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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**The Effects of Physiological Age on Bone Marrow-Derived
Mesenchymal Stem Cells**

A thesis submitted in partial satisfaction of the requirements
for the Master of Science degree

in

Bioengineering

by

Caitlin Buckspan

Committee in charge:

Professor Shyni Varghese, Chair
Professor Gaurav Arya
Professor Shankar Subramaniam

2010

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Chair

University of California, San Diego

2010

DEDICATION

To my amazing family members, who have supported me in all my endeavors.

EPIGRAPH

“For reason, ruling alone, is a force confining;

And passion, unattended, is a flame that burns to its own destruction.

Therefore let your soul exalt your reason to the height of passion, that it may sing;

And let it direct your passion with reason, that your passion may live
through its own daily resurrection, and like the phoenix rise above its own ashes.”

-Kahlil Gibran “The Prophet”

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ABSTRACT OF THE THESIS

**The Effects of Physiological Age on Bone Marrow-Derived
Mesenchymal Stem Cells**

by

Caitlin Buckspan

Master of Science in Bioengineering

University of California, San Diego, 2010

Professor Shyni Varghese, Chair

Mesenchymal stem cells (MSCs) have great potential in tissue engineering as they can be easily isolated, differentiated into several lineages (cartilage, bone, fat), and allogeneically transplanted without immune response. For developing

therapies to be clinically translated, it is imperative to understand how donor characteristics, such as age, affect the efficacy of MSC-based treatments. The overall aim of this study was to characterize age-related changes in MSCs from bone marrow using a murine model. First, the conditions for isolation and *in vitro* expansion were optimized for bone marrow-derived MSCs; MSCs were isolated from C57Bl/6 mice of varying age (three-week, two-month, six-month, ten-month, and thirteen-month old). Second, donor age-dependent changes in expansion ability were determined by investigating the population doubling time and colony-forming capability of MSCs from various age groups. Cell populations from older mice proliferated more quickly and produced more colonies than their younger counterparts. Finally, age-related changes in MSC functionality and differentiation potential were investigated. Migration rate, antioxidant levels, and cytoskeletal dynamics of each population were determined. It was found that the older populations had faster migration and more dynamic cytoskeletons, while the younger cells had higher antioxidant activity. The cells were also subjected to chondrogenic, osteogenic, and adipogenic assays. The intermediate-aged cells consistently underwent more adipogenesis, while osteogenesis increased until a peak age then declined, and chondrogenesis was not affected by age.

Chapter 1: INTRODUCTION

Stem cells are defined by two characteristics: 1) the ability to self-renew, and 2) the ability to differentiate into more specialized cells¹. Such pluripotent cells can generally be categorized into the following: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and adult stem cells that are isolated from post-natal tissue. Adult stem cells can be either multipotent or unipotent. ESCs and iPSCs can be differentiated into all three germ layers while adult stem cells are more specialized and at least partially committed to certain tissue lineages^{1, 7}.

Adult stem cells serve the purpose of maintaining and repairing tissue of the organism⁷. Most somatic tissues contain adult stem cells responsible for tissue homeostasis. Adult stem cells are subject to the Hayflick phenomenon and will senesce after multiple cell divisions⁷.

Mesenchymal stem cells (MSCs) are one kind of multipotent adult stem cell, defined by their plastic-adherence, groups of positive and negative surface markers, and ability to differentiate into multiple cell types such as osteoblasts, adipocytes, and chondrocytes⁹. They also have the capacity to form other tissue types, such as muscle and tendon. MSCs have been isolated from almost all adult tissues, but are considered to be naturally occurring stromal cells of the bone marrow and are most commonly isolated from bone marrow and adipose tissue for research purposes¹⁵.

MSCs are also known to have the unique ability to regulate cells of the immune system, which enhances their therapeutic appeal²³. These cells have been shown to modulate the action of many cells of the reactive immune system including T and B cells, dendritic cells, and natural killer (NK) cells^{25,30,31,32}.

The multi-potentiality of MSCs, along with their immunomodulatory ability, has made them the focus of several clinical trials in the field of regenerative medicine⁸⁶. However, MSCs are heterogenous and their behavior is known to be dependent on intrinsic factors; even cells from animals of the same species demonstrate strain-dependent differences in phenotype and differentiation potential⁴⁹.

It is known that many connective tissues of mesenchymal origin deteriorate with age and also lose the ability to properly repair^{39,40}. Cartilage and bone tissue, as load-bearing tissue systems, are constantly broken down and restructure themselves with production of fresh extracellular matrix (ECM). With age, these tissues lose the ability to reproduce the normal ECM, which results in weakness and aberrant mechanical properties⁴⁰. Osteoporosis and osteoarthritis are examples of musculoskeletal disorders with increased prevalence in the aged population.

MSCs have been shown to regulate homeostasis and repair of the connective tissues, and the age-associated changes in these tissues have piqued interest about the changes that MSCs undergo with aging and how that affects their function. Previous studies have investigated the effects of intrinsic MSC aging and have found conflicting results, such as in the relationship of donor age to MSC density in the bone marrow, MSC colony formation ability, and MSC proliferation capacity⁴⁸. Similarly, some studies have shown an age-dependent decrease in osteogenic or chondrogenic potential of MSCs from bone marrow while others show no age-associated relationship at all^{35,51,52,53,54}. These differences could possibly be due to

differences in experimental conditions like species, animal strain, culture conditions, age grouping, etc⁴⁸.

It is important to understand how donor age contributes to differences in intrinsic function of MSCs in order for MSCs to be successful in clinical applications; thus, the overall aim of this study is to characterize donor age-mediated changes that MSCs in the bone marrow undergo and how that affects their phenotypic and functional properties.

The first specific aim of this study is to optimize the isolation and culture conditions for bone marrow-derived murine MSCs. MSCs were isolated from bone marrow of variably aged female C57Bl/6 mice. The culture conditions were optimized for a number of conditions such as cell plating density, medium composition, and frequency of medium change. The effect of culture condition on cells was monitored for cell survival and proliferation. The conditions that resulted in the most viable cell populations were used for the subsequent studies.

The second aim is to understand how age affects the ability of MSCs to expand *in vitro*. This aim was achieved by determining the population doubling times, colony formation abilities, and onset of senescence of MSCs from the various murine age groups. Experiments were performed at both passage 2 and passage 4 to elucidate the effect of *in vitro* aging and to investigate any correlation between physiological and passage-dependent aging.

The third specific aim is to understand age-related changes in MSC functionality and differentiation potential. The differentiation potential was examined for osteogenesis, chondrogenesis, and adipogenesis. The experiments

were carried out simultaneously and the cells from the same donor groups were used for functional characterization. First, functionality of the variably aged populations was characterized by studying antioxidant activity, migration, and cytoskeletal dynamics. Cytoskeletal dynamics investigated the polymerization dynamics of both actin and tubulin structures.

These studies proved a connection between MSC age, expansion ability, cell functionality, and differentiation ability.

The following summarizes the results of this study:

1. Optimal conditions for cell viability and growth were found to be higher initial cell plating density (roughly 10^5 mononuclear cells/cm²), delaying initial medium change (allow 3-4 days to elapse after plating mononucleated cells), and higher serum in medium (100 mL of FBS in 500 mL of DMEM).
2. MSCs at higher passage proliferated more quickly, produced more colonies, and had a higher proportion of senescent cells.
3. MSCs from older mice were more predisposed to spontaneous transformation, which caused aberrant morphology and behavior.
4. MSCs from older mice proliferated more quickly and formed more colonies than those from younger mice.
5. MSCs from older mice had a higher proportion of senescent cells than those from younger mice.
6. MSCs from older mice migrated faster than MSCs from younger mice.
7. MSCs from younger mice had higher antioxidant levels than those from older mice.

8. MSCs from older mice had more dynamic cytoskeletons than those from younger mice.
9. Osteogenesis and adipogenesis both peaked in MSC populations at intermediate ages (two months and ten months respectively) then declined with age.
10. MSCs from mice of all ages were capable of undergoing chondrogenic differentiation when pellet-cultured in chondrogenic media without discernible difference among age groups.

Chapter 1 is in part being prepared for submission for publication in “Systematic analysis of physiological donor age on the functional performance of bone marrow-derived mesenchymal stem cells”, of which the thesis author is the primary author and is co-authored by Dr. Ramses Ayala and Dr. Shyni Varghese.

Chapter 2: LITERATURE REVIEW

2.1. Stem cells

2.1.1. Overview of stem cells

Stem cells have been the focus of much scientific and medical research over the past couple of decades. Stem cells can be categorized by their source and their differentiation lineages, but all stem cells have in common two specific characteristics: 1) the ability to self-renew, or to preserve their *status quo* phenotype through multiple cellular divisions, and 2) the ability to alter their phenotype and become a cell of a specific tissue lineage¹. Stem cells can be categorized into two broad subtypes that scientists currently study *in vitro*: embryonic stem cells and adult stem cells. Recently, pluripotent stem cells have been developed from differentiated cells¹. These embryonic-like cells are called induced pluripotent stem cells (iPSCs).

2.1.2. Embryonic stem cells

Embryonic stem cells were first isolated from mouse embryos in 1981 and subsequently were isolated from human embryos in 1998¹. These cells are derived from blastocysts, small clusters of embryonic cells that eventually develop into complete organisms with all 3 germ layers (endoderm, mesoderm, and ectoderm). Thomson et al. showed that embryonic stem cells, or ESCs, that are isolated from human blastocysts have highly active telomerase and normal chromosomal karyotypes². These ESCs maintain the ability to divide into all three germ layers indefinitely in culture. The uses of ESCs abound; they are studied in order to determine how tissue systems organize as an organism develops from the

preliminary zygote³, and since their differentiation potential encompasses all cell types of a fully developed organism, they are investigated as a cell-source for diseased or injured tissues in regenerative medicine and tissue engineering^{1,2}.

Despite their potential biomedical applications, embryonic stem cells have been the center of several legal and ethical debates due to their origin from human embryos requiring the destructive isolation from the blastocyst.

2.1.3. Induced pluripotent stem cells

Within the past decade, cell lines have been created which obviate the ethical debate surrounding ESCs while maintaining a source of pluripotent cells for research purposes. Induced pluripotent stem cells (iPSCs) were first created in 2006 from murine cells by Shinya Yamanaka's research team at Kyoto University, and iPSCs have been studied extensively for uses similar to ESCs¹. iPS cells can be created from differentiated, adult tissues such as fat and skin by the addition of certain factors to the cells and culturing them in embryonic stem cell conditions⁴. It has been shown that there are several possible combinations of factors required for functional iPSC transformation, but the ES-related gene *Nanog* is a requisite for complete transformation². These iPSCs are embryonic cell-like because they express the same surface markers, have highly active telomerase, can divide indefinitely in culture, and have the pluripotent potential to divide into all cell lines of the adult organism⁴. Researchers in 2007 demonstrated that these cells can be used to treat sickle cell anemia in a mouse model⁵, and Nelson et al. showed in 2009 that iPSCs can be used to repair heart tissue following cardiac infarction⁶.

2.1.4. Adult stem cells

Adult stem cells are also called somatic stem cells because they are found within fully developed tissues and are more specialized than the embryonic stem cells isolated from the blastocysts. Adult stem cells contribute to the repair and maintenance of adult tissues. Somatic stem cells have been found as precursors for most tissue types, including skin, gut, neural, pancreatic, hepatic, cartilage, blood, etc. Adult stem cells are not as heavily debated as embryonic stem cells because they are isolated from postnatal, developed tissue and thus do not require the destruction of any part of a human embryo; however, they can not be used for all the same scientific and medical purposes as ESCs, since they are partially committed to specific tissue lineages. Adult stem cells are classified as unipotent, bipotent, tripotent, or multipotent, depending on the number of cell types that they can form⁷. For example, hematopoietic stem cells are multipotent stem cells isolated from bone marrow that can be induced to differentiate into all of the cells of the blood tissue lineage. There are also unipotent stem cells such as the satellite cell responsible for muscle maintenance, which can be differentiated exclusively into myoblasts⁸. In contrast to the immortality of ESCs, adult stem cells are subject to the Hayflick limit of cell division and will senesce during passing in *in vitro* culture. Each type of cell can be distinguished by a specific combination of surface markers and have various responses to growth factors and other differentiation-inducing agents⁷.

2.2. Mesenchymal stem cells

2.2.1. Definition

The mesenchymal stem cell, MSC, is a type of multipotent adult stem cell with fibroblastic morphology that has also been termed mesenchymal progenitor cell or mesenchymal stromal cell and is primarily responsible for maintaining connective tissues such as cartilage, fat, and bone (Figure 1). Mesenchymal stem cells and the bone marrow-derived hematopoietic stem cells are some of the most extensively studied and widely used adult stem cells. MSCs are heterogenous and loosely-defined, and no single surface marker or property has been shown to identify them; therefore the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has determined three requisite criteria to distinguish human MSCs from other cell lines: 1) cells are plastic-adherent, 2) cells express CD105, CD73, and CD90 while they do not express CD45, CD34, CD14, CD11b, CD79 α , CD19, and human leukocyte antigen class II, and 3) cell must demonstrate by positive histology *in vitro* differentiation into osteoblasts, adipocytes, and chondrocytes⁹.

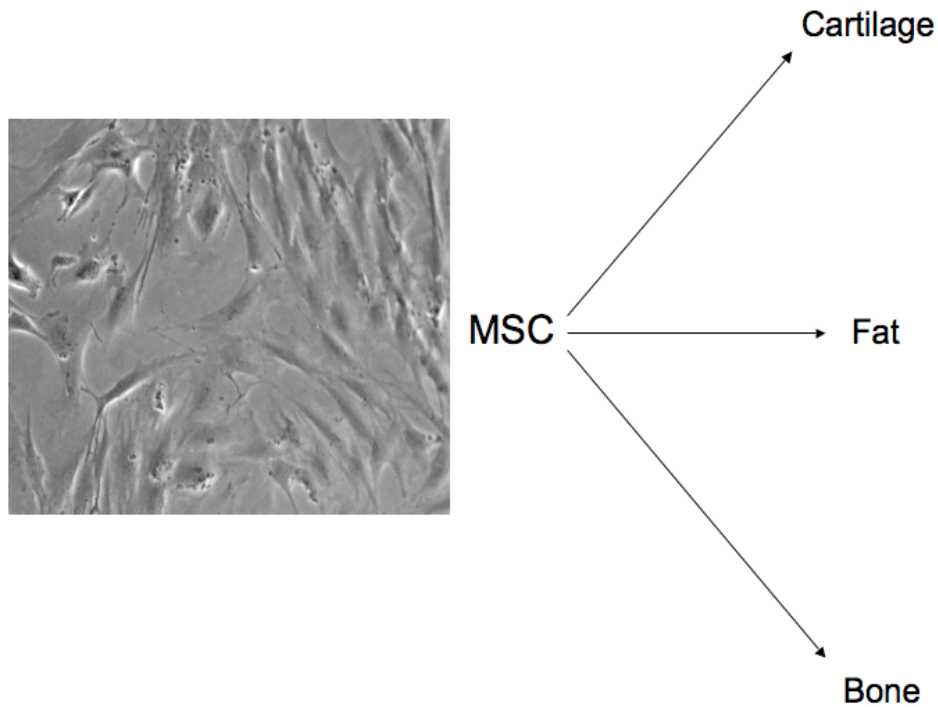


Figure 1: MSCs differentiate into cartilage, fat, and bone.

2.2.2. Cell sources

Mesenchymal stem cells provide the cells for the maintenance and repair of the various and widespread connective tissues, thus it is not surprising that they can be located in a wide range of *in vivo* tissue types. These cells are reliably and commonly isolated from the bone marrow¹⁰ but have also been shown to be available from many other tissues, and may even be found in all postnatal tissues¹¹. MSCs have been isolated from adipose tissue, synovial fluid¹², human monocytes¹³, and human umbilical cord perivascular cells¹⁴, among others. The ubiquity of MSCs has raised questions about their *in vivo* niche and requirements for survival and maintenance¹⁵.

2.2.3. Self-renewal

One of the defining characteristics of all stem cells is the ability to undergo multiple cell divisions and to renew the stem-like state of the cells, and mesenchymal stem cells are known to self-renew their *status quo* phenotype throughout a number of population doublings. The ability to self renew is ultimately important to the function of MSCs because it maintains a constant source of progenitor cells which differentiate into the mesodermal tissue lineages, while preventing the depletion of the source.

The MSC self-renewal capacity is regulated and maintained in a complicated orchestration by a combination of various growth factors and cytokines, including leukocyte inhibitory factor (LIF), fibroblast growth factor 2 (FGF2), and various members of the Wnt family^{16,17,18}. These factors suppress tissue-specific differentiation and may also be involved in tumorigenesis¹⁸.

2.2.4. Differentiation

Mesenchymal stem cells are most noted for their ability to differentiate into precursors of the bone, cartilage, and fat tissues, though they have been shown to form other connective tissues as well, and some studies claim to have produced non-connective cells such as neurons from MSCs¹⁹. *In vitro*, differentiation of these cells to targeted cells can be achieved by incubating them in medium containing soluble factors that are known to promote tissue-specific differentiation.

Since MSCs are a heterogenous population, questions have been raised as to whether a single cell has multi-lineage potential or if the population is a mixture of

uni-lineage potential cells¹⁵. One study has shown that clones from a single cell maintain the ability to differentiate into all three tissue lines²⁰, while another study has shown that MSCs differentiate hierarchically into the various lineages²¹. Muraglia et al demonstrated that only one-third of clones were tripotent while most could differentiate into osteoblasts and chondrocytes. In this study, an overwhelming majority (99.5%) of clones could undergo osteogenesis, while no clones exclusively underwent adipo or chondrogenesis. This study also showed that during *in vitro* culture, clones lose the ability to undergo adipo and chondrogenic potential while osteogenic potential is maintained²¹.

2.2.5. Immunomodulation

In addition to their extensive differentiation ability, MSCs are being investigated for their immunomodulatory ability. Bartholomew et al. has shown that no immune response is induced in lymphocytes when they are introduced into the environment of allogenic MSCs²². Furthermore, transplanted MSCs have been shown to reduce immune response of a recipient²³ by interacting with the efficacy of immune system T cells. The MSCs can interfere with T cell proliferation and can also hinder the ability of memory T cells to recognize the proper antigen^{22,24}.

Studies have shown that MSC interaction with T cells is essential for immunosuppression, suggesting that T cells elicit MSCs to produce immunomodulatory compounds²⁵. Ren et al. reports that MSCs are stimulated by certain combinations of inflammatory cytokines that are released by activated T cells; they found that interferon- γ (INF- γ) must be present as well as one more

inflammatory factor, specifically either tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), or interleukin- β (IL- β). Once the MSCs are stimulated, they produce T cell attractants and nitrous oxide (NO), the latter of which produces a direct immunosuppressive effect on the T cells²⁵. NO has previously been shown to reduce the activity of T cells, but it can only produce such an effect when highly concentrated^{26,27}; therefore the attractive chemokines secreted by the MSCs are essential in order to bring T cells proximal to MSCs (the NO source) so the gas is concentrated enough to suppress the T cells. Other studies suggest that T cell function is disrupted by heme oxygenase 1 or by a depletion of tryptophan^{28,29} and MSCs have also been shown to decrease the proliferation and activity of immune B cells, dendritic cells, and natural killer cells^{30,31,32}. The variable results in these studies may prove that many factors are responsible for immunomodulation²³.

MSCs are a vital component of the bone marrow stroma in which white blood cells, the foundation of the immune system, are found *in vivo*. It has been shown that MSCs disrupt the proliferation and activity of cells of the immune system, and it is thought that the MSCs assist in regulating the natural immune response and the behavior of stromal cells through this immunomodulatory capacity²². This property has also been investigated as a basis for MSC therapeutic potential. MSC injection has been proposed as a possible treatment for several autoimmune diseases, such as graft-versus-host disease (GVD), diabetes, systemic lupus erythematosus, multiple sclerosis, systemic sclerosis, etc³⁴. These diseases involve an autologous or allogenic (in GVD) immune assault on tissues caused by

over active immune cells. Since MSCs suppress the activity of immune cells such as T and NK cells, they are natural candidates for therapeutic inactivation of these cells.

2.2.6. Tissue engineering applications

MSCs are being investigated for use in treating a myriad of diseases and injuries; both their differentiation potential and immunomodulatory properties may be useful in therapeutic applications, and these characteristics may work in concert with each other. Additionally, MSCs are relatively easy to derive and expand.

