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The University of Southern Mississippi

Characterization of Embryonic Stem Cell-Differentiated Cells as Mesenchymal Stem  
Cells

by

Rachael Nicole Kuehn

A Thesis  
Submitted to the Honors College of  
The University of Southern Mississippi  
in Partial Fulfillment  
of the Requirements for the Degree of  
Bachelor of Science  
in the Department of Biological Sciences

December 2015



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## ABSTRACT

Embryonic stem cells (ESCs), due to their ability to differentiate into different cell types while still maintaining a high proliferation capacity, have been considered as a potential cell source in regenerative medicine. However, current ESC differentiation methods are low yielding and create heterogeneous cell populations. If transplanted in the human body, differentiated ESCs could be rejected by the immune system, form tumors, or may not function normally within the human body. On the other hand, mesenchymal stem cells (MSCs), a type of adult stem cell typically derived from bone marrow, have proved to be excellent candidates in clinical applications due to their defined differentiation capacity and immunoregulatory properties. However, MSCs lack sufficient expansion capacity and can only be derived from limited tissues. This project entails characterizing ESCs differentiated through retinoic acid induction as MSCs. It is speculated that these cells are MSCs due to the extensive similarities in behavior and differentiation capacity. To complete the characterization, the morphology of our MSCs was compared to naturally differentiated MSCs, and a cell cycle analysis was performed. The tentative MSCs were spontaneously differentiated into osteocytes, adipocytes, and chondrocytes, the three distinct cell lineages that characterize MSCs differentiation capacity. Based on the results, our cells were determined to be MSCs, thereby identifying them as ESC-MSCs. This is significant, because it allows for the formation of cells that bypass many of the challenges mentioned above. ESC-MSCs express combined advantages from both ESCs and MSCs, making them even better cell sources for future therapeutic applications.

**Key Words:** mESCs, transcription factors, MSCs, differentiation, regenerative medicine

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## LIST OF ABBREVIATIONS

<b>ATCC</b>	American Type Culture Collection
<b>ASC</b>	Adult Stem Cell
<b>C3H</b>	C3H10T1/2 (cell line of Mouse Mesenchymal Stem Cells)
<b>cDNA</b>	Complementary DNA
<b>C/EBP</b>	CCAT-enhancer-binding protein
<b>COL2A1</b>	Collagen, Type II, Alpha-1
<b>EC</b>	Embryonic Carcinoma
<b>ESC</b>	Embryonic Stem Cell
<b>ESC-MSC</b>	Embryonic Stem Cell-derived Mesenchymal Stem Cell
<b>DEPC</b>	Diethylpyrocarbonate
<b>DMEM</b>	Dublecco's Modified Eagle Medium
<b>FBS</b>	Fetal Bovine Serum
<b>hESC</b>	Human Embryonic Stem Cell
<b>hMSC</b>	Human-derived Mesenchymal Stem Cell
<b>ICM</b>	Inner Cell Mass
<b>iPSC</b>	Induced Pluripotent Stem Cell
<b>LIF</b>	Leukemia Inhibitory Factor
<b>mESC</b>	Mouse Embryonic Stem Cell
<b>mMSC</b>	Mouse-derived Mesenchymal Stem Cell
<b>MSC</b>	Mesenchymal Stem Cell
<b>OCN</b>	Osteocalcin
<b>PBS</b>	Phosphate-buffered Saline
<b>PPAR</b>	Peroxisome Proliferator-Activated Receptor
<b>RA</b>	Retinoic Acid
<b>RT-qPCR</b>	Reverse Transcription Quantitative Polymerase Chain Reaction
<b>RUNX2</b>	Runt-related transcription factor 2
<b>SOX9</b>	Sex Determining Region Y-box 9
<b>TB</b>	Toluidine Blue

## **INTRODUCTION**

Stem cells are characterized as cells that have the ability to continuously proliferate (self-renewal), while also maintaining the capacity to differentiate into different cell lineages (potency). The self-renewal capacity and potency properties of stem cells are what make them different from ordinary somatic cells and exciting tools for cell research. There are two main categories of stem cells including embryonic stem cells (ESC) and adult stem cells (ASC).

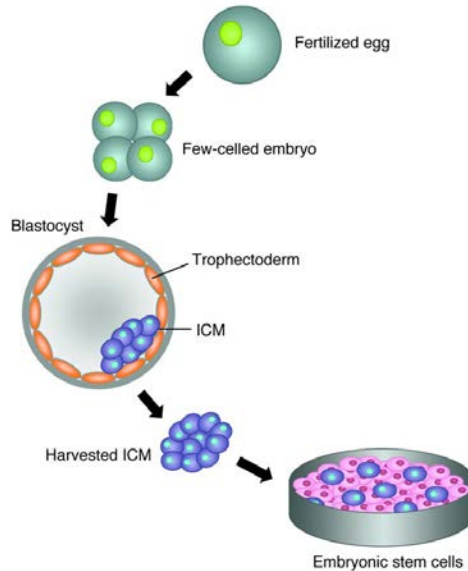
### **Embryonic Stem Cells**

#### ***Background***

About twenty-five years ago, the discovery of ESCs sparked an impressive advancement in cellular biology and medicine (Keller, 2005). Research on ESCs began when the very first stem cells, the embryonic carcinoma (EC) cells, were established in the early 1970s. EC cells were first formed as cell lines from germ line tumors known as teratocarcinomas. Isolated EC cells displayed differentiation abilities and were able to transform into the derivatives of the three primary germ layers: endoderm, mesoderm, and ectoderm. Furthermore, EC cells were found to participate in embryonic development when the cells were transferred to the inner cell mass (ICM) of early chimeric mice embryos. However, after this process occurred, the EC cells lost their pluripotent capabilities and were no longer able to differentiate into specialized cells. Many of these cells also showed chromosomal abnormalities. This setback sparked the search for an alternate stem cell line. It was this investigation that led to the development of ESCs.

In hopes of avoiding the chromosomal alterations associated with teratocarcinoma growth, mouse ESCs (mESC) were isolated in 1981. Using different techniques, two scientists, Evan and Kaufman, were able to successfully cultivate cell lines from mouse blastocysts that preserved the cells differentiation abilities (Wobus & Boheler, 2005). In 1998, Thompson, *et al.* were able to derive human ESC (hESC) cell lines from blastocysts that were generated from in vitro fertilized human eggs.

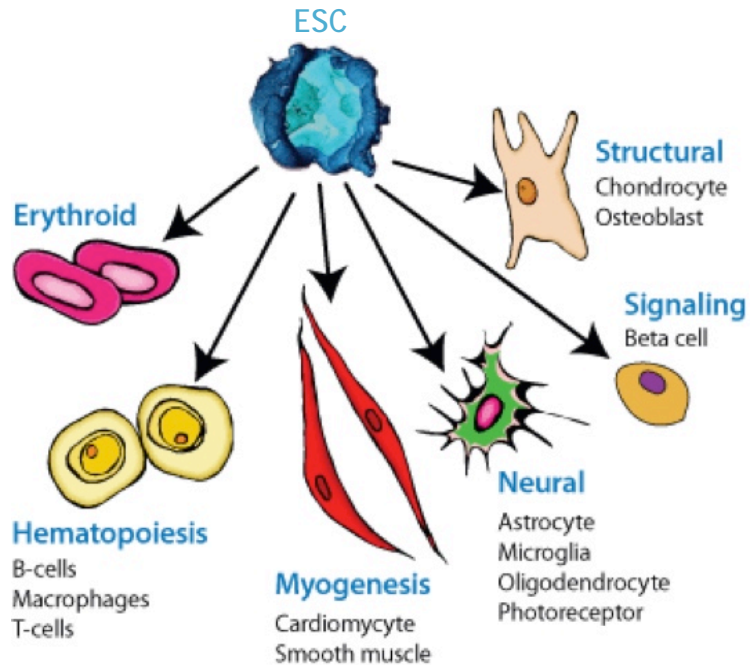
A fertilized egg, the first entity of life, has totipotent capabilities, meaning it is able to produce an entire organism. Totipotency persists in the zygote until about the eight-cell stage, which is also known as the morula. About three to four days after fertilization takes place, a blastocyst is formed through cell differentiation. The blastocyst is composed of outer trophoblast cells, a trophectoderm layer that provides nutrients to the embryo, which further develops into the placenta, and inner undifferentiated cells, which composes the ICM. The cells of the ICM will form all other tissues and organs. They are pluripotent, which is defined as the capacity for a cell to develop into any type of cell given the proper conditions. It is at the stage of the ICM where ESCs are derived as shown in Figure 1 (Wobus & Boheler, 2005).



**Figure 1:** Isolation and Culture of ESCs. After fertilization, the blastocyst forms including both the trophoctoderm layer and the ICM. The ICM is harvested five to seven days after fertilization and embryonic stem cells are cultured (Landry & Zucker, 2004).

The pluripotency of ESCs were demonstrated when they were able to differentiate into all three primary germ layers after implantation into a host blastocyst (Keller, 2005). The primary germ layers include the endoderm, mesoderm, and ectoderm. The cells of the endoderm give rise to the epithelial lining of major body systems including respiratory, gastrointestinal, and urinary. The mesoderm forms connective tissue, cartilage, and bone, and also gives rise to important organs including the kidneys, ovaries, and spleen. Whereas the ectoderm forms the central and peripheral central nervous systems, epidermis, mammary glands, and sensory tissue of the eye, ear, and nose. Together, the three germ layers are able to form every organ in the body (Panski, 1982). When a pluripotent cell becomes a tissue cell of one of the germ layers, it then becomes multipotent. This means it has limited differentiation potential and is restricted to differentiating into cells of specific tissue types. For example, the cells of the inner

germ layer or endoderm are only capable of becoming cells such as pancreas or liver cells. The middle or mesoderm layer cells can only differentiate into cell types such as muscle, bone, or cartilage. The outer germ layer or ectoderm cells are only capable of becoming cell types including epithelial and nerve cells (Wobus & Boheler, 2005). An example of ESC's differentiation potential is shown in Figure 2.



