CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF RAT BONE MARROW MESENCHYMAL STEM CELLS INTO CARDIAC-LIKE CELLS

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ABSTRACT

Heart diseases are the leading cause of death worldwide. Despite the development of a broad array of treatment options, current therapies only delay progression of the disease and failed to prevent myocardial scar formation and replace the lost cardiomyocytes (cardiac muscle cells). Over the past decade the use of adult stem cells, particularly bone marrow derived mesenchymal stem cells, to safely facilitate recovery of cardiac function after myocardial infarction has received a lot of interest. Mesenchymal stem cells (MSCs), which are adherent stromal cells of a non-hematopoietic origin, have great differentiation potential and under appropriate *in vitro* culture conditions can trans-differentiate into cardiomyocyte cells. This study investigated the characterization of rat bone marrow derived-mesenchymal stem cells (BM-MSCs) and *in vitro* differentiation potential of them into cardiomyocyte-like cells by two DNA-demethylating agents, 5-azacytidine and zebularine. MSCs were isolated from *Sprague Dawley's* bone marrow and cultured in complete Dulbecco's Modified Eagle Medium (DMEM). Morphological characteristics of MSCs were analyzed by phase contrast microscopy. Selected surface antigens CD44, CD117, known MSCs markers, and CD34, a hematopoietic marker (negative marker), were analyzed by immunocytochemistry. In addition, CD45, known hematopoietic marker (negative marker) and CD44 were analyzed by flow cytometry for the MSC cell population count. Passage 1 (P1) cultured MSCs were then treated in separate culture flasks for 24 hours with a 3 μM optimized concentration of 5-azacytidine and zebularine. After 20 days, treated cells were analyzed for the expression of rat cardiac specific genes; namely, alpha-myosin heavy chain (CAMHC), cardiac troponin-T (cTnT), and cardiac transcription factor (GATA-4) by reverse transcriptase polymerase chain reaction (RT-PCR). The endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an

internal standard gene for normalization of mRNA. The isolation of MSCs from rat bone marrow was successfully completed. Isolated MSCs exhibited spindle-shaped morphology with adherence ability to the surface of flasks and proliferated in the culture medium. Immunocytochemistry results showed that cell surface antigen expression was observed to be positive for CD44 and CD117. However, MSCs were negative for CD34 (hematopoietic marker); hence, confirming the absence of hematopoietic cells. Furthermore, CD44 was found to be >85% positive, while CD45 was more than 60% negative in MSCs after flow cytometry cell population analysis. Upon induction with 5-azacytidine and zebularine, the morphology of the MSCs changed and the cells showed extended cytoplasmic processes with ball-like appearance. After 20 days, they were connected with adjoining cells forming myotube-like structures. The mRNAs of CAMHC, cTnT and GATA-4 were detected in both treated and untreated cells. However, RT-PCR analysis for the expression of cardiac specific genes showed that treated MSC cells expressed cTnT, CAMHC and GATA-4 significantly higher compared to untreated cells. While there were no significant differences between 5-azacytdine and zebularine treated cells, zebularine could be a good replacement for 5-azacytidine as it is more stable and less toxic to biological system. These results showed that bone marrow mesenchymal stem cells (BM-MSCs) could differentiate *in vitro* towards a cardiomyogenic lineage.

ABSTRAK

Penyakit jantung adalah punca utama kematian di seluruh dunia. Walaupun terdapat kemajuan dalam pelbagai opsyen rawatan, terapi semasa hanya dapat melambatkan perkembanga penyakit dan gagal untuk menghalang pembentukan parut miokardium dan menggantikan kardiomiosit sel-sel yang hilang. Sepanjang dekad yang lalu, penggunaan sel stem dewasa, terutamanya sel-sel stem mesenkimal yang diperolehi dari sum-sum tulang untuk memudahkan pemulihan fungsi jantung dengan selamat selepas infarksi miokardium telah mendepat banyak pnumpuan. Sel-sel stem mesenkimal (*Mesenchymal Stem Cells*) yang merupakan sel stromal *adherent* yang bukan berasal dari hematopoietik, mempunyai potensi yang besar dalam pembezaan/diferensiasi dan di bawah keadaan *in vitro* kultur yang sesuai boleh trans-diferensiasi untuk menjadi sel kardiomiosit. Kajian ini menyiasat tentang pencirian sel-sel mesenkimal yang diperolehi dari sum-sum tulang tikus (BM-MSCs - *Bone Marrow Mesenchymal Stem Cells*) dan potensi diferensiasi *in vitro* sel-sel tersebut menjadi sel-sel mirip kardiomiosit dengan menggunakan dua ejen demetilasi DNA, 5-azacytidine dan zebularine. MSCs telah diasingkan daripada sum-sum tulang *Sprague Dawley* dan dikultur di dalam *Dulbecco's Modified Eagle Medium* (DMEM) yang lengkap. Ciri-ciri morfologi MSCs dianalisa dengan menggunakan mikroskop fasa-kontras. Antigen permukaan yang terpilih CD44, CD117, yang dikenali sebagai penanda bagi MSCs, dan CD34, suatu penanda bagi hematopoietik (penanda negatif), dianalisa dengan immunositokimia. Sebagai tambahan , CD45, iaitu penanda bagi hematopoietik (penanda negatif) dan CD44, dianalis dengan *flow cytometry* untuk mendapatkan bilangan populasi sel-sel MSC. Pasaj 1 (P1) MSC yang telah dikulturkan kemudiannya dirawat di dalam kelalang kultur yang berasingan selama 24 jam dengan kepekatan 3µM 5-azacytidine dan zebularine yang telah dioptimakan. Selepas 20 hari, sel-sel yang telah

dirawat dianalisa untuk ekspresi gen-gen spesifik jantung tikus; iaitu *alpha-myosin heavy chain* (CAMHC), *cardiac troponin-T* (cTnT), dan *cadiac transcription factor* (GATA-4) dengan menggunakan *reverse transcription polymerase chain reaction* (RT-PCR). BM-MSC yang telah diasingkan mempamerkan morfologi berbentuk gelendong dengan keupayaan melekat kepada permukaan kelalang dan telah berkembang biak dalam medium kultur. Keputusan immunositokimia menunjukkan bahawa ekspresi antigen permukaan sel diamati positif untuk CD44 dan CD117. Walau bagaimanapun, MSC adalah negatif untuk CD34 (penanda bagi hematopoietik), oleh itu, mengesahkan ketiadaan sel-sel hematopoietik. Tambahan pula, CD44 didapati > 85% positif, manakala CD45 (penanda hematopoietik); adalah lebih daripada 60% negatif dalam MSC melalui analisis populasi sel menggunakan *flow cytometry*. Setelah induksi menggunakan 5-azacytidine dan Zebularine, morfologi MSC telah berubah dan sel-sel mempamerkan unjuran proses sitoplasm dengan penampilan seperti bebola. Selepas 20 hari, sel-sel yang bersebelahan telah berhubung dan membentuk struktur seperti miotiub. mRNA bagi CAMHC, cTnT and GATA-4, dan GATA-4 telan dikesan dalam kedua-dua sel dirawat dan tidak dirawat. Walau bagaimanapun, analisis RT- PCR untuk ekspresi spesifik gen kardiak menunjukkan bahawa sel-sel MSC yang dirawat mempamerkan kehadiran cTnT, CAMHC dan GATA-4 yang signifikannya lebih tinggi berbanding sel-sel yang tidak dirawat. Manakala, tidak ada perbezaan yang signifikan di antara sel-sel yang dirawat 5-azacytidine dan zebularine. Zebularine boleh menjadi pengganti yang baik untuk 5-azacytidine kerana ia lebih stabil dan kurang toksik kepada sistem biologi. Keputusan ini menunjukkan bahawa sel sum-sum tulang mesenkima (BM- MSC) boleh didiferensiasi secara *in vitro* menjadi kumpulan kardiomiogenik.

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LIST OF ABBREVIATIONS

ASCs   Adipose Stem Cells

BM-MSCs   Bone Marrow Stem Cells

bp   base pair

CD   Cluster of Differentiation

CD34   Hematopoietic progenitor cell surface antigen

CD44   Homing-associated cell adhesion molecule

CD45   Leukocyte surface antigen

CD117   c-kit or stem cell factor receptor

cDNA   complementary Deoxyribonuccleic acid

DMEM   Dulbecco's Modified Eagle Medium

EPCs   Endothelial Progenitor Cells

ESCs   Embryonic Stem Cells

GAPDH   glyceraldehydes-3 phosphate dehydrogenase

HSCs   Hematopoietic Stem Cells

IDV   Integraded Density Value

IgG   Immunoglobulin G

iPSCs   induced Pluripotent Stem Cells

MI   Myocardial Infarct

mRNA   messenger Ribonucleic acid

MSCs   Mesenchymal Stem Cells

PBS   Phosphate Buffer Saline

RNA   Ribonucleic acid

RT-PCR   Reverse Transcriptase-Polymerase Chain Reaction

UNIT OF MEASUREMENT

cm^3	cubic centimeter
^oC	degree celcius
mL	miliLiliter
uL	microLiter
mg	milligram
ug	microgram
uM	microMolar
min	minute
mg/mL	miligram /milliliter
mM	miliMolar
ug/mL	microgram/milliliter
rpm	revolutions per minute
V	Volt

CHAPTER 1

INTRODUCTION

Heart disease is one of the most leading causes of mortality globally (Mendis et al., 2011). It is estimated that around 23.6 million people will die from cardiovascular diseases by 2030 (Elnakish et al., 2012). Diseases such as diabetes, coronary artery diseases, and myocardial infarction are best known by the irreversible loss of specific cell types that lead to dysfunction of heart's tissue, which will cause limitation of heart for self-renewal and regeneration (Woodbury et al., 2000; Melo et al., 2004; Lu et al., 2006).

Due to high mortality and morbidity rate associated with coronary heart diseases novel methods to improve their function are highly demanded (Kumar et al., 2012; Naeem et al., 2013). More than a century of researches into the etiology, pathophysiology and pathology of acute myocardial infarction have given rise to many mechanical and pharmacological approaches to improve the quality of life for sufferers and extend their healthy lifespan. However, these treatment methods are limited to delaying or reducing the functional decline experienced by patients and cannot restore lost function (Davy, 2011; Elnakish et al., 2012).

Within the last decade, scientists have tried to find ways to cure and regenerate lost myocardium and restore cardiac function. In order to overcome these obstacles scientists have introduced cell-based therapeutic approaches to treat the damaged heart (Psaltis et al., 2008). The discovery of differentiated potential of stem cells has opened new windows in the field of regenerative medicine. Regenerative medicine or stem cell therapeutics is a rapidly emerging field and gaining substantial attention for research and clinical applications. Regenerative medicine involves the repair or regeneration of an organ tissue or cells in order to restore an impaired function of the tissue (Psaltis et al., 2008). The

goals of stem cell based cellular therapy are the same as all regenerative medicine strategies; which is to recover function in a damaged or diseased organ or system that lacks sufficient regenerative capacity to heal unaided. The main goal is to repair and cure injured and diseased organ and tissue with living, home-grown replacement, not with mechanical devices like insulin pumps and titanium joints. It would be the beginning of a new era of regenerative medicine, one of the holy grails of modern biology.

The use of stem cells in medicinal therapy is a promising therapeutic approach for a variety of diseases including heart diseases (Mendis et al., 2011; Elnakish et al., 2012). For instance, acute and chronic heart disease related functional losses are most probably the biggest targets of cellular therapy research and clinical trials to date. Although some previous works had suggested that adult stem cells offered myocardial regenerative potential, research into the efficacy and mechanisms involved exploded only in the last decade.

Multipotent adult stem cells, such as bone marrow mesenchymal stem cells (BM-MSCs) have become one of the interesting and important candidates in cardiac cellular therapy. The unique properties of MSCs are that they could be easily isolated and proliferated from the bone marrow (Caplan & Dennis, 2006), immunologically tolerated as an allogeneic transplant (no immune rejection) (Uccelli et al., 2008) and their multilineage potential (Pittenger et al., 1999). These characteristics of MSCs have lead to intense investigation as a cell-based therapeutic for cardiac repair.

1.1 General Objective:

The main aim of this study is to isolate, proliferate and characterize bone marrow mesenchymal stem cells (BM-MSCs) and explore cardiomyogenic differentiation potential of these cells through induction by optimized concentration of two compounds, namely 5-azacytidine and zebularine.

1.1.1 Specific Objectives:

The general objective will be achieved through specific objectives as follows:

1. Isolation and proliferation of bone marrow derived mesenchymal stem cells (BM-MSCs) from rat bone marrow.

2. Characterization of (BM-MSCs) by:

   (a) Immunocytochemistry
   (b) Flow Cytometry

3. Trans-differentiation of BM-MSCs into cardiac-like cells by treating with:

   (a) 5-azacytidine
   (b) Zebularine

4. Analyze gene expression level of selected cardiac specific genes of treated and untreated MSC cells by reverse transcriptase (RT) PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical Overview:

Highlighted by several historical breakthroughs, stem cell biology saw its rebirth at the end of the last century. In 1997, the world was surprised by Wilmut and his group, who demonstrated that the nucleus of a somatic cell showed full genetic potential by giving birth to Dolly sheep after injecting it into a denucleated oocyte. A year later, Thomson *et al.* (1998) developed an isolation and culture method to maintain human embryonic stem cells *in vitro*.

In the field of adult stem cell research, Friedenstein and his colleagues (1970) were the first investigators to demonstrate that bone marrow consist of a mixed population of hematopoietic stem cells (HSCs) and a rare population of plastic-adherent stromal cells, which is now commonly called mesenchymal stem cells (MSCs). Friedenstein identified the importance of MSCs in controlling and supporting the hematopoietic niche and he also demonstrated the differentiation ability of MSCs into mesodermal derived tissue. Piersma *et al.* (1985) and Caplan (1986) showed differentiation of MSCs into osteoblasts, chondrocytes, and adipocytes. During the 1990s, differentiation of MSCs into a myogenic phenotype was shown (Wakitani et al., 1995). Ferrari *et al.* (1998) first reported the trans-differentiation of bone marrow stem cells into muscle tissue and the same year Shi *et al.* (1998) followed by reporting the endothelial tissue from bone marrow.

In early 21st century, differentiation of MSCs into endodermal derived cells and cardimyocytes *in vivo* were studied (Toma, 2002; Sato et al., 2005). Within this time, Di and colleagues (2002) stated MSCs can suppress T-lymphocyte proliferations. This study

attracted scientists' attention for application and potential of MSCs therapy for allogenic transplantation and immunomodulatory. MSC therapy has been recently moved to pre-clinical and clinical trials for cardiovascular disease (CVD) (Hare et al., 2009). These reports of the adult stem cell multipotency changed the view of the old paradigm in cell biology and opened new possibilities for treating human diseases. With the findings of adult stem cell plasticity, it becomes possible to replace the injured or senile tissues by either stimulating the proliferation of endogenous adult stem cells, or grafting allogenic progenitors derived from an exogenous source (Williams et al., 2011).

2.2 What are Stem Cells

Stem cells are defined as undifferentiated cells that have the ability to self-renew (self-replicate) and differentiate themselves into other types of cell such as blood, muscle, skin and brain cells. They can self-replicate for indefinite periods in the human body through process of "proliferation". When cells replicate themselves many time over it is called "proliferation" (Swanepoel, 2006). During human development, after fertilization, the fertilized egg (zygote) ultimately give rise to more than 200 cells types such as blood cells, liver cells, skin cells and neural cells that make up the human body. This process, which less specialized cells turn into more specialized cell types, is called "differentiation" (Enmon, 2002; Kumar et al., 2012). Stem cells can replicate and differentiate many times, unlike muscle cells, blood cells and nerve cells which do not normally replicate themselves. One of the characteristic of a stem cell is that it does not have a tissue-specific structure that allows it to perform specialized functions (Ma, 2010). For instance, unlike heart muscle cells which works together systematically with a complete heart structure to pump blood or red blood cells which carry molecules of oxygen through blood stream, a stem cell could not do this sort of work on its own. However unspecialized stem cells, by

coordinating their gene expression in an elaborate and complex pattern span many generation of cells, have the ability to differentiate to complex cells or tissue, such as heart muscle cells, blood cells, nerve cells and many other types of cells (Swanepoel, 2006; Ma, 2010).

2.3 Importance of Stem Cells

Self-renewal, proliferation and differentiation potential of stem cells into other cell types have made them as a leading candidate in order to repair and replace damaged tissue and organs. Scientists hope to overcome and treat many common diseases, including heart, kidney, liver and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases by using these cells. A better understanding and control of stem cell proliferation and differentiation *in vitro* would benefit many other areas such as drug testing, cancer research and fundamental research on embryonic development. Researchers also hope that stem cell research will help lead to the application of other therapeutic treatment called gene therapy. By understanding the human genome, scientists can identify genetic inconsistencies that lead to disease and modify them by introducing a corrective genetic remedy. Stem cell treatment, unlike most conventional drug treatment, has the potential to become a lifelong cure. There is almost no realm of medicine that would not be touched by this innovation. It is not too unrealistic to say that this research has the potential to revolutionize the practice of medicine and improve the quality and length of life. However, to exploit and apply the therapeutic potential and promises of stem cells, extensive researches need to be done on the risks and benefits of their use and applications (Swanepoel, 2006).

2.4 Types of Stem Cells

There are different sources of stem cell which differ in their potential for differentiation and in the number of cell types to which they can normally give rise. They are usually categorized in term of how committed they are to becoming any particular type of cells, namely "Totipotent stem cells", "Pluripotent stem cells" and "Multipotent stem cells"(Thomson et al., 1998; Laurie, 2004;Takahashi et al., 2007)(Figure 2.1).

2.4.1 Totipotent Stem Cells

As mentioned earlier, Stem cells are defined as having two essential properties: the ability to self-renew and reconstitute their own population, and the ability to differentiate into multiple different types of mature daughter cells. The latter ability is referred to as the cell's potency, and several different levels of potency exist during the development of an organism. Human cells can be divided into sex or germ cells; (eggs and sperm cells); and somatic cells (the rest of body cells). When a sperm cell and an egg cell unite, they form a fertilized egg or zygote. After fertilization, zygote starts dividing to initially form two, then four, then eight identical cells. These cells are totipotent, which means they have the ability to give rise to any and all human cells, such as heart cells, brain cells and liver cells as well as the extra-embryonic tissues such as the placenta or yolk sac (Thomson et al., 1998). Totipotent cells exist for a short time between fertilization and the formation of the blastocyst.