MSCs can regularly undergo osteogenesis and chondrogenesis, so they are being extensively investigated as a cell source for injury and disease in bone and cartilage. To obtain these tissues from MSCs, various growth factors and a scaffold with optimal biomechanical properties are generally used³⁵. Bone regeneration is challenging because stabilization is necessary for proper re-growth, making scaffold implantation into a defect a necessity. MSC implantation has been shown to enhance bone tissue regeneration in polymer-based scaffolds³⁶. Cartilage has a low cell density and thus is a difficult tissue to repair, so incorporating MSC transplantation as part of therapies can improve the regeneration of cartilage tissue³⁵. MSCs may be especially useful because of the close interaction of bone and cartilage tissue, and MSCs have a unique bipotentiality, which could prove useful in the engineering of interfacial tissue.

Some studies suggest that MSCs have applications in muscle and cardiac tissue engineering. MSCs have been differentiated into muscle tissue precursors,

therefore they may be able to produce cardiomyocytes and offer a cell source for repair of heart injuries. A study by Fukuda showed that some bone marrow-derived MSCs cultured in 5-azacytidine lost their fibroblastic morphology and began to form myotubes after 1 week³⁷. By 3 weeks in culture, Fukuda observed synchronized beating in these cells. Additionally, injected MSCs may produce both muscular and vascular components in a myocardium defect³⁸, further enhancing their regenerative potential.

2.3. Musculoskeletal tissue aging

It is well known that aging affects all tissues of the body, and physiological age is a risk factor for multiple diseases and disorders. Some of the most prevalent and widespread of these age-associated ailments affect the musculoskeletal tissue³⁹, and MSCs are closely tied to the homeostasis of these tissues. Long-term suffering of musculoskeletal disorders often leads to disability³⁹, thus aging is a significant issue, both in terms of health and finances. Common impairments such as osteoporosis, osteoarthritis, and sarcopenia are most common in the elderly, and these diseases involve abnormal breakdown and insufficient repair of musculoskeletal tissues. As musculoskeletal tissue ages, it is more prone to disease but it also loses strength, making the tissue susceptible to repetition-based injury and also slowing the healing process⁴⁰ (Figure 2).

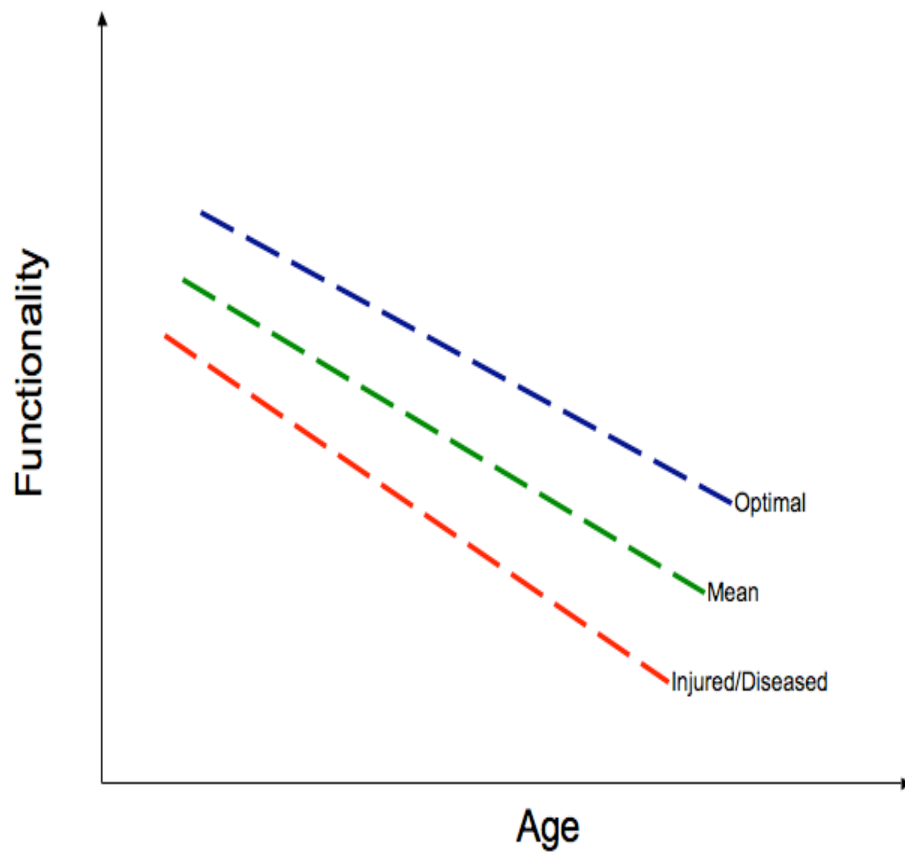


Figure 2: Representation of how age increases susceptibility to tissue impairment. Adapted from (40).

Age-related changes in the musculoskeletal system are due to widespread changes in physiology. The number of cells in a tissue may decrease, the extracellular matrix breaks down through years of wear-and-tear, the functionality of cells' repair mechanisms may dwindle, levels of growth factors change, and tissue-influencing hormone levels change drastically⁴⁰. Individual cells, both in the

differentiated and undifferentiated state, may begin to senesce and lose their ability to proliferate or otherwise function properly⁴⁰.

2.3.1. Aging of bone tissue

The human skeleton is made of two types of bone: intramembranous flat bones and endochondral bone with centralized bone marrow⁴¹. Bone development in a human embryo is a dual-path process; one results in intramembranous bone formed in mesenchyme from the direct differentiation of MSCs into osteoblasts⁴², while endochondral bone is created first from MSC differentiation into chondrocytes, followed by chondrocyte differentiation into osteoblasts and mineralization of the cartilaginous ECM^{42,43}. Bone tissue is continually broken down by osteoclasts, specialized cells formed from macrophages, while osteoblasts form new mineralized tissue to replace the old tissue⁴². During development, osteoblast activity is most prevalent, resulting in tissue accumulation and growth. As a person grows, these processes continue until a point of peak bone mass, around the third decade of life⁴³. After this point, osteoclast action is favored and bone tissue begins to weaken, resulting in osteopenia, osteoporosis, and finally increased fracture risk⁴³.

Interestingly, MSC-based adipogenesis is also important to the formation of the skeleton. Fat begins to accumulate in the post-natal bone marrow of the feet and hands, and fatty bone marrow continues to form in the skeleton progressively toward the torso with age⁴³. The total amount of fat in bone marrow has been shown

to increase with increasing age⁴³, which may contribute to age-related skeletal weakening.

2.3.2. Aging of articular cartilage

Articular cartilage is the load-bearing tissue found between skeletal bones. It is a heavily hydrated connective tissue composed of a ground substance (extracellular matrix) that is produced by the interspersed chondrocytes^{44,45}. The ECM of cartilage is made of proteoglycans and collagen proteins in an aqueous environment that give cartilage the ability to withstand compression and tension and thus protect the skeletal bones. The proteoglycans and collagens form a tight network that contributes to the tissue's mechanical properties⁴⁵. Like bone, this tissue is constantly being remodeled, and ECM maintenance is the responsibility of the chondrocyte cells^{44,45}.

Articular cartilage, like bone, also undergoes several compositional changes as it ages. These changes include increased overall stiffness, overall tensile weakening, smaller proteoglycan aggregates, and post-translational modification of protein resulting in crosslinked and stiff collagens^{40,46}. The most profound age-related cartilaginous disorder is osteoarthritis a disease in which the cartilage wears and breaks down, causing many morphological and mechanical alterations resulting in significant pain and incapacity. It is thought that aging chondrocytes senesce and lose their ability to repair cartilage damage⁴⁶, which in turn contributes to osteoarthritis.

2.4. Mesenchymal stem cells and aging

It is commonly presumed that the aging and disorders of connective tissues result from aberrant behavior of the aged cells that produce the extracellular matrix. For example, both bone and cartilage weaken over time as osteoblasts and chondrocytes lose their ability to replace worn tissue. Bone has also been shown to have an increasing proportion of fat within the bone marrow as people age⁴⁷. Since osteoblasts, chondrocytes, and adipocytes all originate from MSCs, it has been postulated that aging of MSCs may contribute to the tissue manifestations of aging. Studies do indicate that progenitor cells lose function and contribute to tissue aging, but it is also known that age-related changes in the native environment affect MSC behavior⁴⁸ (Figure 3). It is unknown to what extent intrinsic and extrinsic factors such as changes in the tissue environment due to aging affects the MSCs. Studies often report conflicting results as to the specific changes of MSCs with age, which may be consequential from using different species or strains, experimental conditions, etc used in the numerous studies⁴⁸. Peister et al. has shown that even cells from animals of the same species demonstrate strain-dependent differences in phenotype and differentiation potential⁴⁹.

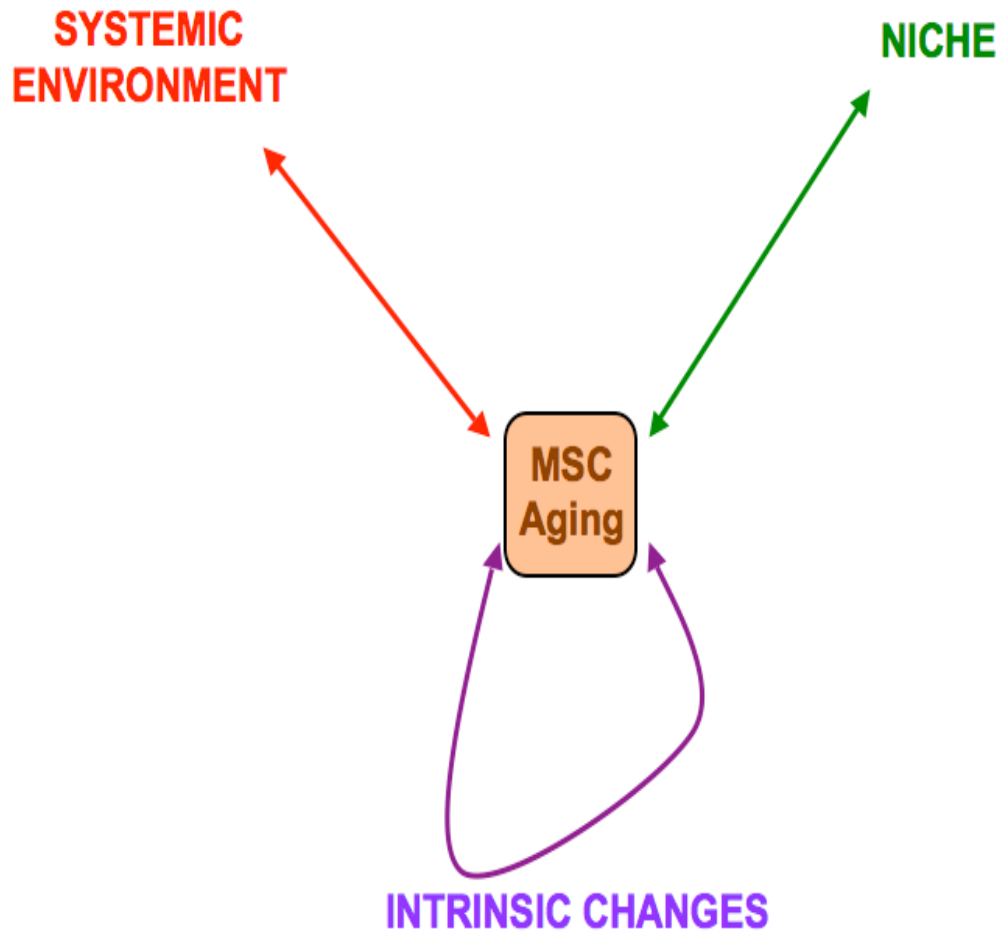


Figure 3: Causes of stem cell aging. Adapted from (50).

Age-associated changes in MSCs could be the result of intrinsic changes where they lose their phenotypic characteristics, thereby losing functionality. If MSCs lose their ability to divide, migrate, and form colonies during aging, it could explain why their progeny are less functional as well. Some reports show that age-induced loss of MSC functionality does indeed exist, but others show no loss of function or even increased functionality. Studies give conflicting results as to

whether CFUF count increases or decreases as an MSC population ages⁴⁸. The relationship between growth rate and age is also ambiguous. A study by Stenderup et al showed that human MSC proliferation decreased with increasing age, while Bergman et al found that MSC from older mice grew at triple the rate of young MSCs^{48,51}. Scharstuhl et al. found that MSCs from young and old human donors were indistinguishable in respect to bone marrow mononuclear cell density, cell size, and ability to proliferate⁵².

Differentiation potential of MSCs also may change with age; studies give conflicting results as to the age-related changes of MSC osteogenesis, adipogenesis, and chondrogenesis⁴⁸. Zheng et al investigated the chondrogenic capacity of bone marrow-derived MSCs from rats of various ages, and they demonstrated that the aged cells (from 12-week and 1-year-old animals) had lost *in vitro* chondrogenic potential compared to the young 1-week-old rats⁵³. This was verified by histology and RT-PCR. Stolzing et al also found that chondrogenic potential declined in aged MSCs from human donors⁵⁴. However, Scharstuih et al found that MSCs isolated from older human donors were just as capable of producing chondrocytes as MSCs from younger donors⁵².

The results from studies regarding aging and osteogenic potential are just as contradicting as those investigating chondrogenic potential. Stenderup et al. observed no difference in osteogenic potential between MSCs isolated from young and old human donors⁵¹, while Chen found that osteogenic potential of rat-derived MSCs does decrease with increasing age³⁵. Supporting Chen's findings, Stolzing et

al also found that osteogenic potential decreased with age in human bone marrow-derived MSCs⁵⁴.

Researchers have questioned whether individual MSCs have an inherent capacity to favor either osteogenesis or adipogenesis, which could “switch” at some point to favor fat over bone. This theory is supported by the accumulation of fatty tissue in the skeleton as people age^{47,55}. Some studies have confirmed this theory and shown that osteogenic potential declines with age while adipogenic potential increases⁵³, while others show that no decrease in either lineage is observed⁵⁵.

Studies have sought to identify the source of the possible switch, with many studies finding that regulating either an adipo or osteo pathway will inversely affect the other pathway. Jaiswal et al found that osteogenic differentiation involves several different pathways including the mitogen-activated protein kinase (MAP kinase) and extracellular signal-regulated kinase (ERK) pathways, and when these osteogenic paths are blocked by external factors, adipogenic differentiation ensues⁵⁶. Multiple studies have pinpointed peroxisome proliferator-activated receptor- γ (PPAR- γ) as a potential source of the “adipogenic switch”^{57,58}. Moerman explains that PPAR- γ , already known to be a key transcription factor for adipogenesis, also inhibits osteogenesis, which could account for the mutual exclusivity of the osteo and adipo pathways⁵⁷. Akune et al. supports such an argument, showing that cells with homozygous deficiency of the PPAR- γ gene spontaneously differentiate into osteoblasts, and cells with only one missing PPAR- γ allele have enhanced osteogenesis⁵⁷. Additionally, whether an MSC population

prefers to undergo adipo or osteogenesis has been shown to depend on various hormone levels and many other factors (including PPAR γ), and titers of these factors are known to change with aging⁵⁸. The adipogenic switch may thus contribute to osteoporosis (Figure 4), and similar mechanisms may contribute to other age-related degenerative disorders of the connective tissues.

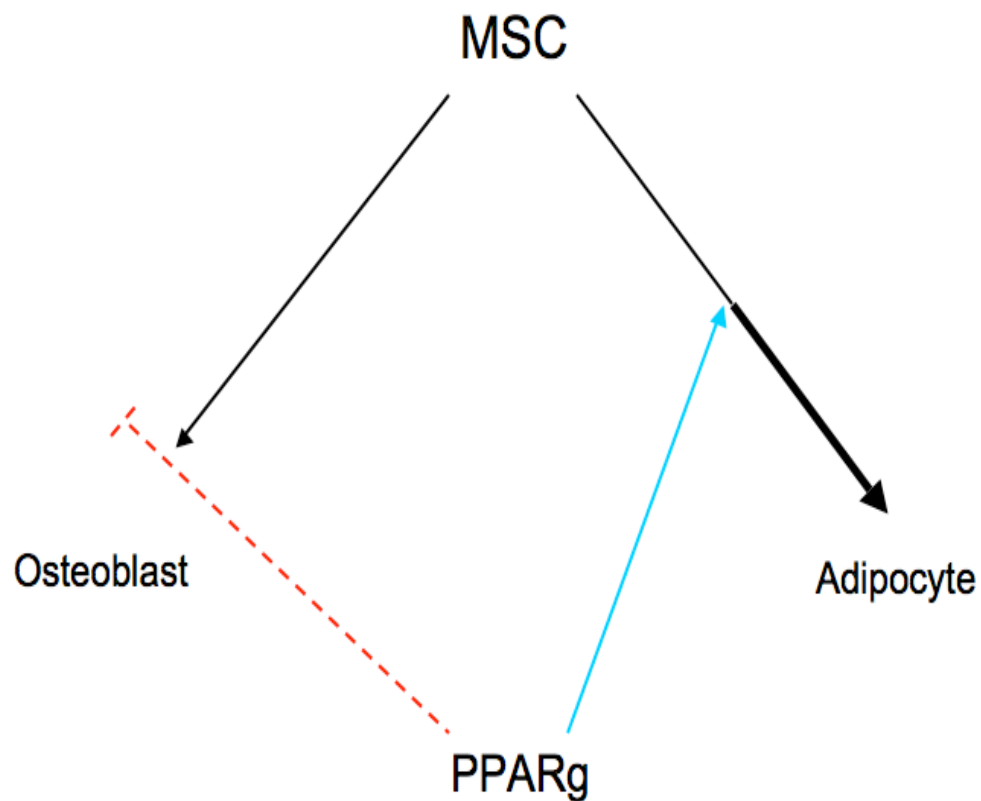


Figure 4: Diagram depicting that PPAR γ promotes MSC adipogenesis while hindering osteogenesis. Adapted from (47).

There is sufficient evidence to suspect that MSCs do intrinsically change with physiological age, but due to variable experimental conditions and methods, it is difficult to distinguish what specific changes occur in terms of functionality and differentiation potential. Therefore, in this study, MSC were isolated from mice of varying age where the cells were isolated and cultured concurrently and consistently, then subjected to various functional and differentiation assays to determine how multiple cellular characteristics change with physiological age.

Chapter 3: MATERIALS AND METHODS

3.1. Optimization of MSC isolation and culture

The methods of this study involve isolation and characterization of bone marrow-derived MSCs from mice of varying age. Cell isolation and culture were performed using variable conditions, and MSC populations were monitored for viability. The following protocols were observed to be the most optimal in producing the most viable cell populations.

3.1.1. MSC isolation

3.1.1.a. Bone marrow collection

1. C57Bl/6 female mice were obtained from Harlan Sprague Dawley, Indianapolis, IN through the UCSD Animal Acquisitions Office per animal use protocol #S07411
2. Mice were euthanized by CO₂ chamber in groups of 3-4, sprayed thoroughly with 75% ethanol, and placed in a biological flow hood
3. All following procedures were performed under sterile conditions
4. Cervical dislocation was employed to ensure euthanization
5. Each hind leg was removed by holding the foot with forceps and cutting at the hip joint with dissection scissors
6. Each foot was removed by cutting at the ankle with dissection scissors
7. Soft tissue was removed from bone with forceps and scissors
8. Femurs and tibias were separated at the patella and epiphyses were removed
9. 1-mL syringe was filled with ~0.3 mL of growth media [high-glucose Dulbecco's Modified Eagle Medium (Invitrogen, catalog# 11960) with 20%

fetal bovine serum (Atlanta Biologicals, lot #), 1% penicillin-streptomycin (Invitrogen, catalog# 15140), and 1% L-glutamine (Invitrogen, catalog# 25030)] through an attached 27-gauge needle

10. While holding bone with forceps, needle was inserted into each bone cavity and bone marrow was flushed from the orifice with growth media into a 50-mL conical tube
11. Each bone was flushed a total of three times
12. Marrow was stored in growth media on ice after collection.

