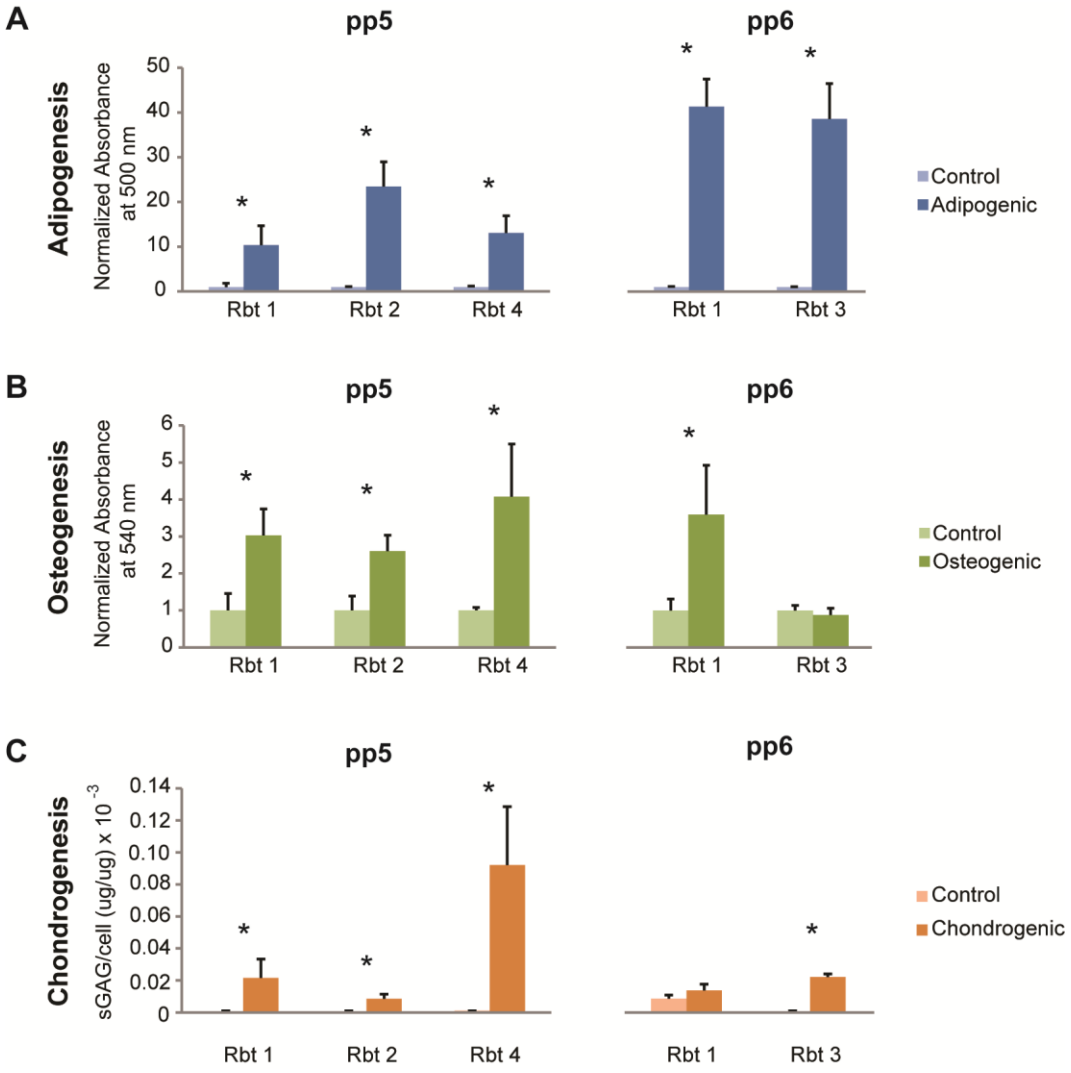

Figure 7.2.1 Multilineage differentiation of muscle cell preplate populations. Induced values of all preplate 5 (pp5) rbt donor cells were significantly higher than respective controls for (A) adipogenesis, (B) osteogenesis, and (C) chondrogenesis. Conversely, induced values of all pp6 rbt donor cells were only significantly higher than control values for adipogenesis ($p < 0.05$). Asterisk denotes significance between control and induced values ($p < 0.05$).