2.4.2 Pluripotent Stem Cells

During embryonic development, on the fourth day, the ball of cells forms into an outer layer known as a "blastocyst". The blastocyst is a small, hollow ball that consists of

around hundreds relatively undifferentiated cells. Each blastocyst consists of two layer, the outer cell mass, which develops into extra embryonic tissue such as placenta and other tissue needed for fetal development in the uterus, and the inner cell mass, which is a group of about 30 cells that will produce tissues for the resulting child (Swanepoel, 2006). These cells are pluripotent cell, which have the potential to develop into any of the 200 cell types that make up the human body (Laurie, 2004). Thus, pluripotent cells in the inner cell mass of the blastocyst are a source of human embryonic stem cell lines (Thomson, 1998; Laurie, 2004). Pluripotent cells could eventually differentiate into any bodily tissue, but they cannot develop into a human being themselves, because they are unable to give rise to the placenta and other tissues required for full human development. Therefore, they would not be able to develop into a fetus if placed in a woman's uterus. These cells have lost the ability to form the extra-embryonic tissues, but still have the ability to form all three germ layers of the developing embryo (endoderm, mesoderm, and ectoderm). The pluripotent stem cells further specialize into other type of stem cells. These cells, which can only develop into a few tissues, are called multipotent stem cells (Enmon, 2002).

2.4.3 Multipotent Stem Cells:

Multipotent stem cells exist in many organs and tissues, including bone marrow, fat, peripheral blood, skeletal muscle, skin, heart, liver and even the brain. Adult stem cells are characterized as multipotent stem cells. These multipotent stem cells can differentiate *in vitro* and currently being studied for their purpose use in regenerative medicine (Enmon, 2002; Ma, 2010). Multipotent adult stem cells are attractive stem cell resources for the replacement of damaged tissue or organ in regenerative medicine. They can give rise to cells that have a particular function. For example, hematopoietic stem cells give rise to red

blood cells, white blood cells and platelets, while skin stem cells give rise to the different types of skin cells. These organ specific stem cells form during fetal development and remain in adult individuals. They are undifferentiated cells with the capability of self-renewal and a high rate proliferation which have potential to differentiate into specialized cells with specific functions (Pittenger et al., 1999). Despite of limitation and restriction of using multipotent adult stem cells for differentiation into a particular lineage such as mesodermal, endodermal or ectoderm, they have the potential and ability to differentiate into distinct somatic cell types with appropriate stimulation. Unlike pluripotent embryonic stem (ES) cells, adult stem cells can avoid some ethical issues associated with ES cells, resulting in a more timely approval for research and therapeutic use. Another advantage of using adult stem cells is that the derivation and transplantation of these cells believed to be less likely to initiate rejection when they transplanted. Although adult stem cells believed to be a promising candidate for the treatment of many diseases in the field of regenerative medicine and cellular therapy, many aspects remain to be explored in order to guarantee appropriate quality assurance and control of these cells, such as avoiding inappropriate gene expression in transplanted cells or the undesirable traits of tumorigenesis (Reik, 2007).
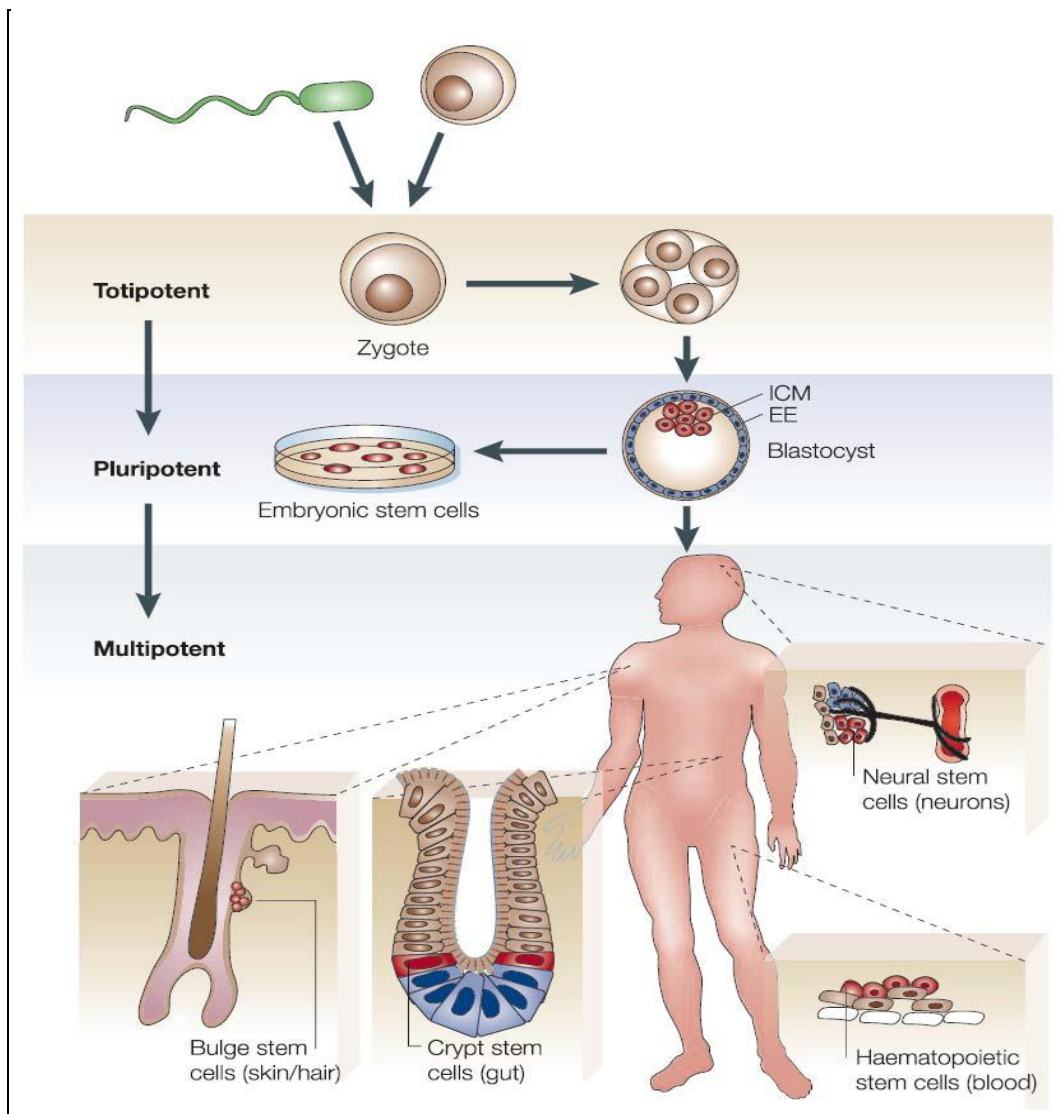
Figure 2.1: Different types of stem cells: Totipotent zygote gives rise to the blastocyst. Pluripotent embryonic stem cells derived from the inner cell mass of the blastocyst. Multipotent adult stem cells exist in many mature tissues, used as a reservoir of renewing cells (Retrieved from Netanely, 2006).

2.5 Sources of Stem Cells

Until 2006 based on development stage, scientists dealt with two kinds of stem cells namely, embryonic stem cells (ESCs) and adult stem cells (ASCs). However, in 2006, scientists made breakthrough by introducing new kind of stem cell by identifying conditions that allow some specialized adult cells to be reprogrammed genetically to assume a stem cell-like state. This new type of stem cell, called "Induced Pluripotent Stem Cells" (IPSCs). Therefore, there are now three kinds of stem cells: embryonic stem cells (Adewumi et al., 2007), adult/somatic stem cells (ASCs) (Young & Black, 2004) and induced pluripotent stem cells (iPSCs), which is recently discovered by Japanese scientist (Takahashi et al., 2007). Although these cells carry overlapping properties, they are different in their general properties and potential which will be discussed in later sections.

2.5.1 Embryonic Stem Cells (ESCs):

Embryonic stem cells (ESCs) are derived from early embryos that can be propagated indefinitely in the primitive undifferentiated state while remaining pluripotent. Specifically, ESCs are derived and isolated from embryos that developed from fertilization of eggs *in vitro* at *in vitro* fertilization (IVF) clinics. They are never derived from eggs fertilized in a woman's body. They are isolated from the inner cell mass of blastocyst, which comprises 16 to 140 cells. These stem cells could also be obtained from aborted fetuses and could also be derived through somatic cell nuclear transfer techniques for therapeutic purposes (Adewumi et al., 2007).

Murine embryonic stem cells were first isolated in 1981 (Evans & Kaufman, 1981) and human embryonic stem cells were isolated in 1998 (Thomson, 1998). Embryonic stem cells exhibit normal and stable karyotype, express embryonic cell surface markers and can

be cultured in vitro for very long periods in an undifferentiated state and yet retain their pluripotent differentiation potential. Upon induction by specific differentiation compounds, cultured ESCs can differentiate *in-vitro* into a variety of mature cell types, including: neurons, skin cells, blood, muscle, cartilage, endothelial cells, cardiac cells and pancreatic cells (Adewumi et al., 2007). ESCs have gained a lot of attention because they are immortal and have almost unlimited development potential. However, human embryonic stem cell research which holds the greatest potential for regenerative medicine has proven to involve the greatest difficulties as well. Unfortunately, the generation of human ESCs lines has sparked a great deal of controversy, particularly in certain religious communities (Orive et al., 2003).

2.5.2 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are induced from reprogrammed fibroblasts by the retrovirus mediated introduction of Oct3/4, Sox2, c-Myc and Klf4, transcription factors that unlock all restrictive conditions of a differentiated cell and reverse the biological clock to provide pluripotency (Takahashi et al., 2007; Gonzalez et al., 2011; Robinton & Daley, 2012). Scientists are using iPSC technology for generation of new types of cells by reprogramming adult stem cells with the same potential as ESCs rather than using ESCs which involve many ethical issues. Within last few years, scientists have created iPSCs from multiple human tissues, including lung fibroblasts, keratinocytes (Aasen et al., 2008), fibroblast-like synoviocytes (Takahashi et al., 2007), cord blood (Giorgetti et al., 2009; Haase et al.,2009), peripheral blood (Loh et al., 2009), mesenchymal stromal cells (Oda et al., 2010), oral mucosa fibroblasts (Miyoshi et al., 2010) and T-cells (Loh et al., 2010; Seki et al., 2010) (reviewed by Aránega, 2011). The ability to reprogramme somatic cells into iPSC cells that are pluripotent, self-renewal and

self-replication potential has transformed the field of regenerative medicine (Aranega, 2011). However their complete potential and possible toxicity is yet to be assessed before any future use in clinical setting (Takahashi et al., 2007; Okita et al., 2007).

2.5.3 Adult Stem Cells (ASCs)

Following fetal and childhood development, the organs and tissues of adult humans and animals generally maintain their size and structure. This stable external appearance of tissue hides the fact that tissue maintenance is a steady state process; dying cells must be replaced continuously throughout life. Organs and tissues have distinct rates of turnover, which is related to their function (Rizvi et al., 2005). The term adult stem cell (ASCs) or somatic stem cell refers to the cells found in adult organisms that constantly replenish the somatic cells in the tissue of their origin. Scientists defined them as cells of the body (not the germ cells, sperm or eggs).

ASCs are multipotent stem cells, which are capable of self-renewal throughout the organism's life, and also capable of differentiating into various mature cell types usually through an intermediate cell of increased commitment (progenitor). Therefore, adult stem cells are already committed to a certain cell lineage and, thus, they are restricted in their differentiation range and this characteristic makes them to be referred to as multipotent stem cells. Multipotent adult stem cells reside within mature tissues and serve as a limitless source for new mature cells, enabling maintenance and repair of the tissue by continuously regenerating mature tissues, either as part of normal physiology or as part of repair after injury. Although the existence of these ASCs is beyond doubt in most cases, their isolation and identification proved to be difficult. It is important to assess the *in vitro* differentiation capability of these cells, which may reflect their developmental potential. In recent studies, the concept of multipotency of ASCs has moved to the forefront of stem cell research.

Studies have shown that multipotent ASCs have the ability to retain much of the multipotentiality of their embryonic and fetally-derived counterparts, compared to ESCs with the many ethical issues surrounding its use (Swanepoel, 2006; Li & Clevers, 2010; Humphreys, 2011). Scientist have discovered and derived adult stem cells from many sources including the bone marrow, umbilical cord, adipose tissue, kidney, blood, liver and certain regions of the adult brain. To date, bone marrow stem cells is the most accessible and least invasive source of multipotent adult stem cell (Irons, 2007; Ma, 2010) which is the focus of this thesis.

2.6 Bone Marrow Niche

The bone marrow (BM) is a spongious and fatty tissue that contains a multitude of cell types and niches. BM is a complex tissue consists mainly of two different tissue types, hematopoietic (HSCs) and stromal/mesenchymal stem cells (MSCs) with function of supporting hematopoiesis. Each of these tissues is home to important forms of adult stem cells (ASCs) namely, hematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and endothelial progenitor cells (EPCs), respectively.

HSCs are the major source of cells within bone marrow. The HSCs are developed and supported in the bone marrow microenvironment, termed the hematopoietic niche, and the MSCs are one of the most important cell type that support BM microenvironment (Prockop, 1997; Pittenger & Martin, 2004). MSCs are known as marrow stromal cells because they were originally identified as forming a tiny proportion of the non-hematopoietic stromal tissue. The rest of the stroma consists of fibroblasts, macrophages, adipocytes, osteoblasts and endothelial cells. They contribute to connective tissue, defense, nutrient delivery, bone tissue management, and vascular structure, respectively. All of

which contribute in some way to the formation and maintenance of the stem cell niches (Herzog et al., 2003; Davy, 2011).

2.6.1 Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells are a circulating bone marrow cells that contribute to the endothelium and participate in both vasculogenesis and vascular homeostasis (Khakoo & Finkel, 2005). Existence of a bone marrow–derived circulating progenitor for the endothelial lineage called the endothelial progenitor cell (EPC) was first reported in 1997 (Asahara, 1997). They were originally isolated from peripheral blood as CD34+ circulating progenitor cells and were later determined to originate in the bone marrow (Asahara, 1997). Several studies have elucidated and reported the roles of putative bone marrow– derived EPCs in cancer (Young & Black, 2004; Kim et al., 2005; Kaplan et al., 2006;), cardiovascular disorders (Werner et al., 2005; McNeer, 2007), and diabetes (Eizawa et al., 2004; Loomans et al., 2004; Fadini et al., 2007).

2.6.2 Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cell (HSC) is one of the first well-known and most-studied ASCs in bone marrow. It is also the most successful example of "stem cell therapy" (Kuznetsov et al., 2001). HSCs are multipotent adult stem cells that give rise to all the myeloid and lymphoid cells of the blood. They give rise to cell progenies that constitute the lympho-hematopoietic system, responsible for the cell-mediated immunity such as monocytes, macrophages, cytotoxic T cells or natural killer cells and adaptive immunity (B cells), or cells initiating clotting (platelets). HSCs can also be found in peripheral blood in adults (Kuznetsov et al., 2001) or in umbilical cord blood of newborns and are critical in the study of blood-related malignancies (Lee et al., 2004). The first report of adult stem

cell differentiation into cardiomyocytes and transplantation into infarcted mice heart was done by bone-marrow-derived hematopoietic stem cells (Leri et al., 2005). However, some other studies have not demonstrated the differentiation of haematopoietic progenitor cells into cardiomyocytes (Balsam et al., 2004; Murry et al., 2004).

2.6.3 Mesenchymal Stem Cells (MSCs)

As described earlier, several progenitor cells can be found in bone marrow niche and one class of progenitor's cells in BM is known as mesenchymal or stromal stem cell (MSCs). The term "MSC" is introduced by Caplan (1991). However, seminal studies by Friedenstein (1970), Owen (1988), Tavassoli and Crosby (1970) identified what was initially referred to as bone marrow-derived 'mechanocytes' or stromal fibroblasts. Bone marrow mesenchymal stem cell (BM-MSC) was first described by (Friedenstein et al., 1966) around 40 years ago.

2.6.3.1 Isolation and Characteristics of MSCs

The first and the most important characteristic of MSCs is their tendency. MSCs were originally isolated from bone marrow (BM) aspirate based on their tendency, which allow spindle-shaped or fibroblast-liked cells to adhere to a plastic substrate in the cell culture plate. In contrast, most other bone marrow derived cells, like the highly researched HSCs that also reside in the bone marrow, do not possess this plastic-adherence property (Friedenstein, 1995). MSCs display stable phenotype in long-term culture and retain the potential for adipogenic, chondrogenic and osteogenic lineage differentiation *in vitro* and they are typically involved in the healing of damaged tissues such as bone, cartilage, muscle, ligament, tendon, and stroma *in vivo* (Pittenger et al., 1999; Psaltis et al., 2008).

Although, there is a very small fraction of MSCs (0.001–0.01%) in bone marrow, they can be isolated and expanded with high efficiency and induced to differentiate into multiple lineages under defined culture conditions (Pittenger et al., 1999). They have been isolated almost from every type of tissue, including peripheral blood (Kuznetsov et al., 1997), umbilical cord blood (Lee et al., 2004), dental pulp (Gronthos et al., 2000), amniotic fluid (Anker et al., 2003), fetal blood (Noort et al., 2002), lung (Fan et al., 2005), liver (Campagnoli et al., 2001) adipose tissues (Zuk et al., 2002), intestine (Bjerknes & Cheng, 2006) and hair follicle (Amoh et al., 2005).

In experimental animals, bone marrow aspirates are normally taken from the tibias and femurs. In human marrow donors, they are often harvested from the superior iliac crest of the pelvis (Digirolamo et al., 1999; Barry & Murphy, 2004). Frequently, the marrow sample is subjected to fractionation via density gradient centrifugation and cultured in a medium such as Dulbecco's modified Eagle's medium (DMEM), containing 10-20% fetal bovine serum. Primary cultures are usually maintained for 16-21 days and are then detached by trypsinization, followed by sub-culturing (Pittenger et al., 1999; Barry, 2003). In the recent development of regenerative medicine, MSCs have been the favorite sources of stem cells for transplantation because of their potent differentiation capability, and also the accessibility and possible autologous transplantation to eliminate immuno-rejection (Dezawa et al., 2004; Kolf et al., 2007). The unique immunophenotype characteristics of MSCs which coupled with powerful immunosuppressive activity have made MSCs as a leading candidate for allogeneic transplant (Sato et al., 2005; Krampera et al., 2006; Gimble et al., 2008). The potential of the putative functions for MSCs in regenerative medicine are such that hundreds of human trials involving MSCs are currently underway all across the world (Williams et al., 2011). However, despite the great interest, the MSCs remains enigmatic as both its identity and qualification as a true stem cell remains

uncertain and this uncertainty results from lack of universally defined cell surface markers to characterize the MSCs in the manner of the hematopoietic stem cell (Devine, 2002; Baksh et al., 2004; Rastegar et al., 2010).

2.6.3.2 MSCs Marker

One of the obstacles in defining MSCs is that there are no immunophenotypic markers that are uniquely and specifically expressed by MSCs up to date (Rastegar et al., 2010; Williams & Hare, 2011). Scientists have made many attempts to develop a cell-surface antigen profile for the better purification and identification of MSCs. However, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has came up with some criteria to define human MSC which is as follow: MSC must be plastic-adherent when maintained in standard culture condition; MSC must express CD105, CD73 and CD90 and lack of CD45, CD34, CD14, CD79, CD19 and HLA-DR expressions; MSC must differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici et al., 2006). In general, MSCs do not express cell surface markers such as CD11b (an immune cell marker), CD31 (expressed on endothelial and hematopoietic cells), CD34 (the primitive hematopoietic stem cell marker), and CD45 (a marker for all hematopoietic cells) (Haynesworth et al., 1992; Majumdar et al., 2003).