3.1.1.b. Marrow processing

1. Conical tubes were centrifuged for 10 minutes at 1000 RPM separate bone marrow fractions
2. Marrow was washed three times with warm growth media, carefully aspirating previous wash while tilting conical tube to avoid marrow pellet
3. At least 5 mL of media always remained in tube during washing
4. Marrow was resuspended in 20 mL of media per conical tube

3.1.1.c. Counting mononuclear cells with hemocytometer

1. 1 μ L of 10x cell lysis buffer (Cell Signaling Technology, catalog# 9803) was diluted with distilled water in a microfuge tube to produce 1x buffer
2. 10 μ L of cell suspension was added to 1x cell lysis buffer and mixed while pipetting to lyse multinucleated cells
3. 10 μ L of lysed cell suspension (containing only intact mononuclear cells) was pipetted under the coverslip of a hemocytometer
4. Cell count was taken from a set of 3 1-mm² squares and averaged

5. Mononuclear cell concentration in cell suspension was calculated by

$$\text{Cell concentration (cell/mL)} = \text{Average cell count} \times 10^4$$

3.1.2. MSC culture

1. Cells were plated at 1×10^5 mononucleated cells/cm² in tissue culture flasks
2. Media was added to flasks as follows: 20 mL in T-175 flasks, 10 mL in T-75 flasks, and 3 mL in T-25 flasks
3. Flasks were incubated at 37°C and 5% CO₂
4. After 3 days, flasks were washed with PBS and 2% penicillin-streptomycin before adding fresh media
5. Media was then changed twice per week without washing
6. Cells were passaged at 90% confluency

3.1.3. Trypsinization

1. Media was aspirated from cell culture flasks
2. Flasks were washed with 37°C autoclaved and filtered PBS (approximately half of media volume)
3. 37°C trypsin (0.25% trypsin-EDTA, Invitrogen, catalog# 25200) was added to flasks in the following volumes: 7 mL for T-175 flasks, 4 mL for T-75 flasks, and 1.25 mL for T-25 flasks
4. Trypsin was left on cells for 2-3 minutes at room temperature while flasks were agitated to shear cells off the flasks
5. 37°C growth media was added to the flasks in volumes greater than or equal to the volume of trypsin

6. Cell solution was collected by serological pipette into conical tubes and 100 μL was removed to measure cell concentration by Beckman Coulter Z2 Coulter particle count and size analyzer
7. Tubes were centrifuged at 1000 RPM for 10 minutes to pellet cells
8. Trypsin and media were aspirated and cells were resuspended in growth media to a concentration of 10^5 - 10^6 cells/mL
9. Cells were plated in flasks at ~ 1500 cells/cm²

3.1.4. Cell freezing

1. Excess cells were pelleted by centrifuging at 1000 RPM for 10 minutes in growth media
2. Media was aspirated and replaced with freezing media at a final concentration of approximately 1×10^6 cells/mL
3. Cell suspension was transferred to labeled cryotubes and stored at -80°C in a styrofoam container or Nalgene 5100 1°C Freezing Container to ensure slow cell freezing
4. Cryotubes were transferred to liquid nitrogen after 1-7 days

3.1.5. Cell thawing and plating

Protocol was optimized for cell plating density. Note that at P0, initial plating density should be approximately 3000-4000 cells/cm² while plating density can be lower at later passages.

1. Cryotube was removed from liquid nitrogen and the bottom half of tube was submerged in 37°C water

2. Tube was agitated until cell suspension was approximately 75% thawed then transferred to biological flow hood
3. Cell suspension was transferred to 15-mL conical tube containing 5-10 mL of warm growth media
4. Suspension was mixed with media by inverting tube several times
5. Conical tubes were centrifuged at 1000 RPM for 10 minutes to pellet the cells
6. Media was aspirated and cells were resuspended in ~10 mL of warm media
7. Cells were plated overnight at $\sim 10000 \text{ cells/cm}^2$ in tissue culture flasks or 10-cm dishes in order to remove dead cells
8. Cells were passaged by treating for 2 minutes with 37°C trypsin (0.25% trypsin-EDTA) before adding warm media and collecting solution in conical tubes
9. Cell solution was mixed by inversion and 100 μL of cell solution was removed and used to measure cell concentration using a Beckman Coulter Z2 Coulter particle count and size analyzer
10. Conical tubes were centrifuged at 1000 RPM for 10 minutes to pellet the cells
11. Media was aspirated and cells were resuspended in warm growth media to a final concentration of $\sim 10^5\text{-}10^6 \text{ cells/mL}$
12. Cells were plated at $\sim 3000\text{-}4000 \text{ cells/cm}^2$ at P0 or $\sim 1500 \text{ cells/cm}^2$ at later passages for expansion in tissue culture flasks and media was added for a

total volume of 20 mL for T-175 flasks, 10 mL for T-75 flasks, and 3 mL for T-25 flasks

3.1.6. Surface marker immunostaining

1. Cells at passage 2 (P2) were plated at 4000 cells/cm² in 24-well plates and allowed to expand for 3 days in growth media
2. Media was aspirated and wells were washed once with PBS
3. Monolayers were fixed for 10 minutes with 4% paraformaldehyde (PFA)
4. PFA was aspirated and wells were washed three times with PBS for 10 minutes each wash
5. Cells were treated with permeabilizing blocking solution [3% w/v bovine serum albumin and 0.5% v/v Triton-X (Fisher Scientific, catalog# 9002-93-1) in PBS] for 30 minutes
6. Blocking solution was aspirated and wells were washed three times with PBS for 10 minutes each wash
7. Primary antibody solution was added to wells (1:50 v/v dilution for surface markers CD34 (H-140), CD73 (H-300), and CD106 (H-276) in 3% w/v bovine serum albumin in PBS) and incubated at room temperature for 1 hour
8. Immunostain was aspirated and wells were washed three times for 10 minutes with PBS
9. Samples were counterstained with 1:250 dilutions of AlexaFluor 568-conjugated secondary antibody (Invitrogen, catalog# A11004) and 1:20 AlexaFluor 488-phalloidin (Invitrogen, catalog# A12379) in 3% w/v bovine serum albumin in PBS for half an hour in the dark

10. Secondary antibody solution was removed and wells were washed three times for 10 minutes in PBS
11. ~50 μ L of VectaShield Mounting Medium with DAPI (Vector Laboratories, catalog# H-1200) was added per well and a glass coverslip was placed in each well
12. Plates were wrapped in aluminum foil and stored at 4°C
13. Immunofluorescence was imaged using the Zeiss confocal microscope
14. All surface marker antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA

3.2. Studies of MSC expansion ability

3.2.1. Population doubling time (PDT)

1. At passage 2 and passage 4 (P2 and P4), cells were plated at 2000 cells/cm² in 12-well plates and allowed to proliferate for 5-6 days in growth media
2. Monolayers of cells were washed with warm PBS and trypsinized for 2 minutes (0.25% trypsin/EDTA) before adding warm growth media and collecting media
3. Cell concentration was counted using a Beckman Coulter Z2 Coulter particle count and size analyzer
4. Population doubling time in hours was calculated as:

$$[\log_2 / \log (N1/N2)] \times \Delta t$$

where N1 and N2 are cell counts at beginning and end of the assay and Δt is proliferation time in hours

3.2.2. Colony forming unit-fibroblast (CFUF)

Protocol was optimized for plating density.

1. At passage 2 and passage 4 (P2 and P4), 1000 cells/well were plated in triplicate into 6-well plates and allowed to expand for 12 days in growth media
2. Media was changed twice a week during expansion
3. Media was aspirated and wells were washed with PBS
4. Monolayers were stained with 3% w/v crystal violet (Sigma, catalog# C0775) in methanol for 5-10 minutes
5. Wells were washed thoroughly with distilled water
6. Colonies containing 20 or more cells were counted per well

3.2.3. Senescence

1. Senescence was characterized using the Senescence β -galactosidase Staining Kit (Cell Signaling Technology, catalog# 9860)
2. All solutions were prepared in polypropylene plastic containers as indicated by the manufacturer
3. At passage 2 and passage 4 (P2 and P4), cells were plated at 8000 cells/cm² in 12-well plates and allowed to proliferate for 3 days in growth media
4. The provided 10X fixative and staining solutions were diluted to 1X with distilled water
5. 20mg of X-gal was dissolved in 1 mL of dimethylformamide
6. Media was aspirated from the cells and wells were washed once with PBS
7. Cells were fixed with 1X fixative solution for 10 minutes

8. Meanwhile, working staining solution was prepared with 465 μL of 1X staining solution, 5 μL of staining supplement A, 5 μL of staining supplement B, and 25 μL of 20 mg/mL X-gal solution per well
9. Fixative was removed and wells were washed twice with PBS
10. 500 μL of working staining solution was added to each well and plates were incubated overnight at 37°C and 5% CO_2
11. Plates were imaged by the Zeiss confocal microscope for blue-stained β -galactosidase residues
12. Stain was removed and 70% glycerol was added to wells for storage at 4°C

3.3. Studies of MSC functionality and differentiation

3.3.1. Antioxidant activity

Antioxidant activity was quantified using either the Sigma Aldrich Antioxidant Assay Kit (catalog# CS0790-1KT) or the Cayman Scientific Antioxidant Assay Kit (catalog# 709001).

3.3.1.a. Sigma Aldrich Antioxidant Assay Kit

1. The kit was used to determine antioxidant activity of cell lysates following manufacturer protocol
2. Approximately 2×10^4 cells/ cm^2 were plated at passage 4 and allowed to adhere overnight
3. Monolayers were removed with plastic cell scrapers and sonicated on ice in the provided buffer solution
4. 2 mL of 10x assay buffer was diluted in 18 mL of nanopure water to produce 1x assay buffer

5. One vial of lyophilized myoglobin was suspended in 285 μL of nanopure water and vortexed to produce myoglobin stock solution
6. Myoglobin working solution was produced by diluting myoglobin stock solution 100-fold with 1x assay buffer and vortexing
7. Trolox working solution was made by adding 2.67 mL of 1x assay buffer to one vial of lyophilized Trolox and vortexing
8. ABTS substrate solution was made by adding one ABTS tablet and one phosphate-citrate buffer tablet to 100 mL of nanopure water and vortexing well
9. Trolox standards of various concentrations were produced by diluting the reconstituted Trolox in 1x assay buffer and storing on ice
10. ABTS working solution was prepared by mixing 10 mL of ABTS substrate solution with 25 μL of 3% hydrogen peroxide solution (used within 20 minutes)
11. To obtain a standard curve, triplicates of the following were added to wells in a 96-well plate: 10 μL of Trolox standard and 20 μL of myoglobin working solution
12. To obtain sample measurements, triplicates of the following were added to well in the 96-well plate: 10 μL of cell lysate solution and 20 μL of myoglobin working solution
13. 150 μL of ABTS working solution was added to each well using a multi-channel pipette

14. Plate was covered and incubated on a shaker for 5 minutes at room temperature
15. 100 μ L of room temperature stop solution was added to each well with a multi-channel pipette
16. The absorbance of the plate at 405 nm was read immediately
17. Antioxidant concentrations in Trolox equivalents of the cell lysates were determined using the standard curve and normalized to the DNA content, determined using the Quant-iT PicoGreen dsDNA Kit (Invitrogen, catalog# P7589) per manufacturer instruction

3.3.1.b. Cayman Scientific Antioxidant Assay Kit

1. The kit was used to determine antioxidant activity of cell lysates per manufacturer instruction
2. Approximately 2×10^4 cells/cm² were plated at passage 4 and allowed to adhere overnight
3. Monolayers were removed with plastic cell scrapers and sonicated on ice in the provided buffer solution
4. 3 mL of antioxidant assay buffer concentrate was diluted with 27 mL of nanopure water
5. One vial of antioxidant assay chromogen was resuspended in 6 mL of nanopure water, vortexed, and kept on ice
6. One vial of antioxidant assay metmyoglobin was resuspended in 600 μ L of 1x antioxidant assay buffer, vortexed, and kept on ice

7. One vial of antioxidant assay Trolox was resuspended in 1 mL of nanopure water, vortexed, and kept on ice
8. Trolox standards of various concentrations were produced by diluting the reconstituted Trolox in 1x assay buffer and storing on ice
9. 10 μ L of the provided hydrogen peroxide solution was diluted with 990 μ L of nanopure water to obtain hydrogen peroxide stock
10. Hydrogen peroxide working solution was made by diluting 20 μ L of stock with 3.98 mL of nanopure water
11. To obtain a standard curve, triplicates of the following were added to wells in a 96-well plate: 10 μ L of Trolox standard, 10 μ L of metmyoglobin solution, and 150 μ L of chromogen solution
12. To obtain sample measurements, triplicates of the following were added to the 96-well plate: 10 μ L of cell lysate solution, 10 μ L of metmyoglobin solution, and 150 μ L of chromogen solution
13. Oxidation reactions were triggered by the addition of 40 μ L of hydrogen peroxide working solution to each well using a multi-channel pipette
14. Plate was covered and incubated on a shaker for 5 minutes before reading absorbances at 405 nm
15. Antioxidant concentrations in Trolox equivalents of the cell lysates were determined using the standard curve and normalized to the DNA content, determined using the Quant-iT PicoGreen dsDNA Kit per manufacturer instruction

3.3.1.c. DNA quantification

1. DNA content was determined using the Quant-iT PicoGreen dsDNA Kit (Invitrogen, catalog# P7589)
2. 20X TE Buffer was diluted to 1X with DEPC-treated water
3. PicoGreen working solution was made by diluting the PicoGreen stock solution 1:200 in TE Buffer
4. 2 $\mu\text{g/mL}$ of DNA standard was made by diluting the double-stranded DNA stock 1:50 in TE Buffer
5. Various standards were prepared by diluting the 2 $\mu\text{g/mL}$ DNA standard in TE Buffer
6. Sample solutions were prepared by diluting cell lysate 1:1 in TE buffer
7. 100 μL of either standard or sample was added to wells of a 96-well plate (standards in triplicate and samples in one well each)
8. 100 μL of PicoGreen working solution was added to each well with a multi-channel pipette and plate was incubated for 5 minutes at room temperature in the dark
9. Fluorescence emission intensity was measured at 520 nm
10. Sample DNA content was calculated from the standard curve

3.3.2. Migration scratch test

1. At passage 4, cells were plated at 5×10^4 cells/well in triplicate in 12-well plates and expanded in growth media for 5 days to confluency
2. Each well was scraped with a plastic pipette tip to form a gap 550-650 μm in width

3. Images were taken sporadically over 3 days of approximately the same area in each well to monitor cell migration into the gaps
4. Three gap lengths were measured per image to calculate average migration rate per age

3.3.3. Cytoskeletal dynamics

3.3.3.a. Fibronectin-coating coverslips

1. Glass coverslips were incubated in 1 $\mu\text{g/mL}$ of fibronectin in PBS at 37°C for 2 hours
2. Coverslips were washed three times with PBS for 10 minutes at room temperature
3. For sterilization, coverslips were immersed in ethanol and suspended in a flame for several seconds
4. Coverslips were then placed in wells of 24-well plates

3.3.3.b. Cytoskeletal protein depolymerization

1. 5000 cells/ cm^2 of P4 cells were plated onto fibronectin-coated glass coverslips and expanded for 5 days in growth media containing 10% FBS
2. Growth media was aspirated before treating cells with an actin-depolymerizing drug (10 μM cytochalasin D; Sigma, catalog# C8273) or a tubulin-depolymerizing drug (5 $\mu\text{g/mL}$ nocodazole; Sigma, #M1404) in high-glucose DMEM for 3 hours
3. After 3 hours, the drug was removed from all of the wells and was replaced with warm DMEM

4. At various time points after recovery was initiated, the DMEM was removed and wells were washed with PBS
5. Cells were then fixed with 4% PFA
6. After 10 minutes of fixation, the PFA was removed and replaced with PBS until all wells had been fixed
7. Wells were washed three times with PBS for 10 minutes at room temperature

3.3.3.c. Cytoskeletal protein immunofluorescent staining

1. PBS was aspirated and cells were put into blocking solution (3% w/v bovine serum albumin and 0.5% v/v Triton-X in PBS) for 30 minutes at room temperature
2. Cells were immunostained overnight with either AlexaFluor 488-phalloidin (Invitrogen, catalog# A12379) diluted 1:20 in blocking solution or cy3-beta-tubulin (Sigma, catalog# C4585) diluted 1:100 in blocking solution at 4°C
3. Coverslips were mounted with VectaShield Mounting Medium with DAPI onto glass slides
4. Coverslips were sealed to the glass slides with fingernail polish along the edges and imaged with the Zeiss confocal microscope
5. Slides were stored at -20°C

3.3.4. Chondrogenesis

1. 250,000 cells were pelleted in growth media in 15-mL conical tubes by centrifuging at 1000 RPM for 10 minutes
2. Pellets were stored in growth media for 2 days at 37°C and 5% CO₂

3. Growth media was aspirated and replaced with chondrogenic media [high-glucose DMEM supplemented with 1% penicillin-streptomycin, 100 $\mu\text{g/mL}$ sodium pyruvate (Invitrogen, catalog# 11360), 1x ITS-Premix (BD Biosciences, catalog# 354351), 100 nM dexamethasone (Sigma, catalog# D2915), 40 $\mu\text{g/mL}$ proline (Sigma, catalog# P5607), 50 $\mu\text{g/mL}$ ascorbic acid-2-phosphate (Sigma, #A8960)]
4. 10 ng/mL TGF- β 1 (Fitzgerald, catalog# 30R-AT027) was added to chondrogenic media immediately prior to media change to preserve its functionality
5. Chondrogenic media was replaced twice a week for 3 weeks

3.3.5. Osteogenesis

1. 10^4 cells/cm² were plated and allowed to expand to confluency in growth media for 5 days
2. Growth media was aspirated and replaced by osteogenic media [high-glucose DMEM containing 2% penicillin-streptomycin, 1% sodium-pyruvate, 10% fetal bovine serum, 50 μM ascorbic acid-2-phosphate, 1.8 mM B-glycerophosphate (Calbiochem, catalog# 35675) and 100 nM dexamethasone]
3. Osteogenic media was changed twice a week

3.3.6. Adipogenesis

1. Cells were plated at 10^4 cells/cm² and allowed to expand to confluency in growth media for 5 days.

2. Confluent monolayers were cultured in adipogenic media [GM supplemented with 1 μ M dexamethasone, 200 μ M indomethacin (Sigma, catalog# 17378), 0.5 mM isobutylmethylxanthine (Sigma, catalog# I5879), and 5 mg/mL insulin (Sigma, catalog# 16634)]
3. Adipogenic media was changed twice during the 10-day differentiation period

3.3.7. Optimization of myogenic media

During the initial planning for this study, it was decided to additionally test myogenesis since mesenchymal stem cells have been shown to differentiate into the myoblast lineage under certain culture conditions. Previously published studies describe several different media components for myogenic media, including a low-serum growth media without additional components, and so several test medias were designed and tested on monolayers of MSCS from 2-month-old mice at passage 5 to find the most successful media, according to the following protocol.

1. 10^5 cells/cm² were plated in two 6-well plates and growth media was added to a total volume of 2 mL
2. Cells were allowed to expand to ~90% confluency in growth media for 4 days
3. Growth media was aspirated and replaced by 2 mL of the following media types (2 wells per media type):
 - a. Control in standard 20% FBS growth media
 - b. 0.1 μ M dexamethasone
50 μ M hydrocortisone (Sigma, catalog# H0888)

- 2% FBS media with 1% penicillin-streptomycin and 1% L-glutamine
- c. 2% FBS media with 1% penicillin-streptomycin and 1% L-glutamine
no additional components
- d. 10 μ M 5-azacytidine (Sigma, catalog# A1287)
10 ng/mL basic FGF (Invitrogen, catalog# 13256-029)
2% FBS media with 1% penicillin-streptomycin and 1% L-glutamine
- e. 1x ITS + Premix (BD Biosciences, catalog# 354351)
0.1 μ M dexamethasone
2% FBS media with 1% penicillin-streptomycin and 1% L-glutamine
- f. 10 μ M 5-azacytidine
5 ng/mL IGF (Invitrogen, catalog# 13245063)
2% FBS media with 1% penicillin-streptomycin and 1% L-glutamine

4. Media was changed twice during the one-week assay

3.3.8. Chondrogenic histology

At the conclusion of the 3-week-long period of pellet culture in chondrogenic media, the pellets were fixed, cryosectioned, and stained with safranin O for glycosaminoglycans or immunostained for collagens I and II.