**Figure 2:** The differentiation potential of ESCs. ESCs can differentiate into different cell types that can be used for different purposes including structural (cartilage and bone cells) and signaling (beta cells.) These cells then become specialized with their own specific functions. (Wu & Belmonte, 2014)

### *Characteristics*

One of the hallmark features of ESCs in culture is their ability to remain in the pluripotent state while dividing indefinitely. In vitro, the cells can remain in this state for several years if cultured under the appropriate conditions, but at the molecular level, the maintenance of this undifferentiated state is more complex. It is maintained by several transcription factors working together to promote proliferation and prevent differentiation

(Niwa, 2007). The three main transcription factors involved in this process are Oct4, Nanog, and Sox2. They work by activating target genes that encode self-renewal and pluripotency and repressing the signaling pathways involved in promoting differentiation. There are more than three hundred genes that are simultaneously being either expressed or repressed by these three transcription factors alone (Chan, Yang and Ng, 2011).

The unique cell cycle of ESCs is yet another factor contributing to their stem cell state. Somatic cell cycles involve four discrete stages that conclude in the formation of two daughter cells each with identical contents. During the synthesis phase or S phase of the cell cycle, the genetic information is replicated. The actual physical division of the two cells occurs during the mitotic phase or M phase. Between these two phases exists the gap phases ( $G_1$  and  $G_2$ ), where cell growth and preparation for division occurs. The notable differences in ESC's cell cycle include much shorter gap phases and a longer S phase. In all pluripotent cells, about 60% of the cells are in the S phase, but as ESCs begin to differentiate, their cell cycles become more like that of somatic cells (White & Dalton, 2005).

### ***In Vitro Maintenance of Pluripotency***

The cultivation of ESCs begins five to seven days after fertilization when the ICM is isolated from the blastocyst. The ICM is then cultured in a specific medium to maintain their pluripotent nature (Wobus & Boheler, 2005). Initially, cultivation required ESCs be grown in a culture medium containing both bovine serum and mouse embryonic fibroblasts, a type of feeder cell thought necessary for ESC undifferentiated state (Amit *et al.*, 2006). However, recently it was found that the leukemia inhibitory factor (LIF), and not the feeder cell layer, was responsible for promoting mESC proliferation and

suppressing differentiation in vitro. A part of the interleukin-6 family of cytokines, LIF is a soluble glycoprotein that activates Stat3, promoting the undifferentiated state in mESCs, but not in hESCs (Wobus & Boheler, 2005). It has been noted that the activation of Stat3 is not sufficient to maintain the stem cell state of hESC. This suggests that there is a fundamental difference between the human and murine mechanisms for maintaining pluripotency (Humphrey *et al.*, 2004).

### ***In Vitro Differentiation of ESCs***

Various methods have been established to differentiate ESCs in vitro. Spontaneous differentiation of ESCs is among the most commonly used. When the factors maintaining their stem cell state, such as LIF, are removed, ESCs will spontaneously differentiate into various cell types. By allowing ESCs to spontaneously differentiate in culture, several cell types have been formed including cells from each of the three germ layers, endoderm, mesoderm, and ectoderm (Keller, 2005). Another common ESC differentiation method includes the use of growth factors or cytokines to induce differentiation into specific cell lineages. For example, in order for ESCs to differentiate into endothelial cells, vascular endothelial growth factor and basic fibroblast growth factor are typically used. Though these differentiation methods are capable of forming several different cell lineages, both lead to low-yielding and heterogeneous cell populations that cannot be used for medical application. Due to these deficiencies, other methods of differentiation are being researched.

In 2006, Takahashi and Yamanaka discovered induced pluripotent stem cells (iPSCs). They were able to successfully reprogram mouse somatic cells into pluripotent cells using key transcription factors (Puri & Nagy, 2012). This was an extraordinary



breakthrough in stem cell research, and it is from this discovery that we now know that somatic cells have the potential to be reprogramed to pluripotency. This brought upon this new idea of cell reprogramming, and how specific transcription factors can be used to induce differentiation. This method is believed to be able to differentiate unlimited cell lineages from iPSCs generated from a patient (Daubman, 2011).

These transcription factors have already been identified for a number of cell lineages, including the factors directing differentiation into bone, fat, and cartilage. Transcription factor Runt-related transcription factor 2 (RUNX2) has been named the essential transcription factor for osteogenic differentiation, bone matrix gene expression, and bone mineralization (Nakahara *et al.*, 2010). During adipogenesis, there are a few different transcription factors that play a major role while directing differentiation. Peroxisome Proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been identified as the master regulator for adipocyte formation. Without its expression, precursor cells cannot differentiate into an adipocyte phenotype. Also members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors have also been found to play a role in the late stages of adipogenic differentiation (Siersbaek *et al.*, 2010). In chondrogenic differentiation, the Sex Determining Region Y-box 9 (SOX9) has been identified as the essential transcription factor for chondrogenic differentiation (Akiyama *et al.*, 2004). By utilizing these key transcription factors that control cell fate, it is possible to direct ESCs to differentiate into desired cell lineages.

### ***Biomedical Applications***

Cells in the human body can be dysfunctional for a variety of reasons including injury, genetics, disease, or aging. ESC's pluripotency and ability to proliferate in vitro through an extraordinary, nearly unlimited, self-renewal process make them excellent candidates for cell sources in regenerative medicine. ESCs have the potential to treat several diseases including cancers, Parkinson's disease, spinal cord injuries, muscular dystrophies, diabetes, and several others (Murnaghan, 2014). In cell culture, ESCs have the ability to be grown indefinitely and to be manipulated genetically. Several differentiation strategies for various cell lineages have already been established. Through the manipulation of cell culture conditions and genetics, ESCs could be differentiated into nearly any cell type to be used as treatments for various human diseases and disorders (Wobus & Boheler, 2005).

Though hESCs have a lot of potential, differentiated hESCs exhibit several complications. Currently, the differentiation methods for hESCs are low yielding and create heterogeneous cell populations, meaning the cell populations are not uniform in nature. If transplanted into the human body, the differentiated hESCs could be rejected by the immune system, form tumors, or may not even function normally within the body. Due to different histocompatibility complexes between the patient and donor, immunorejection of ESCs could potentially occur (Drukker, 2004). Also as mentioned earlier, the attenuated immune response of ESCs and their derivatives could cause problems if exposed to pathogens during transplantation (Wang *et al.*, 2013). It is vital that these problems be resolved before hESCs can be used for various cell therapies (Wobus & Boheler, 2005).

### ***Similarities and Differences between mESCs and hESCs***

The majority of the current knowledge regarding the use of ESCs in cell-based therapies comes from the study of animal models such as mESCs. While these models can be helpful in determining the prospective use of ESCs in treatment of human diseases, the use of hESCs would be much more beneficial. Although very similar, differences do exist between mESCs and hESCs. One notable difference is that, unlike mESCs, LIF is not sufficient to inhibit differentiation of hESCs. Some studies show the application of extracellular matrix factors can be used to maintain the stem cell state of hESCs. It has also been found that hESCs possess a longer than average population doubling time in comparison to mESCs. Also, unlike mESCs, hESCs are able to differentiate into trophoblast-like cells. Subtle differences in morphology, expression of differentiation markers, cell cycle, cell-death regulating genes, and patterns of embryonic antigen immunostaining were also found between mESCs and hESCs (Ginis *et al.*, 2003). Because of these differences, more research is necessary to fully understand the application potential of hESCs in cell therapy.

Both mESCs and hESCs have been tested and analyzed in various animal models for human diseases. The first therapeutic demonstration involved the transplantation of mESC-derived cardiomyocytes into the ventricular myocardium of adult mice with muscular dystrophy. Only 7 weeks after implantation, the cells showed several cell markers inferring their differentiation into mature cardiomyocytes. The injected mice were reported to have increased left ventricular function shortly after implantation. This study verified the potential use of ESCs for cardiac therapy. Also, endothelial cells

derived from hESCs were found to form microvessels after their implantation into mice with an immunodeficiency disorder (Wobus & Boheler, 2005).

Although several studies suggest ESC-derived cells to be therapeutically useful, it is still unknown whether these cells could function normally or exhibit long-term functionality within the human body. Currently, there are no ESC therapies being tested with human models. In addition to the technical and biological barriers mentioned for mESCs, the social and ethical concerns as discussed below have delayed the medical application of hESCs.

### ***Ethical/Social Concerns and Alternatives for hESCs***

Because of their embryonic origin, research dealing with ESCs has many ethical and social concerns as well. It is true that blastocysts have the potential to develop a complete and functional organism. The debate lies on the fine line of whether or not a 5-day-old embryo is yet a human being. It is without doubt this debate is based on several misconceptions. It is commonly misconceived that ESCs alone can form an entire organism. A blastocyst is composed of about 100 cells with 30 to 34 of those cells being the ICM. The remaining cells make up an outer layer known as the trophectoderm. This layer is essential for the blastocyst to develop to maturity and for the production of the placenta. In ESC research, the trophectoderm is extracted, and therefore ESCs are only considered pluripotent. They are able to form nearly every tissue type of a human body, but without the ability to form the extraembryonic tissue, a fully, functional organism can never be created in vitro (Gilbert, 2004).

Also, a common belief is that ESCs are derived from aborted fetuses, which is not true. In fact, there is no connection between ESCs and abortion. The current ESC lines

were derived from blastocysts that were voluntarily donated from people participating in In Vitro Fertilization. For In Vitro Fertilization, hundreds of eggs are fertilized in a petri dish with the hopes that one will form a child. Five days after fertilization, a few of these fertilized embryos are then transplanted into the uterus. If the transfer is successful, the remaining embryos are then discarded. These embryos, that would normally be discarded, are what we use today in ESC research if consent from the donor is provided (Gilbert, 2004).

Since the use of ESCs is sometimes referred to as “therapeutic cloning,” some people mistake this for “reproductive cloning,” which is an entirely different mechanism. Human cloning involves the transfer of DNA from the cells of one individual into an egg to form an embryo. Reproductive cloning occurs when this very same embryo is then transplanted into a mother’s uterus, and the developing baby, being genetically identical to the original donor, is brought to full term. On the other hand, therapeutic cloning uses only the blastocysts of these embryos, which then prevents the embryo from developing beyond the blastocyst stage (Gilbert, 2004).