On the other hand, cells from MSCs culture are known to be positive for the surface peptides SH2, SH3, SH4 (monoclonal antibodies), the surface receptors CD35 (trans-membrane protein), CD73 (5'ectonucleotidase), CD90 (Thy1), CD123 (interleukin-3 receptor) and CD117 (a hematopoietic stem/progenitor cell marker), CD271 (neurotrophic growth factor) and Stro-3-positive mesenchymal precursor cell (Kuçi et al., 2010). Other cell types such HSCs also express these markers. Thus, it would be preferable if there is truly a unique marker to identify the most immature and, therefore, the most highly potent

MSCs (Kuci et al., 2010; Kim & Ahn, 2012). In addition, with minor differences in expression patterns from one tissue source to another, all MSCs express embryonic cell markers such as Oct4, Nanog, and stage specific embryonic antigen-4 (SSEA-4) (Gang et al 2007; Christensen, 2010). In order to further distinguish MSCs from HSCs, the cultured cells can be selected against the hematopoietic characteristic markers CD34, CD45 and CD14 (Haynesworth et al., 1992; Majumdar et al., 2003). However, since there is no currently known MSC-specific cell surface markers that exclusively identify MSCs; therefore, isolated MSC populations are still not entirely homogenous (Peister et al., 2004; Rastegar et al., 2010; Williams & Hare, 2011; Asumda, 2013).

2.6.3.3 Differentiation Potential of Mesenchymal Stem Cells

Upon induction by specific compounds, cultured MSCs can differentiate into a variety of mature cell types (Figure 2.2). Friedenstein and his colleagues (1970) isolated first MSCs and differentiated them into bone and cartilage *in vitro* 40 years ago. Several groups have demonstrated that long-term cultured MSCs can be induced to differentiate into pancreatic (Lee et al., 2004), neural lineages (Woodbury et al., 2000), bone (Haynesworth et al., 1992), cartilage (Yoo et al., 1998), muscle (Wakitani et al., 1995), marrow stroma (Majumdar et al., 1998), tendon and ligament (Young et al., 1998), fat (Dennis et al., 1999), and a variety of other connective tissues (Studeny et al., 2004).

BMMSCs have been shown to ameliorate tissue damage and to improve function after myocardial infarction (Iso et al., 2007; Cho et al., 2011), lung injury (Ortiz et al., 2007; Curley et al., 2012), kidney disease (Kunter et al., 2006; Alfarano et al., 2012), diabetes (Lee et al., 2006; Si et al., 2012), liver injury (Kanazawa et al., 2011; Zhao et al., 2012) and neurological disorders (Edalatmanesh et al., 2011). Several studies have shown that involvement of BM-MSCs is a promising therapeutic option for the treatment of heart

disease (Orlic et al., 2003; Antonitsis et al., 2008; Garcia et al., 2008; Psaltis et al., 2008). Moreover, numerous studies have shown differentiation potential of MSCs have attracted significant attention to their possible role in elucidating differentiation pathways and promoting tissue engineering as gene vectors and immunomodulators in autoimmune diseases in recent years (Rastegar et al., 2010). From review of many reports, it can be finalized that MSCs have the potential to differentiate into several different mesenchymal lineages such as muscle, bone, cartilage, fat, tendon, and marrow stroma, upon induction by different compounds. It is discovered that under certain culturing conditions, MSCs can differentiate into mature, specialized cells other than those of the mesenchymal tissues, including cardiomyocytes. During differentiation of a stem cell into a mature cell, the cell changes its phenotype as it becomes committed to a certain function. The discovery of genes whose expression is changed along differentiation into a certain lineage may shed light on biological pathways associated with that specific differentiation process and its induction methods. For instance, studies have shown that during differentiation of MSC into cardiomyocyte, some cardiac specific genes such as myosin heavy chain (MHC), cardiac troponin T (cTnT), NKx2.5 and GATA4 become upregulated and expressed (Reik, 2007). The transcription factor GATA4 is a critical regulator of cardiac gene expression, modulating cardiomyocyte differentiation and adaptive responses of the adult heart (Oka et al., 2007; Heineke et al., 2007). GATA4 is also expressed in the adult heart where it is thought to function as a key transcriptional regulator of numerous cardiac genes including atrial natriuretic factor (ANF), b-type natriuretic peptide (BNP), MHC, and many others. MHC and cTnT are the two major contractile proteins which playing important roles in the regulation of skeletal and cardiac muscle in most of the vertebrates and mammals heart (Willie et al., 1999).
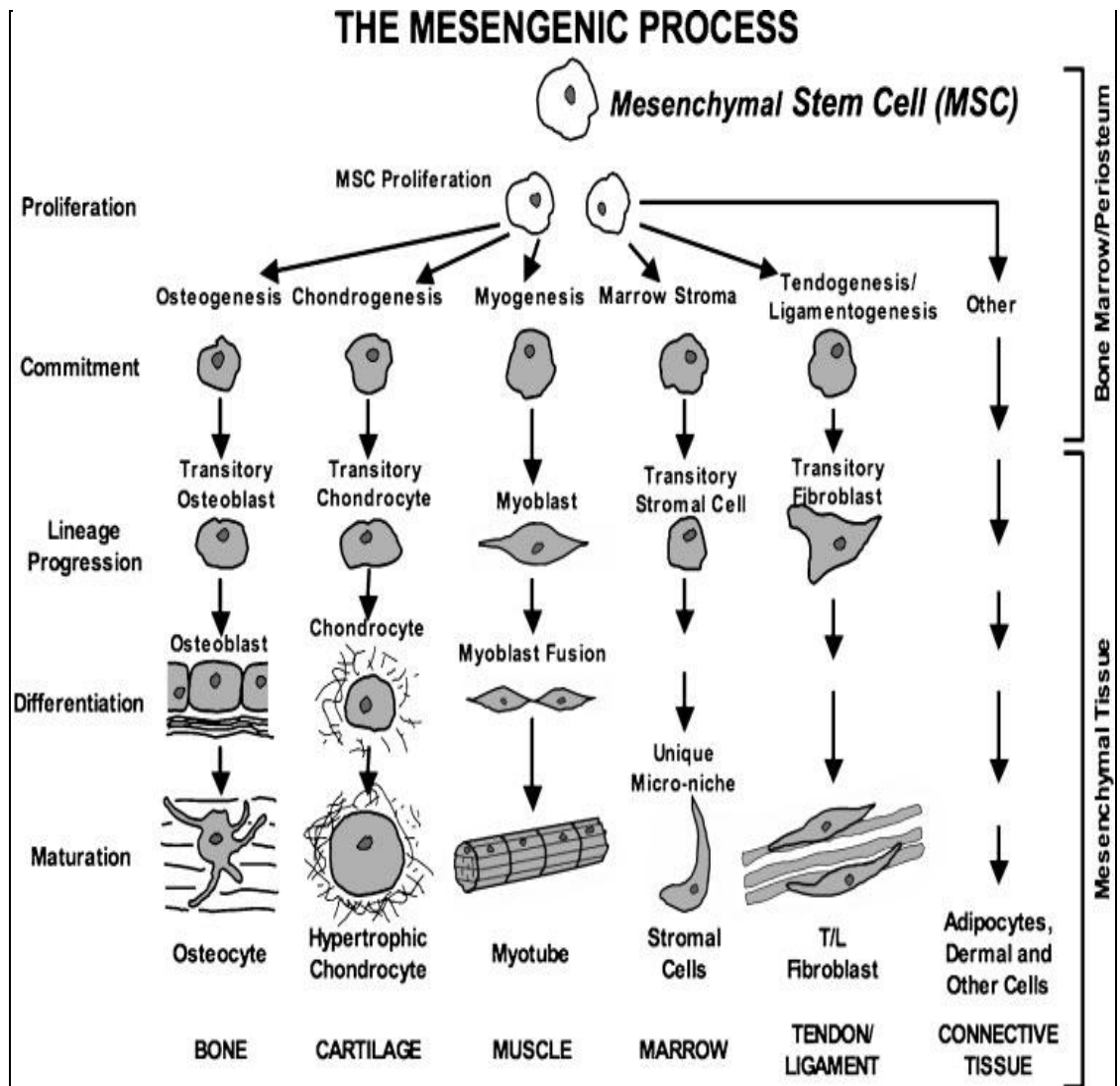
Figure 2.2: Differentiation potential of bone marrow-derived mesenchymal stem cells. They are capable of replicating and having its progeny differentiate to produce bone, cartilage, muscle, marrow stroma, tendon/ligament, and other connective tissues (Caplan & Dennis, 2006).

2.7 DNA Methylation:

DNA methylation constitutes major mechanisms that are responsible for epigenetic regulation of gene expression during development and differentiation (Li, 2002; Cedar & Bergman, 2009). DNA methylation is an important epigenetic mechanism, which has been reported to be involved in gene expression, chromosome inactivation, genomic imprinting and endogenic gene silencing (Sulewska et al., 2007). DNA methylation is also important in maintaining pluripotency and self-renewal of stem cells. To maintain pluripotency of cells, genes are usually activated during hypomethylation and genes that are associated with differentiation are repressed by hypermethylation (Fouse et al., 2008).

The most well studied and widely used drugs to inhibit DNA cytosine methylation and reactivate silenced is 5-azacytidine (Taylor & Jones, 1980; Harris, 1982). Zebularine is also another DNA methyltransferase inhibitor, which was developed as a more stable and less toxic drug recently (Yoo et al., 2004). Zebularine and 5-azacytidine (Figure 2.3) were originally developed as cancer chemotherapeutic agents (Vesely & Cihak, 1975) and are powerful inducers of genes silenced by DNA methylation (Jones, 1985). In this study, these two synthetic compounds were used for induction of MSCs into cardiac-like cells.

2.8 5-azacytidine and Zebularine as Cardiomyogenic Inducer

One of the most important and well characterized DNA demethylating agents is 5-azacytidine (Jüttermann et al., 1994; Naeem et al., 2013). DNA methylation inhibitors such as 5-azacytidine (5-aza-CR) and its deoxy analog, 5-Aza-2'deoxycytidine (5-Aza-CdR) have been studied for decades. However, both drugs are toxic *in vitro* and *in vivo*, and have been difficult to administer due to their low stability in aqueous solution (Taylor & Jones, 1982). 5-azacytidine incorporates into DNA and forms a covalent irreversible

complex with DNA methyltransferase (DNMT) preventing the enzyme from methylating position 5 of cytosines clustered in regulatory CpG islands (Cheng et al., 2003).

Several studies reported BM-MSCs can be induced with 5-azacytidine treatment to express cardiac-specific markers and exhibit spontaneous beating and measurable action potential, consistent with a myocyte lineage (Toma, 2002; Xu et al., 2004; Dimarakis et al., 2006; Ye et al., 2006; Antonitsis et al., 2007; Naeem et al., 2013). However, cardiogenic differentiation of stem cells with the use of 5-azacytidine is still controversial. Zhang *et al.* (2007) reported that the cardiomyogenic differentiation potential of bone marrow mesencymal stem cells was passage-restricted. Their result showed that treatment of mesenchymal stem cells with 5-azacytidine expressed cardiac specific markers and myotubes formation at only passage 4 (P4). In addition, Liu *et al.* (2003) reported that when cells are only immortalized, 5-azacytidine can induce rat bone marrow stromal cells to differentiate into cardiomyogenic cells.

Zebularine, a cytidine analog containing a 2-pyrimidinone ring, is another novel DNA methyltransferase (DNMT) inhibitor, which was developed as a more stable and less toxic drug compare to 5-azacytidine (Yoo et al., 2004). Zebularine was originally developed as a cytidine deaminase inhibitor. It lacks an amino group at position 4 of the pyrimidine ring (Kim et al., 1986; Driscoll et al., 1991). Despite of many reports of using 5-azacytidine as MSCs inducer, there are a few studies which reported the potential of zebularine as cardiogenic inducer. The ability of zebularine to inhibit DNA methylation was widely studied in microbial system, cancer therapy, as well as mammalian cell lines (Irelan & Selker, 1997; Cheng et al., 2003). Cheng and colleagues (2003) reported that both zebularine and 5-azacytidine induced the expression of the myogenic phenotype in mouse embryonic fibroblast cells and inhibited the methylation of specific loci in both the mouse CII-d and human p16 promoter. Naeem and his colleagues (2013) also stated that

zebularine can be used as a new candidate for cardiogenic inducer. However they reported that the extent of muscle cell formation in cultures treated with zebularine was less than that induced by 5-azacytidine. More studies need to be done, in order to investigate and explore more potential of these two compounds. In this study, the cardiomyogenic differentiation potential of BM-MSCs in response to 5-azacytidine and zebularine treatment was investigated.
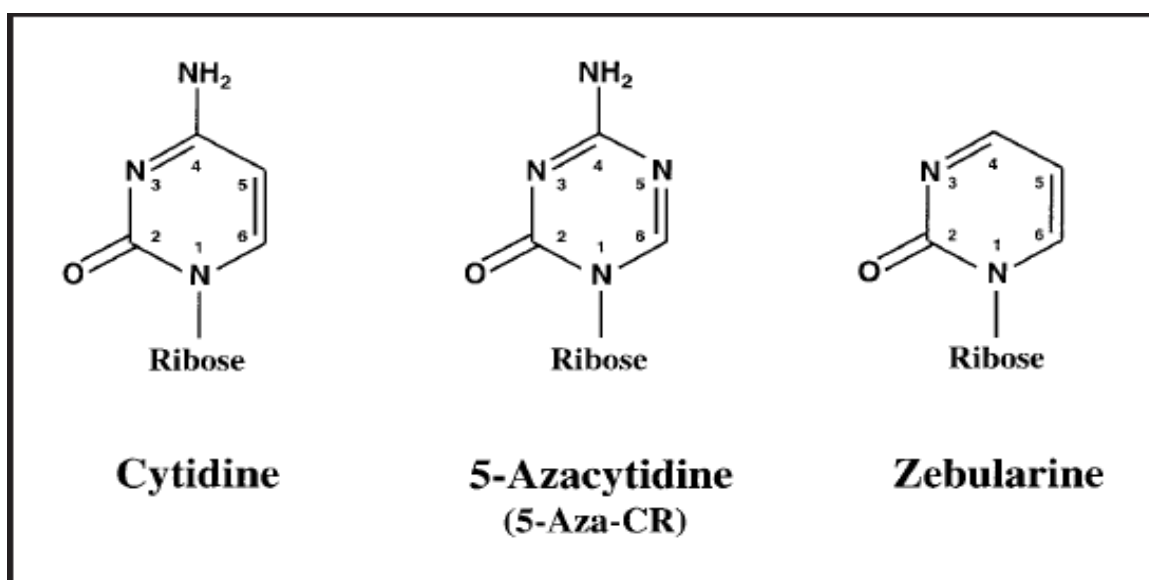
Figure 2.3: Structures of 5-azacytidine and zebularine: 5-azacytidine contains a nitrogen in position 5 and zebularine contains a 2-(1H) pyrimidinone ring (Taylor and Jones, 1982; Zhou et al., 2002).

2.9 Therapeutic Uses of MSCs

Several possible therapeutic functions exist for MSCs. First, they may directly participate the cell repopulation via expansion and differentiation. Disease caused by physical or chemical damage may be treated and cured by directing the differentiation of a patient's own stem cells into the depleted cell types and introducing them into the affected tissue (Christensen, 2010). The hypothesis that MSCs could reconstitute a population of stem cells in adipose, bone, or cartilaginous tissues has been put forward for many years (Prockop, 1997), and continually investigated till now (Mareddy et al., 2007). Moreover, as stated previously, MSCs are under investigation for direct repair of many other tissues such as heart, kidney, brain and skin.

A second possible role of MSCs is as a vessel for delivering a therapeutic transgene. The dysfunctional alleles that may be responsible for a disease can be circumvented by the insertion of a functional gene into the patient's stem cells, followed by transplantation into an appropriate tissue where they can propagate and produce the therapeutic gene products (Reiser et al., 2005).

Transplanted MSCs have been reported to stably reside in several tissue types including cardiac (Kraitchman et al., 2005), bone (Lee et al., 2001), and neural tissues (Torrente et al., 2008). Because of MSCs ability to migrate, they have been shown to be an effective and important therapeutic agent to fight the tumor glioblastoma multiforme (GBM). MSCs engineered to express tumor necrosis factor apoptosis ligand (TRAIL) were shown to migrate toward GBM cells. There, they remained undifferentiated and non-expansive, and stably expressing and secreting TRAIL, effectively reducing the tumor burden and increasing survival time in a mouse model (Sasportas et al., 2009).

The third therapeutic role for MSCs is as an immune system modulator. Several studies have shown that allogeneic transplantation of MSCs does not appear to induce immune response (Devine et al., 2001; Le et al., 2004; Rastegar et al., 2010). As discussed earlier, MSCs produce an immunomodulatory effect by interacting with both innate and adaptive immune cells. MSCs have been shown to suppress most of the innate immune cells such as neutrophils, dendritic cells (DCs), natural killer cells, eosinophils, mast cells, and macrophages (Rastegar et al., 2010). MSCs have also shown to suppress adaptive immune cells such as T and B lymphocytes (T-cell, B-cell) proliferation in a mixed lymphocyte culture (Di et al., 2002; Aggarwal & Pittenger, 2005; Christensen, 2010; Rastegar et al., 2010). Suppression of lymphocyte proliferation is mediated through cytokines released by MSCs that equally suppress the proliferation of cytotoxic and helper T cells (Di et al., 2002). Overall, the possibility of transplanting allogeneic MSCs, removing the need to harvest cells from a patient if it may cause undue risk. Also, expansion of MSCs can take place prior to need, and universal donors may be utilized for many patients. Importantly, MSCs harvested from adult rhesus monkey bone marrow have shown decreased potential for self-replication and differentiation when compared to MSCs from younger age groups (Lee et al., 2006; Hacia et al., 2008). Therefore, future therapeutic approaches for adult patients may prove to be more effective when utilizing allogeneic cells from younger donors (Gracia et al., 2008).

A fourth possibility for MSCs in tissue repair is an indirect role in support of other cell types. MSCs are known to support hematopoiesis in bone marrow by acting as part of the stroma and allogeneic. MSC transplants have been shown to enhance engraftment of HSCs (Almeida et al., 1999). MSCs supply physical support and cytochemical direction by producing growth factors and cytokines, likely providing the essential cues for cell

proliferation and differentiation (Ball et al., 2008). MSCs given to patients who experienced tissue toxicity after receiving HSC transplants have been shown to aid in clearing severe haemorrhagic cystitis, pneumo-mediastinum, as well as diverticulitis and peritonitis caused by steroid-resistant graft versus host disease (GVHD) (Ringden et al., 2007). There is a similar role for those MSCs found to reside in other tissues undergoing repair and re-growth. MSCs have been shown as home to areas of hypoxia and cause rapid revascularization after tissue injury (Rosova et al., 2009). This ability is particularly important for the treatment of a myocardial infarction (MI). Ischemic tissue regeneration studies utilizing MSCs have included stroke models (Li et al., 2005), skeletal muscle ischemia (Nakagami et al., 2005; Kim et al., 2006), and a MI model (Tang et al., 2006). The utilization of MSCs for cardiac repair is one area of regenerative medicine where all of these cells' putative therapeutic capabilities have been explored.