3.3.8.a. Pellet cryosectioning

1. After 21 days in chondrogenic media, pellets were fixed in 750 μ L of 4% w/v PFA for 3 hours
2. 4% PFA was removed from pellets and replaced with 750 μ L of PBS

3. Pellets were stored in PBS at room temperature
4. Pellets were moved to histomolds and embedded in Tissue-Tek Optimum Cutting Temperature medium (Tek Pella, catalog# 27050)
5. Pellets were frozen in liquid nitrogen
6. Frozen pellets were cryosectioned to 10 μ M sections, mounted on glass slides, and stored at -20°C

3.3.8.b. Glycosaminoglycan (GAG) staining

1. Slide-mounted pellet sections were hydrated in distilled water for 5 minutes at room temperature
2. Slides were stained sequentially at room temperature in staining jars (5 minutes per jar) filled with 100 mL of the following:
 - i. 1 jar of 0.1% safranin-O in distilled water (0.1 g of safranin-O powder in 100 mL of distilled water)
 - ii. 3 jars of distilled water
 - iii. 2 jars of 95% ethanol
 - iv. 3 jars of 100% ethanol
 - v. 3 jars of xylene
3. Immediately following the last xylene wash, sections were mounted with 150 μ L of Permount Mounting Medium (Fisher Scientific, catalog# SP15) and sealed with a glass coverslip
4. Slides were allowed to dry overnight at room temperature before imaging with a Zeiss confocal microscope for presence of red GAG
5. Stained slides were stored at -20°C

3.3.8.c. Collagen I and II staining

1. Slide-mounted pellet sections were rehydrated for 3 minutes in distilled water at room temperature
2. Sections were blocked with 10-20 μ L of permeabilizing blocking solution (3% w/v bovine serum albumin and 0.5% v/v Triton-X in PBS) for 15 minutes at room temperature
3. Permeabilizing blocking solution was aspirated and replaced with 20 μ L of primary antibody solution for collagen I (Fitzgerald, catalog# 70R-CR007X) and collagen II (Fitzgerald, catalog# 70R-CR008X) (1:250 antibody dilution in 3% w/v bovine serum albumin and 0.1% v/v Triton-X in PBS) for 1 hr at room temperature
4. Each slide was washed with 1 mL of PBS and allowed to sit for 10 minutes
5. PBS wash was performed a total of 3 times, pouring off previous PBS before fresh PBS was added
6. Final PBS wash was aspirated from slides before adding 15 μ L of secondary antibody solution [1:300 v/v dilution of Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, catalog# A11008) in 3% w/v bovine serum albumin with 0.1% v/v Triton-X in PBS] per section
7. Slides were immunoconjugated with secondary antibody solution for 45 minutes in the dark
8. Each slide was washed once with PBS then washed twice with distilled water for 10 minutes each wash, pouring off previous wash between washes

9. Water was aspirated before adding 2 μ L of DAPI Mounting Medium per section and covering sections in glass coverslips
10. Coverslips were sealed to slides with clear fingernail polish along the edges and allowed to dry overnight
11. Slides were imaged with a Zeiss confocal microscope for immunofluorescence (collagens fluoresce green while nuclei are blue)
12. Slides were stored at -20°C

3.3.9. Osteogenic histology

Osteogenesis was characterized at 3 time points: 1, 2, and 3 weeks. At the conclusion of each of these time periods, the monolayers were fixed and stained for extracellular alkaline phosphatase or with alizarin red S for calcium.

3.3.9.a. Preparation of alkaline phosphatase (ALP) staining solution

1. 30 mL of distilled water was added to a 50-mL conical tube
2. The contents of one Fast Blue RR salt capsule (Sigma, catalog# FBS25) were added to the water and the tube was homogenized
3. 2 mL of Naphthol solution (Sigma, catalog# 855-20mL) was added to solution and the tube was homogenized
4. The tube was filled with distilled water to 50 mL and was stored in the dark at room temperature prior to use

3.3.9.b. ALP staining

1. Osteogenic media was aspirated from plates and wells were washed once with PBS

2. Monolayers were fixed with citrate working solution [0.8% v/v Citrate Concentrated Solution (Sigma, catalog# C8540) and 60% v/v acetone] for 30 seconds
3. Citrate working solution was aspirated and monolayers were washed twice with PBS
4. ALP staining solution was added to each well
5. Plates were wrapped in aluminum foil and stored in the dark while staining for 45 minutes
6. ALP staining solution was aspirated and wells were washed twice with PBS
7. Plates were imaged with a Zeiss confocal microscope for presence of ALP stained blue

3.3.9.c. Preparation of alizarin red S (ARS) staining solution

1. 0.2 g of alizarin red S powder (Sigma, catalog# A5533) was added to 10 mL of distilled water in a 50-mL conical tube
2. Tube was homogenized for ~1 minute
3. NaOH was added to the acidic stain until the pH was between 4.3 and 4.6

3.3.9.d. Alizarin red S staining

1. Osteogenic media was aspirated from plates and wells were washed once with PBS
2. Monolayers were fixed for 10 minutes with 4% PFA
3. 4% PFA was aspirated and wells were washed twice with PBS
4. PBS was removed and monolayers were stained with ARS staining solution for 10 minutes

5. Stain was aspirated and wells were washed ~6 times with PBS to remove excess stain
6. Plates were imaged with a Zeiss confocal microscope for presence of extracellular calcium deposits stained red

3.3.10. Adipogenic histology

At the conclusion of the 10-day culture in adipogenic media, monolayers were fixed and stained with oil red O for presence of intercellular lipid droplets.

3.3.10.a. Preparation of oil red O (ORO) staining solution

1. 0.075 g of oil red O powder (Fluka Chemical Corporation, catalog# 75087) was mixed with 25 mL of isopropanol to make the ORO stock solution
2. 6 mL of stock was mixed with 4 mL of distilled water and allowed to stand for 10 minutes
3. ORO working solution was filtered under vacuum filtration and used within 1 hr
4. Excess working solution was discarded

3.3.10.b. Oil red O staining

1. Adipogenic media was aspirated from plates and wells were washed once with PBS
2. Monolayers were fixed with citrate working solution (0.8% v/v Citrate Concentrated Solution and 60% v/v acetone) for 30 seconds
3. Fixative was aspirated before washing wells with distilled water for 45 seconds
4. Monolayers were stained with oil red O working solution for 15 minutes

5. Stain was removed and wells were washed ~6 times with distilled water
6. Plates were imaged with a Zeiss confocal microscope for presence of red lipid deposits

3.3.11. PCR

3.3.11.a. RNA extraction

1. Cells in monolayers were removed from culture plates using ~1 mL TRIzol reagent (Invitrogen, catalog# 15596-018) per 1×10^6 cells and stored at -80°C
2. TRIzol-cell solutions were thawed by hand and all surfaces and instruments were wiped with RNase Away solution
3. 200 μL of chloroform per mL of TRIzol was added and tubes were shaken by hand for 15 seconds then incubated at room temperature for 3 minutes
4. Tubes were centrifuged at 14000 RPM for 15 minutes at 4°C
5. The top, aqueous phases were removed without disturbing interphases and placed in separate tubes
6. 500 μL of isopropanol was added to each tube and incubated for 10 minutes at room temperature
7. Tubes were centrifuged at 14000 RPM for 10 minutes at 4°C
8. Supernatant was removed and 1 mL of 100% ethanol was added
9. Samples were briefly vortexed and centrifuged at 11000 RPM for 5 minutes at 4°C
10. Ethanol was removed from tubes and the RNA pellets were allowed to air dry to ~5 minutes

11. RNA was resuspended in 15 μ L of diethylpyrocarbonate (DEPC)-treated water and drawn several times through pipette tip to mix
12. Tubes were stored at 4°C overnight to thoroughly dissolve RNA
13. RNA was quantified using Nanodrop

3.3.11.b. cDNA synthesis

1. cDNA was synthesized from 1 μ g of RNA using the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad Laboratories, catalog# 170-8891) for a total volume of 20 μ L
2. Nuclease-free water was added to tubes in quantity=(15 μ L-required volume of RNA)
3. 4 μ L of reaction mix was added to each tube
4. RNA solution was added to tubes in varying volumes so that each contained 1 μ g of RNA
5. 1 μ L of reverse transcriptase was added and the tubes were shaken by hand and briefly centrifuged to collect solution
6. Complete reaction solutions were incubated at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and finally held at 4°C
7. cDNA was stored at -20°C

3.3.11.c. Conventional PCR reaction

1. PCR was performed using Applied Biosystems GeneAmp Fast PCR Master Mix (Applied Biosystems, catalog# 4359187) and cDNA
2. cDNA was diluted 1:5 in DEPC-treated water

3. For each sample, 0.5 μL of each 10 mmol primer (forward and reverse) was combined with 13 μL of DEPC-treated water, 6 μL of GeneAmp Master Mix, and 5 μL of diluted cDNA in a microtube
4. PCR reactions were run in an Applied Biosystems Veriti 96-well thermal cycler at the following temperature profile:

95°C for 1 min, 34 cycles of 95°C for 30 seconds followed by annealing temperature for 30 seconds and 72°C for 1 second, 72°C for 2 minutes, then reaction was held at 15°C indefinitely
5. Annealing temperature varied and was optimized for the primers

3.3.11.d. Conventional PCR gel electrophoresis

Protocol was optimized for ethidium bromide staining duration and method.

1. 2% w/v agarose was combined with TAE buffer in an Erlenmeyer flask, heated in a microwave, and swirled to combine
2. Agarose solution was poured into an electrophoretic gel mold and allowed to cool for several hours
3. On parafilm, one droplet of 0.5 μL of 10x Blue Juice dye (Invitrogen, catalog# 10816-015) was created per sample
4. For DNA standards, 0.5 μL of 100bp DNA ladder and 5 μL of TAE buffer were added to each dye droplet
5. For samples, 5 μL of PCR product was added to each dye droplet
6. The droplets were transferred to the wells of the agarose gel and run at 110 V for 50 minutes

7. Gels were soaked in 1X ethidium bromide in TAE buffer in the dark at room temperature for 30 minutes-1 hour
8. DNA was visualized with UV image system
9. Bands were normalized to β -actin intensity

3.3.11.e. Quantitative real time PCR

1. qRT-PCR was performed in triplicate per sample with SYBR Green PCR Master Mix (Applied Biosystems, catalog# 4367659) and cDNA produced using the Bio-Rad iScript cDNA Synthesis Kit
2. A master PCR reaction mixture was produced for each sample by combining 7.75 μ L of each 10 mmol primer (forward and reverse), 38.75 μ L of SYBR Green PCR Master Mix, and 23.25 μ L of cDNA diluted 4:1 in DEPC-treated water
3. 25 μ L of the master PCR reaction mixture was transferred to triplicate wells per sample in a 96-well plate
4. Plate was covered with transparent optical film and centrifuged briefly to collect reaction mixtures
5. PCR reactions were run by the Applied Biosystems 7300 real time PCR system according to the following temperature profile:

50°C for 2 minutes, 95°C for 10 minutes, followed by 41 cycles of 15 seconds at 95°C and 1 minute at 60°C, and 1 cycle of 15 seconds at 95°C and 15 seconds at 60°C and Ct values were measured
6. β -actin was used as the housekeeping gene while undifferentiated cells of the 3W age group served as the controls

7. Fold-increase of expression was calculated using the $2^{-\Delta\Delta C_t}$ method⁷⁸ and standard deviation was calculated⁷⁹
8. The GraphPad Prism 5 software package was used to perform one- and two-way analyses of variance (ANOVA) as well as Bonferroni's Multiple Comparison Tests
9. The critical p-value for significance was 0.05

Chapter 3 is in part being prepared for submission for publication in "Systematic analysis of physiological donor age on the functional performance of bone marrow-derived mesenchymal stem cells", of which the thesis author is the primary author and is co-authored by Dr. Ramses Ayala and Dr. Shyni Varghese.

Chapter 4: RESULTS

4.1. Optimization of MSC isolation and culture

4.1.1. Conditions for viable cell populations

While testing various isolation and culture conditions for the murine bone marrow-derived MSCs, it was observed that the best conditions for viable cell populations increased the concentration of signaling molecules in the environment. Three specific conditions were noted: (1) After initial plating of bone marrow, it was essential that the culture flasks were left for 4-5 days before the first medium change. (2) Cells grew best when plated at high cell density, which was especially crucial for the initial plating and early passages. (3) Cells grew best in a higher serum culture medium; in this case both 10% and 20% FBS media were tested, and 20% FBS medium resulted in more successful isolations. It was observed that if one of the previous three conditions were not followed, the population might not survive the *in vitro* culture. However, viable cell populations always resulted when all of the three conditions were followed.

4.1.2. Verification of MSC isolation

MSCs were isolated from the primary bone marrow tissue of C57Bl/6 mice, which contains a mixed population of cells including hematopoietic stem cells. Mononucleated cells were plated at a density of approximately 1×10^5 cells/cm² in tissue culture flasks and allowed to adhere for 3 days, and MSCs were sequestered from other cell types by washing off non-plastic adherent cells during medium change (Figure 5). A previous study has shown that non-MSC cell types are further removed from bone marrow culture during passage⁵⁷.

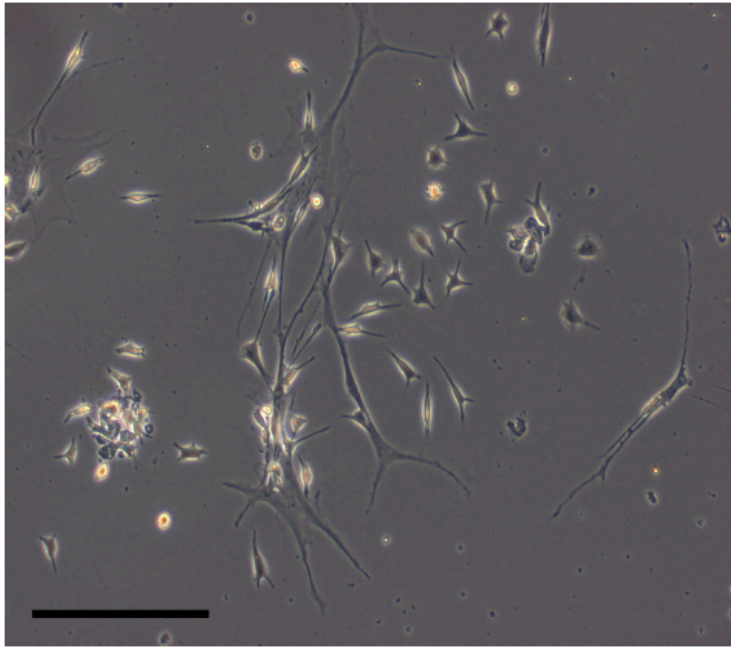


Figure 5: Primary MSCs shown 9 days post-isolation and after media change. Scale bar represents 200 μm .

The isolation was visually confirmed by the fibroblastic morphology that is expected of mesenchymal stem cells (Figure 6). Immunostaining for mMSC surface epitopes revealed that at passage 2, cells were positive for CD106 and negative for CD34 as expected of MSCs.

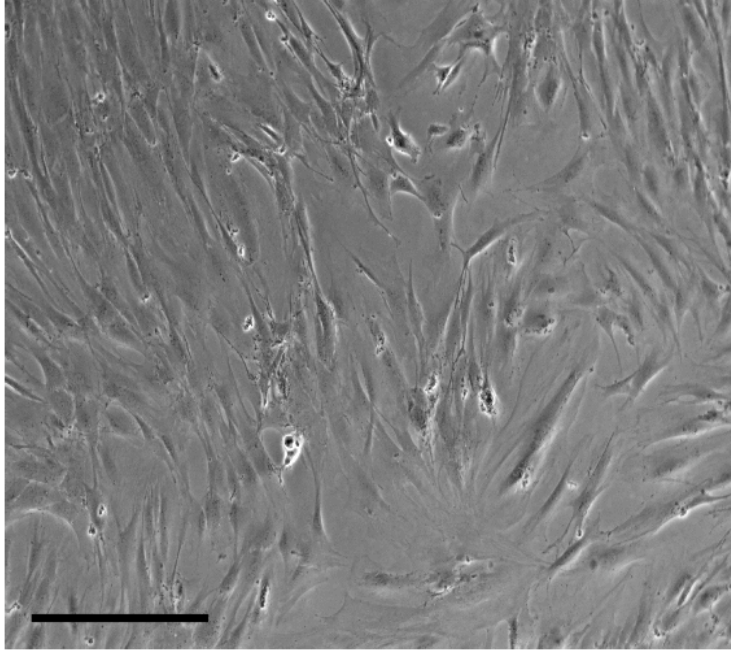


Figure 6: MSCs from 6-month-old mice at passage 5. Scale bar represents 200 μm .

4.2. Age-related differences in MSC *in vitro* expansion

4.2.1. Spontaneous transformation

During *in vitro* passage, it was observed that some groups of MSCs spontaneously transformed, resulting in abnormal behavior (Figure 7). These groups were MSCs obtained from older mice, specifically from a population of 6-month and a population of 13-month-old female mice. These cells were omitted from some of the functional and differentiation assays as their behaviors will be incomparable to the normal MSC groups; however, all data that was obtained from these groups is included herein to give a comprehensive description of the studies and to show how the aberrations manifest in the behavior of these transformed cells.

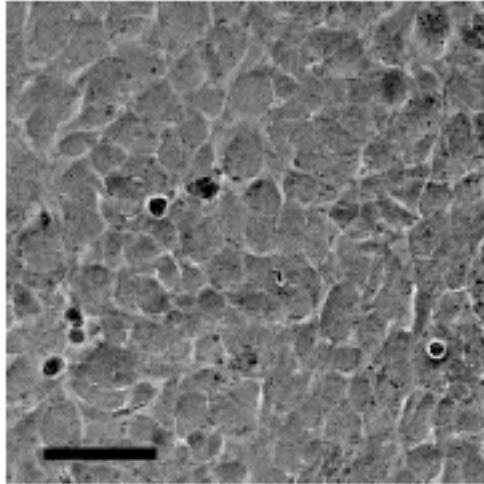


Figure 7: Spontaneous transformation and aberrant morphology of MSCs from 6M mice at P4. Scale bar represents 100 μm .

4.2.2. Effect of passage on cell population doubling, colony formation, and senescence

It is necessary to understand how both *in vitro* aging (cell passaging in culture) as well as physiological aging affect cells in order for MSC-based therapies to be clinically translatable. Both types of aging may similarly involve an increased number of population doublings resulting in shortened telomeres, accumulation of genetic damage, etc. Some of the functional assays were performed at both *in vitro* passage 2 and passage 4 in order to determine how the aging of a cell population in culture affects the population's functionality so a comparison could be made between *in vitro* and *in vivo* aging. It was found that passaging does affect the behavior of the cell groups, as expansion potential was different between passages 2 and 4.

A similar trend in population doubling time was observed between P2 and P4, with the younger (3W and 2M) cells proliferating more slowly than the older (6M and 10M) cells; however, the actual doubling times decreased for each age from P2 to P4 (Figure 8), showing that the proliferative ability is enhanced during passaging.

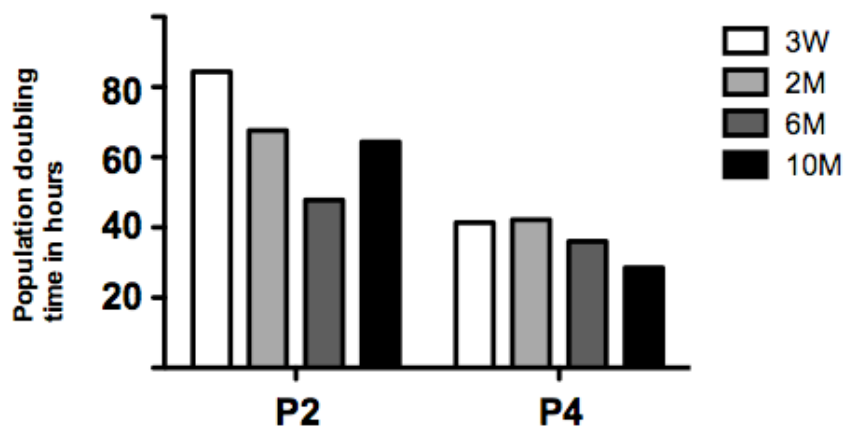


Figure 8: Population doubling times (hours) at P2 and P4.

In the colony forming unit assay, the 3W population had the fewest colonies at both P2 and P4, while all age groups had an overall increase in colony number during passaging (Figure 9). A two-way ANOVA study showed that passage is a significant source of variation in CFUF count ($p < 0.0001$). This data reflects observations of cell culture in which cells plated at later passages formed more colonies than cells at earlier passages. Additionally, when cells were plated at 100

cells/10-cm dish they would not produce colonies. However, cells did produce colonies when plated at 1000 cells/10-cm dish.