Due to the ethical and social concerns, countries have passed bioethical regulations regarding ESC research (Wobus & Boheler, 2005). The National Institute of Health provides support and funding for public stem cell research in the United States. The institute articulates a clear distinction between “using” ESC and “deriving” ESC. They will only fund research on ESCs already derived from discarded embryos formed through In Vitro Fertilization with uncompensated consent of the donor. NIH will not under any circumstances support research on new ESC lines.

It is because of these issues that adult stem cells (ASCs) are now more commonly used instead of hESCs for therapeutic use. For example, allogeneic ASCs in bone marrow are transplanted as a treatment for Leukemia patients (Wobus & Boheler, 2005). Another alternative to ESCs is the newly found induced pluripotent stem cell (iPSC). In 2006, Takahashi and Yamanaka were able to successfully reprogram mouse somatic cells into pluripotent cells using key transcription factors (Puri & Nagy, 2012) as previously mentioned. Although iPSCs and hESCs exhibit several similarities including morphology, proliferation, surface markers, gene expression, and in vitro differentiation, genetic differences have been detected as well as other concerns. Further study of iPSCs is critical before they can replace hESCs in clinical applications (Takahashi *et al.*, 2007).

### **Adult Stem Cells**

As development of the embryo in the womb continues, organ development becomes required to form a complete and functional organism. It is at this point in development when ASCs are first seen. These cells maintain their self-renewal properties, but are restricted in their potency. After birth, ASCs reside in specific “stem cell niches” throughout the body where their primary purpose is to maintain tissue homeostasis. They do this by consistently replacing damaged cells after natural cell death (apoptosis) or injury (Li & Xie, 2005). They can remain in a non-dividing state for several years, but can then become activated in response to an injury or disease. ASCs exhibit multipotent abilities, meaning they are only able to differentiate into a few specific cell types. They can be found in several mature tissues within the human body including brain, skeletal muscle, bone marrow, teeth, heart, and liver (NIH, 2014).

## **Mesenchymal Stem Cells**

### ***Background***

Research on ASCs began in the 1950s, when it was discovered that two types of stem cells were present in bone marrow. The first type, hematopoietic stem cells, gives rise to all the blood cells in the body including red blood cells, white blood cells, and platelets. The second type of stem cell was found to be bone marrow stromal cells or mesenchymal stem cells (MSC) that are capable of differentiating into a few cell types, primarily bone cells (osteoblasts and osteocytes,) cartilage cells (chondrocytes,) and fat cells (adipocytes) (NIH, 2014). In 1976, Fridenstein *et al.* were the first to describe MSCs as clonal, plastic adherent cells that act as a source for fat, bone, and cartilage. Within the bone marrow, MSCs primary function is to secrete extracellular matrix proteins, growth factors, chemokines, and cytokines, thereby creating a tissue framework for the hematopoietic cell system (Bobis, Jarocha and Majka, 2007).

### ***Characteristics***

In terms of their morphology, physiology, and expression of surface antigens, MSCs create a heterogeneous cellular population. Their functionality largely differs depending on their environmental factors. When MSCs are given sufficient room to grow, they give rise to several fibroblastic colonies. Stem cells that are able to form these colonies are referred to as colony unit forming stem cells. Findings regarding MSC cultures are not entirely consistent. Past studies of MSCs derived from bone marrow have shown homologous colonies expressing only a single cell type, while more recent studies find heterogeneous colonies containing more than one cell type. The proliferation potential of MSCs have also been found to differ from small and rapidly renewing to large and slowly renewing. However, the most recent studies reveal MSC colonies

containing as many as three cell types: some being small and spindle shaped, some flat and cuboidal, and the third type having a large nucleus to cytoplasm ratio, rapid self-renewal properties, and great potential for multilineage differentiation (Bobis *et al.*, 2007).

A single specific cell marker that is mutually expressed throughout all MSCs has yet to be identified. On the other hand, they do express a wide variety of adhesion molecules, cytokines, growth factor receptors, and extracellular matrix proteins. MSCs isolated from bone marrow express several markers in common including: CD44, CD105, CD106, CD166, CD29, CD73, CD90, CD117, STRO-1, and Sca-1. These cells also do not express cell markers specific to hematopoietic and endothelial cell lineages such as CD11b, CD14, CD31, CD33, CD34, and CD45 (Bobis *et al.*, 2007). It is more specifically the lack of antigens CD14, CD34, and CD45 that enable scientists to distinguish MSCs from hematopoietic precursor cells (Minguell, Erices and Conget, 2001).

MSCs have been found to greatly decrease with age. Newborns express the highest amount of MSC with their levels decreasing to about half by the age of eighty (Bobis *et al.*, 2007). MSCs can be found in the previously described “stem cell niches” where they remain inactive until they are confronted with injury, disease, or aging. This is when their self-renewal capacity takes over, and they are able to efficiently replace damaged cells (Minguell *et al.*, 2001). Research is still determining why exactly MSCs remain in this undifferentiated state within their niches. However, some studies indicate that a family of signaling proteins known as Wnt proteins for maintaining MSC’s stem cell state (Bobis *et al.*, 2007).



Though MSCs reside primarily in bone marrow, recent studies suggest they can be found in several other tissues as well. These tissues include trabecular bone, adipose tissue, synovium, skeletal muscle, lung, deciduous teeth, and human umbilical cord perivascular cells (Baksh, Song and Tuan, 2004). It has yet to be established whether or not these MSCs act the same as bone marrow-derived MSCs. It has also been seen that the differentiation potentials of MSCs vary depending on the isolation origin of the cells (Barry and Murphy, 2004).

### ***In Vitro Growth and Tri-Lineage Differentiation Potential***

MSCs growth in-vitro is characterized by three distinct phases. The beginning phase, known as the lag phase, lasts about three to four days, followed by an extensive growth period called the log phase, and finally a stationary phase proceeds. In ideal conditions, bone marrow-derived MSCs can be maintained in vitro for about twenty to thirty population doublings, while still retaining the ability to differentiate (Bobis *et al.*, 2007). The cell cycle profile of MSCs include approximately 10% of the cells in the S, G2, and M phases of the cell cycle and 90% in the G0 and G1 phases (Minguell *et al.*, 2001).

In order for MSCs to differentiate in vitro, a variety of factors including specific differentiation factors, growth factors, basal nutrients, and cytokines are needed. Other factors such as the cell density, mechanical forces, and spatial organization of cells also control MSC's differentiation potential. Studies suggest that the same factors can elicit different differentiation results on various species. For example, when both human derived MSCs (hMSC) and mouse derived MSCs (mMSC) are treated with

dexametasone, hMSCs form osteogenic cell lineages while mMSCs form adipogenic lineages (Bobis *et al.*, 2007).

The exact mechanism through which MSCs differentiate has yet to be determined. It is not clear whether there is a single multipotent MSC that gives rise to each cell lineage or if MSCs make up a mixture of progenitor cells each committed to their own distinct lineage (Bobis *et al.*, 2007). To characterize MSCs, the cells must be able to differentiate into three distinct tissue lineages: adipocytes, osteocytes, and chondrocytes, but MSCs have also been found to exhibit a high degree of plasticity (Kimbrell *et al.*, 2014, Minguell *et al.*, 2001). They have been found to also produce some non-mesenchymal cell types including neural cells, endothelial cells, and muscle cells. The specific factors that promote these types of differentiation in vitro have yet to be identified (Bobis *et al.*, 2007). During tissue growth and repair in vivo, the body displays an elevated demand for cell progenitors. MSCs, being uncommitted progenitors, have been reported to travel to other tissues as a cell source. For example, this was exhibited when MSCs in the bone marrow traveled to muscles to aid in skeletal muscle repair (Minguell *et al.*, 2001).

### ***Biomedical Applications***

MSCs make up only about .001% to .01% of the cells found in bone marrow. For research purposes, it has been found easy to collect a mixture of cells from the adult bone marrow that includes MSCs. However, being that MSCs make up such a small fraction of bone marrow, it is extremely complicated to isolate a pure culture of MSCs, and scientists have yet to find a successful way of doing this (Barry and Murphy, 2004). Though several gaps exist in the research of MSCs, their easy isolation and culture, high ex vivo

expansion potential, and multi-lineage differentiation potential give MSCs a promising future in a wide range of clinical applications including regenerative medicine, gene therapies, and tissue engineering (Minguell *et al.*, 2001).

Several studies have already been conducted to demonstrate MSCs potential in biomedical applications. It has been found that transplanted MSCs are stable when engrafted into various tissues of animal models. Engrafted MSCs were even observed to migrate to specific sites of injury including bone fractures, myocardial infarction, and cerebral ischemia. MSCs also have excellent potential for gene therapies. In one study, genetically modified MSCs were used successfully to transfer a therapeutic gene into a mouse model. The MSCs were genetically modified through transduction with a viral vector, and the transfer to the donor revealed 74% gene transfer efficiency. Genetically altered MSCs have also been tested clinically in humans to treat hemophilia. MSCs were modified to carry coagulation factors VII and IX and then transferred into hemophiliac patients (Bobis *et al.*, 2007).

MSCs have been used successfully for the treatment of bone defects in animal models. Scaffolds containing recombinant bone morphogenetic proteins were used to gather local MSCs to induce bone formation in rats. Furthermore, MSCs are currently being used in several clinical trials to treat osteogenesis imperfecta, a genetic disorder involving over 150 mutations that together cause many abnormalities especially in collagen formation and bone structure. Some of the clinical trials involve engraftment of purified populations of MSCs, MSC gene therapies, and even transplantation of MSCs in utero in some severe cases of osteogenesis imperfecta (Bobis *et al.*, 2007).