2.10 Therapeutic Potential of MSCs for Heart Diseases:

Heart diseases including myocardial infarction (MI) (heart attack), coronary and ischemic heart diseases are leading cause of morbidity and mortality in the world (Psaltis et al., 2008; Mendis et al., 2011). These acute and chronic heart diseases endanger millions of peoples in developed and developing countries and are predicted to be the leading cause of death by 2030 (Humphreys, 2011; Elnakish et al., 2012).

Mutipotent adult MSCs have shown that to have great potential as treatment for many diseases and clinical applications of tissue regeneration, including myocardial regeneration (Qian et al., 2012). Several researches including preclinical and clinical studies have suggested that isolated or cultured bone marrow derived stem cells can be used for treatment of injured cardiac (Toma et al, 2002; Williams et al., 2011).

Several preclinical studies on large animal species such as swine (Shake et al., 2002; Quevedo et al., 2009), sheep (Hamamoto et al., 2009), and dogs (Silva et al., 2005; Perin et al., 2008) have been used to investigate the effects of MSC therapy for heart diseases particularly myocardial infarction (MI). For instance, a study by Quevedo *et al.* (2009) showed BM-MSCs exhibit the ability to differentiate into cardiomyocytes, smooth muscle cells, and endothelium in a swine model of chronic ischemic cardiomyopathy. Another study by Miyahara *et al.* (2006) on rats showed MSCs transplantation improve cardiac function and also significantly increase survival rates in post-MI. To note, there are some methods for delivering stem cells to the heart including peripheral intravenous infusion, direct surgical injection during open heart surgery, or via a catheter-based intracoronary infusion and retrograde coronary venous infusion (reviewed by Williams & Hare, 2011). Studies have shown intravenous fusion of MSCs is the easiest and most practical method for delivery, though the MSCs must travel through the pulmonary circulation, where entrapment of cells is a concern (Barbash et al., 2003). However, many studies showed low retention of stem cells in the heart by any mentioned delivery route. Despite low retention of stem cells in the heart, preclinical results of MSC therapy have shown highly promising results for cardiac diseases. Based on the review by Williams and Hare (2011), there was a significant improvement of left ventricle function, reduction of scar size and increscent of myocardial tissue perfusion in post-MI large animal models, regardless of delivery method or species.

MSC therapy for acute MI and ischemic cardiomyopathy also showed safe result and has favorable effects on cardiac structure and function based on early-phase clinical trial data (Chen et al., 2004; Williams et al., 2011). Phase I/II clinical data have reported using intravenous therapy (Hare et al., 2009), intracoronary infusion (Chen et al., 2004),

and intramyocardial injection (Williams et al., 2011). Many other studies have also shown that use of bone marrow- derived MSCs (BM-MSCs) is a promising therapeutic option for the treatment of heart disease (Obradovic et al., 2004; Miyahara et al., 2006; Hare et al., 2009; Williams et al., 2011). Stamm and colleagues (2003) demonstrated the delivery of bone marrow cells into the infarct zone in patients following MI.

In a randomized, double-blinded study, patient receiving an IV infusion of MSCs post-MI demonstrated significant enhancement in cardiovascular function (Hare et al., 2009). The similar therapeutic benefit was also reported in patients receiving intracoronary MSC administration compared to placebo (Dill et al., 2009). The result of this treatment was a dramatic improvement in global heart function which indicates that stem cell therapy can be useful in treating coronary artery disease. In summary, despite of the great potential of MSCs for heart disease's treatment, novel strategies and methods are needed to be developed for delivery and enhancement of stem cell retention in the heart. The exact mechanism action of MSCs differentiation into cardiomyocytes is still poorly understood and unresolved (Quevedo et al., 2009; Hatzistergos et al., 2010).

CHAPTER 3

MATERIALS & METHODS

All lab works were carried out in the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan, in the Stem Cell Lab Headed by Assoc. Prof. Dr. Asmat Saleem. Procedures for sacrificing the animals were performed in accordance with the guidelines for animal experimentation by the ICCBS's Institutional Animal Care and Use Committee (IACUC).

3.1 Chemicals and Materials

All chemicals, apparatus and materials used in this study are listed in Appendix A. The details of different chemical solutions preparations are given in Appendix B.

3.2 Bone Marrow Sample

Bone marrow sample for isolation of mesenchymal stem cells (BM-MSCs) were obtained from adult 200-300 grams *Sprague Dawley* (SD) rats. Anesthetized SD rats were sacrificed and tibia and femur bones were dissected out, and subsequently cleaned from muscle and connective tissues. The bones were then placed in a sterilized petri dish containing sterile phosphate buffered saline (PBS) (Sigma, USA) before being transferred to the cell culture room for isolation. The culturing of bone marrow mesenchymal stem cells (BM-MSCs) was subsequently carried out in cell culture room (Figure 3.1).
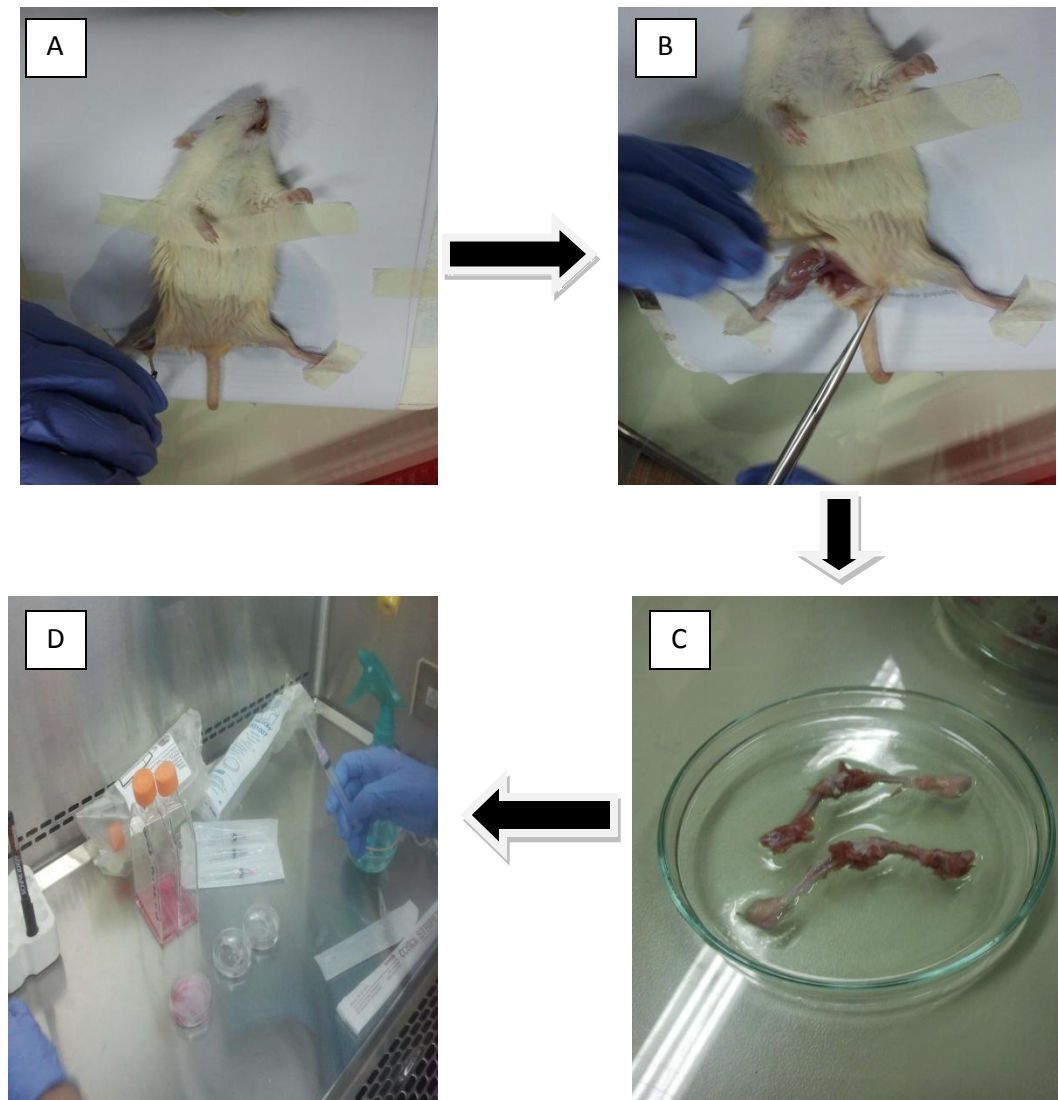
Figure 3.1: Bone marrow isolation from *Sprague Dawley* (SD) rats. Anesthetised SD rat was sacrificed and tibia and femur bones were dissected out (A and B) and cleaned from muscle and connective tissue (C). The bones were then placed in a sterilized petri dish containing sterile phosphate buffered saline (PBS) before being transferred to the cell culture room. The marrow cavities of these bones were flushed by using a 1 ml disposable syringe with a 26-gauge needle with complete medium (D).

3.3 Isolation, Expansion and Maintenance of BM-MSCs

BM-MSCs were isolated from fresh bone marrow samples as follows: The marrow cavities of the bones were flushed by using a 1 ml disposable syringe with a 26-gauge needle with complete Dulbecco's Modified Eagle Medium (DMEM) (Figure 3.1). DMEM contained high glucose, and was supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, 1mM sodium pyruvate and 4mM L-glutamine (Gibco, USA). Isolated cells were then plated at a density of 5×10^6, 2×10^7 cells per T75 flasks and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 7 days. This culture is termed as the primary culture (Passage 0 or P0 cells).

3.4 Changing the Culture Medium

As described earlier in the literature review, MSCs have the ability to adhere to the plastic surface of the flask. Non-adherent hematopoietic cells were removed using frequent medium change. During the expansion and maintenance of MSCs, the medium was replaced three times per week until almost complete removal of dead and hematopoietic cells and 70-80% confluence of MSCs, which took between 16 to 20 days. Flasks were kept back in the CO_2 incubator, which has a humidified environment with 5% CO_2 at 37^oC.

3.5 Sub-culturing /Passaging

After 70-80% confluence of MSCs which took around 16-20 days, MSCs were sub-cultured. Initially, Medium was aspirated from the tissue culture flask. Cells were washed 2-3 times with 4-6ml PBS by swirling the flask carefully so that all medium was removed from the flask. 10X (2.5%) trypsin-EDTA (Gibco, USA) was diluted to 1X (0.25%) concentration using PBS, and 3-4ml 1X trypsin-EDTA was then added to the flask. The flask was swirled to distribute trypsin-EDTA evenly and incubated for 5 minutes at 37^oC.

The cells were observed under phase contrast microscope (Nikon, Japan) for detachment from the surface of the flask. After complete detachment, approximately 2-3ml complete DMEM was added into the flask to stop the reaction. The dissociated cell suspension was transferred into 15 ml falcon tube and centrifuged at 1200 g for 8 minutes (Eppendorf, Germany). The supernatant was then discarded and approximately 1 ml fresh medium was added to the pellet. Pelleted cells were mixed well and equally distributed in two 75cm^2 flasks (Passage 1 or P1). So basically, one flask of MSCs was divided to two flasks. The flasks were labeled and incubated at 37°C in the incubator containing 5% CO_2. For all experiments passage 1 or passage 2 cells were used.

3.6 Characterization of Mesenchymal Stem Cell

3.6.1 Immunocytochemistry

Passage 1 (P1) MSCs, were grown in chambered glass slides (Iwaki, Japan) using 10,000 cells/200µl per well and incubated in a humidified environment at 37°C in the incubator containing 5% CO_2. On the next day, the medium was removed from the chambered slides and washed 2-3 times with 0.1% PBS. Cells were fixed by adding 250-300µl 4% paraformaldehyde (PFA) in PBS and were permeablized with 0.1% tritonX-100 (250µl) in PBS for 20 minutes at room temperature. Cells were again washed with PBS three times for 3-5 minutes. Cultures were blocked by blocking solution (250µl), which contained 2% bovine serum albumin (BSA); 2% goat serum and either 0.2% Nonidet P40 or 0.1% Tween 20) at 37°C to block non-specific binding sites (Table 3.1).

Next, 2.5µl Primary antibodies (Bioscience, USA) were incubated overnight at 4°C against the following proteins: CD44, CD117 (positive marker, 1:100), CD34 (negative marker,

1:100). Negative controls were obtained by omission of the primary antibody. Next day, cells were washed three times with PBS for 5 minutes and then incubated with secondary antibody conjugated to either Alexa Fluor 488, or Alexa Fluor 568 (1:200; Molecular Probes) for 1 hour at room temperature. The solution was then discarded and washed three times with PBS for 5 minutes. Nuclei of MSC cells were counter stained with DAPI to preserve fluorescence. MSCs were washed five times with PBS for 5 minutes and finally rinsed with distilled water for 1 minute. Finally MSC cells were mounted with mounting medium (Merck, Germany) and were examined under fluorescent microscope (Nikon, Japan).

Table 3.1 Blocking solution preparation for immunocytochemistry

Components	volume
2% BSA*	0.02 g
2% goat serum	200 μL
0.1% Tween 20	1 μL
Distilled water	1000 μL

* BSA: bovine serum albumin

3.6.2 Flow Cytometry (FACS- Fluorescence-activated cell sorting)

Flow cytometry analyses (FACS) were performed in the presence of cell surface markers on BM-MSCs by using Flow cytometer (FACS Calibur, Becton Dickinson, USA). Cells were stained for the expression of CD44 (positive marker), CD45 (negative marker), blank control (no antibodies) and negative control (only secondary antibody) using Goat anti-mouse antibodies coupled with flurochromes as following details:

Details regarding preparation of flow cytometry solutions are listed in Table 3.2. BM-MSCs were grown in tissue culture flask and washed two times with PBS. The cells were detached by adding 3-5 ml cell dissociation buffer, enzyme free; Hank's based (Gibco, USA). It was then incubated at 37°C for 30-45 minutes. The dissociated cell suspension was transferred into a 15 ml falcon tube and centrifuged at 800 rpm for 8 minutes at 4°C. Supernatant was removed and 400 µl cold FACS solution (PBS containing 1% BSA, 1 mM EDTA and 0.1% sodium azide) was added to the pellet. 400 µl of cell suspension were equally divided into four non pyrogenic polystyrene FACS tubes (to be used for different primary antibodies) for which each tube contained 100 µl cell suspensions. 5 µl blocking solution (PBS containing 1% BSA) was added into each tube, mixed well by vortexing and incubated at room temperature for 2 minutes. The cells were then incubated at 4°C for 30 minutes in dark with primary antibodies against CD44 and CD45 at 1:40 dilution. Cells were washed twice with a 2ml FACS solution, vortexed and centrifuged at 800 rpm for 10 minutes. The cells were then treated with Alexa Fluor 546 goat-anti mouse secondary antibodies at 1:500 dilution (100 µl for each tube), vortexed and incubated in dark for 30 minutes on ice. Cells were again washed twice with 2 ml FACS solution, vortexed and centrifuged at 800 rpm for 5 minutes. Finally 500 µl FACS solution was added to each tube and mixed well and analyzed through flowcytometer. FSC was selected as the

threshold parameter and the threshold was set to a value of 52 which eliminated small debris. Data was evaluated using BD Cell Quest Pro software.

Table 3.2: Preparation of FACS solution, Blocking solution and PBS 1X

FACS solution	Blocking solution	PBS 1X
0.5g of 1% BSA	10 ml of 1% BSA	8 g of NaCl
0.0146g of 1 mM EDTA		0.2g of KCI
0.05g of 0.1% sodium azide		1.15g of $Na_2 HPO_4$
		0.2g of KH_2PO_4

BSA: bovine serum albumin

KCI: potassium chloride

KH_2PO_4: Potassium dihydrogen phosphate

NaCl: sodium chloride

$Na_2 HPO_4$: sodium hydrogen phosphate

PBS: phosphate buffered saline

3.7 Differentiation of BM-MSCs into Cardiomyocytes-like Cells

3.7.1 Treatment of MSCs with 5-Azacytidine and Zebularine

Passage 1 (P1) MSCs and 3μM optimized concentration of 5-azacytidine and zebularine were used in this experiment following protocol established by Naeem and colleagues (2013). MSCs were divided into three separate groups as follow: (1) untreated control, (2) treated with 3 μM of 5-azacytidine, (3) treated with 3 μM of zebularine. Details regarding preparation of stock and working solutions of 5-azacytidine, zebularine are given in Appendix B. After 70-80% confluence of passage 1 (P1) MSCs, which may take around 16-20 days, old media was discarded. Subsequently, 3μM of each compound solution was added directly in DMEM, which was then added to the tissue culture flask and incubated at $37^{o}C$ in an incubator containing 5% CO_2. After overnight incubation (24 hour), cells were washed twice with PBS and medium was replaced with the complete culture DMEM medium.

3.7.2 Expression Analysis of Cardiac Specific mRNA

3.7.2.1 Isolation of RNA from Treated and Untreated MSCs

Total RNA was extracted from normal and treated BM-MSCs by spin method according to the manufacturer's instructions (Promega, USA). After 80-90% confluence of cells, the medium was discarded from the flasks. Cells were washed twice with 5-10ml of PBS. Next, 3ml of trypsin (0.25%) (v/v) was added to the flasks and incubated for 5-7 minutes at a 37°C using air jacketed CO_2 incubator. After the complete detachment of cells, 7-8ml of complete DMEM was added to the flask. The dissociated cell suspension was transferred to a 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes to pellet the cells.

Supernatant was removed and 600 μl of RNA lysis buffer (RLT) with freshly added β mercaptoethanol (βME) (10 μl per 1 ml buffer RLT) were added to disrupt the pellet. Cell lysate was transferred directly onto a QIA shredder spin column placed in a 2 ml collection tube and centrifuged at maximum speed. A series of steps followed: 600 μl of 70% ethanol was added to the lysate and mixed well by pipetting; 700 μl of this mixture was applied to the RNeasy mini column placed in a 2 ml collection tube, centrifuged at 10500 g for 15 Seconds. 700 μl buffer RNA Wash solution (RW1) was added to RNeasy column and centrifuged at 10500 g for 15 Seconds. Flow through was again discarded along with the collection tube. RNeasy column was transferred to a new collection tube. 500 μl buffer RPE was added onto the RNeasy column and centrifuged at 10500 g for 15 seconds. Similarly, Flow through was discarded. Another 500 μl buffer RPE was added onto the RNeasy column and centrifuged at 10500 g for 2 minutes to dry the RNeasy silica gel membrane. Flow through was again discarded. RNeasy column was then placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute. Finally, RNeasy column was transferred to a new 2 ml collection tube. An amount of 50 μl RNase-free water was added directly onto the RNeasy silica gel membrane to elute the RNA. The eluted RNA was stored at -20°C till further steps in the experiments.