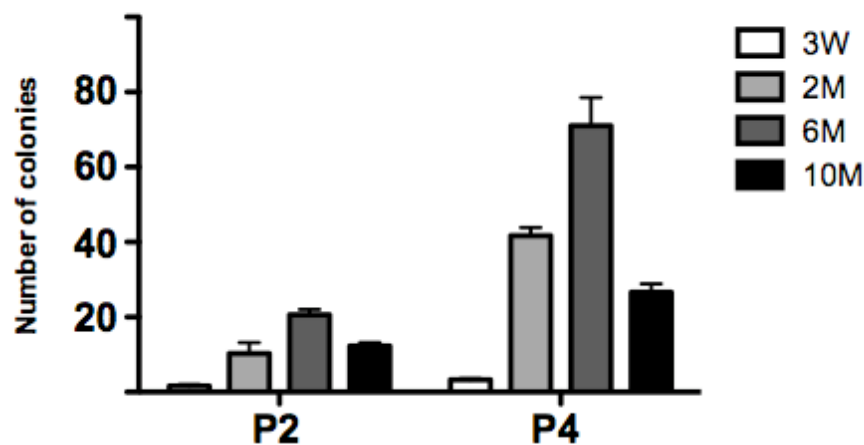


Figure 9: Colony forming units (# of colonies) at P2 and P4.

Senescence, visualized by B-gal staining of pH 6-dependent B-galactosidase activity, was also seen to change during passaging (Figure 10). As expected, a greater percentage of cells became pH 6-dependent B-gal positive (stained blue) at passage 4 than at passage 2. The images show that *in vitro* culture induces senescence in murine mesenchymal stem cells. The age-related trend is also preserved between passages, where the density of B-gal positive cells is proportional to physiological age of the cells.

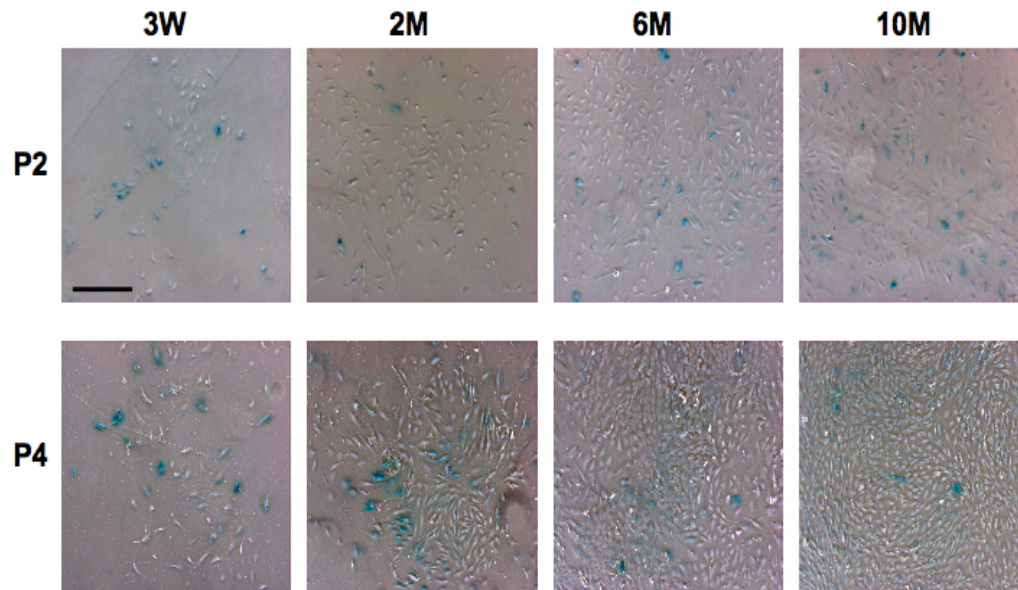


Figure 10: B-galactosidase senescence staining (blue) of MSCs at P2 and P4. Scale bar represents 500 μ m.

4.2.3. Effect of physiological age on population doubling, colony formation and senescence

In order for MSCs to be used in successful reparative therapies, age-dependent changes in cell expansion potential must be elucidated. It is essential to understand how physiological age of a cell population is related to proliferation, measured as population doubling time (PDT). Cells from age groups 3W, 2M, 10M, and a transformed 6M were counted, plated, then trypsinized and counted at a later time in order to quantify their doubling times. Population doubling times were found to decrease with age (excluding the transformed 6M cells), as the younger cells proliferated more slowly than the older cells at P2 and P4 (Figure 8). The transformed 6M cells exhibited the fastest population doubling at P2, and these cells also had a faster doubling time than the 3W and 2M cells at P4.

In addition to PDT, it is important to know how well a population's cells can colonize in culture, and this was quantified by a colony forming unit fibroblast (CFUF) assay. Cells were counted, plated, and then stained at a later time to count colonies of 20 or more cells. Similarly to the PDT, there was an age-dependent difference in colony formation between P2 and P4, excluding the 6M data (Figure 9). At P2, the two-month-old (2M) and ten-month-old (10M) populations had significantly more colonies than the three-week-old (3W) population ($p=0.0105$), but the 2M and 10M CFUF counts were not significantly different from each other. The 3W cells had the fewest colonies and the 2M population had the most colonies at P4, while the 10M had a median colony count. However, colony size increased with age, so that the 10M colonies were much larger than the 2M and 3W colonies (one-way ANOVA; $p<0.0001$), and by the conclusion of the assay the 10M colonies were practically confluent and hard to quantify. The transformed 6M cells had the most colonies at both passages.

Senescence was difficult to qualify between the age groups, as the older (6M and 10M) populations had much higher cell densities than the younger populations, and cells showed variable intensities of blue staining, blurring the definition of positive and negative senescence (Figure 10). The 6M and 10M populations do show a higher extent of staining, as a majority of cells in these populations are tinted blue, but the intensity of the blue staining is very low. Counter to that, the younger (3W and 2M) populations show a higher percentage of cells with intense blue staining than in the older populations, but the young cells overall show less blue staining. Therefore it is difficult to qualify the actual senescence trend that is

occurring, but it will be said that positive β -galactosidase staining increases with age.

4.3. Age-related differences in MSC functionality and differentiation

4.3.1. Effect of age on antioxidant activity

In order for a cell population to be successful in tissue regeneration and repair, it must be able to withstand stress induced by oxidation. Oxidative toleration is predominantly performed by active antioxidants within the cells. Antioxidant activity was measured compared to Trolox standards and normalized to DNA per sample in 3W, 2M, 10M, and transformed 6M populations. At passage 4, antioxidant activity was found to be the greatest in the young 3W cells and lowest in the 6M cells (Figure 11). Disregarding the 6M population (with the knowledge that the 6M cells are transformed and behaving aberrantly), antioxidant activity decreased with physiological age; however, neither one-way ANOVA ($p=0.2397$), Bonferroni post-tests, nor Tukey multi comparison test show significant differences among the antioxidant levels of the various age groups.

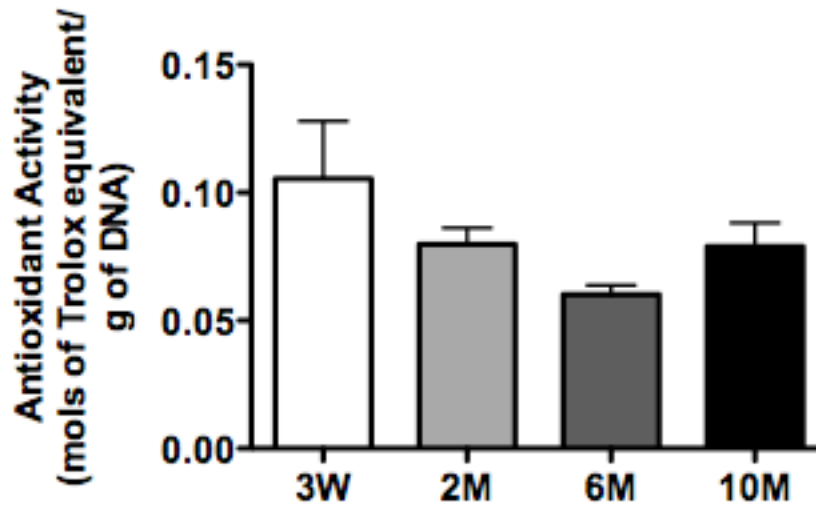


Figure 11: Antioxidant activities (mol of Trolox equivalent per g of DNA) at P4.

4.3.2. Effect of age on migration

Migration is a good indicator of a population's ability to repair or regenerate because MSCs must migrate from cell source to injury site *in vivo*. Treatments involving cell transplantation may require these cells to migrate through tissue layers as well.

Confluent monolayers were plated before scratching gaps 550-650 μm wide into the monolayers. MSCs migrated into the gaps and migration was observed over 3 days (Figure 12). Figure 13 shows that that migration speed was significantly and directly proportional to physiological age of the 3W, 2M, and 10M MSCs (two-way ANOVA; $p < 0.0001$). The 10M cells were the fastest migrating population, whose gap was filled in about 25 hours at approximately 19 $\mu\text{m/hr}$, despite having the widest initial gap of the cell populations. It took over 45 hours for the 2M cells to fill in the gap at approximately 13.8 $\mu\text{m/hr}$ while the 3W cells filled in the gap in

roughly 54 hours at $10.6 \mu\text{m/hr}$. The transformed 6M cells showed little migratory capacity and had a significant gap remaining even after 45 hours (Figure 12).

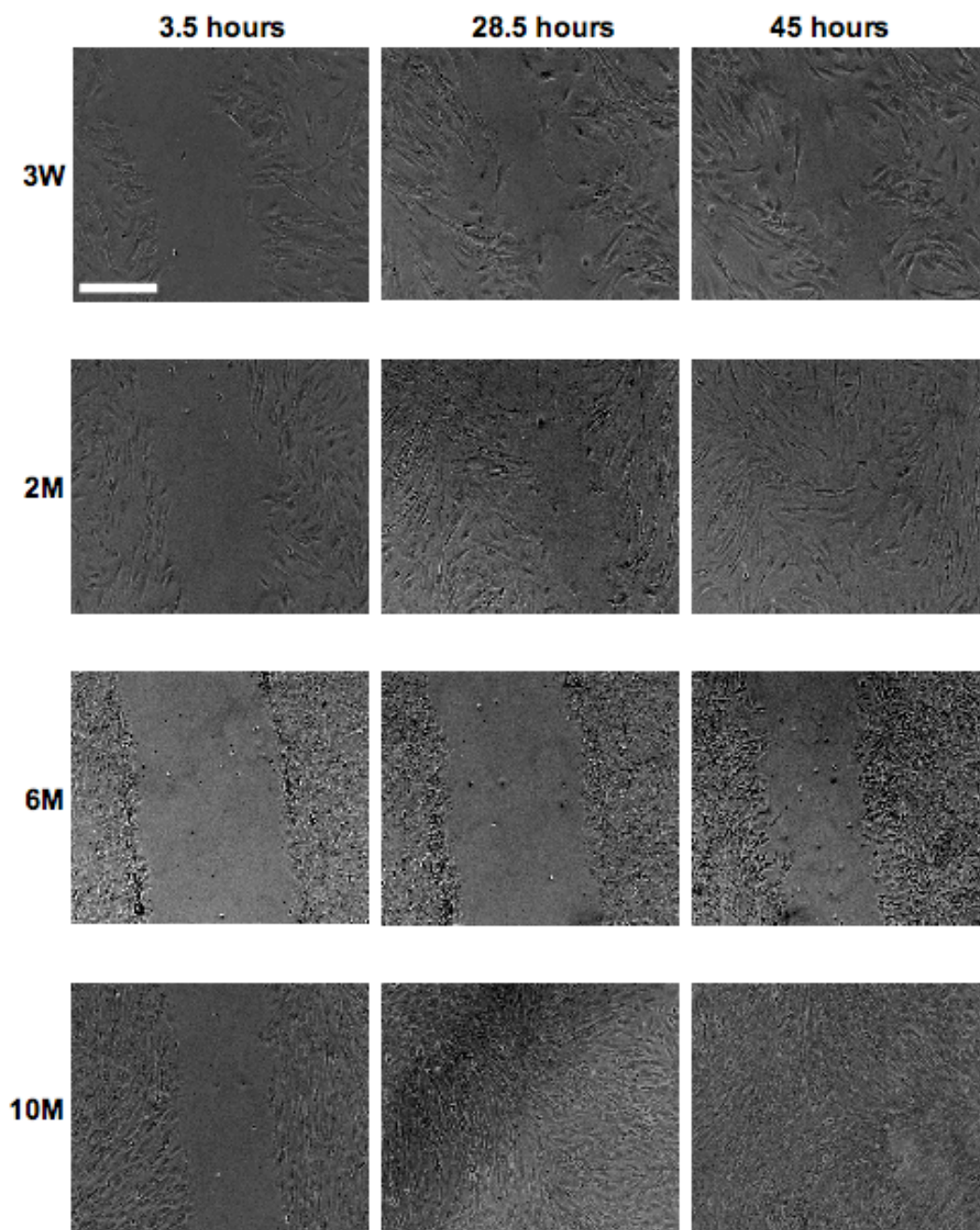


Figure 12: Migration scratch test of MSCs at P4. Scale bar is $500 \mu\text{m}$.

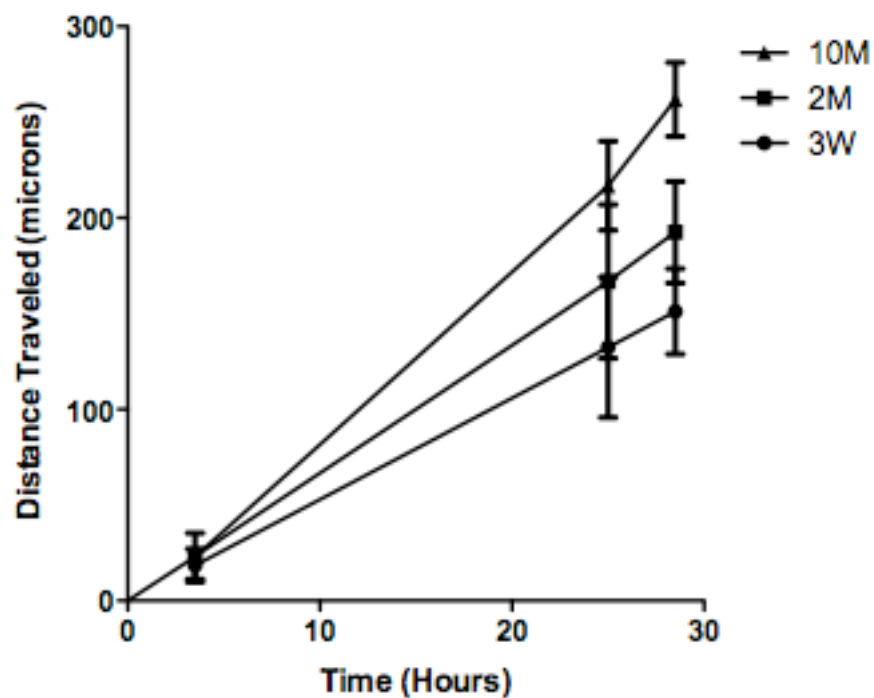


Figure 13: Average distance traveled by MSCs versus time.

4.3.3. Effect of age on cytoskeletal dynamics

Dynamic cytoskeletal proteins, most notably actin and tubulin, are responsible for cell migration and division. The differences in migration rate and proliferation between the MSCs from 3W, 2M, 10M, and transformed 6M age groups were significant, indicative that the cytoskeletal dynamics may also have age-related differences.

The MSCs did demonstrate age-dependent actin repolymerization after 3-hour treatment with the actin-depolymerizing drug cytochalasin D. Immediately after the drug was removed from the medium, the filamentous actin in cells of all

ages had collapsed to globular actin (Figure 14). However, similar to the age-related differences in migration rate, the 10M cells repolymerized the actin in less time than the younger cells. After 15 minutes of recovery, the actin filaments in the 10M cells appeared to be longer and more pronounced than the filaments in the 3W and 2M populations. The 10M cells began to demonstrate a mostly reformed actin structure by 30 minutes while the 2M cells took around an one hour to repolymerize the actin and the 3W cells did not experience as much recovery as the older cells. The 6M cells showed the weakest actin formation at 15 min, but were comparable to the 10M population after 1 hr, indicating that the 6M cells have functional actin dynamics.

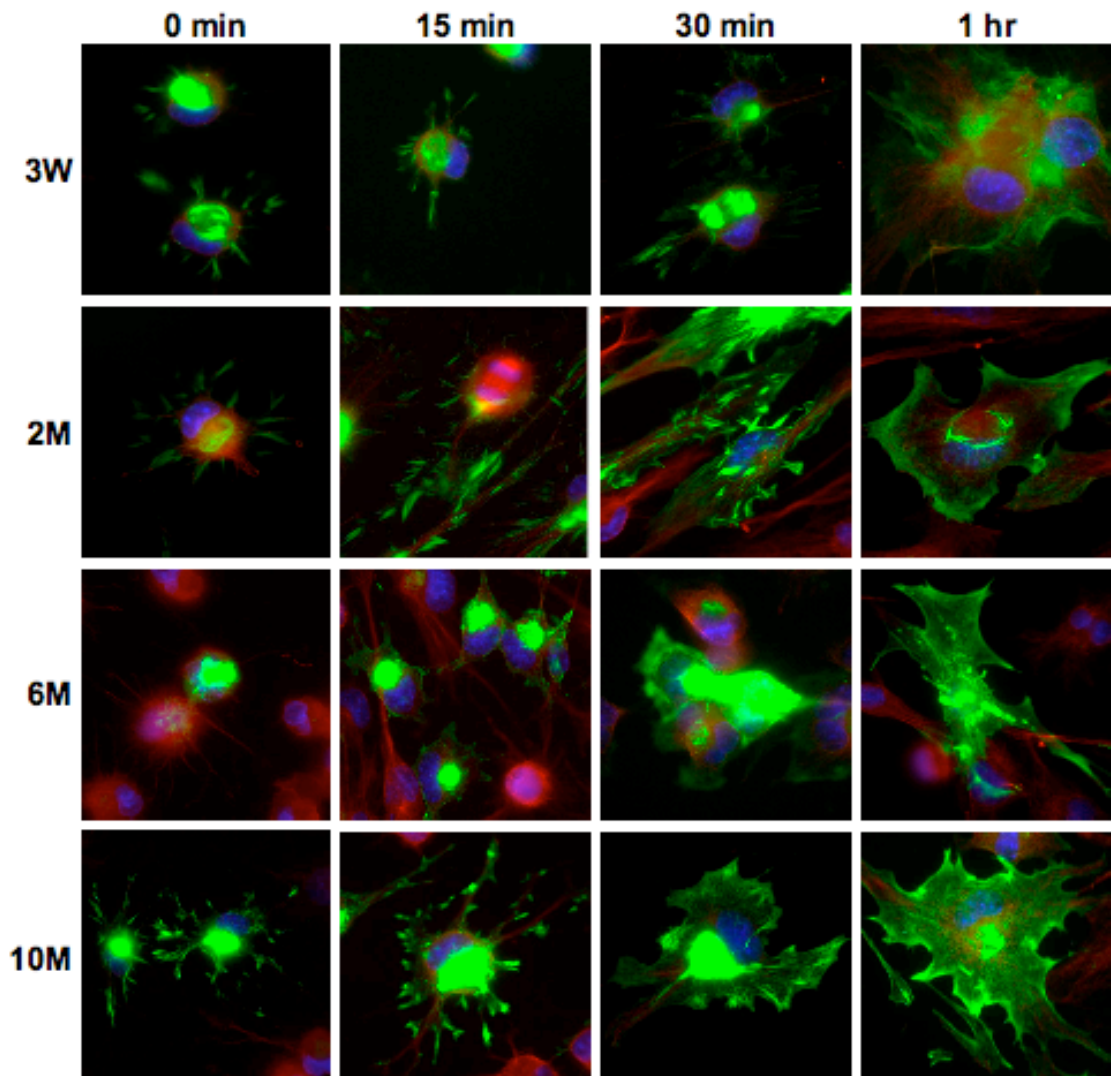


Figure 14: Actin repolymerization after treatment with cytochalasin D.

The microtubule repolymerization profiles after nocodazole treatment also showed age-related differences. A 3-hour treatment with the tubulin depolymerizing drug nocodazole caused microtubules to collapse in all MSC populations (Figure 15), but the older 10M cells proved to have the quickest tubulin repolymerization. Short microtubule fragments were seen in the cytoplasm and near the centrosomes

at 30 minutes post-treatment. While the rate of tubulin polymerization appeared to be consistent among ages, the 10M cells began to transport the microtubule fragments to the periphery of the cytoplasm by 30 minutes, and fragment transportation was further at 1 hour. The fragments in the 3W and 2M cells had not been transported to the periphery of the cell and were mainly contained near the centrosomes even at the later time points.

Similarly to the actin dynamics, the transformed 6M cells again showed that they have functional tubulin dynamics and by 1 hr, they had similar formation and distribution of microtubules to that of the 10M cells.