MSCs have also been used for tissue engineering in the treatment of cartilage lesions. Wakitani *et al.*, (1994) used collagen sponges filled with MSCs to fill mechanically induced cartilage lesions within white rabbits. Though active chondrocytes and a cartilaginous matrix did indeed form, the new tissue and host tissue were found to be discontinuous. Since then, MSCs stimulated with the growth factors BMP-2 and IGF-1 have been used successfully to repair knee joints (Bobis *et al.*, 2007).

In addition to these uses, MSCs have also been found successful in several *in vivo* tissue repairs including kidney, muscle, and lung repairs. Also, MSCs have been utilized to promote angiogenesis and to treat chronic skin wounds. The biomedical use of MSCs posses fewer limitations than ESCs. Unlike ESCs, MSCs have less ethical concerns and are less probable to trigger tumor formation (Bobis *et al.*, 2007).

#### ***ESC-derived MSCs***

Harvesting MSCs from the body can be a difficult process that requires both a perfect match donor and invasive procedures to extract the cells. Also, only a limited number of MSCs can be isolated from a single donor, and these cells do not have the capacity to proliferate for long periods of time. It is because of these reasons that researchers sought out for a new source of MSCs other than adult tissues. Being that ESCs have the capacity to produce unlimited specialized cells, several studies have reported the differentiation of hESCs into cells that have very similar characteristics to adult tissue-derived MSCs (Hematti, 2011). The potential of ESC-derived MSCs (ESC-MSC) could provide an unlimited source of MSCs for various clinical applications. Due to the inconsistencies regarding the definition for MSCs, the International Society for Cellular Therapy recently put together a list of widely accepted criteria based on

phenotypic and functional characteristics as well as specific culture properties. The criteria include the tri-lineage differentiation into fat, cartilage, and bone tissues. MSCs must be plastic-adherent when grown in standard culture condition. It also includes that MSCs do not express the surface molecules CD34, CD45, CD14, CD11b, CD79 $\alpha$ , CD19, or HLA-DR, but include the surface makers CD73, CD90, and CD105 (Dominici, *et al.*, 2006). In 2005, Barberi, Willis, Socci and Studer. were able to characterize ESC-MSCs using the above criteria after 40 days of coculture hESCs with murine bone marrow stromal cells known as OP9 cells. The gene expression analysis of their ESC-MSCs also showed 579 transcripts in common with human adult MSCs (Barberi *et al.*, 2005). In 2011, Olivier and Bouhassira were able to derive MSCs from ESCs through the “Raclure” method. This method produced ESC-MSCs without the coculture of OP9 cells or any feeder layer (Olivier *et al.*, 2011). Other methods of creating ESC-MSCs include using embryoid body for formation, the plating of hESCs on MSC media, and inhibiting TGF $\beta$  and MAPK signaling pathways (Kimbrel *et al.*, 2014).

ESC-MSC’s potential in biomedical applications has been tested in a few studies using disease models. ESC-MSCs were found to bring therapeutic benefits to mice with trinitrobenzenesulfonic acid-induced colitis. Another study showed ESC-MSCs enhancement of hematopoietic stem cell engraftments. Kimbrell *et al.*, 2014 tested their effects on mice with lupis nephritis and uveitis, two different autoimmune disorders. For lupis nephritis, the ESC-MSCs helped preserve kidney function, thus leading to an increased average lifespan of the mice. The cells were also found to decrease the severity of uveitis in the mice models. They concluded that ESC-MSCs are an excellent alternative to adult tissue-derived MSCs (Kimbrel *et al.*, 2014).

## **RATIONALE, HYPOTHESIS, AND OBJECTIVES**

Due to their self-renewal properties and differentiation potential, ESCs are a promising cell source for regenerative medicine. Though ESCs have great potential, several challenges must be overcome before these cells can be used. Current methods of ESC differentiation produce low-yielding, heterogeneous cell populations that are not sufficient for biomedical applications. Differentiated ESCs have also been found to have an attenuated immune response (Wang *et al.*, 2013), giving us doubt to whether or not they will function normally within the human body.

On the other hand, MSCs, a type of multipotent ASC found within bone marrow, also have excellent characteristics for therapeutic applications. MSCs have already advanced far in clinical applications due to their defined differentiation abilities and immunoregulatory properties. However, these cells can only be derived from limited sources and lack sufficient expansion capacity needed for use in biomedical applications (Baksh, Song and Tuan, 2004).

The research question investigated in this project is to determine if mESC-derived fibroblasts that were previously characterized (Wang *et al.*, 2014) have characteristics in common with MSCs. By characterizing these cells with properties defined for adult tissue derived-MSCs, we will obtain novel information regarding their future use in therapeutic applications. This will allow us to generate cells that possess advantages of both ESCs and MSCs, thus creating a new and improved cell source for future biomedical applications. Two graduate students, William D'Angelo and Chandan Gurung led this research project under Dr. Guo's direction. By participating in this research project, my

objective is to gain a greater understanding of stem cell biology and to learn basic research skills including laboratory techniques and procedures.

## **EXPERIMENTAL DESIGN AND METHODS**

### **Experimental Design**

Through retinoic acid (RA) induction, a cell line has been differentiated from mESCs in Dr. Guo's lab. Initially identified as fibroblasts (Wang *et al.*, 2014), these cells are now believed to share several characteristics with MSCs. The goal of this research project is to characterize these cells as MSCs by examining cell morphology, proliferation rate, cell cycle profile, and tri-lineage differentiation capacity (osteocytes, adipocytes, and chondrocytes; NIH 2014). By characterizing these cells' MSC properties, we aim to show that the ESC-MSCs that are generated will be able to bypass several complications that exist when using adult tissue-derived MSCs for biomedical applications.

### **Cell Culture Techniques**

#### ***Background***

By definition, a cell culture is when cells are isolated from tissues or organs of an organism during the cultivation process. The cells are then grown in an in vitro environment such as a culture dish, containing a medium that provides the essential nutrients for cell survival and growth such as glucose and amino acids. In 1907 Ross Harrison was the first to create an animal cell culture, but it was not until the late 1940s that the cell culture technique was developed enough to be readily used by scientists. Several advances took place before this technique was perfected. First, the discovery and use of antibiotics helped avoid contamination that plagued the early efforts of cell culture.

Then enzymes such as trypsin were discovered to disassociate the cells from their culture dish, enabling scientists to grow continuous cell lines. Lastly, culture media were optimized to allow for sufficient cell growth in vitro. Continuing into the 1960s and even today, commercialization of culture technology is expanding the use and efficiency of cell cultures (Ryan, 2008).

The first step of creating a cell culture is producing the primary culture by removing the cells from the desired tissue and placing them into the appropriate culture environment. This can be done by one of two methods: explant culture or enzymatic dissociation. In an explant culture, tissue pieces are isolated from the source and attached to a culture dish. Culture medium is added to the dish. Cells are then able to move from the tissue explant to the surface of the dish where they begin to divide. The second method, enzymatic dissociation, is more commonly used. For this method, digestive enzymes, such as trypsin, are used to dissolve the extracellular material holding the cells together and form a suspension layer of cells that is then added to a culture dish with medium. The cells are then able to grow and divide (Ryan, 2008).

To allow for continued growth and continuous cell lines, the cells of the primary culture must be subcultured. When the cells from the primary culture have filled the available space of the culture dish, trypsin is used to detach the cells from the culture dish, and the cells are replated at a lower density. Extra cells that are not presently needed can be treated with cryoprotective agents such as glycerol or dimethylsulfoxide, frozen, and then usually stored in a liquid nitrogen tank below  $-130^{\circ}\text{C}$  until they are needed. Rather than establishing a new cell line through primary cultures, cell cultures can also be



purchased from organizations such as the American Type Culture Collection (ATCC) (Ryan, 2008).

Most cell lines derived from normal tissues contain cells that are anchorage-dependent. This means they are only able to grow and divide when attached to an appropriate substrate, a glass or plastic surface of a cell culture dish. Cells such as these require specific attachment factors such as collagen or gelatin, which are used to coat cell culture dishes and allow for better attachment. The culture medium is also very important and must provide all necessary nutrients for cell growth, such as glucose and essential amino acids. The medium should regulate the environment by optimizing pH and osmolality through the use of buffer solutions. Growth factors and hormones are also used within the medium to control the cells' growth rate. These factors are usually provided in the form of 5% to 20% animal sera, such as fetal bovine serum (FBS). Cells are typically stored near 37°C in mammals or the body temperature of the organism in which they were derived (Ryan, 2008).

### ***ESC Culture***

A D3 mESC cell line from ATCC (Toumadje *et al.*, 2003) was cultured in dishes coated with 0.1% gelatin and supplied with Dulbecco's Modified Eagle Medium (DMEM), supplemented with 15% FBS, 1000 U/mL LIF, non-essential amino acids, and 100 µg/mL streptomycin. The cells were then stored in a humidified incubator at 37°C in 5% CO<sub>2</sub>. Once the cells reached 100% confluence, meaning that the surface of the culture dish was completely covered with a monolayer of cells, they were subcultured. Trypsin, phosphate-buffered saline (PBS), and DMEM containing 15% FBS was heated for 5 to 10 minutes at 37°C. The medium was removed from the culture dish by a suction

pipette. PBS was added to the culture dish to wash the cells, and then also removed by the suction pipette. Trypsin was added for 3-5 minutes to detach cells from the dish and then an equal volume of DMEM with 15% FBS was added to deactivate the trypsin. The cells were then placed in 1.5mL tubes and centrifuged at 1,100 rpm for 3.5 minutes. The supernatant was removed, and the remaining cell pellet was resuspended in 1 mL of fresh medium. The cells were replated on new dishes coated with gelatin at 50% to 60% confluence and maintained as described above.

## **In Vitro Differentiation of mESCs**

### ***Retinoic Acid Induction***

Retinoic acid (RA), a vitamin A derivative that regulates many developmental processes during embryogenesis (Keller, 2005), was used to induce mESC differentiation. Cell differentiation was begun by adding 1 $\mu$ M RA to mESCs in a gelatin-coated culture dish. The cells were differentiated over a 10-day period with the cell medium being refreshed three times over that period. Once the cells were differentiated, they were trypsinized and replated in a culture dish without gelatin. The differentiated cells were able to attach within 30 to 45 minutes. They express fibroblast markers and show extensive similarities to 10T1/2 cells (Wang *et al.*, 2014), therefore they are tentatively defined as MSCs, and are referred to as D3-MSCs hereafter.