3.7.2.2 Quantitative Measurement of RNA's Concentration

The purity of the total RNA was assessed spectrophotometrically at 260 and 280 nm, with an A_{260} : A_{280} ratio of 1.8–2.0 considered acceptable. RNA was diluted (1:200) in deionized water and deionized water was used as blank. The ratio A_{260}/A_{280} was measured to check the purity of RNA samples.

Concentration of RNA was calculated using the following formula:

$$[RNA]\ \mu g/ml = OD_{260}\ /OD_{280}\ x\ Dilution\ Factor\ x\ *40.0$$

* The absorbance of 1 unit is equivalent to 40 μg of RNA per ml

Total RNA yield was calculated by the following formula:

$$Total\ yield = concentration\ of\ RNA\ sample\ x\ volume\ of\ sample\ in\ milliliters$$

3.7.2.3 cDNA Synthesis

First strand cDNA was synthesized by using first Strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. 1μg of total RNA was subjected to cDNA synthesis by using 50μM oligo (dT)$_{18}$ primer. The amount of RNA that is equivalent to 1 μg was then taken in a 0.2 ml tube.

RNA/ primer mixture was next prepared (Table3. 3). The mixture of RNA/Primer was adjusted by adding Diethylpyrocarbonate (DEPC) treated water to 10 μl and incubated at 65°C for 5 minutes and placed on ice for 1 minute. The cDNA synthesis mixture was also prepared separately (Table 3.4). To the RNA/primer mixture, 10μl of cDNA synthesis mixture was added and incubated at 50°C for 50 minutes. The reaction was terminated at 85°C for 5 minutes and chilled on ice. The cDNA synthesis mixture was stored at -20°C.

Table 3.3: Components used in RNA/primer mixture

Components	volume
Total RNA	1 μg
50 μM Oligo (dT)$_{20}$ primer	1 μl
10 mM dNTP mix	1 μl
DEPC treated water	up to 10 μl

DEPC: Diethylpyrocarbonate
dNTP: Deoxynucleotide mix

Table 3.4: Components of cDNA synthesis mixture

Components	volume
10 X buffer	2 μl
25 mM MgCl$_2$	4 μl
0.1 M DTT	2 μl
RNAse OUT (40 U/ μl)	1 μl
Superscript TM III RT (200 U/ μl)	1 μl

DTT: Dithiothreitol

MgCl$_2$: Magnesium chloride

3.7.2.4 Amplification by Reverse Transcriptase (RT) PCR

Transcriptional expression of glycer aldehyde-3-phosphate dehydrogenase (GAPDH), myocardium cardiac alpha heavy chain (CAMHC), Cardiac troponin-T (cTnT), and cardiac transcription factors (GATA-4) genes were determined by reverse transcriptase (RT) PCR according to the manufacturer's instructions (Thermo Scientific, USA).

Primers were synthesized by Integrated DNA Technologies (IDT) and designed by using the primer3 design program (http://frodo.wi.mit.edu/primer3). Information on the primers details is summarized in Table 3.6. All primers were reconstituted in 10 mM Tris-EDTA buffer (TE buffer; pH 8.0) prepared from (10X) 1M stock.

The volume of the TE buffer was calculated for 100 μM stock of primer as follows:

Volume of TE buffer in ml = (x)[*] μMoles x 1000ml /100 μMoles

[*] (x) μMoles = Amount of oligo in μg/ molecular weight

cDNA (1 μg) was amplified by using Fermentas Life Science's PCR kit (Thermo Scientific, USA) according to the manufacturer's instructions and PCR mixture was prepared in RNase/DNase free 0.2 ml microcentrifuge tube on ice. 50 μl reaction volume was prepared and the components were added in described in Table 3.5.

All the components were mixed, briefly centrifuged and the microcentrifuge tubes were placed in Thermal Cycler (Eppendorf, Germany) for amplification. Rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer was used as an internal standard in all experiments. Reverse transcription reaction products were denatured for 1 minutes at 94°C, followed by 35 cycles of amplification: denaturation at 94°C (1 minute), annealing at 50-

60°C (1 minute), and extension at 72°C (1 minute) and a final extension at 72°C for 10 minutes. The amplified products were stored at -20°C.

Table 3.5: Components used in PCR mixture

Components	Amount	Final concentration
Q solution	10 µl	
10X PCR buffer containing $MgCl_2$	5 µl	1X, 1.5mM $MgCl_2$
10mM dNTP mix	1 µl	0.2 mM
Forward primer (100µM)	1 µl	2 µM
Reverse primer (100µM)	1 µl	2 µM
cDNA	1 µg	<0.5 µg/50 µl
Taq DNA polymerase (5U/µl)	0.5 µl	0.05 unit
DEPC treated water	29.5 µl	
Final volume	50 µl	

DEPC: Diethylpyrocarbonate

dNTP: Deoxynucleotide mix

Table 3.6: summary of primers involved in RT-PCR experiments

Gene PCR Primer	Accession #	Primer Sequence (5'-3')	Annealing Temperature (°C)	Product size (bp)
GAPDH (F) GAPDH (R)	BC09593	GGAAAGCTGTGGCGTGATGG GTAGGCCATGAGGTCCACCA	60	414
cTnT (F) cTnT (R)	NM_012676	TTCGACCTGCAGGAAAAGTT GTGCCTGGCAAGACCTAGAG	57	206
GATA-4 (F) GATA-4 (R)	NM_144730	TCTCACTATGGGCACAGCAG CCGAGCAGGAATTTGAAGAG	60	245
CAMHC (F) CAMHC (R)	X15938	TGATGACTCCGAGGAGCTTT TGACACAGACCCTTGAGCAG	57	234

CAMHC: alpha myosin heavy chain

cTnT: cardiac-specific troponin T

GAPDH: glyceraldehyde phosphate dehydrogenase

GATA-4: cardiac transcription factor

3.7.2.5 Agarose Gel Electrophoresis

The amplified products were separated using 1% (w/v) agarose gel electrophoresis. Details regarding preparation of agarose gel and TBE buffer preparations are presented in Appendix B. One fifth of each PCR product was electrophoretically resolved on 1% agarose gel containing 1.5 µl of 0.3 µg/ml ethidium bromide.

To start, 0.5 gm agarose was dissolved in 10ml 5X TBE buffers, by heating the mixture in microwave oven for 1 minute. The solution was allowed to cool approximately to 60°C. 1.5 µl ethidium bromide was added and swirled gently. The gel was poured into the horizontal gel casting unit and allowed to polymerize for approximately 30 to 40 minutes. The tank was filled with 5X TBE buffer before. 10 µl DNA ladder and PCR products were loaded into the wells.

3.7.2.6 Densitometry and Statistical Analysis

Densitometry analysis was performed to measure the integrated density value (IDV) of each gene. The IDV of each band was compared with the corresponding GAPDH band, which was used to normalize the level of mRNA. The related information regarding densitometry was obtained by gel documentation system (Alpha Innotech, USA). Data obtained were presented as mean ± standard error of the mean (SEM) and calculated using Microsoft Excel. Statistical significance (*$p<0.05$) was determined by using SPSS software and data were subjected to t-test to determine significant differences in gene expression level between differentiated and undifferentiated MSCs.

CHAPTER 4

RESULTS

4.1 Identification and Characterization of BM- MSCs

4.1.1 Characteristics of Isolated and in Vitro BM-MSCs

Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from the tibia and femur of adult Sprague Dawley (SD) rats and cultured according to the adhesive property of mesenchymal stem cells (MSC) as described by Peister *et al.* (2004) and Soleimani and Nadri (2009). When isolated BM-MSCs were seeded in culture flask, initially roundish or polygonal cell types appeared in bone marrow dissociates culture, which was a mixture of MSCs and non-adherent cell populations such as hematopoietic stem cells (HSCs). The HSCs were removed after frequent changing the culture medium. After 2-3 days, MSCs adhered to the wall in small quantity and scattered about showing spindle-shaped or fibroblast-liked morphology as previously reported by (Colter et al., 2001; Peister et al., 2004; Soleimani & Naderi, 2009) (Figure 4.1). This result showed that bone marrow heterogeneity can be broken down in culture by prolonged growth and in a time dependent manner, resulting in distinct morphology such as fibroblast-liked phenotypes. Along with morphological characteristics, reverse transcriptase (RT) PCR, flow cytometry and immunostaining were also performed to confirm BM-MSCs which will be explained in the next sections.

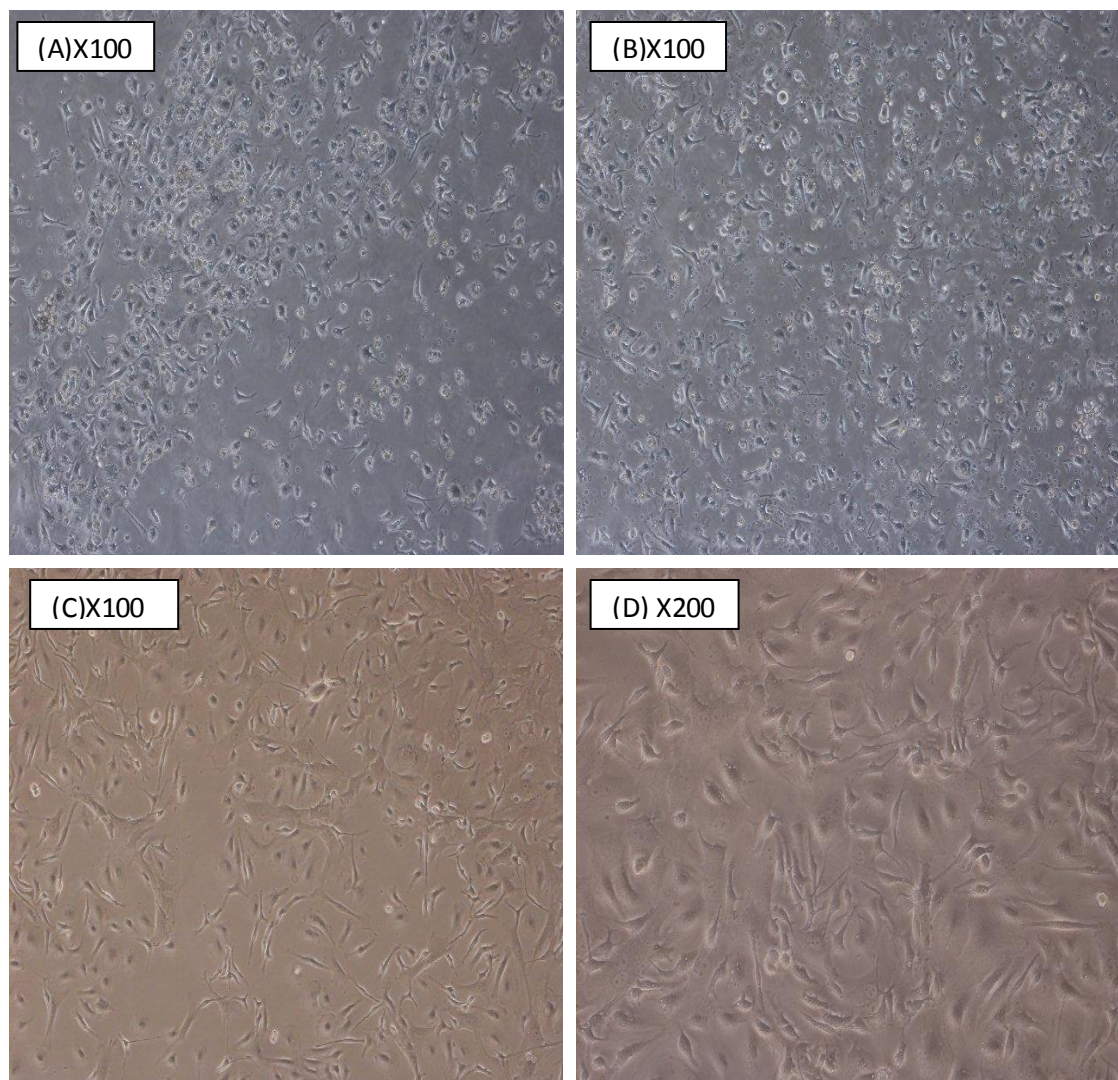**Figure 4.1:** Morphology of undifferentiated BM-MSCs. BM-MSCs exhibited fibroblast-liked morphology with their characteristic property of attaching to plastic culture dishes. At passage 0(P0), MSCs appeared as a mixture of small or spindle shaped cells (A and B). As MSC cells reached 70-80% confluence within 16-20 days, homogenous population of cells with uniform fibroblast-liked morphology was observed (C) and (D).

4.1.2 Molecular Analysis of BM-MSCs

Molecular analysis was initially performed to validate the expression of glyceraldehyde phosphate dehydrogenase (GAPDH) in MSCs. GAPDH is a housekeeping gene, which has always been activated by all mammalian cells whether differentiated or undifferentiated. The total RNA was extracted from the first passage of MSCs and was subjected to RT-PCR analysis (Figure 4.2). Expression of GAPDH which served as an internal control was detected and amplicon (414 bp) was observed in untreated BM-MSCs indicating GAPDH remains activated in untreated MSCs.
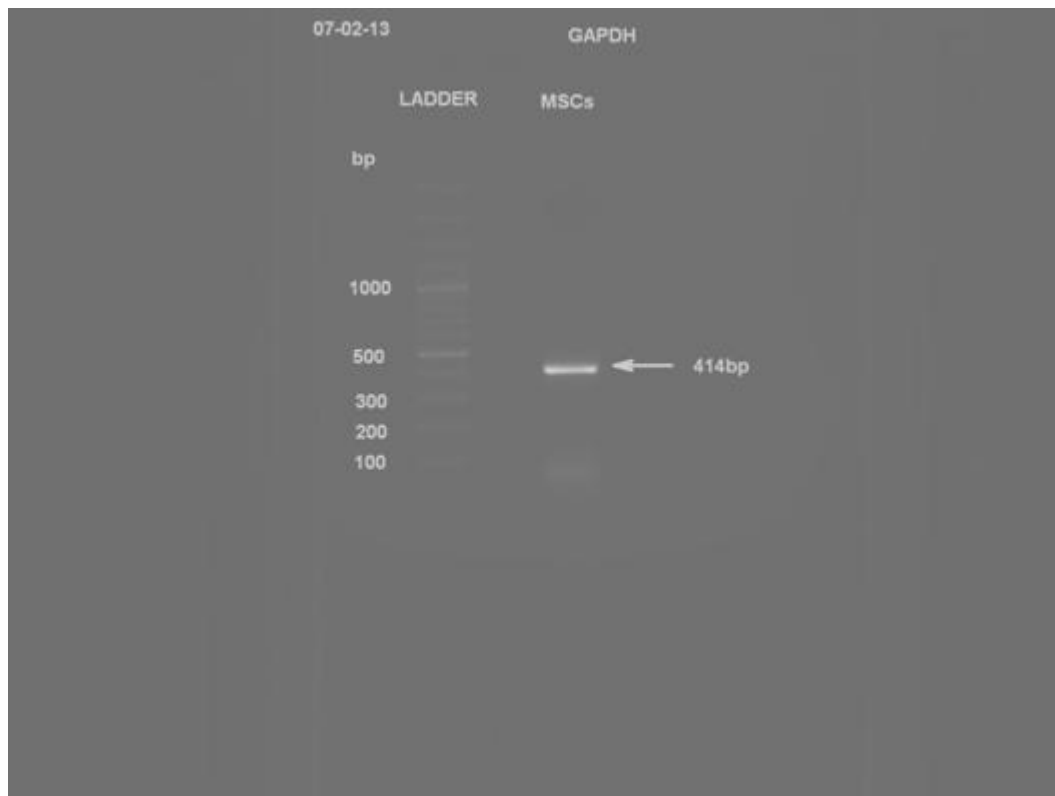

Figure 4.2: RT-PCR expression of GAPDH in undifferentiated BM-MSCs: GAPDH as an internal control was detected in undifferentiated BM-MSCs.

4.1.3 Immunocytochemistry Analysis of BM-MSCs

In order to further distinguish mesenchymal stem cells from hematopoietic stem cells, immunocytochemistry and flow cytometry were used to determine the presence of cell specific surface markers on MSCs. MSCs were first characterized for the positive presence of CD44, CD117 and negative presence of CD34 by immunocytochemistry. MSC cells were also treated only with secondary antibody (negative control). DAPI indicated the nucleus and merged indicated the positive or negative reactivity of marker proteins in immunocytochemistry analysis. Secondary antibodies employed were Alexa fluor 546 (red) conjugated IgGs.

Immunocytochemistry analysis revealed that expression of cells surface marker were negative when cells treated only with secondary antibodies (negative control), indicating that in the absence of primary antibody, no reaction occurs between cells and secondary antibodies (Figure 4.3). Cell surface antigen expression was observed to be positive for CD44 (Figure 4.4) and CD117 (Figure 4.5) which are known as rat MSC markers. In addition, cells were negative for hematopoietic cell surface marker CD34, which validating that cells were MSCs (Figure 4.6).