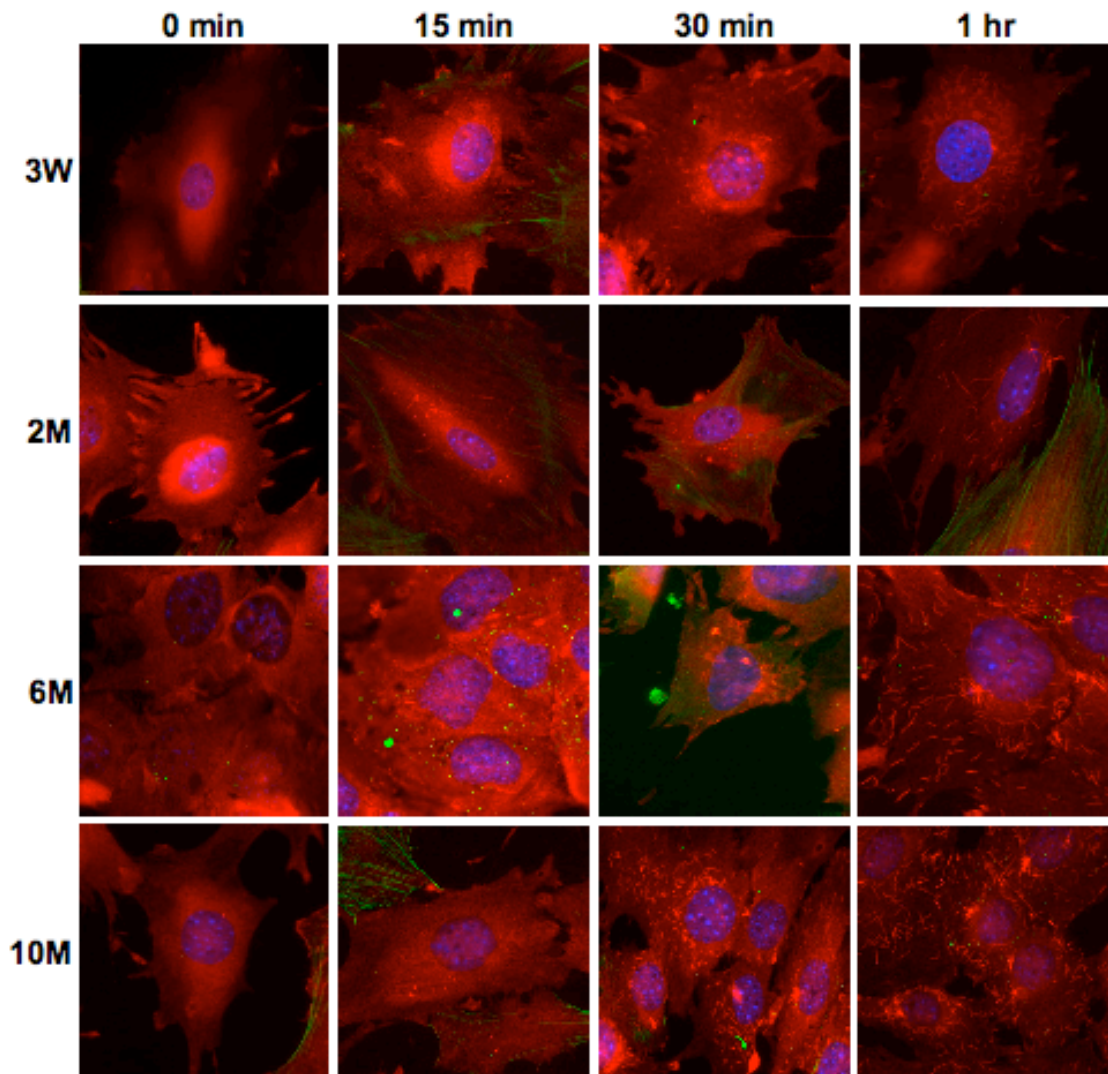


Figure 15: Microtubule repolymerization after treatment with nocodazole.

4.3.4. Effect of age on chondrogenesis

To investigate chondrogenic potential, MSCs were pellet-cultured in chondrogenic media containing TGF- β for 3 weeks. Pellets were cryosectioned and stained for glycosaminoglycans and type II collagen by safranin-O staining and

immunofluorescence. Pellet sections were also immunofluorescent stained for collagen I, an osteogenic marker, for comparison purposes.

It was found that MSCs from both 2-month and 13-month female mice, which had been previously cultured in 10% FBS medium, successfully underwent chondrogenesis when pellet-cultured in chondrogenic media for 3 weeks (Figure 16). There is no distinguishable difference in the degree of staining, so it can be concluded that both age groups have the ability to differentiate into chondrocytes in the described chondrogenic conditions.

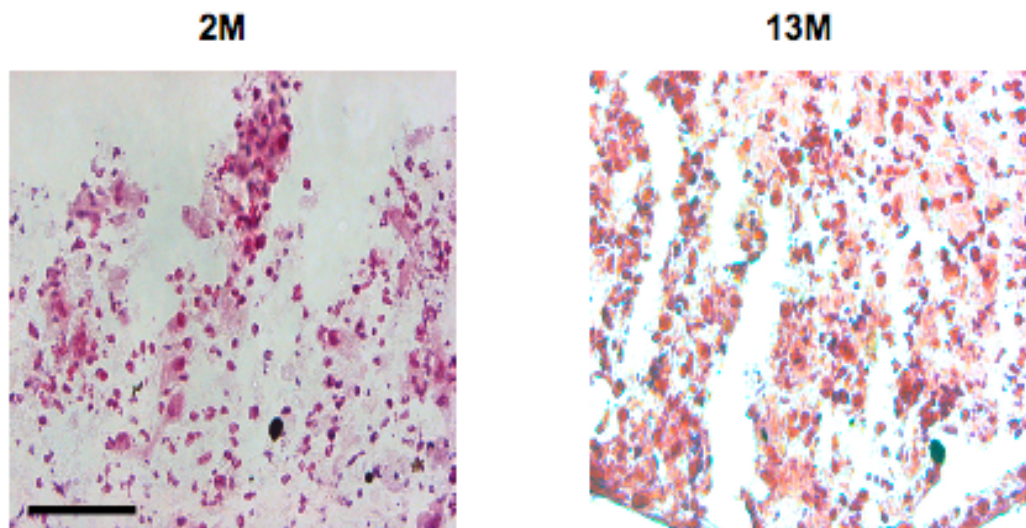


Figure 16: Results of chondrogenic assay. Safranin O staining for GAGs. Scale bar is 50 μ m.

Chondrogenesis was repeated using MSCs from 3W, 2M, and 10M mice, which were previously cultured in 20% FBS medium prior to differentiation. The results of chondrogenesis using these cells are shown in Figure 17. Sections from each age group stained positive for proteoglycans, collagen type II, and collagen

type I. The trend shows no age-related differences in MSC chondrogenic potential based on the histological results, demonstrating that MSCs from all tested age groups underwent chondrogenesis. There was also no difference in chondrogenic potential based on percentage of FBS in growth medium prior to pellet culture.

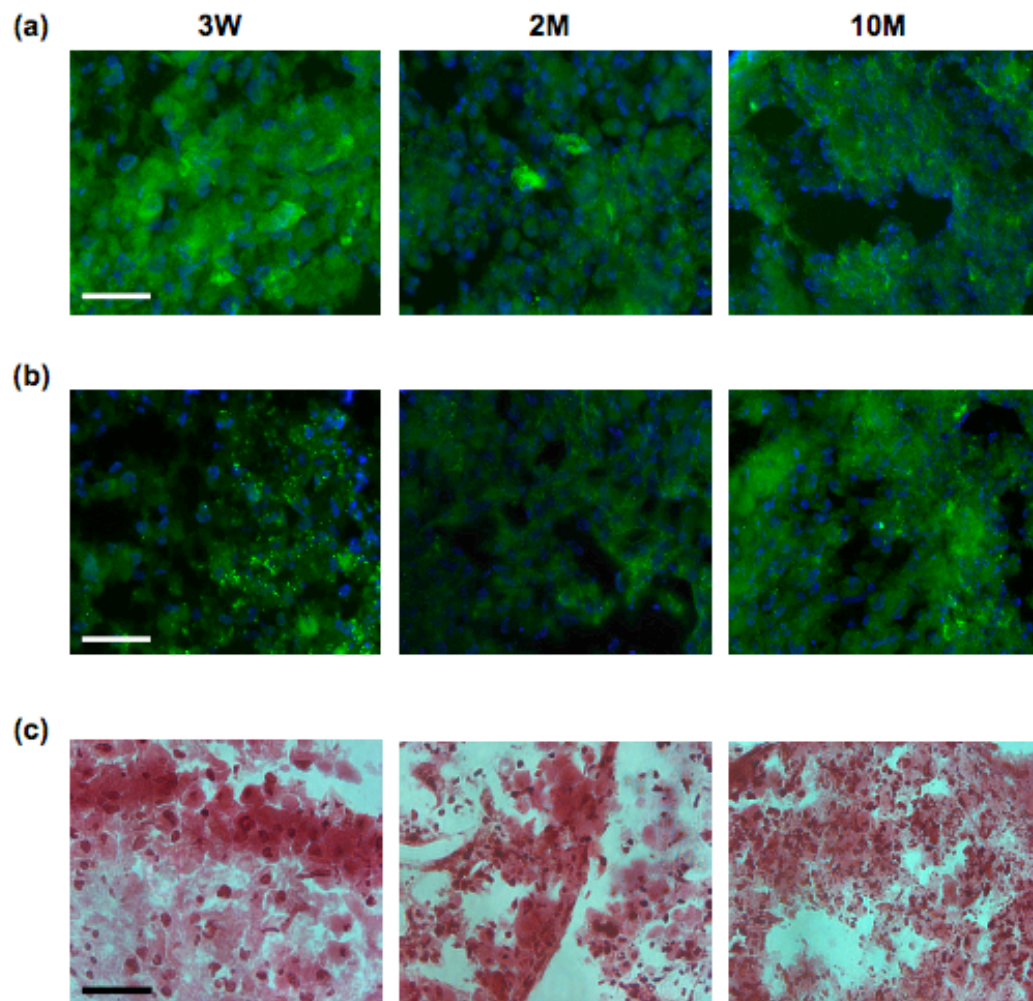


Figure 17: Chondrogenic staining results. (a) Collagen II immunofluorescent staining. (b) Collagen I immunofluorescent staining. (c) Glycosaminoglycan staining by safranin-O. Scale bar in all images represents 50 μm.

Results of qRT-PCR showed that each of the age groups 3W, 2M, and 10M had upregulated expression of aggrecan, collagen I, and collagen II compared to the 3W control cells (Figure 18). There was no significant difference in collagen I expression among the groups (two-way ANOVA; $p=0.5350$) while the expressions of aggrecan and collagen II were significantly different among the different age groups (two-way ANOVA; $p=0.0020$ and $p=0.0112$ respectively). Notably, the 10M control cells had upregulated expression of both collagen I and collagen II, which were repeatable results and need to be investigated further.

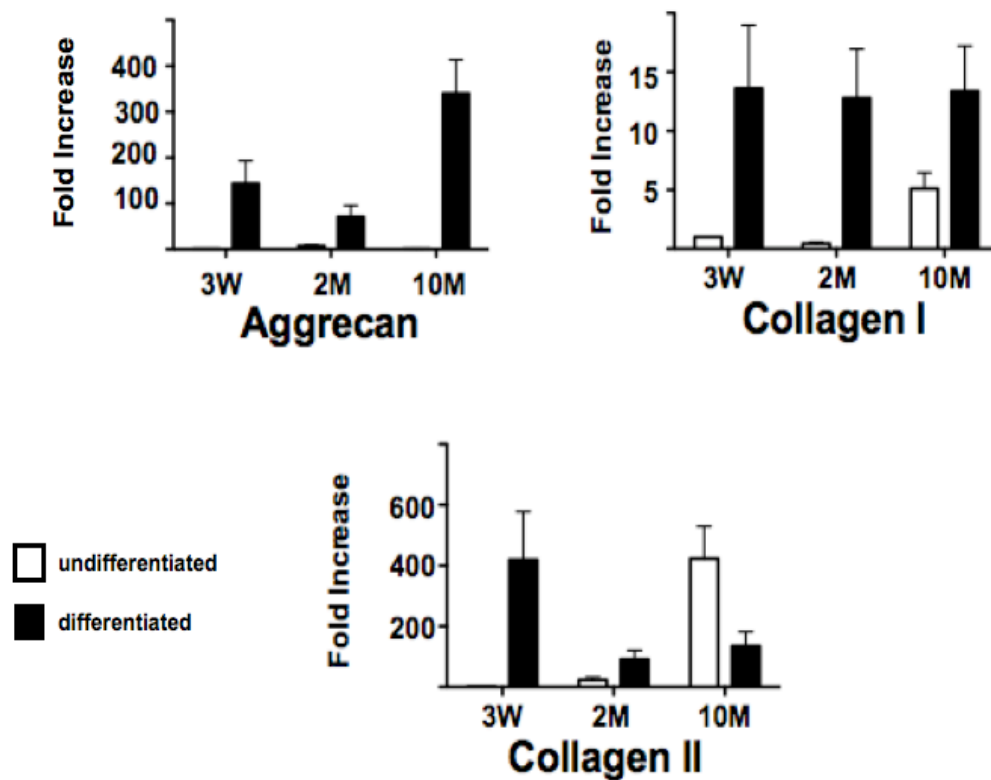


Figure 18: qRT-PCR for chondrogenic markers. All fold-increases are relative to the 3W undifferentiated cell expression.

4.3.5. Effect of age on osteogenesis

Osteogenic potential was also investigated. Confluent monolayers were cultured in osteogenic medium with ascorbic acid, dexamethasone, and β -glycerophosphate for 3 weeks. The monolayers were fixed and at the end of each week for the alkaline phosphatase enzyme (ALP) and calcium with alizarin red S (ARS) staining.

The results of osteogenesis in 2M and 13M populations, in which the MSCs were cultured in 10% media prior to differentiation, are shown in Figure 19 after three weeks of culture in osteogenic media. As seen, the two age groups have different staining patterns. The older MSCs from 13-month-old mice showed the strongest staining for ALP (Figure 19.a), a marker that is positive during the early stages of osteogenesis, but had relatively little calcium staining compared to the 2M sample (Figure 19.b). The younger MSCs from 2-month-old female mice showed stronger calcium staining via alizarin red S but less ALP staining. Since calcium is a marker of later-stage osteogenesis than ALP, these results indicate that the cells from 2-month-old mice have an enhanced ability to undergo osteogenesis in the described differentiation conditions compared to cells from 13-month-old mice.

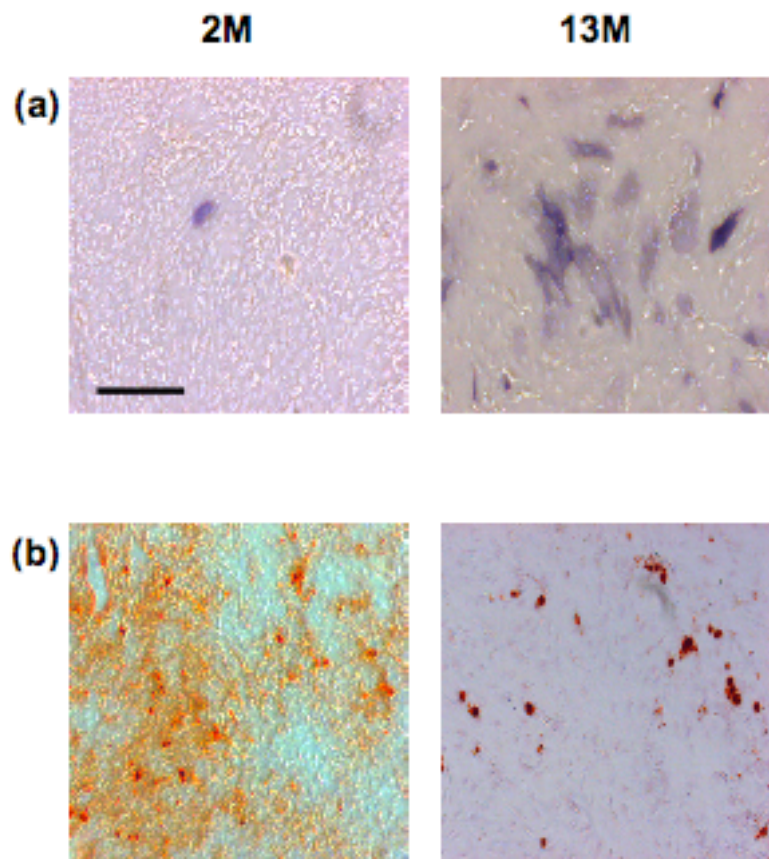


Figure 19: Osteogenic results. (a) ALP staining, (b) ARS staining. Scale bar represents 500 μm .

Osteogenesis was then tested in 3W, 2M, 6M, and transformed 13M cells, and the results are shown in Figure 20 after three weeks of culture in osteogenic media. These cells were isolated and cultured in media containing 20% FBS prior to differentiation. There is both an increase in ALP staining (Figure 20.a) and in calcium staining (Figure 20.b) with increasing age, with the transformed 13M cells showing the strongest staining. The cells appear to have an increase in cell density with increasing age, correlating with the increase in ALP and ARS; due to the almost complete lack of osteogenesis in the 3W cells, it is believed that the age-

associated increase in staining is not an artifact due to the increase in cell number with age and that the older cells have indeed undergone more osteogenesis.

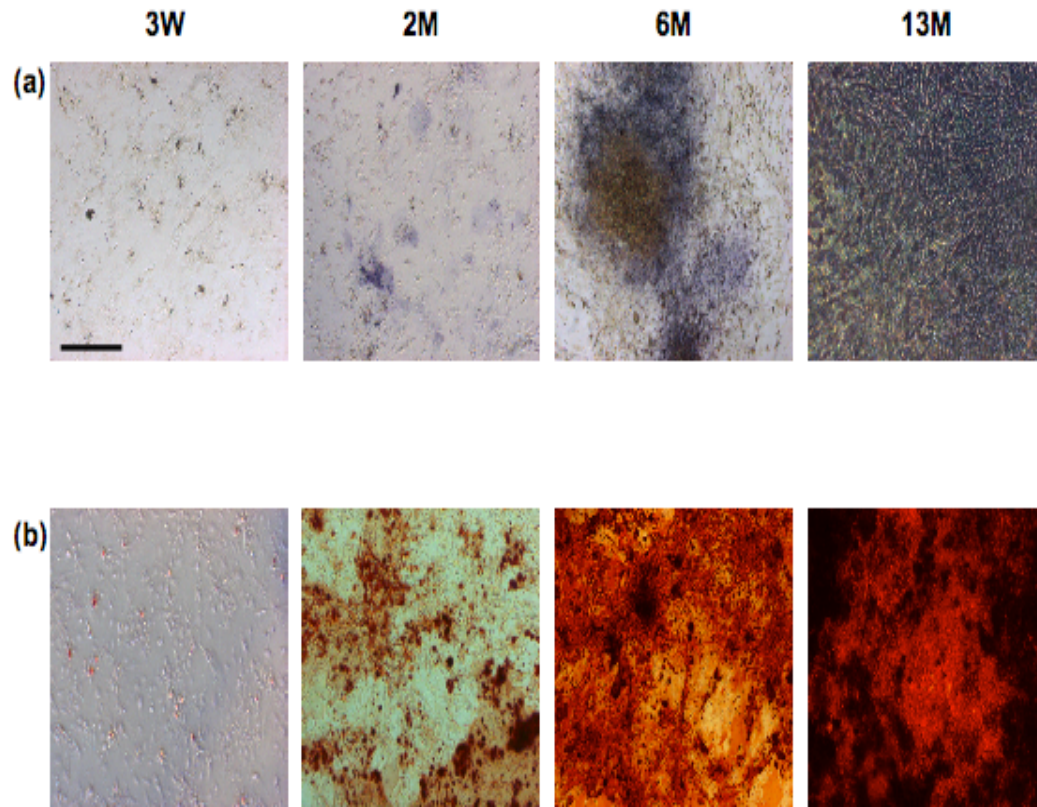


Figure 20: Osteogenic staining of MSCs. (a) ALP staining and (b) alizarin red S staining. Scale bar is 500 μm .

These results were repeated using MSCs from 3W, 2M, 10M, and transformed 6M age groups. After 3 weeks in osteogenic media, the 10M cells demonstrated high quantities of ALP and calcium stainings (Figure 21 a and b). The 3W and 2M samples did not have as extensive ALP or calcium staining as the 10M populations, while these younger populations showed very similar calcium staining to each other. The 3W group was mostly negative for ALP activity, and the 2M

ALP staining was only slightly elevated compared to the 3W staining. The transformed 6M-old cells were strongly positive for calcium staining and were moderately positive for ALP staining.