### ***MSC Culture***

D3-MSCs and C3H 10T1/2 cells (10T1/2, a line of mMSCs isolated from 14-17 day mouse embryos, ATCC) were cultured in DMEM + 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL of streptomycin. The cells were maintained at 37°C in a humidifying incubator in a 5% CO<sub>2</sub> environment. The subculture procedure was identical to that of

ESCs, except the culture dishes were not coated with 0.1% gelatin, as the cells have no problem adhering to plastic. 10T1/2 cells have been used as a model for studying MSC differentiation (Haas & Tuan, 2000) and are used in this project as a positive control. .

## **Toluidine Blue Staining**

### ***Principle of TB Staining***

Toluidine blue (TB) was first discovered in 1856 when a chemist, William Perkin, was attempting to synthesize quinine. Rather than his expected result, he produced a blue substance with considerable staining properties. TB, the first synthetic organic chemical dye, is a polychromatic dye that absorbs various colors depending on which tissues it binds. It can selectively bind to acidic cellular components, having a very high affinity for nucleic acids including both DNA and RNA. On the other hand, TB has less of an affinity for proteins, thus minimizing the background staining of cells (Sridharan and Shankar, 2012). Due to its relatively simple procedure, TB staining is commonly used as a method to determine the number of cells and visualize morphology (Perry, 2014).

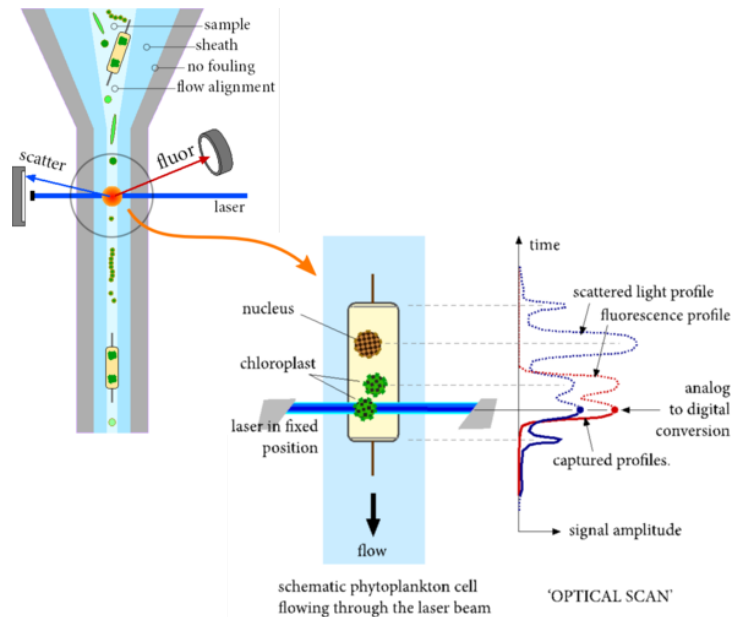
### ***TB Staining Protocol***

D3-ESCs, D3-MSCs, and 10T1/2 cells grown in a 48-well plate were fixed with methanol for 5 to 10 minutes at room temperature. The methanol was then aspirated and cell were air dried for 10 minutes. Cells were stained with 150  $\mu$ l of TB for 30 minutes. A bulb pipette was used to aspirate the stain from each well, and the wells were washed with tap water 2-3 times. The stained cells were visualized using an Olympus CKX31 microscope.

## Cell Cycle Analysis by Flow Cytometry

### *Principle of Flow Cytometry*

Flow cytometry is a technique used to analyze both the physical and chemical properties of cells suspended in a thin stream of fluid and passed single file through a laser. This technology is particularly useful in biological laboratories to study cellular components as shown in Figure 3. Preferred components (i.e. DNA, specific proteins, etc.) are labeled with fluorescent dyes that are then excited by a laser to emit light at varying wavelengths, which is detected and digitized to enable software analysis. One of the first practical uses of flow cytometry was cell cycle analysis through the quantitative measurement of DNA content within cells (Ormerod, Tribukait and Giaretti, 1998).

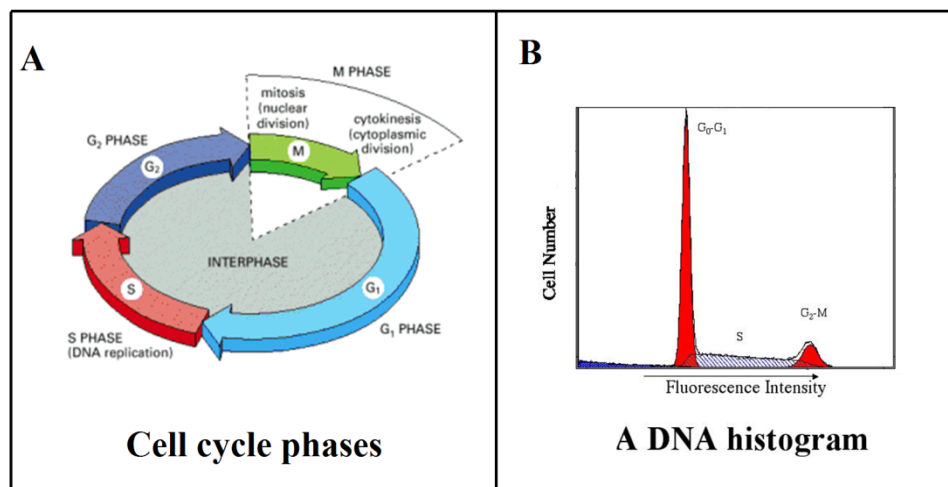


**Figure 3:** The basic principle of flow cytometry. A phytoplankton cell flowing through Flow Cytometry lasers with an exemplary scan (retrieved from <http://www.cytobuoy.com/faq/frequently-asked-questions/>).

The cell cycle is split into distinct phases known as interphase and mitosis.

Interphase can be further broken down into three sub-phases: G1, S, and G2 as shown in

Figure 4A. As discussed earlier, S phase is when the DNA synthesis takes place to replicate the genome. The G1 and G2 phases, also known as the gap phases, involve cell growth and preparation before mitosis, or M phase, can take place. The DNA within the cell can be stained through a variety of DNA binding dyes including propidium iodide (PI), 7-aminoactinomycin-D, as well as several others. These particular dyes are special in that they are stoichiometric, meaning they are able to bind proportionately to the amount of DNA in the cell. Cells in G2 or M phases (after DNA replication) will have double the amount of DNA of cells in G1, and therefore fluoresce twice as brightly, while cells in S phase will have an intermediate amount of amount of DNA and thus fluorescence. This principle can be seen in Figure 4B (Ormerod, 1998).



**Figure 4:** The Phases of the Cell Cycle (A) and an example of a diploid DNA histogram measured through Flow Cytometry (B) (Tabll and Ismail, 2011).

### *Cell Cycle Analysis Protocol*

D3-ESCs and D3-MSCs were harvested at 30% to 80% confluence in 6-well dishes. They were then washed in PBS, detached with 0.4 mL trypsin, and collected in a 1.5 mL tube. The wells were each washed with 0.4 mL of medium, which was then

combined with the trypsinized cells. The cells were then centrifuged at 1200 rpm for 3 minutes. The supernatant was removed, and the pellet was broken up and resuspended in 200  $\mu$ l of PBS+0.1% FBS. The cells were then fixed in 800  $\mu$ l of 100% ethanol at 4°C for 30 minutes. Fixation is necessary to prevent deterioration of the cells and allow permeabilization of the membrane to dyes or antibodies. After fixation, the cells were centrifuged again at 1500 rpm for 5 minutes, and resuspended in 0.5 mL of PBS+0.1% FBS to wash. After another centrifugation, cells were resuspended in 50  $\mu$ L of PBS plus 1  $\mu$ L of RNase and incubated at room temperature for 10 minutes. The RNase breaks down all RNA within the cell, so only the DNA is measured allowing for fewer distortions in the results. 100  $\mu$ L of FACS buffer, containing both PBS and 2% FBS, and 10  $\mu$ L of PI (1 mg/mL) were added to the cells. They were then incubated at 4°C for 30 minutes. The results were analyzed using a flow cytometer. Both the forward and side scatter of the cells were measured to identify single cells.

### **Tri-lineage Differentiation Techniques**

#### ***Principle of Spontaneous Differentiation***

The principle of mESC differentiation is that, in the absence of LIF, mESCs begin to spontaneously differentiate into various cell types. For effective cell type-specific differentiation, specialized media that contain specific growth factors or cytokines are commonly used. Although not very effective, it has been demonstrated that MSCs can spontaneously differentiate in the absence of growth factors or specific environmental conditions. Naruse *et al.*, 2004, allowed MSCs obtained from fetal rat circulation to differentiate in a normal medium without any additional factors. Both chondrocytes and osteocytes were formed through spontaneous differentiation of the MSCs (Naruse *et al.*,

2004). As an initial assessment, D3-MSCs were spontaneously differentiated for 4 weeks to test their tri-lineage differentiation potential into osteocytes, adipocytes, and chondrocytes.

### ***Differentiation Protocol***

D3-MSCs and 10T1/2 cells were plated in DMEM + 10% FBS and allowed to grow for 4 weeks without subculturing. During this time, the medium was changed regularly, approximately every 2 to 3 days. The cells were positively stained with respective dyes for detection of cellular products. To compare levels and intensity of staining, cells were stained after 3 days to use as an undifferentiated control and again after 4 weeks of growth.

### ***Osteocyte Staining***

Osteocytes, or bone cells, have the capacity to undergo mineralization, which is when cells produce extracellular calcium deposits when grown in vitro. The formation of calcium deposits indicates successful osteogenic differentiation. Alizarin Red can be used to stain calcium deposits a bright orange-red color to test for successful bone formation (Gough, Jones and Hench, 2004). Prior to staining, the cells were fixed in methanol, and 2% Alizarin Red S was filtered before use. The cells were washed twice in PBS, then stained with 2% Alizarin Red S for 5 minutes. They were then washed 3 times in distilled water and visualized with a microscope.