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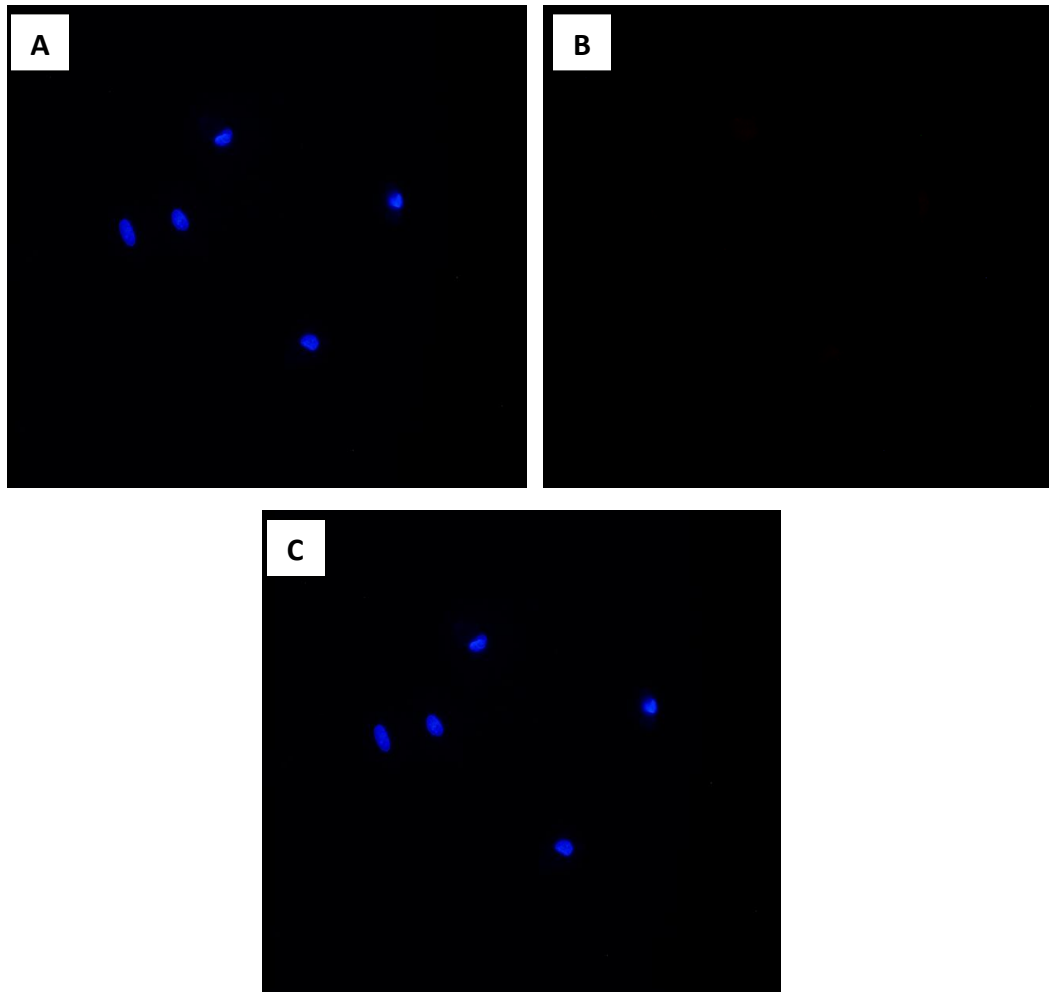
Figure 4.3: Immunostaining identification of BM-MSCs on the basis of surface marker expression (**Negative Control**): A) The nuclei stained with DAPI (blue), B) Secondary antibodies employed were Alexa fluor 546 (red) conjugated IgGs, which was negative, and C) Merged image indicated the negative reaction of the marker protein in the absence of primary antibody (magnifications: X200).
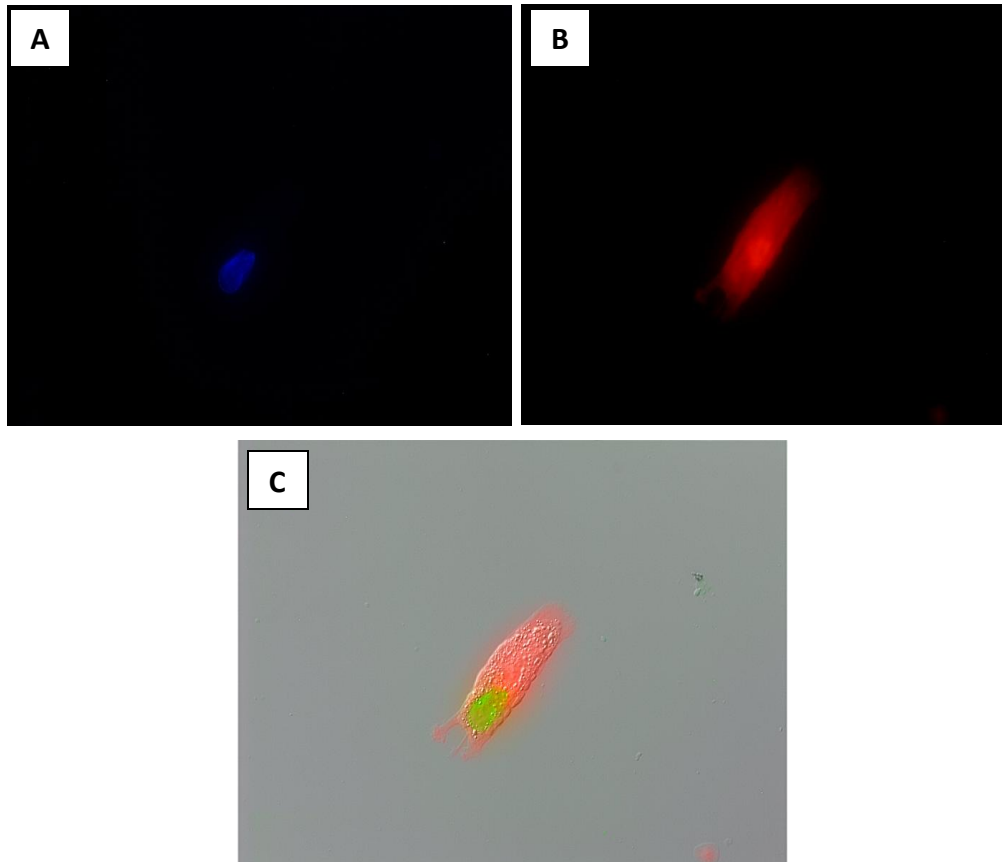
Figure 4.4: Immunostaining identification of a BM-MSC on the basis of **CD44** positive expression, (magnifications: X400). A) The single nuclei stained with DAPI (blue), B) Secondary antibodies employed were Alexa fluor 546 (red) conjugated IgGs and C) Merged image indicated the positive reactivity of marker proteins. This image showed cells were positive for CD44, which validated that the cells were MSCs.
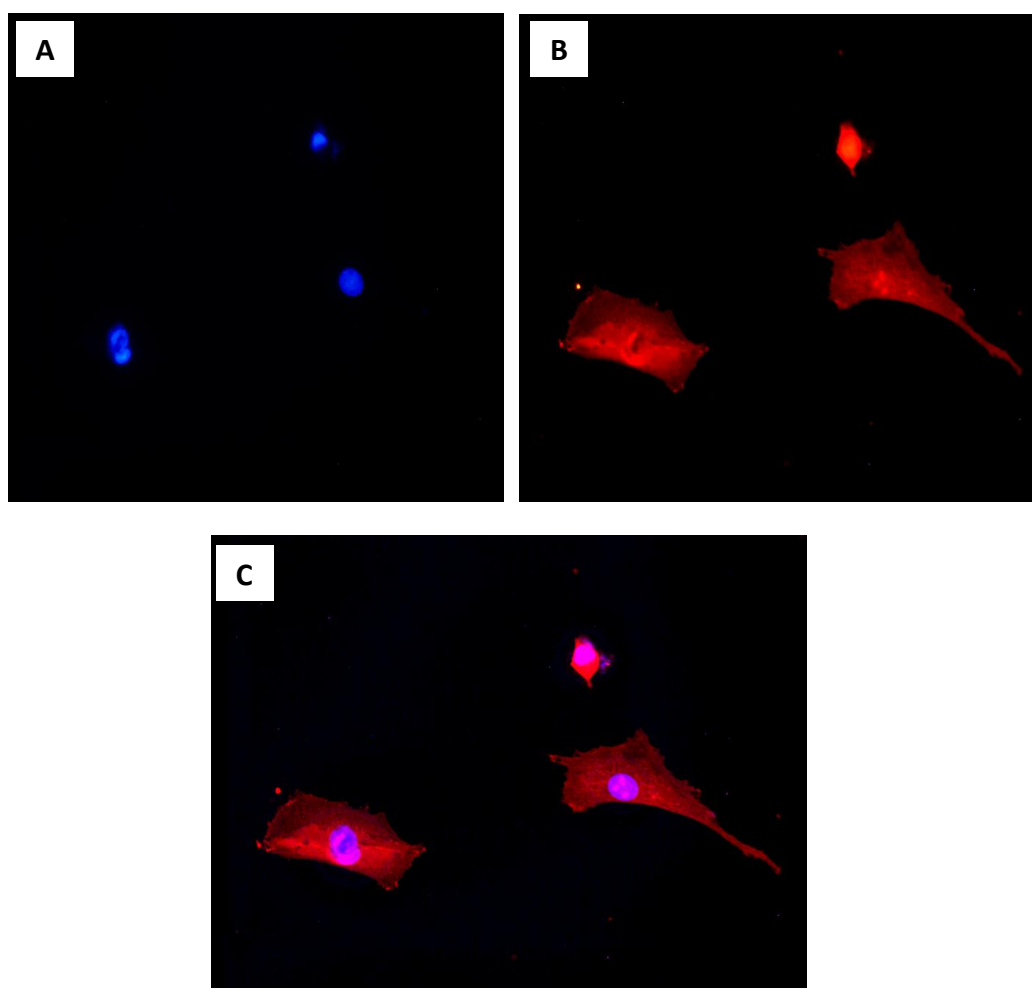
Figure 4.5: Immunostaining identification of MSCs on the basis of **CD117** positive expression, (magnification: X400). A) The nuclei of MSCs stained by DAPI (blue), B) Secondary antibodies employed were Alexa fluor 546 (red) conjugated IgGs and C) Merged indicated the positive reactivity of marker proteins. Cells were positive for CD117, which confirmed the fibroblast-liked cells were MSCs rather than hematopoietic stem cells.
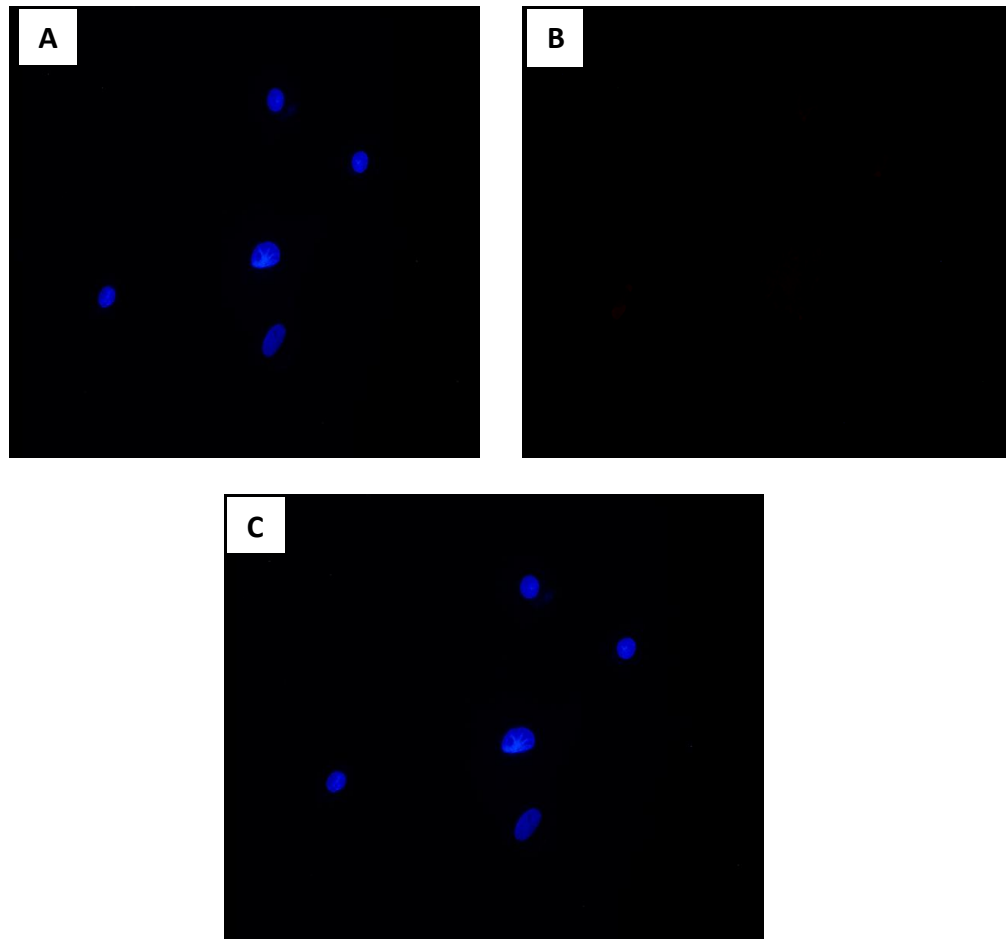
Figure 4.6: Immunostaining identification of BM-MSCs on the basis of **CD34** negative expression, (magnification: X400). A) DAPI stained the nuclei blue, B) Secondary antibodies did not bind to primary antibodies, and C) merged image indicated the negative reactivity of marker proteins. Secondary antibodies employed were Alexa fluor 546 (red) conjugated IgGs. Hence, undifferentiated BM-MSCs were negative for CD34, a known cell surface marker for hematopoietic cells. This result also validated that the fibroblast-liked cells were MSCs rather than hematopoietic stem cells.

4.1.4 FACS Analysis of BM-MSCs

Fluorescence-activated cell sorting (FACS) analysis was further performed for the cell population count of MSCs against CD44 (known MSCs marker) and CD45 (hematopoietic marker). To note, unlabeled cells (blank control) and cells with only secondary antibody (negative control) were used as a reference in this study where both of them should give about the same results. Cells were trypsinized, labeled with antibodies against the indicated antigens and analyzed by FACS in triplicates. Green Alexa Flour 488 goat anti-mouse was used as secondary antibody.

The FACS analysis revealed that untreated MSCs were more than 85% positive for CD44 (Figure 4.7 (B)). While MSCs showed to be more than 60%, 85% and 82% negative for CD45, unlabeled cells (blank control) and negative control (only secondary antibody) respectively. These results, along with morphological characteristics, indicated that the cultured BM-MSCs populations were almost mesenchymal stem cells and most of the HSCs had been removed.
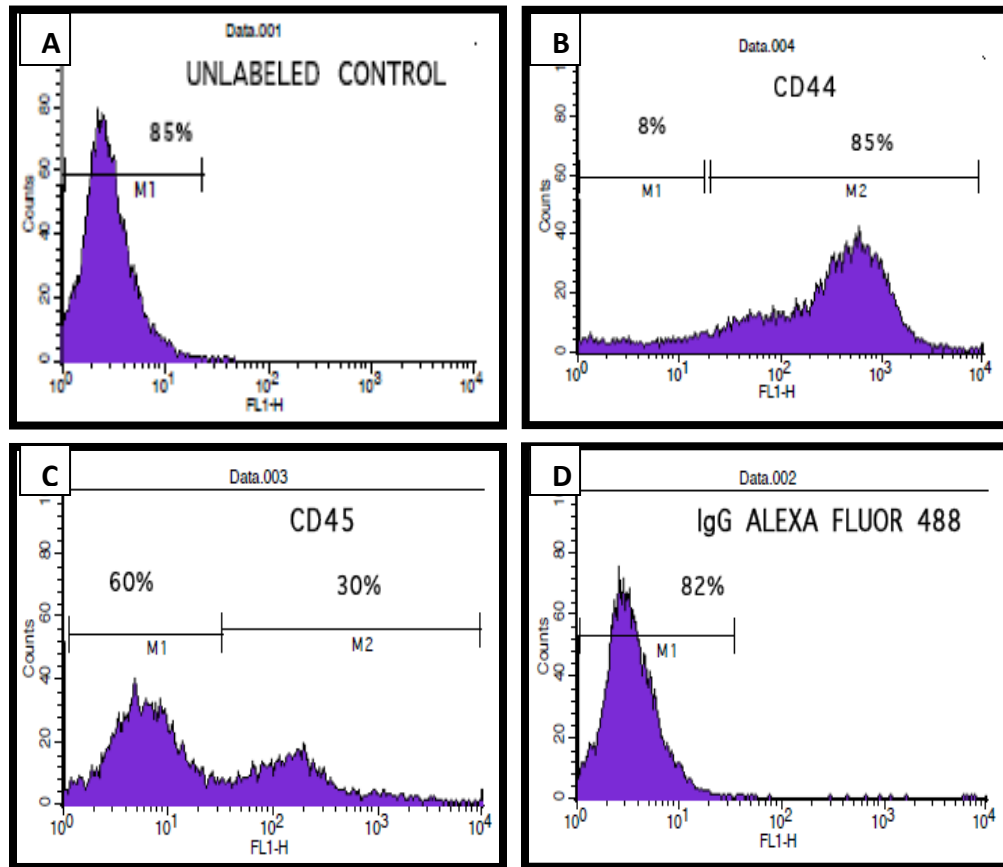
Figure 4.7: FACS analysis of cell surface markers (CD44) and (CD45) of BM- MSCs. The filled histograms refer to specific surface markers. (A) Blank control or unlabeled cells with no antibodies: cells showed about 85% negative, (B) cells were positive (85%) for CD44 which confirmed that more than 85% of cells were mesenchymal stem cells. (C) MSC cells were 60% negative for CD45 with partially 30% positive, (D) cells were treated only with secondary antibodies (IgG Alexa Flour 488) were 82% negative which confirmed the purity of the antibody stock that had been used.

4.2 Differentiation of BM-MSCs into Cardiomyocytes-like Cells

4.2.1 Characteristics of Differentiated MSCs after Treatment

The induction of cardiomyocyte-like cells from MSCs was successfully performed. In this study, to induce myocardial differentiation, MSCs at 70-80% confluence were incubated in serum-containing medium (DMEM) supplemented with 3μM 5-azacytidine and zebularine separately for 24 hours, and subsequent culturing in complete medium (DMEM) up to 20 days. After exposure to 5-azacytidine and zebularine, changes in morphology were observed. During exposure, some adherent cells died, and the surviving cells began to proliferate and differentiate. The morphology of the MSCs changed, with the remaining adherent cells enlarging and forming a ball-liked appearance, or lengthening in one direction forming a stick-like morphology (Figure 4.8). Towards the end, the cells changed into myocyte-like cells along with cluster-like aggregates.
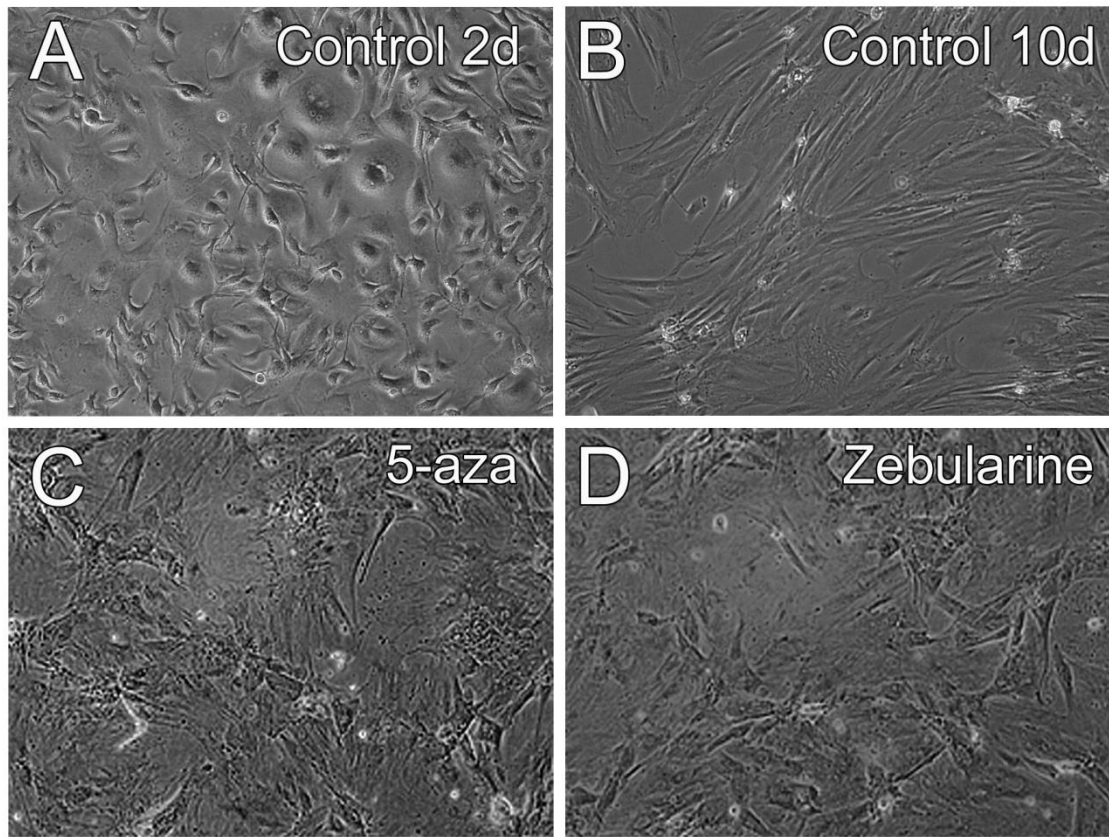
Figure 4.8: Phase contrast imaging of the morphological modification of the BM-MSCs before and after treatment with 5-azacytidine and zebularine. **(A-B):** Primary BM-MSCs showing spindle-like morphology after being seeded in complete DMEM. **A:** 48 hours; **B:** 10 days after seeded (scale bars: 100 μM). (**C-D):** Morphology of BM-MSCs after treatment with 5-azacytidine and zebularine respectively. After exposure, the morphology of the MSCs changed, some adherent cells died, and the surviving cells began to proliferate, differentiate and started to enlarge showing ball-like or stick-like cells morphology after 20 days of treatment (scale bars: 100 μM).