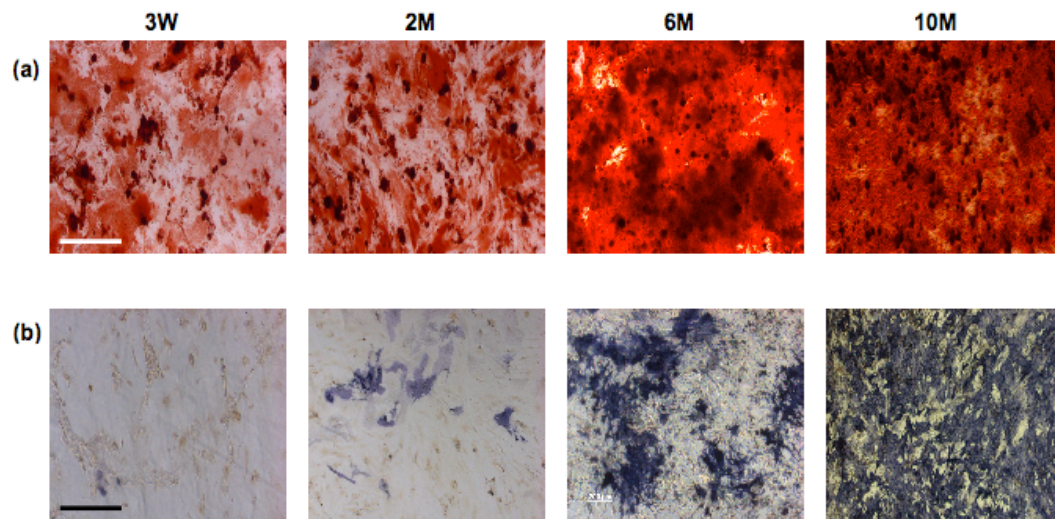


Figure 21: Osteogenesis results. (a) Alizarin red staining and (b) ALP staining. Scale bars represents 500 μm .

Results of qRT-PCR also demonstrated a significant age-related differences in osteogenesis among the cell groups (two-way ANOVA; $p < 0.0001$), as the collagen I, osteocalcin, and core binding factor-1 expressions were highest in the 10M cells and similar between the 3W and 2M populations (Figure 22).

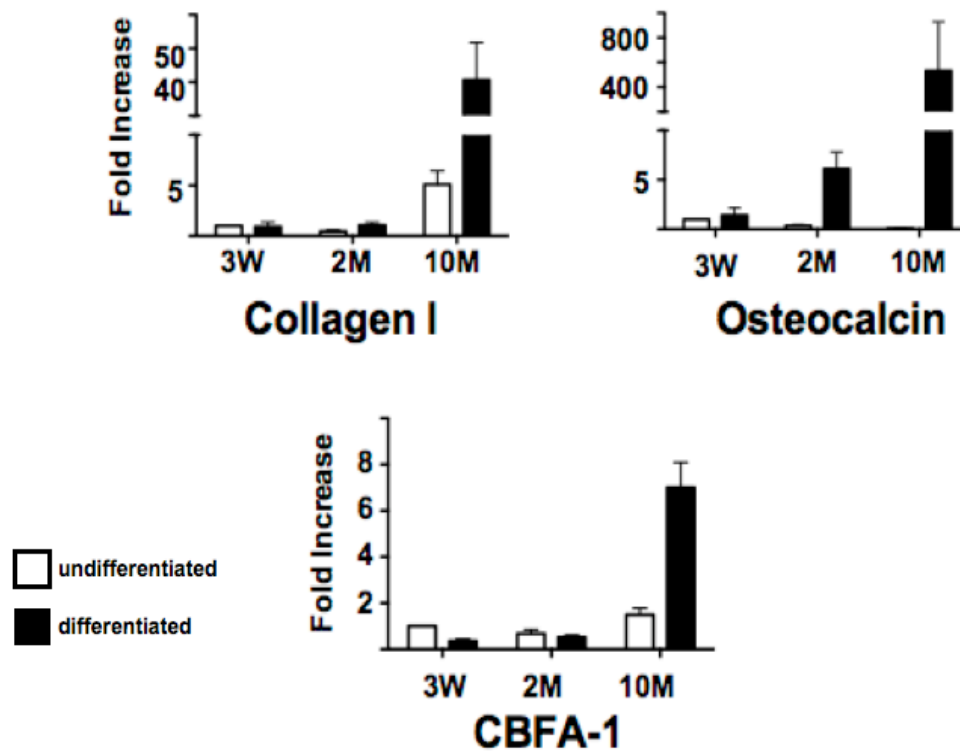


Figure 22: qRT-PCR results for osteogenic markers. All fold-increases are relative to the 3W undifferentiated cell expression.

The results of the osteogenic assays indicate that osteogenic differentiation potential in the nontransformed cells may increase from 3W MSCs to 10M MSCs, and then decline with age as seen in the nontransformed 13M cells. Additionally, the transformed 6M and 13M cell groups showed enhanced osteogenesis.

4.3.6. Effect of age on adipogenesis

The effects of age on MSC differentiation into adipocytes were also studied. MSCs from 2M and 13M mice, in which the cells were cultured in 10% FBS growth media prior to differentiation, were differentiated in adipogenic media for 7 days

before fixing and staining lipid deposits with oil red O. The results of the adipogenesis are shown in Figure 23. Both the 2M and 13M cells show extensive positive staining for lipid deposits (Figure 23.a), indicating that both age groups are capable of undergoing adipogenesis. The 2M cells show stronger staining than do the 13M cells, and the cells of each group are clustered differently. The 2M cells are more aggregated than the spread-out 13M cells. The groups both show morphological changes as well (Figure 23.b). The ORO-positive cells have lost their fibroblastic appearance and are more spherical. Lipid granules can clearly be seen within the ORO-positive cells of both age groups (Figure 23.b).

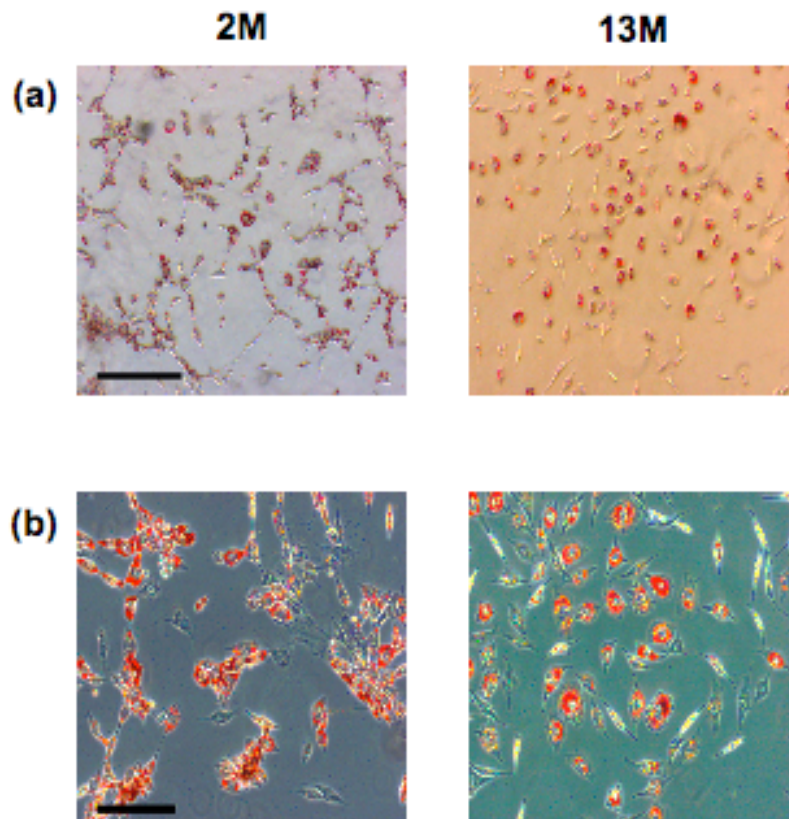


Figure 23: Adipogenesis results. (a) 5x oil red O staining. Scale bar is 500 μm . (b) 10x oil red O staining. Scale bar is 200 μm .

Adipogenesis was repeated using cells from 3W, 2M, 6M, and transformed 13M mice and the results are shown in Figure 24. These cells were cultured in 20% FBS prior to differentiation in adipogenic media for 7 days. The cells were then fixed and stained with oil red O for lipid deposits. The older (6M and 13M) cell groups show fewer ORO-positive cells than the younger (3W and 2M) cells (Figure 24). Both the 3W and 2M cells are almost completely differentiated, compared to the older cells with few differentiated cells. Interestingly, the transformed 13M cells have also continued to proliferate in the adipogenic media, and have become overly confluent. The image of the 13M cells shows some black debris, which are not lipid deposits within the cells, only residues of the oil red O stain that was not well filtered before staining and proved difficult to remove from the fixed monolayer.

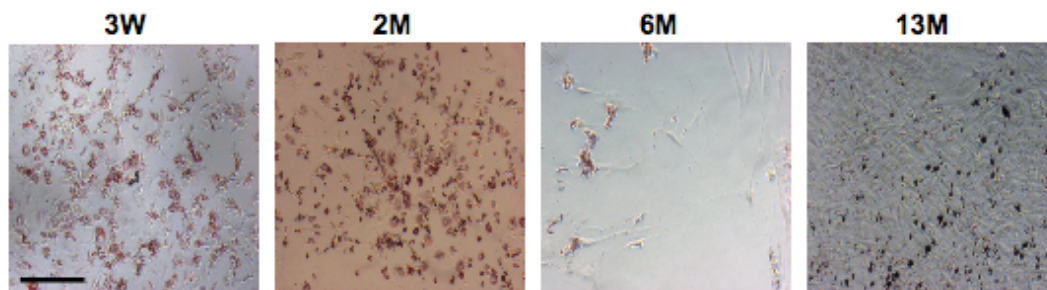


Figure 24: Adipogenesis results. Images taken at 5x. Scale bar is 500 μ m.

Adipogenesis was repeated again using MSCs from 3W, 2M, 10M, and the transformed 6M age groups. Again, the 2M intermediate-aged population had the

greatest adipogenic potential demonstrated by extensive oil red O staining and adipocyte-like morphology after 10 days in adipogenic media (Figure 25.a and b). The 3W and 10M cells had minimal lipid deposits and maintained fibroblastic morphologies. The transformed 6M cells showed little ORO staining. Additionally, the 3W and 2M populations showed a decline in cell number during the 10-day study, while the 10M population and 6M transformed population proliferated in adipogenic media.

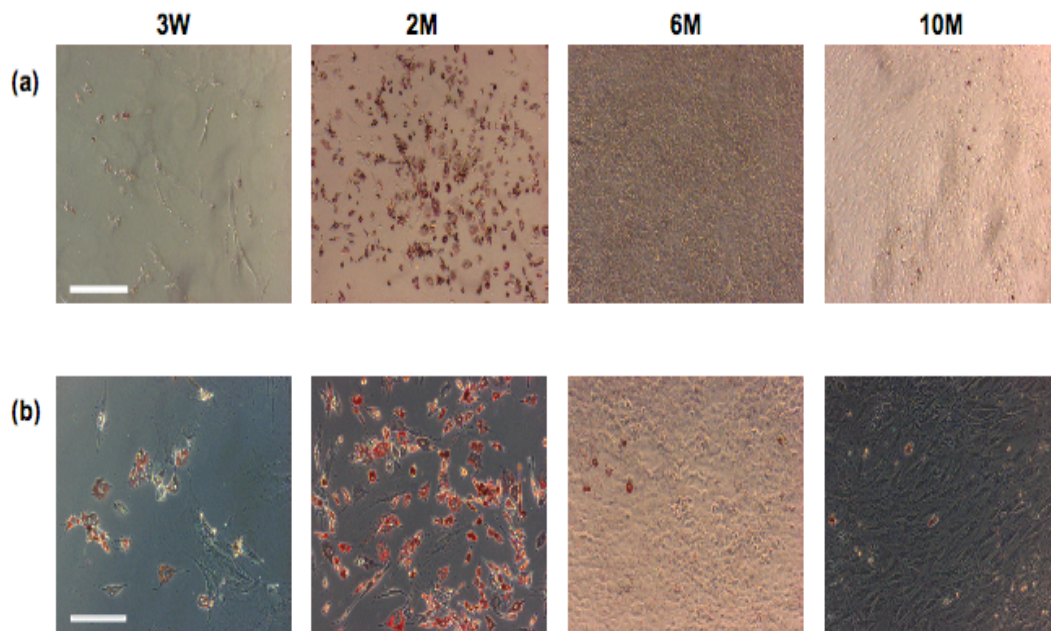


Figure 25: Adipogenesis results. (a) 5x oil red O staining after 10 days in adipogenic media. Scale bar is 500 μm . (b) 10x oil red O staining after 10 days in adipogenic media. Scale bar is 200 μm .

Results of qRT-PCR showed that expressions of lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor- γ (PPAR- γ) (Figure 26) were significantly higher in the intermediate 2M cell population than in the 3W and 10M

cell populations (two-way ANOVA; $p=0.0002$ and $p=0.0007$ respectively). These PCR results support the findings in histology demonstrating that adipogenic potential was highest in the 2M cells.

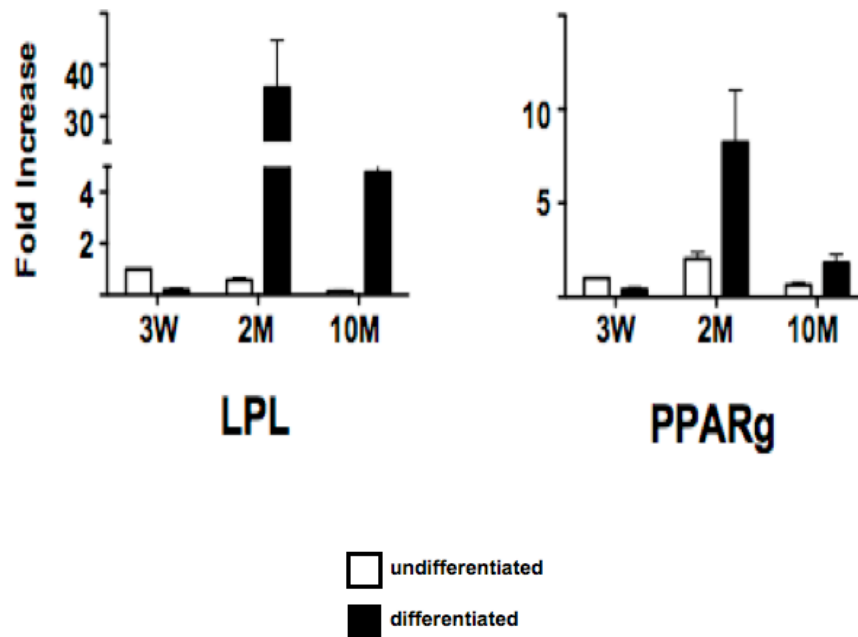


Figure 26: qRT-PCR of adipogenic markers. All fold-increases are relative to the 3W undifferentiated cell expression.

The results of all three repetitions indicate that adipogenic differentiation was consistently best at the intermediate age group of 2M MSCs. The transformed 6M and 13M cell groups were inhibited for adipogenesis and showed very few cells positive for lipid deposits.

4.3.7. Myogenic media

Images were taken of the cells after 3 days in the test myogenic medias, and successful myogenesis was gauged by morphological changes. Histology would

have been performed, but the cells lost their plastic-adherence after a week in myogenic media, before histology was attempted, and thus they could not be immunostained for myosin heavy chain and myogenic differentiation 1 protein (MyoD).

The morphology of the differentiated cells was compared to that of C2C12 cells, which are myoblast precursor cells of human origin with a tri-pointed morphology. Based on morphology after 3 days in differentiation media, it was determined that the best media for myogenic differentiation of murine bone marrow-derived MSCs contained 0.1 μ M of dexamethasone and 50 μ M of hydrocortisone in low-serum (2% FBS) media (Figure 27.b.). MSCs cultured in this media were more triangular in shape, similar to the C2C12 cells, than the others (Figure 27.c-f) and had mostly lost the spindle-shaped morphology of the control MSCs (Figure 27.a).

Though the myogenic media was successfully optimized, myogenesis was abandoned for the remainder of the study because the number of cells was limited and *in vitro* myogenesis of MSCs is less characterized than chondrogenesis, osteogenesis, and adipogenesis.

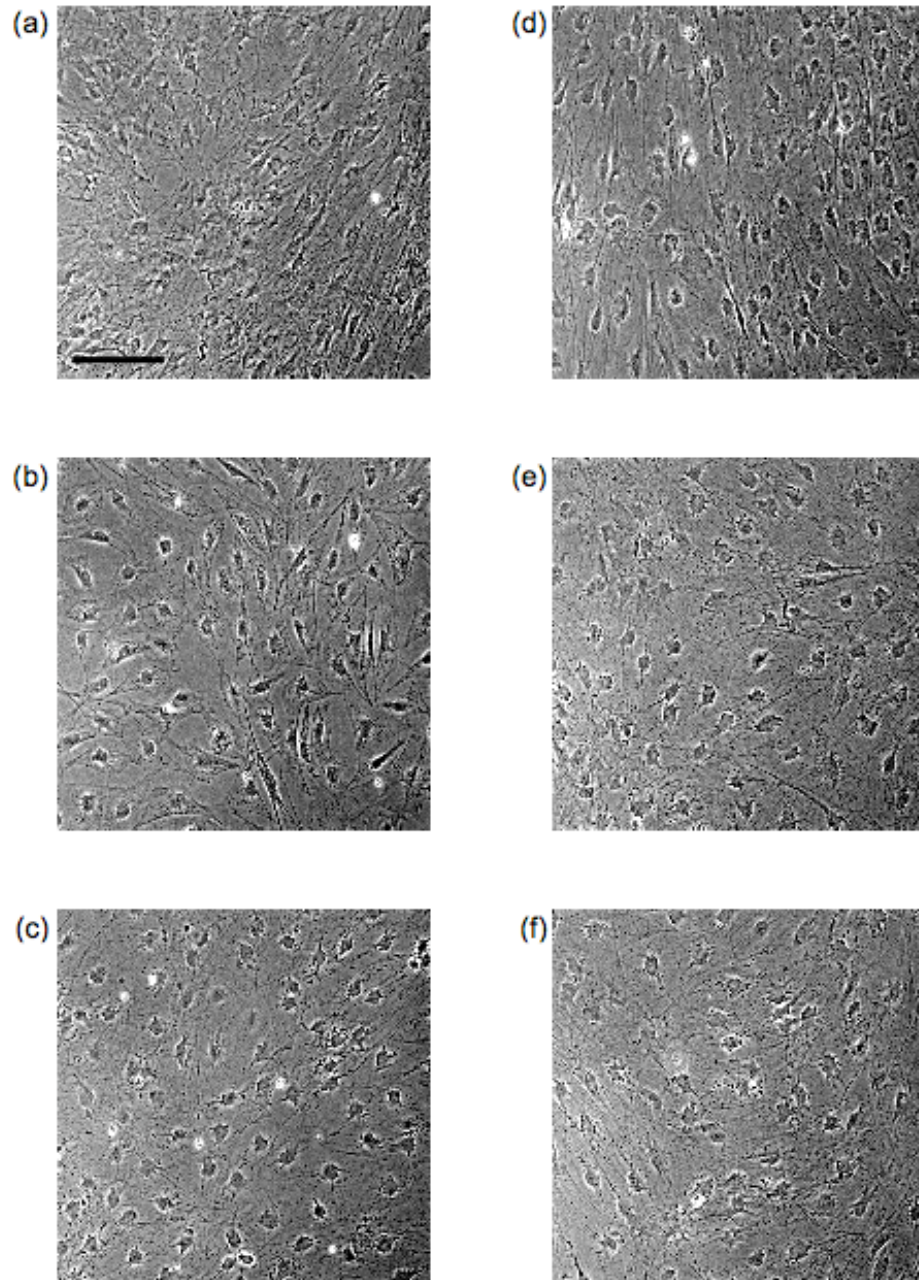


Figure 27: Results of myogenic media optimization. Scale bar represents 200 μm . Cells were grown in media: a) Growth media, b) dexamethasone and hydrocortisone in low-serum media, c) low-serum media only, d) 5-azacytidine and bFGF, e) ITS+Premix and dexamethasone, f) 5-azacytidine and IGF.