### ***Adipocyte Staining***

Oil Red O is a stain that can be used to detect the extent of adipocyte differentiation. It does so by staining the intracytoplasmic lipids within the cells (Ramirez-Zacarias, Castro-Munozledo and Kuri-Harcuch, 1992). Both D3-MSCs and

10T1/2 cells were stained to confirm adipocyte formation. Prior to staining, the cells were fixed in methanol, and 0.5% Oil Red O was diluted in a 3:2 ratio (stain: distilled water). The stain was mixed, left to stand for 10 minutes, and then filtered before use. The cells were washed twice in PBS stained with 0.5% Oil Red O for 5 minutes, washed 3 times in PBS, and visualized with a microscope.

### ***Chondrocyte Staining***

Safranin O is a cationic dye that is used to quantify the amount of proteoglycans present in cartilage. When chondrogenic differentiation occurs, extracellular matrix proteins including type II collagen and the proteoglycan aggrecan begin to form. The higher the intensity of the safranin staining, the higher the proteoglycan content (Camplejohn and Allard, 1988). To test for successful chondrocyte formation, both D3-MSCs and 10T1/2 cells were stained with Safranin O. Prior to staining, the cells were fixed with methanol and washed three times with PBS. The cells were stained with 0.1% Safranin O for 5 minutes, washed with distilled water, and visualized with a microscope.

### **RNA Extraction and RT-qPCR**

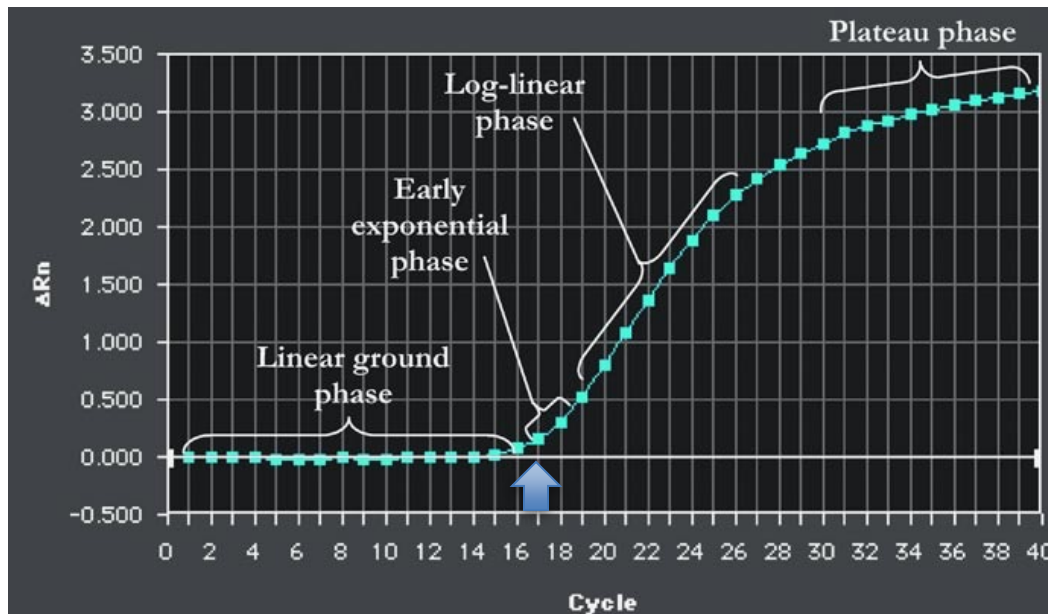
#### ***Principle of PCR-based Gene Expression Analysis***

Real time quantitative polymerase chain reaction (RT-qPCR) is a procedure that measures amplified DNA through the use of fluorescent molecules. DNA binding chemicals such as SYBR green bind to double stranded DNA and become fluorescent. As DNA is amplified, double stranded DNA begins to increase, and in turn so does the fluorescence. This allows for the measure of DNA amplification. RT-qPCR can be split into four phases: the linear ground phase, early exponential phase, log-linear phase, and plateau phase as shown in Figure 5. During the linear ground phase, there is not enough



DNA for an increase in fluorescence to be detected, but the baseline fluorescence can be calculated. In the early exponential phase, the fluorescence has reached a threshold where an increase can be detected. The cycle in which this occurs is called  $C_t$ , indicated by the arrow in Figure 5. The  $C_t$  value is used as a measure of the original amount of complementary DNA (cDNA). In the log-linear phase, DNA reaches optimal amplification and is approximately doubled during each cycle. Finally, the plateau phase is when the reaction components, such as nucleotides, become scarce, DNA amplification slows down, and the fluorescence intensity is no longer needed for calculations. The relative expression of genes of interest can be determined by comparing  $C_t$  values to housekeeping genes, or genes that are stably expressed across all samples and treatments being tested. Some common housekeeping genes are  $\beta$ -actin and GAPDH (Wong & Medrano, 2005). For analysis, one of the most common methods to use is the comparative  $C_t$  method. This method uses a formula to compare the relative expressions of the gene of interest and housekeeping gene. The formula is as follows (Pfaffl, 2001):

$$\frac{\text{Expression in experimental group}}{\text{Expression in control group}} = \frac{2^{(C_t \text{ ref} - C_t \text{ gene of interest})_{\text{experimental}}}}{2^{(C_t \text{ ref} - C_t \text{ gene of interest})_{\text{control}}}}$$



**Figure 5:** Phases of RT-qPCR. The arrow indicates the  $C_t$  value (modified from Wong & Medrano, 2005).

### ***RNA Extraction***

Sigma Tri-reagent was used to isolate RNA from the cells. 0.6mL was added to each well in the culture dish (6-well plate). The cells were then transferred to 1.5mL microfuge tubes. 0.2mL of chloroform per mL of Tri-reagent was then added to each microfuge tube. The cells were vortexed for 15 seconds and then incubated at room temperature for 10 minutes. After incubation, the cells were centrifuged at 10,000 rpm for 10 minutes at 4°C, and three different phases were formed: aqueous phase, interphase, and organic phase. The aqueous phase containing the RNA was transferred to another tube. Due to the amphipathic characteristics of proteins, they were attracted to both the organic and aqueous phases and were found in the interphase or flocculent. Due to both the acidic conditions and the presence of phenol, DNA ended up in the organic phase and interphase. Furthermore, the phosphate groups of DNA are neutralized by  $H^+$ , causing it to be nonpolar, thus making DNA be attracted to both the organic phase and interphase.

Next, 0.5 mL of 100% isopropanol per mL of Tri-reagent was added to the aqueous phase containing the RNA. The sample was then incubated at room temperature for 10 minutes before centrifugation at 12,000 rpm for 15 minutes at 4°C. After centrifugation, a white pellet of RNA had precipitated. The liquid was removed from the tube, leaving only the RNA pellet, and 700 µL of 75% ethanol were added to wash the pellet. The sample was vortexed and put in the freezer at -20°C for at least 1 hour to allow for the RNA to precipitate. The sample was then centrifuged at 10,000 rpm at 4°C for 10 minutes. The ethanol was removed, and the pellet was dried on ice for 5 minutes. The pellet was dissolved in 20µL of Diethylpyrocarbonate (DEPC) water. Using a spectrophotometer, both the concentration and integrity were determined. The sample was stored at -70°C for later use.

### ***Reverse Transcription Protocol***

In order to generate cDNA for use in RT-PCR, reverse transcription was performed using M-MLV reverse transcriptase. For each sample, 1 µg RNA was diluted to a 11.5 µL total volume with sterile water. Then 1 µL of deoxyribonucleotide mix and 2 µL of oligo(dT) primer was added. Samples were then vortexed, incubated at 70°C for 5 minutes, and placed on ice. Then 4µL of 5X buffer and 1 µL reverse transcriptase were added and samples were placed in a thermal cycler and incubated at 42°C for 1 hour, then 95°C for 10 minutes to inactivate the enzyme. Finally 200µL of sterile water were added and samples were stored at -20°C until later use.

### ***Semi-quantitative PCR Protocol***

A 20 µL solution was made for each sample by combining 10 µL of 2X SYBR mix, 3 µL of DEPC water, 5 µL of cDNA, and 2 µL of primers specific to the gene of

interest. Samples were run for 35 cycles (10 min at 95°C initial denaturation, then 35 cycles of 95°C for 15s for denaturation, and 60°C for 1 min for annealing/elongation) on a Stratagene MX3000P real-time PCR thermal cycler using MxPro software, and data were analyzed through the use of the comparative  $C_t$  method (Pfaffl, 2001). The housekeeping gene used for comparison was  $\beta$ -actin. Primer sequences were as follows:

Gene	Forward Primer Sequence	Reverse Primer Sequence
C/EBP $\alpha$	5'- CAAGAACAGCAACGAGTACCG-3'	5'-GTCACTGGTCAACTCCAGCAC-3'
PPAR $\gamma$	5'-GGAAGACCACTCGCATTCTT-3'	R: 5'-GTAATCAGCAACCATTGGGTCA-3'
RUNX2	F: 5'-GCCCAGGCGTATTTTTCAGA-3'	R: 5'-TGCCTGGCTCTTCTTACTGAG-3'
OCN	5'-CTGACCTCACAGATGCCAAG-3'	R: 5'-GTAGCGCCGGAGTCTGTT-3'
SOX9	F: 5'-AGTACCCGCATCTGCACAAC-3'	R: 5'-ACGAAGGGTCTCTTCTCGCT-3'
COL2A1	F: 5'-GGGTACAGAGGTTACCCAG-3'	R: 5'-ACCAGGGGAACCACTCTCAC-3'