4.2.2 Expression of Cardiac Specific mRNA in Treated and Untreated MSCs

The mRNA expression of cardiomyogenic specific markers of untreated and treated MSCs with 3μM of 5-azacytidne and zebularine were assessed by reverse transcriptase polymerase chain reaction (RT-PCR) analysis (Figure 4.9). The housekeeping gene GAPDH was employed for internal normalization of RNA. The amplicon of GAPDH was found in both undifferentiated and differentiated MSCs indicating that GAPDH remained activated in both treated and untreated cells. RT-PCR analysis revealed the presence of cardiac alpha myosin heavy chain (CAMHC), cardiac troponin-T (cTnT) and cardiac transcription factors (GATA-4) amplicon in treated MSCs. However, very low intensity bands of selected cardiac specific genes were observed in untreated MSCs. In addition cardiac transcription factors (GATA-4) band was not observed in untreated MSCs.

Figure 4.9: Expression of cardiac specific genes in treated BM-MSCs: The amplicon of GAPDH was strongly detected in both untreated and treated MSCs. Reverse Transcriptase (RT) PCR analysis showed the amplicon presence of CAMHC, cTnT and GATA-4 in treated MSCs.

4.2.3 Densitometry Analysis

Densitometry analysis was performed to measure the integrated density value (IDV) of each band. Relative quantification of each band was performed using gel documentation system and IDV of each band was compared with the corresponding GAPDH band. The gene expression level of CAMHC (Figure 4.10 (A)), cTnT (Figure 4.10 (B)) and GATA-4 (Figure 4.10 (C)) were significantly increased after treatment compared to untreated cells. CAMHC had the highest expression, whereas GATA-4 showed the lowest expression compared to CAMHC and cTnT after treatment with 5-azacytidine and zebularine. While selected cardiac specific gene expression level were slightly higher in 5-azacytidine treated cells compared to zebularine, however there were no significant differences between 5-azacytidine and zebularine treated cells. This indicated that besides 5-azacytidine, zebularine could be a good candidate for MSCs induction into cardiomyocytes. Data obtained were presented as average (mean $\pm$ SD; standard deviation) and calculated using Microsoft Excel. Statistical significance (*p<0.05) was determined using SPSS software and subjected to t-test to determine a significant difference of the gene expression level between differentiated and undifferentiated cells.

CAMHC expression in MSCs after 20 days treatment

relative expression of CAMHA/GAPDH

(A)

average

C CAMHC   3uM 5aza CAMHC   3uM Zeb CAMHC

cTnT expression in MSCs after 20 days treatment

relative expression of cTnT/GAPDH

(B)

average

C cTnT   3uM 5aza cTnT   3uM Zeb cTnT

C

GATA-4 expression in MSCs after 20 days treatment

relative expression of GATA4/GAPDH

average

C GATA-4   3uM 5aza GATA-4   3uM Zeb GATA-4
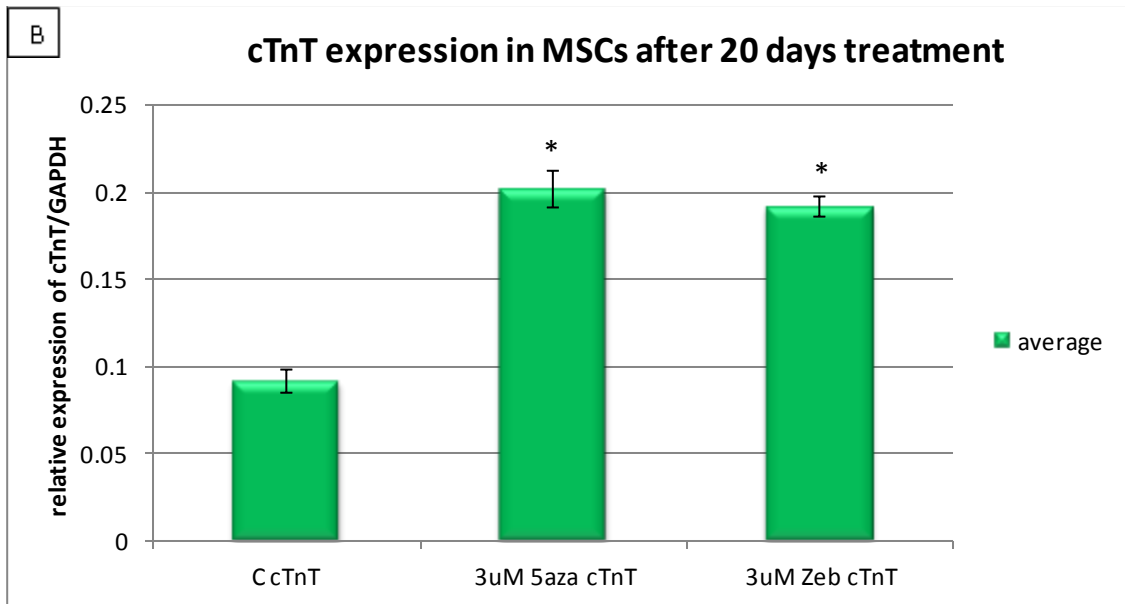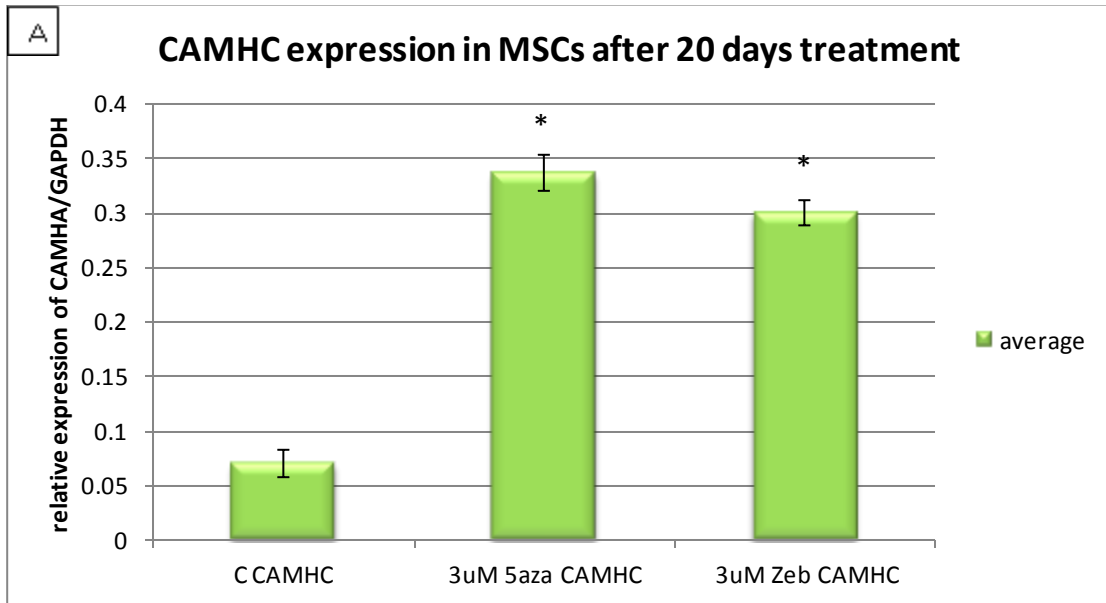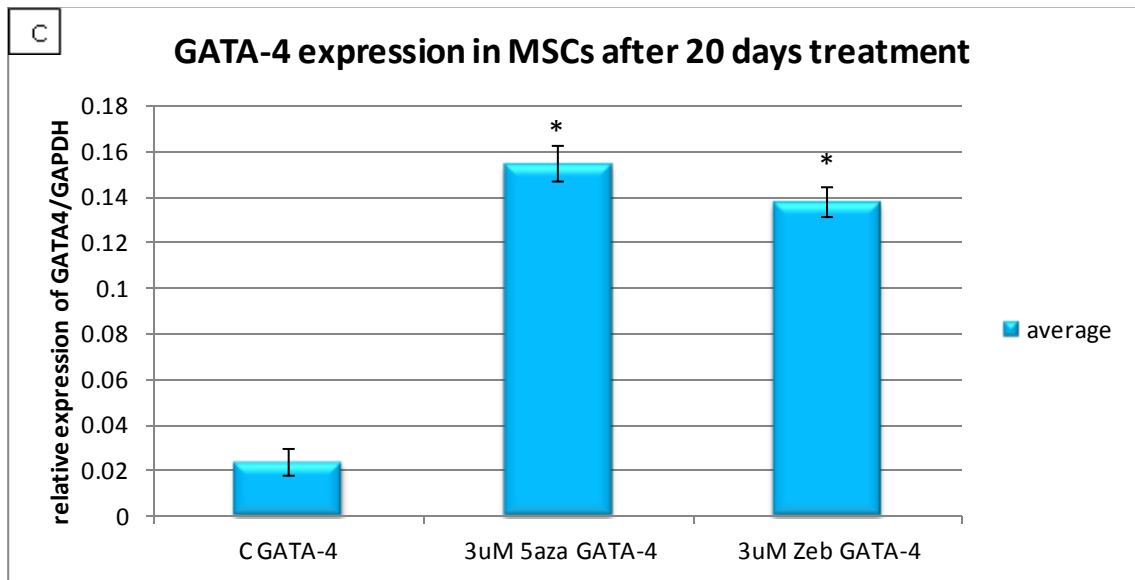
Figure 4.10: Relative gene expression level of cardiac specific genes in treated MSC: (A) CAMHC, (B) cTnT and (C) GATA4 expression level against GAPDH which was used to normalize the cellular mRNA level in untreated and treated MSCs. CAMHC, cTnT and GATA-4 expression were significantly higher in treated MSCs with 5-azacytidine and zebularine compared to untreated MSCs.

CHAPTER 5

DISCUSSION

5.1 General Discussion

Cardiovascular diseases particularly myocardial infarction (MI) is the leading cause of death in the world. This is because most of the cardiomyocytes become differentiated immediately after birth; hence causing limited or no capacity of adult cardiac muscle to regenerate damaged area following MI (Burlacu et al., 2008). The use of bone marrow mesenchymal stem cells (BM-MSCs) and progenitor cells is actively tested as cell-based therapeutics to restore function of post-MI. Therefore, these cell-based therapies are paving a path to novel treatment approaches (Webster, 2005; Tendera et al., 2009). BM-MSCs are adult multi-potential progenitor cells, which have the potential to differentiate into various tissues under appropriate culture condition. BM-MSCs are being aggressively explored for their potential and action in affecting the repair of cardiac tissue after myocardial infarction and have shown the ability to trans-differentiate into cardiomyocytes, both *in vitro* and *in vivo* (Li et al., 2007; Jackson et al., 2007). Cell-based treatment using BM-MSCs have resulted in encouraging and promising results for the treatment of MI (Shi et al., 2011; Kim et al., 2011; Li et al., 2012; Raynaud et al., 2013).

In this study, rat BM-MSCs were successfully isolated by a property of adherence to plastic. After 2-3 days isolation of MSCs and frequent changing of culture medium, BM-MSCs adhered to the surface of flask in small quantity and scattered about showing spindle-shaped or fibroblast-like morphology (Figure 4.1), parallel to previous studies. Naeem *et al.* (2013), Jianquan *et al.* (2012), Antonitsis *et al.* (2007) and Li *et al.* (2002) reported the phase contrast microscopy of MSCs revealed as small, bright with fibroblast-like morphology in primary culture.

While there is a wide range of surface markers that have been tested to characterize MSCs, there is currently no single set of phenotypic markers used to identify a MSC. Therefore, isolated MSC populations are still not entirely homogenous (Peister et al., 2004; Rastegar et al., 2010; Williams & Hare, 2011). In this study, after morphological identification of BM-MSCs, they were further characterized for the positive presence of selected known MSCs markers: CD44, CD117, and absence of hematopoietic markers CD34, CD45 using flow cytometry and immunocytochemistry.

Cytometric analysis showed that the isolated MSCs strongly expressed the known surface markers of MSCs, such as CD44 (85%) (Figure 4.7(B)). The FACS analysis also revealed that unlabeled cells (no antibody) and cells with only secondary antibody (negative control) were observed to be 85% (Figure4.7 (A)) and 82% (Figure 4.7 (D)) negative respectively. Unlabeled cells (blank control) and cells with only secondary antibody (negative control) were used as reference in this study where both of them should give about the same result as both of them used as negative control. MSCs were also more than 60% negative for the hematopoietic surface marker CD45 with partially positive (30%) which could be due to contaminations of other cells for example endothelial cells (Figure 4.7 (C)).

Furthermore, immunocytochemistry results showed cultured cells were positive for the well defined MSCs markers, such as CD44 (Figure 4.4), CD117 (figure 4.5) and negative for CD34 (Figure 4.6), one of the well known hematopoietic surface markers. Cells were also treated only with secondary antibodies (Alexa Flour 546) as a negative control (Figure 4.3). This negative control showed that in the absence of primary antibody, no reaction occurred between cells and secondary antibodies which validated and confirmed completeness of the experimental procedures. The surface marker expression profile accords well with previous studies (Bruder et al., 1998; Colter et al., 2000; Javazon

et al., 2001; Naeem et al., 2013). The absence of CD34 and CD45, have been widely accepted as the major differences between MSCs and hematopoietic stem cells (HSCs) (Colter et al., 2000). Based on the expression of a panel of surface markers, the results of this study support the identity of isolated cells as MSCs. Variable expression of cell surface markers has also been observed due to variation in tissue source, isolation and culture methods (Vacanti et al., 2005; Faast et al., 2006; Ock et al., 2010; Kumar et al., 2012).

Trans-differentiation potential of MSC into cardiac cell types has been explored extensively, with several groups reporting that these stem cells can trans-differentiate into cardiomyocytes (Tomita et al., 1999; Orlic et al., 2001; Rastegar et al., 2010; Williams et al., 2011; Naeem et al., 2013). However, the environmental-driven differentiation of uncommitted stem cells is difficult to anticipate and may result in other cell types (Balsam et al., 2004). One alternative strategy for overcoming ineffective differentiation is the pretreatment of stem cells to turn them into progenitor cells before becoming adult cells (Rosca & Burlacu, 2011). There have been some small molecules such as dexamethasone, ascorbic acid, 5-azacytidine, or all-trans retinoic acid which are capable of inducing the differentiation of stem cells into different cells types (Ding & Schultz, 2004; Rosca & Burlacu, 2011; Naeem et al., 2012).

In the present study, after successful isolation and characterization of bone marrow derived MSCs, the effect of two DNA demethylating agents on differentiation potential of BM-MSCs into cardiomyocytes were investigated. BM-MSCs were treated separately with 3μM optimized concentrations of 5-azacytidine and zebularine, as described by Naeem and coworkers (2013). 5-azacytidine is the most popular chemical for inducing stem cells into cardiomyocytes. 5-azacytidine is analogue of cytidine and it can form covalent conjunction

compound with DNA. It is hypothesized that the treatment of cells by 5-azacytidine makes the cells less responsive to other inductive factors secreted by the microenvironment that might modulate the differentiation (Rosca & Burlacu, 2011). Zebularine, a cytidine analog containing a 2-pyrimidinone ring, is another novel DNA methyltransferase (DNMT) inhibitor, which have been developed as a more stable and less toxic drug compared to 5-azacytidine (Yoo et al., 2004; Naeem et al., 2013). Zebularine could be a good candidate for inducer as an alternative to 5-azacytidine which is less stable and more toxic.

The present study demonstrated that demethylating agents, 5-azacytidine and zebularine could induce changes in BM-MSCs leading to their differentiation *in vitro* and directing them towards the cardiomyogenic lineage. Morphological results showed that fibroblast-liked cells gradually increased in size after exposure to 5-azacytidine and zebularine. It was observed that a certain concentration of 5-azacytidine and zebularine changed rat MSCs morphology and promoted the cells to form mytube structures after two weeks of treatment (Figure 4.8).

Notably, data from reverse transcriptase (RT)-PCR revealed the presence of cardiac-specific genes, including alpha myosin heavy chain (CAMHC), cardiac troponin-T (cTnT) and cardiac transcription factor (GATA4) after treatment with 5-azacytidine and zebularine. However, a very low intensity band of selected cardiac markers were observed in untreated MSC cells. GAPDH is a housekeeping gene, which has always been activated by all mammalian cells by undifferentiated or differentiated cells (Barber et al., 2005). GAPDH amplicon was strongly presented in cells, both before and after differentiation, indicating that GAPDH remains activated in both types of cells (Figure 4.9).

Densitometry analysis showed that selected cardiogenic genes expression levels increased significantly after induction (Figure 4.10). CAMHC which is one of the known structural gene in cardiomyogenesis (Planat-Bernard et al., 2004) showed a modest increase in expression after treatment. After CAMHC, another known cardiac structural gene (cTnT), and cardiac transcription factor (GATA-4) had the highest expression in treated MSC cells respectively. GATA-4 and Nkx2.5 were known as key regulator in cardiac development (Planat-Bernard et al., 2004) and GATA-4 expressed during myocardium development (Charron & Nemer, 1999; Shirinsky et al., 2008). GATA transcription factors are involved in the expression of many genes, which encode for contractile proteins like cardiac troponinT and cardiac alpha actin (Shirinsky et al., 2008).