Chapter 4 is in part being prepared for submission for publication in “Systematic analysis of physiological donor age on the functional performance of bone marrow-derived mesenchymal stem cells”, of which the thesis author is the primary author and is co-authored by Dr. Ramses Ayala and Dr. Shyni Varghese.

Chapter 5: DISCUSSION

5.1. Optimized conditions for MSC growth and survival

Since the focus of this study is to determine the effect of donor age on bone marrow-derived MSC phenotype and function, the isolation and culture conditions for the MSCs had to be optimized, and two important things were noticed: first, that the cells grew much better when the concentration of serum was doubled from 10% of DMEM volume to 20% of DMEM volume; and second, that the cells, particularly at early passage, were more likely to survive in culture and grew faster at later passages when they were initially plated at higher densities (~ 3000 - 5000 cells/cm² critical value).

It is not surprising that higher concentration of fetal bovine serum, containing numerous proteins and factors required by the cells for growth, results in more viable and faster-growing cells. It would also be expected that this effect is most pronounced at the beginning of the isolation process, when the cells have been removed from the native environment and are in a state of shock at the sudden change in extracellular conditions. There evidently is a critical FBS concentration for these cells, and below this value the cells are less likely to grow, whereas at or above this value cells consistently were found to grow and thrive. These findings suggest that both cell-to-cell interactions and FBS concentration are important for cell viability.

5.2. Sex-based differences in MSC culture conditions

During the course of this study, there were many failed attempts to isolate and expand bone marrow-derived mesenchymal stem cells from both sexes and

many ages of C57/Bl6 mice. There were many conclusions drawn from these attempts. One important observation was made that male and female mice have extreme differences in optimal culture method, so much that the MSCs derived from male mice were not successfully cultured in culture conditions used for MSCs from female mice of the same strain. This is not entirely surprising based on knowledge of sex-based *in vivo* differences in mammalian MSCs and tissue produced by MSCs, which are maintained in homeostasis by cytokines and growth factors influenced by sex steroids⁶¹. For instance, in osteogenic differentiation in native tissue, estrogen is known to increase alkaline phosphatase expression and collagen I while androgens are known to enhance the growth of established osteoblasts⁶¹. Thus, MSCs that matured in a certain *in vivo* environment would be selected for their ability to grow and differentiate in presence of a certain composition of chemicals including sex-based hormones. Additionally, many studies have shown that short- or long-term exposure to estrogen confers a protective enhancement to tissues^{62,63}. and may protect cells during serum deprivation⁶⁴. Thus, it is expected that there would be intrinsic differences between the responses of MSCs from both sexes to the various growth factors present in the growth media, and the cells that matured in the female mice may have enhanced ability to grow *ex vivo* due to the higher estrogen levels before isolation.

5.3. Relationship between *in vitro* and *in vivo* aging

This study thoroughly investigated the effects of aging on bone marrow-derived mesenchymal stem cells, and though it focused primarily on physiological or *in vivo* aging, some of the assays were performed at multiple passages in order to

elucidate the effects of passage-dependent or *in vitro* aging. The results of this study prove that both *in vivo* and *in vitro* aging cause significant changes in the behavior of cell populations. The specific changes caused by both types of aging were similar in terms of proliferation, colony formation, and senescence. Since passaging affected expansion ability, passaging would also affect functionality and differentiation potential.

Though the results of this study show that cellular aging causes enhanced functionality of the population, there is reason to believe that this trend would actually begin to reverse at a certain “critical age” where the functionality would decline. Due to the Hayflick phenomenon and the nature of cellular senescence, it is expected that population doubling time and colony formation would hit a peak after so many population doublings and these numbers would decline as the population would senesce and proliferation would reach a halt. Indeed, cellular senescence and decreased population doublings do occur during *in vitro* expansion⁶⁵. During culture, MSCs have been shown to lose the ability to undergo adipogenesis and then eventually lose the ability to undergo osteogenesis⁶⁵. It is expected that the ability of all cells to produce chondrocytes would decline with *in vitro* passage as well.

5.4. Aging and *in vitro* transformation

Two cell lines showed unusual behaviors and morphologies after several weeks of *in vitro* culture in these studies; a population of 13-month-old MSCs and a population of 6-month-old MSCs each began to proliferate at a faster rate and displayed rounder morphology. Fibroblasts normally exhibit contact inhibition of growth and will not grow past confluency⁶⁶, but the suspect cell lines in this report

appeared to lose their contact inhibition and proliferated in growth-reducing conditions, similar to malignant cells⁶⁷. The 6M cells also had low levels of antioxidant activity, reduced migration ability, and unremarkable cytoskeletal dynamics, which may be characteristic of transformed cells. Previous studies do report that this kind of transformation occurs in culture of MSCs⁶⁸. Cell proliferation is generally limited at the onset of senescence, when the telomeres shorten past a certain length, but some cells will continue to divide even with shortened telomeres. Past this point, cells normally encounter a critical point at which apoptosis is inevitable; but, again, in rare cases cells are known to continue dividing past this point. These cells are considered to have spontaneously transformed into malignant cells, bypassing the usual points of cell cycle regulation⁶⁷. It is believed, due to the behavioral and morphological descriptions, that similar transformation did occur in these 13M and 6M cell lines. The molecular cause of the transformation is unknown, but there are many possibilities, such as aberrant cell cycle regulators or a mesenchymal-epithelial transformation.

P53, a tumor suppressing protein, is known to induce cell cycle arrest and senescence in response to cellular stress such as DNA damage, which could otherwise be carcinogenic. Li et al. showed that p53 mutation can occur during *in vitro* culture of mMSCs and cause tumors when the cells are transplanted⁷⁷. It is possible that in the current study, the cells spontaneously transformed during passaging and lost normal p53 activity. However, Rubio et al. showed that titers of many proteins, excluding p53, are altered in transformed cells during the expected late-passage senescence and apoptosis stages⁶⁸. These cells showed increased levels

of DNA repair proteins and unusual expression of cyclins and cyclin-dependent kinases. When senescence was expected, the transformed MSCs had upregulated c-myc and downregulated p16 proteins, then the cells bypassed apoptosis by expressing higher levels of telomerase and Rb tumor-suppressing protein⁶⁸. In order to discern the molecular mechanisms involved in the transformation of the 6M and 13M cells of this report, protein and gene expression analyses would need to be performed.

A recent study has reported *in vitro* transformations of MSCs that are actually a mesenchymal-epithelial transition⁸⁴. During embryogenesis, mesenchymal-epithelial transitions are known to naturally occur, for example during kidney organogenesis⁸⁵. Analysis by Chaffer et al. suggests that flexibility between mesenchymal and epithelial characteristics is also crucial for metastatic carcinomas⁸⁵. Chaffer et al. describes how primary carcinomic tumors transform and accumulate migratory mesenchymal properties in order to metastasize, then undergo a mesenchymal-epithelial transformation (EMT) to establish a secondary tumor⁸⁵. Rubio et al. indicates that this malignant EMT can occur in culture, which he showed by analyzing tumors produced by spontaneously transformed MSCs and identifying them as carcinomas⁸⁴. The transformed 6M and 13M cells in this study demonstrated a “scaly” morphology, enhanced proliferation, reduced ability to migrate, and enhanced plastic adherence, all characteristics that are more epithelial than mesenchymal in nature, so these cells may have also undergone a malignant EMT. Again, proteomics and genomic studies must be performed to prove this theory.

It is not surprising that stem cells could transform into malignant cells; there are similarities and links between stem cells and cancer. They share traits such as the ability to self-renew and to proliferate at length, particularly embryonic stem cells that can proliferate indefinitely. Especially supportive of this relationship are teratomas, cancerous tumors containing tissue from all germ layers and that reportedly result from injection of embryonic stem cells^{1,69,70}.

There is also a relationship between aging, MSCs, and cancer. Aging is a risk factor for cancer due to increased genetic damage, and there may be a connection between MSC aging and cancers of mesenchymal-origin tissues. Tyner et al. showed that accelerated aging was induced in mice mutated to have overactive p53 though they also had very reduced cancer rates⁷¹. Wound healing was also slowed in these mice and osteoporosis was pronounced. It may be that MSCs from older animals would be more prone to spontaneous *in vitro* transformation, as these animals are at higher risk for developing cancer in general.

5.5. Physiological MSC aging and functional behavior

It was found that there are age-related differences in MSC functionality, however we found that the oldest MSCs from the 10-month-old mice demonstrated enhanced performance in terms of proliferation, colony formation, and migration. The enhanced proliferation and migration ability of these cells may be further supported by the faster dynamics of actin and tubulin repolymerization.

Though unexpected, the enhanced ability to proliferate and form colonies in the older populations of MSCs is not entirely inconsistent with previous reports. Bergman et al. found that aging increased the growth rate of murine MSCs, though

it is uncertain why this would occur. A population with a fast growth rate would be expected to also form more colonies due to the increased number of cells, and it may form larger colonies as well due to the faster proliferation. Similar to the previous discussion regarding the enhancement of MSC expansion ability with *in vitro* aging, it would be expected that these trends would begin to reverse with MSCs from much older mice. Cells obtained from very old mice would be expected to have accumulated a lot of genetic damage and would have shorter telomeres, which could prevent these cells from proliferating and colonizing as quickly or to quite the extent seen in this report.

Spontaneous transformation has been seen multiple times in MSC culture, resulting in cell populations known to over-proliferate. Possibly, the cellular mechanisms causing the transformations may also affect the other cells without fully transforming them. Then the apparent enhancement of functionality could actually be due to the decreased activity of certain cell cycle regulators or tumor suppressing genes. 10M cells even proliferated in both adipogenic and osteogenic media in the presence of dexamethasone, indicating the decline of some growth regulation, but the cells are not believed to have transformed due to their normal fibroblastic morphology. This hypothesis could be confirmed by global proteomic or genomic investigation.

The trend in antioxidant activity is consistent with previous reports. Kaspar et al. found that the antioxidant activity of MSCs decreased with donor age in Sprague Dawley rats⁷². In the context of this study, antioxidant activity could decrease with physiological age due to an increase in the amount of reactive oxygen

species (ROS) present in the cell and thus antioxidants in the cytoplasm would be used more quickly than those of a younger cell. It is known that the environment is a source of oxidizing substances, in addition to the significant amount of reactive oxygen species produced during normal cellular processes such as metabolism. As an organism ages, it is increasingly exposed to these toxic ROS and they may even accumulate in the cells or tissues; therefore, an older organism (or cells that have undergone more population doublings) has more oxidative damage and more oxidative substances present than that of a younger organism. These substances, if left unchecked, inflict destruction to cells. Oxidative stress can damage DNA as well as proteins⁷³, all of which can lead to cancer and inflammatory diseases⁷⁴. As more ROS accumulate in the cell, the antioxidants are consumed in order to defend against the ROS. Interestingly, Reverter-Branchat et al. demonstrated that in *Saccharomyces cerevisiae*, cell lines that had been aged *in vitro* had similar responses to oxidative damage to those of the *in vivo*-aged cell lines; specifically, some proteins responsible for stress protection and glucose metabolism had been damaged by protein carbonylation⁷³.

Proliferation, migration, and cytoskeletal dynamics all were enhanced with age, and it is not surprising that these traits are correlated. Mitosis and locomotion are both heavily reliant on actin or tubulin restructuring. Actin is a predominant protein in the cytoskeleton and is required for a cell to move. Cell migration occurs through subsequent extensions, contractions, and releases of focal adhesions between the cell and the surface⁷⁵. To be more specific, in a moving cell, actin is polymerized in the direction of migration in order to form a protrusion that attaches

to the surface, and the adhesions at the far end of the cell dissipate. Concurrently, cytoskeletal myosin proteins provide contraction of the actin, moving the cell toward the direction of the initial protrusion⁷⁵. More dynamic actin could thus explain the faster migration rates of the oldest cells. Additionally, the efficient tubulin formation and transport could explain the enhanced proliferative ability of the 10M cells since microtubules (and actin) contribute to the mechanics for cell division. During mitosis, microtubules are known to be very dynamic and form an organized spindle, which contributes to cell division⁷⁶. Microtubules and associated motor proteins are responsible for separating sister chromatids, while actin and myosin motor proteins form the cleavage in the cell membrane following anaphase which results in cytokinesis and cleavage of the two daughter cells⁷⁶. Efficient actin and tubulin dynamics, as well as the faster distribution of intracellular tubulin, would be necessary for proliferating populations.

5.6. Physiological MSC aging and differentiation ability

The results of this study verified that there are age-associated changes in the differentiation potential of murine MSCs. Chondrogenesis, as verified by histology, was possible in MSCs of the 3W, 2M, 10M, and nontransformed 13M age groups and there was no visible difference among the different ages. However, there were age-related differences in osteogenesis and adipogenesis. In the nontransformed cell groups, osteogenic potential increased from the 3W group to a peak in the 10M group, and then osteogenesis declines in the 13M group. This suggests that osteogenic potential increases in murine MSCs until a “middle-age” when it begins to decline. Adipogenesis also displayed a non-linear relationship with age. Among

the nontransformed cells, the intermediate 2M age group consistently and repeatedly underwent the most adipogenesis, while the younger and older age groups did not consistently differentiate as well. Since osteogenesis and adipogenesis displayed an eventual decline in differentiation with age, it is safe to assume that chondrogenesis would also begin to decline in MSCs from animals aged more than 13 months, which was the oldest group studied in this report. Additionally, the transformed 6M and 13M cells underwent enhanced osteogenesis while they could not undergo adipogenesis.

Some previous studies have claimed that MSCs display an “adipogenic switch”, described as a cell population’s propensity to undergo either adipogenesis or osteogenesis but not both⁵⁵, though it is generally expected that osteogenic potential declines with age while adipogenic potential is enhanced with age⁵⁴. The results of this study do support the putative adipogenic switch, as seen by the mutual exclusivity of the adipogenic and osteogenic paths. The 10M old cells best underwent osteogenesis while the 2M cells best underwent adipogenesis, and the 3W cells did not extensively differentiate into either of those lineages. The trend is reverse of what many other studies demonstrated, but additional studies have shown that as cells are passaged, they lose their adipogenic and chondrogenic potential while maintaining osteogenic potential³⁵. The 10M had faster growth rates than the 3W and 2M populations, so they underwent more population doublings in a given amount of time. Thus, perhaps cells that proliferated more lost their adipogenic potential earlier than slower growing populations, which could also explain why the transformed (and quickly dividing) cells best underwent osteogenesis and could not

undergo adipogenesis. The decreased ability of the 2M population to undergo osteogenesis could then be explained by the adipogenic switch, so that since these populations retained the potential for adipogenesis they also excluded osteogenesis, via the mutually exclusive PPAR- γ pathway or other osteo/adipo pathways.

The transformed cell groups showed very positive staining (ALP and ARS staining) while they were mostly negative for adipogenesis. Chondrogenesis was not evaluated in either of these groups. Since the adipo and osteogenic results of the transformed cells were similar in nature to the results of the older nontransformed populations in this study, it's possible that similar molecular mechanisms are at work. The transformed populations proliferate faster than the other populations, so they may have lost adipogenic potential as they expanded, which concurrently activated the osteogenic pathways.

5.7. Limitations of these studies

These studies were limited by the number of cells available for use and thus assays had to be performed at higher passages in which cells were more available. Assuming that age may affect functionality and differentiation potential, different results may have been seen using cells at earlier passages. Additionally, only female mice were used in this study because MSCs from male mice could not be grown in culture, and the results of these studies may have been different using MSCs from males, as there are verified sex-based differences in musculoskeletal physiology and hormone levels. It also must be acknowledged that varying *in vivo* estrogen levels prior to euthanization may affect the cells from female mice. Estrogen levels are variable with age, and this hormone is known to affect differentiation pathways,

especially osteogenic pathways. Therefore the differentiation results could be affected by previous menstrual cycles, pregnancy history, and other personal histories of the animals not directly related to MSCs.

The limitation with the greatest effects, however, was the definition of “age” in these studies and in previous studies. Previous reports variably group animals and inconsistently define “old” and “young”. Age is not defined simply by a number of months or years of life, and innumerable lifestyle and genetic factors influence the manifestation of age in organisms and in cells. Age is inextricably tied to so many other factors and characteristics that it is impossible to completely isolate age as a single variable in these studies, though every attempt was made to do so; the differentiation trends were repeatable with consistent culture conditions, demonstrating that age may indeed have predominated over other factors.

Chapter 5 is in part being prepared for submission for publication in “Systematic analysis of physiological donor age on the functional performance of bone marrow-derived mesenchymal stem cells”, of which the thesis author is the primary author and is co-authored by Dr. Ramses Ayala and Dr. Shyni Varghese.

Chapter 6: CONCLUSIONS AND FUTURE STUDIES

It has been shown that age does affect both the functionality and differentiation potential of MSCs isolated from murine bone marrow, though the exact age-associated mechanisms are unknown. Cells showed an increase in growth rate, colony formation ability, senescence, migration, cytoskeletal dynamics, and potential for transformation with age. The age-associated enhancement of expansion and functionality may be due to changes in cell cycle regulators with increasing cell division. Some previous studies support this theory, as do the data regarding enhancement in MSC expansion ability with increasing *in vitro* passage. Additionally, it is not surprising that cytoskeletal dynamics are correlated with proliferation and migratory abilities, but it is unknown what age-related mechanisms are influencing the cytoskeleton. Age also affected the differentiation potential of the MSC populations. Osteogenic potential increased with age, peaked in the 10M cells, and declined in 13M cells. Additionally, adipogenic potential was highest in the intermediate 2M age while chondrogenic potential showed no age-dependent changes. It is assumed that chondrogenic potential would peak at an age later than 13M and then decline, similar to the trends in osteogenic and adipogenic potentials.

Findings from this study show a donor age-dependent effect on the functions of bone marrow-derived MCS. This suggests that age of the donor must be considered in the use of MSCs for treatments.

The conclusions of this study support the need for future studies in this field. Though various age-related changes in the behavior of murine MSCs have been identified, the age-associated molecular alterations that cause the behavioral

changes have not been clearly described. The following studies could be performed to help further elucidate the trends and causes of age-associated behavioral changes in murine MSCs.

1. These studies should be performed using multiple biological and technical repetitions from each age group in order to strengthen the observations of age-related trends.
2. The functional and differentiation assays should be performed using a wider range of animal ages i.e. much younger (fetal or immediately post-natal) and much older (≥ 16 months and greater) to determine if any age-related changes in MSC behavior are non-linear.
3. Global proteomic and genomic studies should be performed on undifferentiated cells from all age groups at early and late passages to differentiate between physiological and *in vitro* aging and to identify pathways involved in aging.
4. Proteomic and genomic studies should be performed on undifferentiated and differentiated cells from all age groups to determine the involvement of various pathways in differentiation and any age-related changes in these pathways.
5. Detailed characterization of the transformed cells should be performed, including investigating donor age as a risk factor for MSC transformation.
6. *In vivo* studies should be performed to identify the age-related differences in implanted MSCs in honing and engraftment ability, immunomodulation, and *in vivo* differentiation potential into the various tissue lineages.

Appendix A: Primers Used

Primer Name	Sequence 5'-3'	Length (bases)	T _M (°C)
Lipoprotein lipase (F) ⁸¹	AGTGGCCGAGAGCGAGAAC	19	60
Lipoprotein lipase (R) ⁸¹	CCACCTCCGTGTAAATCAAGAAG	23	57
Peroxisome proliferator activated receptor γ (F) ⁸²	CGCCAAGGTGCTCCAGAAG	19	59
Peroxisome proliferator activated receptor γ (R) ⁸²	GTCAGCGGGTGGGACTTTC	19	59
Osteocalcin (F) ⁸³	GACCGCCTACAAACGCATCT	20	59
Osteocalcin (R) ⁸³	GGGCAGCACAGGTCCTAAATAGT	23	60
Core-binding factor α -1 (F) ⁸⁴	CTGTGGTTACCGTCATGGCC	20	59
Core-binding factor α -1 (R) ⁸⁴	GGAGCTCGGCGGAGTAGTTC	20	60
β -actin (F) ⁸⁴	TGGGTATGGAATCCTGTGGC	20	58
β -actin (R) ⁸⁴	CCAGACAGCACTGTGTTGGC	20	60
Collagen I (F)	GCTCCTCTTAGGGGCCACT	19	59
Collagen I (R)	CCACGTCTCACCATTGGGG	19	59
Collagen II (F)	TTGGCGAGAAAGGCCAGAG	20	61
Collagen II (R)	AGTCAGACCTCGGCCGCCAT	20	64
Aggrecan (F)	CTAAGTTCCAGGGTCACTGTTACCG	25	60
Aggrecan (R)	TCCTCTCCGGTGGCAAAGAAGTTG	24	63

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