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Appendix C

7.3 The Effects of Serum and Induction Factors on Mesenchymal Stem Cell Osteogenesis

Introduction

To assess the multipotent differentiation potential of mesenchymal stem cells (MSCs), cultures are induced along adipogenic, osteogenic, and chondrogenic lineages. While there are established MSC differentiation protocols that include lineage-specific induction factors, cocktails are often specific to tissue source and experimental model. As such, cocktails are optimized and validated within research groups for each experimental cell type.

Osteogenic differentiation is induced using dexamethasone, β-glycerophosphate, and ascorbate-2-phosphate. However, this cocktail is not optimal for all cells or experimental models. Cells respond variably to different combinations and concentrations of these components. Moreover, incorporating bone morphogenic protein (BMP) can enhance osteogenesis for some MSC types [1]. While previous work in our lab has demonstrated increases in calcified matrix production of MDSCs and BMSCs, differentiation can be optimized further. The goal of this study was to determine an

optimal osteogenic cocktail for rabbit muscle, and bone marrow-derived MSCs. For BMSCs, this primarily involved optimization of BMP-2 concentration and source (commercial or lab). For MDSCs, maximizing osteogenesis was investigated through various combinations of lineage-specific induction factors. Identifying an osteogenic cocktail for these cell types is essential for assessing their potential use in regenerative therapies.

Materials and Methods

MSC Isolation and Culture

BMSCs, MDSCs, and ASCs were isolated from one young (4 months) female, New Zealand white rabbit (RSI Farms, Mocksville, NC, USA) as previously described [2-5]. Brief summaries of these isolation procedures are provided below. All animal work was performed in accordance with the local guidelines of the institutions and only after approval by the Institutional Animal Care and Use Committee (Permit numbers: 0015-10 and 0218-12) in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

BMSCs were obtained from the long bone following established protocols, with minor modifications [4-6]. Connective tissue was removed from the femur, which was then cut with bone scissors to expose the marrow cavity. Marrow was collected by centrifuging each half of the long bone at 400 g for 1 minute. Pellets were resuspended in BMSC culture medium (DMEM/F-12, 15% FBS, 1% antibiotic/antimycotic (Hyclone)), centrifuged, and then incubated in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM

K_2CO_3, and 0.1 mM EDTA) at room temperature for 10 minutes. The remaining cells were passed through a 70 µm strainer, centrifuged once more, and plated in BMSC culture medium (passage 0). Cells were counted before plating to determine viability and cell yield. To expand BMSCs, cells were maintained at 37°C, 5% CO_2, and upon reaching 80% confluence, the cells were trypsinized and replated (passage 1). At passage 2, BMSCs were cryopreserved in freezing solution consisting of 80% FBS, 10% culture medium, and 10% dimethyl sulfoxide. Post-thawing, BMSCs were expanded once more (passage 3) before being used for experiments. This procedure (expansion to passage 3, post-thawing) was followed for all MSC types in this study.

MDSCs were isolated from hindlimb skeletal muscle using a modified preplate technique [2]. Briefly, muscle was washed with phosphate-buffered saline (PBS), and connective and fat tissues were removed. The remaining muscle tissue was minced and digested at 37°C in 0.2% type XI collagenase for one hour, then in 2.4 U/mL dispase for 45 minutes, and lastly, in 0.1% trypsin for 30 minutes. The resulting cell pellet was resuspended in MDSC culture medium (high glucose DMEM (DMEM/HG), 10% FBS, 10% horse serum (Hyclone), 0.5% chick embryo extract (United States Biological), and 1% antibiotic/antimycotic) and passed through a 70 µm strainer. Cells were then sequentially drawn through an 18G, 23G, and 27G needle, counted to determine cell viability and yield, and plated in collagen type I-coated flasks (Sigma). After two hours, the cell suspension was replated in new collagen-coated flasks. This process was repeated every 24 hours for an additional 3 days to isolate the slowly adhering cell fraction containing MDSCs. Once preplating was completed, MDSCs were expanded in monolayer and cryopreserved at passage 2 as described above.