Moreover, RT-PCR analysis showed that the cardiac markers gene expression level of 5-aza treated cells were slightly higher than those of zebularine treated cells. However, no significant differences were observed. This supports the procedure that pre-treatment of BM-MSCs with 5-azacytidine or zebularine might facilitate the differentiation towards cardiomyogenic lineage under *in vivo* conditions. Previous studies have also proven the ability of MSC cells to differentiate and express cardiomyogenic genes after *in vitro* treatment with 5-azacytidine. First Makino *et al.* (1999) induced the marrow stromal cells from adult mouse with 5-azacytidine, and obtained self-contractile myotube structure cells agglomerates which the structure looked like embryonic cardiomyocytes. Following this report, several studies showed that MSCs could differentiate into cardiomyocytes after treatment with 5-azacytidine (Fukuda, 2003; Xu et al., 2004; Antonitsis et al., 2007; Burlacu et al., 2008; Naeem et al., 2013).

Although the effects of 5-azacytidine on bone marrow mesenchymal stem cells have been widely studied, there is little evidence of zebularine being directly involved in

stem cell differentiation (Liu et al., 2003; Antonitsis et al., 2007; Burlacu et al., 2008). The results of this study showed that there were no significant differences between gene expression levels of 5-azacytidine and zebularine treated cells, although both increased expression of selected cardiac markers significantly after treatment. However, further studies need to be done to assess the mechanism and differentiation potential of zebularine into cardiomyocytes both *in vitro* and *in vivo*. The potential of zebularine was widely studied in microbial systems, cancer therapy, as well as mammalian cell lines (Cheng et al., 2003). Cheng and colleagues (2003) reported that zebularine and 5-azacytidine can induce the expression of the myogenic phenotype in mouse embryonic fibroblast cells and inhibited the methylation of specific loci in both the mouse CII-d and human p16 promoter. This study agrees with proposed of Naeem and his colleagues (2013) on zebularine that it could be used as a new candidate for cardiomyogeneic inducer. With better stability and less cytotoxic potential, zebularine could be a good replacement for 5-azacytidine for differentiation of MSCs into cardiomyocytes.

It is worth noting that even though the cardiac specific genes were up regulated and expression increased after 5-azacytidine and zebularine treatment, they were also found to be present in untreated cells, in accordance with previous data that suggested a promoting rather than inductive effect of myogenic gene transcription (Burlacu et al., 2008; Rosca & Burlacu, 2011). This is also in accordance with the hypothesis that the transcriptional machinery of adult stem cells is operating at a low level, but is not silenced, such that these cells express a variety of gene families that characterize differentiated progeny (Zipori, 2004; Rosca & Burlacu, 2011). Rosca and Burlacu (2011) stated that treatment of MSC with 5-azacytidine may promote subsequent cardiac differentiation but it is dependent on finding the adequate conditions for cardiomyogenic differentiation. Correct concentration

of 5-azacytidine coupled with the use of growth factors and cytokines may be able to create an adequate conditions for cardiomyogenic differentiation. Rangappa *et al.* (2003) also stated that 5- azacytidine promotes rather than induces the myogenic differentiation of bone marrow progenitor cells as it enhanced the appearance of myogenic markers. According to this hypothesis, adult stem cells are in a standby state, prepared to differentiate at any moment. Taken all together, these results suggested that, 5-azacytidine and zebularine promoted changes in phenotype by expressing markers and activating cardiac specific genes. It can be expected that treated MSCs prior to transplantation may increase the likelihood of successful regeneration of damaged myocardium *in vivo* environment. Because the cells would be still multipotent at the time of transplantation and also more open to the subsequent differentiation stimuli.

5.2 Limitations and Future Studies

Despite of BM-MSCs differentiation and expression of cardiac specific genes by 5-azacytidine and zebularine, treatment by these components alone may not be sufficient to sustain terminal differentiation of MSCs into cardiomyocytes. Pre-treatment of BM-MSCs with 5-azacytidine and zebularine before transplantation could facilitate the differentiation towards cardiomyogenic lineage under *in vivo* conditions. Following this priming step, the cardiac differentiation process could be completed *in vivo* by the factors within the cardiac environment. It would be worthwhile to investigate whether the treatment of multipotent stem cells with demethylating agents such as 5-azacytidine and zebularine prior to transplantation can direct them towards specific cardiomyogenic lineage in the *in vivo* environment.

CHAPETR 6
CONCLUSION

In this study, successfully isolated and cultured cells displayed the typical fibroblast-like morphology and surface antigen profile of bone marrow mesenchymal stem cells (BM-MSCs). Further, distinctive morphological characteristics and the expression of genes specific to cardiac myocytes supported their potential to differentiate *in vitro* into cardiomyocyte-like cells upon exposure to 5-azacytidine and zebularine.

In vitro cardiomyogenic differentiations of rat BM-MSCs, thus, offer a suitable model to understand their molecular and functional identities prior to transplantation. These results suggested that, 5-azacytidine and zebularine induce changes in phenotype by expressing markers and activating specific genes, but its treatment alone may not be sufficient to sustain terminal differentiation of MSCs into cardiomyocytes. Besides 5-azacytidine, which is a well known compound, zebularine as a new candidate could be a good replacement for differentiation of mesenchymal stem cells into cardiomyocytes because of its stability and less toxicity to biological systems. In order to assure the quality of the final therapeutic product, it is however important to evaluate the differentiation potential's stability *in vivo*. This would enhance the rate of bone marrow stem cell differentiation into mature cardiomyocytes in the injured heart**.**

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Appendix A: List of Apparatus, Chemical and Material

1. **Apparatus**

Dissecting set, Disposable syringe, Syringe filters, 26-gauge needles, Electronic shaker, Magnetic stirrer, Microwave oven, Ice cubes, 75 and 25 cm^3 tissue culture flasks with vented cap (Iwaki, Japan), Blue cap tube (Bioscience, Germany), Thermo scientific Nunc Lab-Tek II Chamber Slide System, Falcon tubes (Eppendorf, Germany), Spin columns (Promega, USA), Collection tubes (Promega, USA), Pipettor (Eppendorf, Germany), Elution tube (Promega, USA), Quartz cuvettes (Shimadzu, Japan), Microcentrifuge tube (Eppendorf, Germany), Class II, type A2 Biohazard safety cabinet (ESCO, Singapor), 5% CO2 incubator (NuAir, USA), Phase contrast microscope (Nikon, Japan), Fluorescence inverted microscope (Nikon, Japan), Flow cytometer (Becton Dickinson, USA), Spectrophotometer (Shimadzu, Japan), Thermal cycler (Eppendorf, Germany).

2. **Chemical and Materials:**

- 1X phosphate buffer saline (PBS)

- Alcohol 70%

- Dulbeco's Modified Eagle Medium (DMEM) (Gibco, USA)

- 10% (2.5%) Trypsin-EDTA

- 4% paraformaldehyde (PFA)

- Triton-X100

- 4', 6-Diamidino-2-phenylindone (DAPI), blue dye

- Distilled water

- Cell dissociation buffer, enzyme free, Hank's based (Gibco, USA)

- 1% Bovine Serum Albomin (BSA)

- 0.1% sodium azide

- 14.3 M b-Mercaptoethanol

- 95% EtOH

- Deionized water

- 0.1% Diethylpyrocarbonate (DPEC) treated water

- 10 Mm Tris-EDTA buffer

- M DTT

- 25 Mm MgCl2

- 1% Agarose powder

- 10mg/ml ethidium bromide

- Tris base

- Boric acid

- DNA ladder

3. **Antibodies**

- Primary antibody CD34 (Bioscience, USA)

- Primary antibody CD44 (Bioscience, USA)

- Primary antibody CD45 (Bioscience, USA)

- Primary antibody CD117 (Bioscience, USA)

- Secondary antibody green Alexa Flour, 488 goat-anti-mouse IgG (H+L) 2mg/ml

- Secondary antibody red Alexa Flour, 546 goat-anti-mouse IgG (H+L) 2mg/ml

4. Primers

- Glyceraldehyde-3phosphate dehydrogenase (GAPDH) (IDT, USA)

- Myocardium Cardiac alpha heavy chain (CAMHC) (IDT, USA)

- Cardiac troponin-T (cTnT) (IDT, USA)

- Cardiac transcription factors (GATA-4) (IDT, USA)

5. Kits:

- SV Total RNA isolation system (Promega, USA)

- RevertAid^{TM} first cDNA synthesis kit (Thermo Scientific, USA)

- Fermentas Life Science's PCR kit (Thermo Scientific, USA)

Appendix B: Stock Preparation

1. **Stock solution preparation of 5-azacytidine :**

In order to make 10mM stock solution:

Molecular weight of 5-azacytidine: 244.24 g/mol

10 mM= 0.01 Mole

MW of 5aza = 244.24 g/mol

0.01M x 244.24 g/mol = 2.4424 mg

2.4424 mg of 5-aza dissolve in 1 ml of sterile water $\Longrightarrow$ 2.4424 mg/ml

In order to make 3uM concentration of 5-aza:

$M_1 V_1 = M_2 V_2$

(10) mM x (V_1) = (3)uM (13) ml $\Longrightarrow$ V_1 = 3.9 uL of 5-azacytidine

2. **Stock solution preparation of Zebularine:**

In order to make 10mM of stock solution:

Molecular weight of zebularine: 228.20 g/mol

10 mM= 0.01 Mole

0.01m x 228.20 g/mol= 2.2820 mg

2.2820 mg of zebularine dissolve in 1 ml of sterile water $\Longrightarrow$ 2.2820 mg/ml

In order to make 3uM concentration of zebularine:

$M_1 V_1 = M_2 V_2$

(10) mM x (V_1) = (3) uM (13)ml $\Longrightarrow$ V_1 = 3.9 uL of zebularine

3. 5X TBE buffer preparation for gel electrophoresis

Components	Amount
Tris base	54 gm
Boric acid	27.5 gm
EDTA	1.861 gm
dH$_2$O	up to 1 L

4. Agarose gel preparation

Components	amount
5X TBE buffer	10 ml
Distilled water	40 ml
1% agarose powder	0.5 gm
Ethidium bromide	1.5 uL

Appendix C: Publications

1. Publication:

Noorezatey M, Khairul Fidaa K.B., **Ramin K**. Maizatul Fazilah AR, Anbarieh S, Shamsul Azlin AS, and Durriyyah Sharifah HA. *Visual confirmation of isolated bone marrow mesenchymal stem cells and differentiated cardiomyocyte-like cells.* Malaysian Journal of Microscopy. Vol 9, November 2013. ISSN 1823-7010. pg 145-148.

2. Publications in conference:

1. **Ramin K**, Noorezatey M, Khairul Fidaa KB, Maizatul Fazilah AR, Anbarieh S, Shamsul Azlin AS, Durriyyah Sharifah HA and Asmat S. Differentiation potential of rat bone marrow mesenchymal (BMMSCs) into cardiomyocyte-like cells affer zebularine and 5-azacytidine treatments. *38th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology (MSBMB).* Putrajaya Marriott Hotel & Spa, Putrajaya, Malaysia. 28-29 August 2013.

2. Noorezatey M, Khairul Fidaa K.B., **Ramin K**. Maizatul Fazilah AR, Anbarieh S, Shamsul Azlin AS, Durriyyah Sharifah HA. and Asmat, S. Visual confirmation of isolated bone marrow mesenchymal stem cells and differentiated cardiomyocyte-like cells. *22nd Scientific Conference of Microscopy Society Malaysia (MSM 2013).* Primula Beach Hotel, Kuala Terengganu, Terengganu, Malaysia. 26-28 November 2013.

3. Anbarieh S, Maizatul Fazilah AR, **Ramin K**, Shamsul Azlin AS, Durriyyah SharifahHA Asmat S. Preliminary study: Differentiation of bone marrow mesenchymal stem cells into neuronal-like cells by β-mercatoethanol and confirmation through morphopogical features. *4th **International** NeuroMalaysia Society Symposium.* MonashUniversity Sunway Campus, Malaysia. 28 September 2013.

VISUAL CONFIRMATION OF ISOLATED BONE MARROW MESENCHYMAL STEM CELLS AND DIFFERENTIATED CARDIOMYOCYTE-LIKE CELLS

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ABSTRACT

This project attempted in vitro protocols to isolate, culture, expand and induce bone marrow mesenchymal stem cells (BM-MSCs) into cardiomyocyte-like cells. Mixed hematopoietic and mesenchymal bone marrow cells were isolated from Sprague Dawley rats. They were then cultured for 15 days in complete Dulbecco's Modified Eagle Medium (DMEM), during which the media was changed every 3 days to remove unattached floating hematopoietic cells. Inverted phase contrast microscope was used for morphological detectection of BM-MSCs. Immunocytochemistry was also done to confirm BM-MSCs characteristics based on specific protein markers (positive markers; CD44 and CD117; negative marker: CD34) and secondary antibody IgG Alexa Fluor, visualized by fluorescence microscope. Subsequently, 10-15 days separate treatments of synthetic demethylating agents, 5-azacytidine and zebularine, were conducted to induce differentiation of BM-MSCs into cardiomyocyte-like cells. Acquired mixed bone marrow cells of hematopoietic and mesenchymal cells were seen morphologically roundish/ spherical in shape at the initial culturing. Progressively, more BM-MSCs displaying spindle-shaped fibroblast morphology were observed. Immunostained cells successfully confirmed these BM-MSCs. The separate treatments by selected inducers resulted in morphologically cardiomyocyte-like cells; each having extended cytoplasmic processes and a myotube-like structure. It can be concluded that the protocols used could successfully develop BM-MSCs to cardiomyocyte-like cells.

Keywords: Bone Marrow, Stem Cell, Cardiomyocyte-like Cell

INTRODUCTION

Stem cells are the undifferentiated and unspecialized cells, which not only have the abilities for self-renewal, but also to differentiate into specific cells of various lineages [1, 2]. There are two types of stem cells; embryonic stem cells (ESCs) and adult stem cells. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies with lesser ethical issues than using ESCs [3, 4]. Bone marrow provides a renewable source of adult mesenchymal stem cells (MSCs) that can be expanded rapidly in culture. The culture-expanded and characterized MSCs have been actively studied for their potential to differentiate into several lineages *in vitro* and enhance tissue repair *in vivo* [5, 6]. Since the possibility of MSCs to differentiate into myogenic cells was first reported by Wakitani and associates [7], the transplantation of cultured cardiomyocytes into damaged myo-cardium has been proposed as a potential future therapy for heart treatment [8, 9]. Myocardial cell transplantation research has generated significant interest since myocardial infarction is a leading cause of morbidity and mortality in civilized countries. Adult cardiac muscle unfortunately lacks the ability to regenerate damaged region and post-infarction function is often seriously compromised [10]. The current study attempted *in vitro* protocols to isolate, culture, expand and induce bone marrow mesenchymal stem cells (BM-MSCs) using two de-methylating agents into cardiomyocyte-like cells.

MATERIALS & METHODS

Mixed hematopoietic and mesenchymal bone marrow cells were isolated from tibia and femur of *Sprague Dawley* rats. They were then cultured for 10-15 days in complete Dulbecco's Modified Eagle Medium (DMEM), during which the media was changed every 3 days to remove unattached floating hematopoietic cells. Cultures were maintained in a humidified environment with 5% CO_2 at 37 °C using CO_2 incubator. This culture is termed as the primary culture (Passage 0 or P0 cells). This primary culture contained both hematopoietic and BM-MSCs, with the later having the ability to adhere on to the plastic surface of the flasks. Non-adherent hematopoietic cells were removed by changing the culture medium. Inverted

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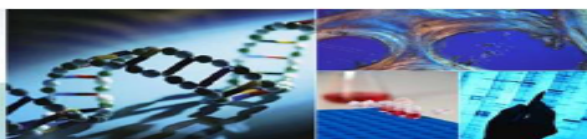
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Malaysia.

Dear **Ramin Khanabdali**,

Thank you for registering for the 38th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology.

Conference   : 38th Annual MSBMB Conference
Day / Date   : Wednesday & Thursday / 28th - 29th August 2013
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Venue   : Putrajaya Marriott Hotel & Spa, Putrajaya

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Thank you.

Best wishes and regards,
M. S. Kanthimathi
Secretariat,
38th Conference of MSBMB

Conference website: http://www.msbmb.org
Conference email: 38msbmb@gmail.com

DIFFERENTIATION POTENTIAL OF RAT BONE MARROW MESENCHYMAL STEM CELLS (BM-MSCS) INTO CARDIOMYOCYTE-LIKE CELLS AFTER ZEBULARINE AND 5-AZACYTIDINE TREATMENTS

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The use of adult stem cells, particularly bone marrow derived mesenchymal stem cells; to safely facilitate recovery of cardiac function after myocardial infarctions has recently received a lot of interest. This study investigated the potential of rat bone marrow-mesenchymal stem cells (BM-MSCs) to differentiate *in vitro* into cardiomyocyte-like cells. MSCs were isolated and cultured in DMEM Medium. Cultured MSCs were first analyzed and confirmed using specific protein surface markers (CD44, CD45, CD117 and CD34) by immunocytochemistry and flow cytometry. Passage 1 cultured MSCs were then treated separately with optimized concentration of two synthetic compounds, zebularine and 5-azacytidine. Treated cells were then analyzed for the expression of cardiac specific gene (GATA-4, cTnT, CAMHC and GAPDH) by RT-PCR. Isolated BM-MSCs exhibited a fibroblast-like morphology and positively stained for CD44, CD45, and CD117. However, they were negative for CD34, hence, confirming the absence of hematopoietic lineage. Upon induction, PCR data indicated higher gene expression level of cardiogenic genes compared to untreated culture. Cardiogenic mentioned gene expression levels were detected to be slightly higher in 5-azacytidine treated culture compared to zebularine.

Preliminary study: Differentiation of bone marrow mesenchymal stem cells to neuronal-like cells through treatment with β–mercaptoethanol and confirmation through morphological features

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Bone Marrow Mesenchymal Stem Cells (BM-MSCs) have extensive self-renewal capability. Mesenchymal Stem Cells (MSCs) could be differentiated *in vitro* into specific cell types through induction by certain compound in the culture medium. This preliminary project investigated the commonly used synthetic compound in cell culture, β-Mercaptoethanol (BME), as inducer in differentiating BM-MSCs into neuronal-like cells. Initial isolation of MSCs from *Sprague Dawley*'s bone marrow was followed by characterizations of confluent BM-MSCs. At P0 unattached and floating cells were seen. Morphologically these cells were roundish and spherical in shape. Within the first couple of days of culturing (P1) spindle shaped cells with projections were observed. Continuous reduction in quantity of the roundish cells and the increase of morphologically altered fibroblast-like cells indicated the development of BM-MSCs. The latter type of cells were subsequently treated with 5μL of BME after reaching 70% - 80% confluency. After a maximum 29 hours of treatment, morphological changes were observed under inverted microscope with magnification of 10X, 20X and 40X. Treated BM-MSCs demonstrated apparent neuronal-like morphological features. Cytoplasm of cells were seen having retracted towards nucleus forming cell bodies, while dendrite-like branches were projected out from cell bodies. Besides the evolvement of multipolar cells, physical connectivities were formed by these neuronal-like cells. Thus, neuronal morphological features and physical connections between cells suggested the differentiation and the existence of neuronal-like cells from BM-MSCs after treatment with BME. This study indicated the ability of BME to serve as a good inducer in differentiating BM-MSCs into neuronal-like cells.