## RESULTS

### Comparison of mESCs, D3-MSCs, and 10T1/2 Cell Morphology

To begin the characterization process, the morphology of D3-ESCs, 10T1/2 cells, and D3-MSCs was compared after the cells were stained with TB. As shown in Figure 6, D3-ESCs exhibit typical ESC morphology of undifferentiated cells growing together in clustered colonies. They also possess a large nucleus to cytoplasm ratio. According to Wobus and Boheler (2005), ESCs typically grow in tight, rounded, multilayered clusters. On the other hand, the D3-MSCs completely lost the morphology of ESCs. They instead expressed flattened, elongated spindle-shaped cells very similar to 10T1/2 cells. Both populations of cells were similar in morphology and grew in a uniform monolayer, very

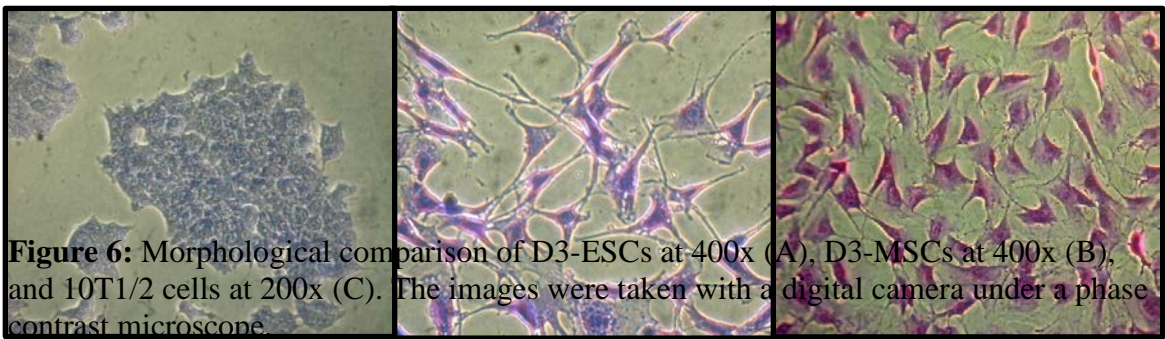
different from the colonies of D3-ESCs. According to Baksh, Song and Tuan (2004), the gold standard assay used to identify MSCs is to identify adherent, spindle-shaped cells.

**A**

**B**

**C**

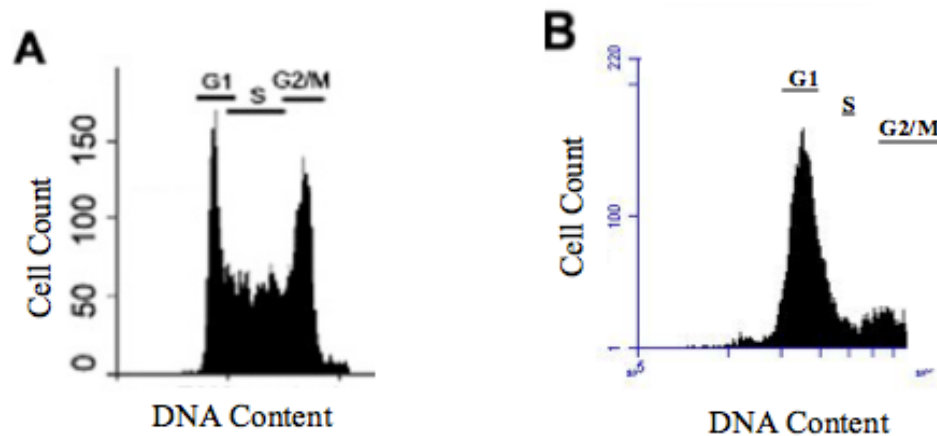
Based on the results in Figure 6, D3-MSCs were confirmed to possess MSC morphology.



### **Cell Proliferation and Cell Cycle Profile**

The cell cycle profiles of D3-ESCs and D3-MSCs were analyzed by flow cytometry. This profile measures both the number of cells at each phase of the cell cycle (Y-axis) and the amount of DNA at that particular phase (determined by fluorescence intensity, X-axis). The first peak in the profile represents cells found in the G1 phase, while the second peak corresponds to cells found in the G2 and M phases of the cell cycle, and cells in between these peaks are in S phase, as labeled in Figure 7. For D3-ESCs, the cell cycle profile included a high percentage of cells in the S, M, and G2 phases of the cell cycle, meaning that a lot of cells were actively dividing and rapid cell division was occurring. mESCs are characterized by their high proliferation rate (Wobus and Boheler, 2005), and their cell profiles typically show about 60% of cells in the S

phase. On the other hand, the cell cycle profile of D3-MSCs show a reduced cell population at the S, G2, and M phases of the cell cycle, indicating MSC's slower proliferation rate when compared with D3-ESCs. MSCs are characterized by having only 10% of the cells in the S, G2, and M phases of the cell cycle and 90% in the G0 and G1 phases, indicating that only a small fraction of MSCs are actively engaged in proliferation (Minguell *et al.*, 2001). Based on this information, the cell cycle profile of D3-MSCs was more similar to the cell cycle of MSCs than ESCs.

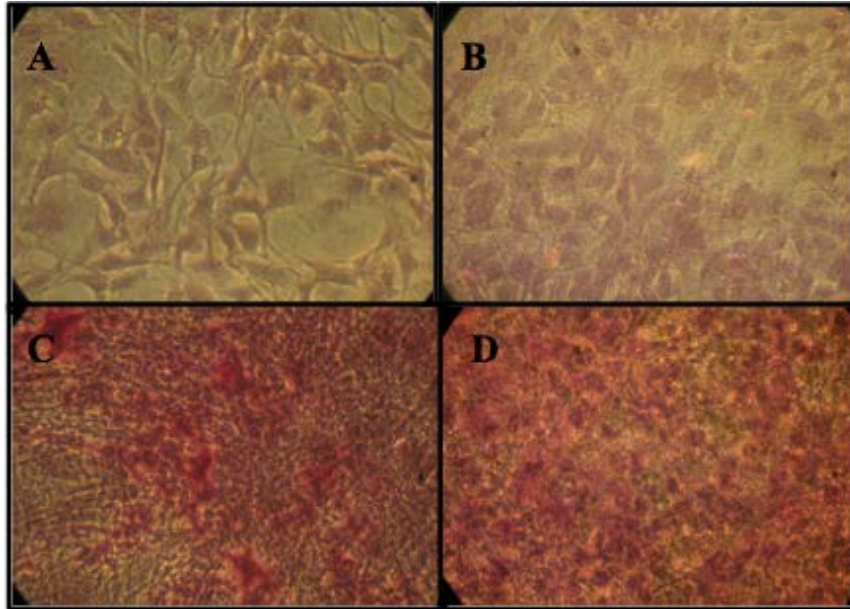


**Figure 7:** Cell Cycle Profiles of D3-ESCs (A) and D3-MSCs (B) determined by flow cytometry.

### Osteogenic Differentiation

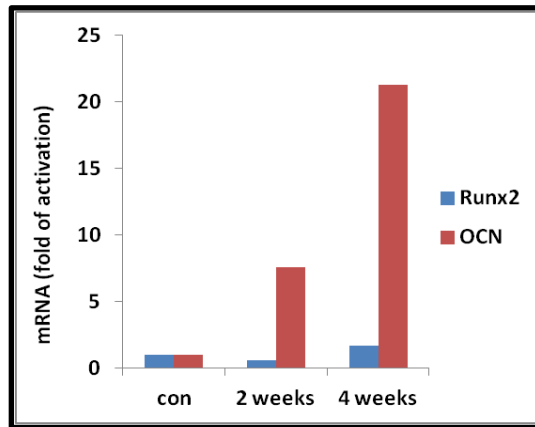
To confirm that osteogenic differentiation had occurred after 10T1/2 cells and D3-MSCs were given 4 weeks to spontaneously differentiate, the cells were stained with Alizarin Red as previously described. They were stained both 3 days into differentiation and again after 4 weeks of differentiation. The results in Figure 8 show strong staining at

the 4 week timepoint compared with undifferentiated controls in both cell types, thus indicating that mineralization had occurred and osteocytes had been formed (Gough *et al.*, 2004).



**Figure 8:** Osteogenic Staining. 10T1/2 cells (A) and D3-MSCs (B) 3 days into spontaneous differentiation after staining with Alizarin Red and 10T1/2 cells (C) and D3-MSCs (D) four weeks into spontaneous differentiation after staining with Alizarin Red. All images are taken at 400x magnification under a phase contrast microscope.

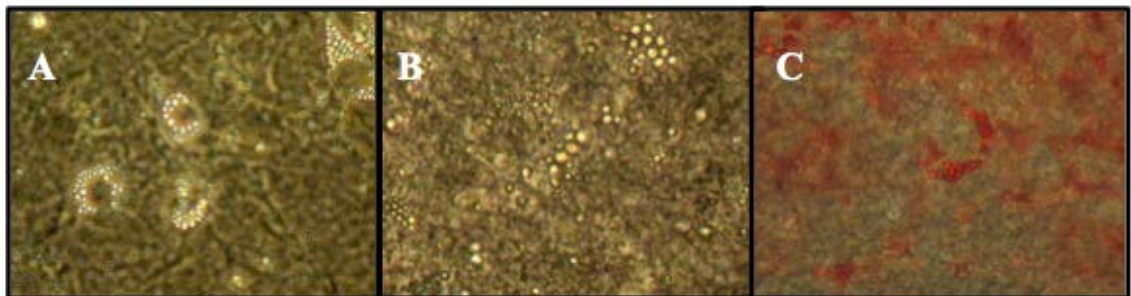
To further confirm that osteogenic differentiation had occurred, the expression of specific transcription factors and structural proteins in D3-MSCs was measured using RT-qPCR. The genes that were measured included the transcription factor, RUNX2, and the structural protein osteocalcin (OCN). RUNX2 has been identified as a primary transcription factor needed for osteocyte differentiation, bone matrix gene expression, and bone mineralization (Nakahara *et al.*, 2010). OCN is used as a marker to identify late stages of osteogenic differentiation (Granéli *et al.*, 2014). As shown in Figure 9, OCN expression increased over the 4 week differentiation period, suggesting that osteogenic differentiation had occurred.