Osteogenic Differentiation

MSCs were plated at 8,000 cells per well in their respective culture medium and induced to differentiate upon reaching confluence 24-48 h later (n = 4 for each experimental group). Monolayers were exposed to various different osteogenic induction media containing all or a combination of the following factors: dexamethasone (Sigma Aldrich), ascorbate-2-phosphate (Sigma Aldrich), 1,15-$(OH)_2$ vitamin D_3 (Sigma Aldrich), β-glycerophosphate (Sigma Aldrich), and bone morphogenic protein-2 [BMP-2, either synthesized in the lab of Dr. Nic Leipzig ($BMP_{Lab)}$ or purchased from R&D systems ($BMP_{R\&D}$)] (Table 7.3.1). Components were added to control medium, consisting

Component	Media [source]			
	1 [7]	2 [7]	3[8]	4[9]
β-Glycerophosphate	10 mM	10 mM	10 mM	10 mM
Ascorbate-2-phosphate	0.120 mM	0.120 mM	0.050 mM	0.150 mM
Dexamethasone	15 nM	15 nM	-	-
1,15-$(OH)_2$ vitamin D_3	10 nM	10 nM	-	-
Bone Morphogenic Protein-2	-	50 ng/mL	50 ng/mL	5-500 ng/mL

Table 7.3.1 Osteogenic Medium Cocktails. This table lists the cocktails and the concentrations of each component that comprises them. Not all cocktails were used for each MSC type.

of DMEM-HG, 10% FBS, and 1% antibiotic/antimycotic. While MDSCs were induced using all of the listed cocktails, it should be noted that BMSCs were only exposed to medium 4 due to previous optimization. Both lab-synthesized and commercially available BMP-2 was evaluated to compare differences in levels of induction. Unless specified, these agents were diluted to a final concentration of 50 ng/mL for induction cocktails.

After 7 days of differentiation, osteogenesis was assessed using a BioVision ALP activity kit according to the manufacturer's protocol (Mountain View, CA). Induced values were normalized to respective controls for comparisons.

Statistical Analysis

A student's T-test was used to make individual comparisons between control and induced conditions. Differentiation was defined as a significant increase in ALP activity.

Results

MSCs were differentiated in various different induction cocktails to investigate their influence on osteogenesis. After testing a range of BMP_{Lab} concentrations on BMSC osteogenesis, it was observed that the normalized RFU of 5 and 50 ng/mL samples were significantly increased from non-induced controls by 190% and 250%, respectively (Fig. 7.3.1A, $p < 0.05$). Conversely, no significant differences were observed between RFU values of control and 500 ng/mL conditions. Induction of BMSCs with 50 ng/mL commercially available or lab-synthesized BMP-2 revealed a 100% increase in normalized RFU of BMP_{Lab} samples over non-induced controls (Fig. 7.3.1B, $p < 0.05$). Conversely, no significant differences were observed between non-induced controls and $BMP_{R\&D}$. Osteogenic differentiation of MDSCs with a variety of cocktails revealed that cocktail 3 (BMP_{Lab}) and 4 ($BMP_{R\&D}$) significantly increased normalized RFU values by 200% and 130%, respectively, over controls (Fig. 7.3.2, $p < 0.05$). However, no significant differences were observed between control and any other media condition.

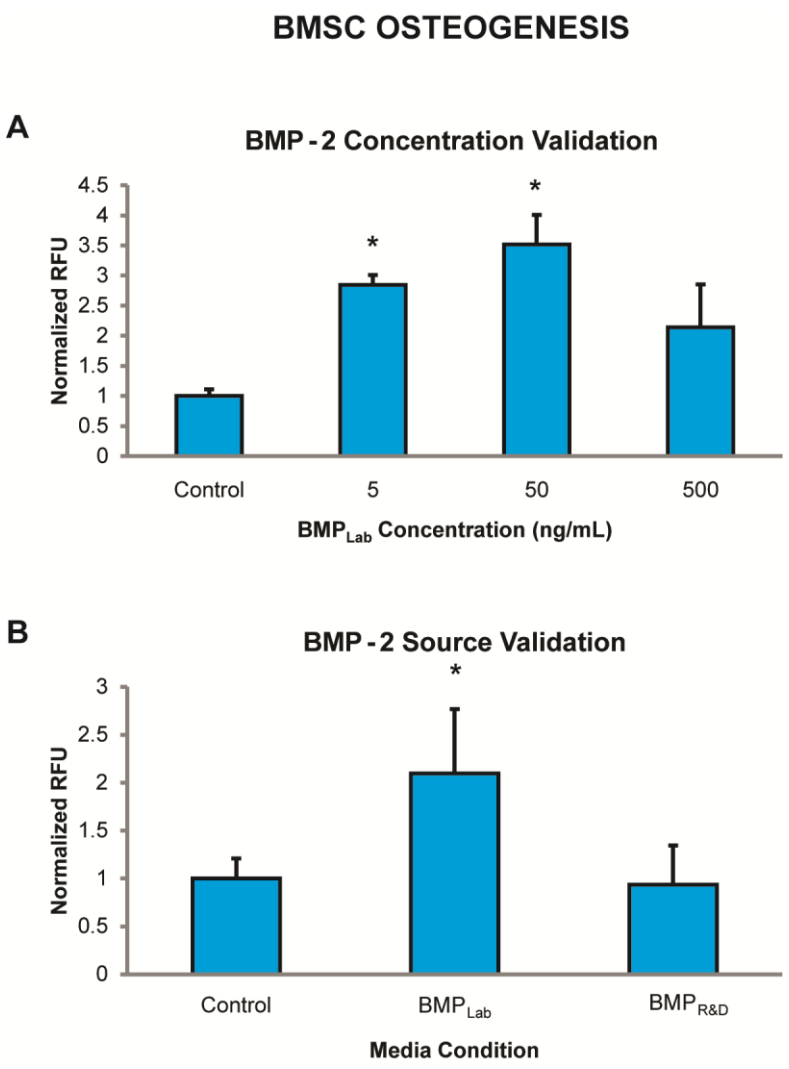

Figure 7.3.1 BMP-2 Concentration and Source Effects on BMSC Osteogenesis. (A) Both 5 and 50 ng/mL BMP_{Lab} increased ALP activity significantly over non-induced controls ($p < 0.05$). No significant differences were observed between control and 500 ng/mL conditions. (B) Similarly, no significant differences were observed between controls and 50 ng/mL of $BMP_{R\&D}$ samples. Asterisk denotes significance between induced and control samples ($p < 0.05$).

Discussion/Conclusion

Rabbit MSC osteogenesis is dependent upon BMP-2 concentration and cocktail composition. For BMSCs, 50 ng/mL of BMP_{Lab} was optimal for osteogenic induction. For MDSCs, media 3 and 4 induced osteogenesis while other cocktails did not. While we hypothesized that increasing BMP-2 concentrations and purchasing commercially

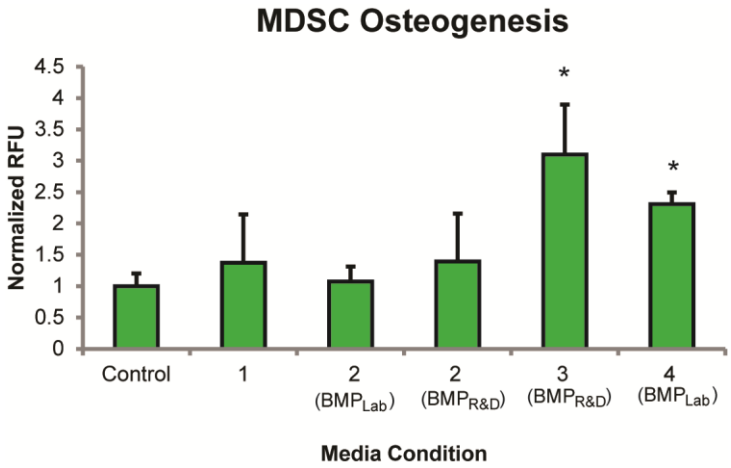

Figure 7.3.2.Effects of Cocktail Composition on MDSC Osteogenesis. Medium 3 ($BMP_{R\&D}$) and Medium 4 (BMP_{Lab}) induced significant increases in ALP activity of MDSCs over non-induced controls ($p < 0.05$). Asterisk denotes significance between induced and control samples ($p < 0.05$).

available BMP-2 would also increase BMSC osteogenesis, this was not the case. In fact, high concentrations had an inhibitory effect on osteogenic differentiation. Moreover, BMP_{Lab} induced differentiation while $BMP_{R\&D}$ did not. For MDSCs, both media 3 and 4 induced differentiation with either BMP-2 agent. Interestingly, these cocktails, in contrast to the others, did not include dexamethasone. Therefore, it is possible that dexamethasone inhibits osteogenic differentiation of rabbit MDSCs. These data help to better understand rabbit MSC osteogenesis, which is essential for investigating the regenerative potential of these cells.

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