**Figure 9:** Expression of Runx2 and OCN in undifferentiated D3-MSCs (con) and 2 weeks and 4 weeks into spontaneous differentiation determined by RT-qPCR.

### Adipogenic Differentiation

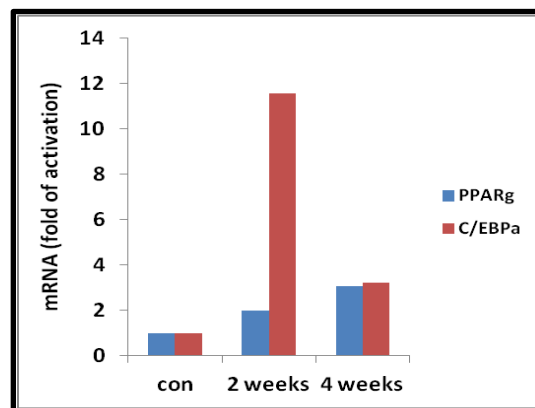
After 10T1/2 cells and D3-MSCs were spontaneously differentiated, lipid droplets became visible in the cytoplasm. Adipocytes in both populations of cells can be seen in Figure 10 (A and B) after spontaneously differentiating for 4 weeks. To confirm that adipogenic differentiation had occurred, D3-MSCs were positively stained with Oil Red O (Figure 10 C). This stain is used for the detection of intracytoplasmic lipids present within cells (Ramirez-Zacarias *et al.*, 1992).



**Figure 10:** Adipogenic differentiation. 10T1/2 cells (A) at 400x magnification and D3-MSCs (B) at 560x magnification observed 4 weeks into spontaneous differentiation, but prior to staining. D3-MSCs (C) positively stained with Oil Red 4 weeks into spontaneous differentiation (400x). All images are taken under a phase contrast microscope.



To further confirm adipogenic differentiation, RT-qPCR was used to measure the expression of transcription factors essential to adipogenic differentiation, including PPAR $\gamma$  and C/EBP $\alpha$ . Both families of transcription factors have been found to play important roles during adipocyte differentiation. Several studies have confirmed PPAR $\gamma$  to be the key transcription factor in adipocyte differentiation in both in vitro and in vivo environments. C/EBP $\alpha$  is expressed during late stages of differentiation and is also one of the key regulators in adipocyte differentiation (Siersbaek *et al.*, 2010). Expression of both transcription factors was somewhat increased in D3-MSCs at both 2 weeks and 4 weeks after spontaneously differentiating as shown in Figure 11, indicating adipogenic differentiation.

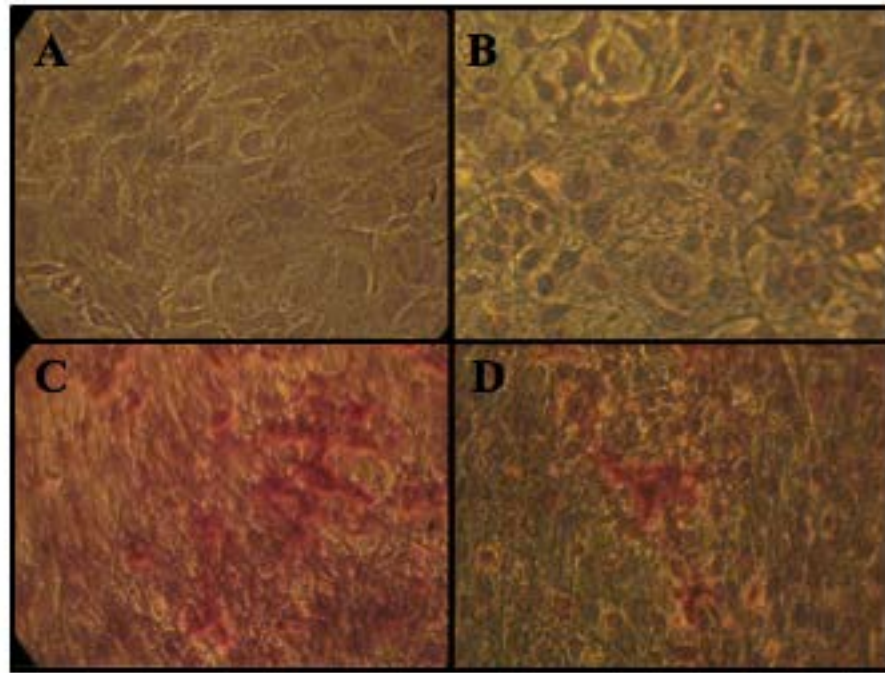


**Figure 11:** Expression of PPAR $\gamma$  and C/EBP $\alpha$  in undifferentiated D3-MSCs (con) and after 2 weeks and 4 weeks of spontaneous differentiation determined by RT-qPCR.

### Chondrogenic Differentiation

As previously described, D3-MSCs and 10T1/2 cells were stained with Safranin O during their spontaneous differentiation to detect whether or not chondrogenic differentiation had occurred. The cells that were stained after only 3 days had not yet differentiated and were not able to retain the Safranin when stained. On the other hand,

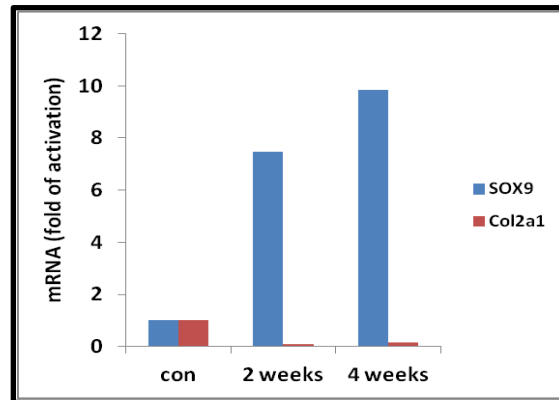
the cells that had been given 4 weeks to spontaneously differentiate stained an intense red color indicating that the cells contained high proteoglycan content and chondrocytes were formed (Camplejohn and Allard, 1988).



**Figure 12:** Chondrogenic differentiation. Undifferentiated 10T1/2 cells (A) and D3-MSCs (B) and cells after 4 weeks of differentiation (C and D) stained with Safranin O. All images are shown at 400x magnification under a phase contrast microscope.

The expression of chondrogenic differentiation markers in differentiated D3-MSCs was measured using RT-qPCR as previously described. These markers included the transcription factor SOX9 and COL2A1, the protein-coding gene that produces type II collagen. SOX9 is a transcription factor that is present in all differentiated chondrocytes and plays an essential role in the early stages of chondrogenic differentiation. This transcription factor is also needed to activate the COL2A1 gene to begin type II collagen formation. All mature chondrocytes have been found to express type II collagen (Akiyama *et al.*, 2004). As shown in Figure 13, the expression of SOX9

was expressed highly in D3-MSCs both 2 and 4 weeks into spontaneous differentiation, indicating that beginning stages of chondrocyte formation had occurred. COL2A1, on the other hand, was not expressed in D3-MSCs indicating a lack of mature chondrocytes in the sample.



**Figure 13:** Expression of SOX9 and COL2A1 in undifferentiated D3-MSCs (con) and the same cells after 2 4 weeks of spontaneous differentiation determined by RT-qPCR.

## DISCUSSION

This goal of this study was to determine whether mESC-derived fibroblasts, that we have previously characterized, have properties of MSCs. By participating in this research project, my objective was to gain a greater understanding of stem cell biology and to become familiar with basic research skills and laboratory procedures.

Based on the morphology comparison in Figure 6, D3-MSCs are clearly more similar in shape to 10T1/2 cells than to D3-ESCs. Both D3-MSCs and 10T1/2 cells possessed a flattened, elongated, spindle-shaped morphology, therefore displaying typical MSC morphology (Baksh, Song and Tuan, 2004.) The results in Figure 7 demonstrated that the cell cycle profile of D3-MSCs was more similar to that of MSCs than ESCs. This was due to the reduced cell population found in the S, G2, and M phases of the cell cycle,

indicating a lower proliferation rate than that of D3-ESCs. When D3-MSCs were spontaneously differentiated for 4 weeks, they were able to form osteocytes, adipocytes, and chondrocytes although these cells are not yet fully matured as indicated by the lack of COL2A1 in chondrogenic differentiation, therefore confirming the tri-lineage differentiation potential characteristic to MSCs. By staining the cells with Alizarin Red, Oil Red, and Safranin and also measuring the expression of differentiation markers through RT-qPCR, we were able to further confirm the identity of the differentiated cells, and thus the tri-lineage differentiation potential. In conclusion, based on the results from morphology, cell cycle profile, and tri-lineage differentiation potential, this research provide strong evidence that D3-MSCs do possess several characteristics in common with MSCs.

Though this study was able to provide several similarities between D3-MSCs and MSCs, it only focuses on some basic aspects of MSC characterization, thus more research is needed to further confirm their true identity, such as tri-lineage differentiation under defined conditions with specific inducers for each of the three cell types. Some other aspects of these cells that should be analyzed include surface antigens and immunomodulatory properties. There is a specific set of surface antigens that are expressed in MSCs. Moreover, MSCs are characterized by having both the presence and absence of specific cell markers. Analysis of marker expression could be performed in D3-MSCs through either flow cytometry or immunocytochemistry. MSCs have been found to exhibit modulatory effects on the immune system. This characteristic brings a lot of interest to their use in future biomedical applications. The innate immunity of D3-

MSCs should be researched to see if they have similar responses to MSCs (Bio-Techne, 2014).

This study is significant, because the ability to generate MSCs from ESCs bypasses many of the complications experienced when using adult tissue derived-MSCs. Adult tissue-derived MSCs, such as bone-marrow-derived MSCs, have a lot of potential for biomedical applications, but the major problem interfering with their use is their limited numbers due to limited tissues of derivation (Baksh, Song and Tuan, 2004). The ability to create MSCs from ESCs could be able to bypass this difficulty, providing an almost unlimited source of MSCs for biomedical applications. In conclusion, the characterization of D3-MSCs and the ability to generate MSCs from ESCs is a significant stride forward towards the use of ESCs in regenerative medicine.

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