CONTRIBUTION OF MESENCHYMAL STEM CELLS IN CELL BASED THERAPIES

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BY

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ABSTRACT

CONTRIBUTION OF MESENCHYMAL STEM CELLS IN CELL BASED THERAPIES

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Ph.D. in Molecular Biology and Genetics

Supervisor: Assist. Prof. Dr. K. Can Akçalı

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Stem cell research evolved as a new hope and has gained tremendous interest in the last two decades to develop new strategies for many of debilitating diseases. Mesenchymal Stem Cells (MSCs) are multipotent cells capable of self-renewal and differentiating into multiple lineages such as osteocytes, adipocytes, chondrocytes, myoblasts, and hepatocytes. MSCs can migrate to the injured tissue and have immunomodulatory effects. Due to these features, MSCs have high therapeutic value in tissue engineering and regenerative medicine. In this thesis, our aim was to investigate the further contribution of the MSCs in different cellular therapies. We used two approaches to accomplish our aim. First, we investigated the possibility of obtaining functional cardiomyocytes from rat MSC within a shorter time period by determining the induction timing of cardiomyocyte differentiation of MSCs. Our data revealed that it is possible to get functional cardiomyocytes from *in vitro* MSC culture in a shorter time period than previously achieved. This reduction in time may provide emergency cases with access to cell-based therapies that may have previously been unavailable. In the second part of this thesis, we examined *in vivo* and *in vitro* effects of a telomerase antagonist, imetelstat (GRN163L) on MSCs. Telomerase activity is essential for the continued growth and survival of malignant cells, therefore inhibition of this activity presents an attractive target for anti-cancer therapy. MSCs also show telomerase activity in maintaining their self-renewal; therefore the effects of telomerase inhibitors on MSCs may be an issue of concern. Our results showed that inhibiting the telomerase activity does not interfere with the self-renewal and differentiation of MSCs under short term *in vitro* culture conditions.

ÖZ

MEZENKİMAL KÖK HÜCRELERİN HÜCRE TEMELLİ TERAPİLERE KATKISI

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Moleküler Biyoloji ve Genetik Doktorası

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Kök hücre araştırmaları tedavisi olmayan hastalıklar için yeni bir umut olmuş ve son 20 yılda hastalıklara tedavi geliştirmek için yeni bir strateji olarak büyük ilgi görmüştür. Mezenkimal kök hücreler (MKH) kendini yenileme kapasitesine sahip, osteosit, adiposit, kondrosit, miyoblast ve hepatosit gibi değişik hücre kökenlerine farklılaşabilen, hasarlı dokuya ulaşabilen ve bağışıklık sistemini baskılayıcı etkiye sahip hücrelerdir. Bu özelliklerinden ötürü MKHlerin doku mühendisliği ve rejeneratif tıptaki terapötik önemi yüksektir. Bu tezde, amacımız MKHlerin hücre terapilerine katkısını incelemekti. Amacımıza ulaşmak için iki yöntem kullandık. İlk olarak, rat MKHlerinden kardiyomiyosit farklılaşmasının daha kısa sürede elde edilebilirliğini, MKHlerden kardiyomiyosit farklılaşmasını indükleme süresini belirleyerek araştırdık. Verilerimiz *in vitro* MKH kültüründen daha kısa sürede fonksiyonel kardiyomiyosit elde edilebildiğini göstermiştir. Süredeki bu kısalma acil vakaların daha önce erişimleri sağlayamadıkları hücre temelli terapilere erişimini sağlayabilecektir. Tezin ikinci bölümünde telomeraz antagonisti olan imetelstat'ın (GRN163L) MKHler üzerindeki *in vivo* and *in vitro* etkilerini araştırdık. Telomeraz aktivitesi malign hücrelerin büyüme ve hayatta kalmaları için gereklidir, bu nedenle bu aktivitenin engellenmesi kanser tedavisi için çekici bir hedef oluşturmaktadır. MKHler de kendilerini yenilemek için telomeraz aktivitesine sahiptirler ve telomeraz inhibitörünün MKHler üzerindeki etkisi kesin olarak bilinmemektedir. Sonuçlarımız, telomeraz aktivitesinin engellenmesinin kısa süreli *in vitro* kültür koşullarında MKHlerin kendini yenileme ve farklılaşma potansiyellerini etkilemediğini göstermiştir.

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ABBREVIATIONS

ALT	Alternative Lengthening of Telomeres
ASC	Adult Stem Cells
BM	Bone Marrow
bp	Base Pair
BSA	Bovine Serum Albumin
BrDU	Bromodeoxyuridine
CCR	Cytokine Receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU-F	Colony Forming Unit Fibroblasts
CXCR	Chemokine receptor
ddH2O	Double distilled water
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	deoxy Nucleotide Triphosphate
ESC	Embryonic Stem Cells
FBS	Fatal Bovine Serum
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GVDH	Graft versus Host Disease
HIF	Hypoxia-Inducible Factor
HLA	Human Leukocytes Antigen
HRP	Horse Raddish Peroxidase

HS	Hot Start
HSCs	Hematopoietic Stem Cells
ICM	Inner Cell Mass
IL	Interleukin
IP3	Inositol-3-Phosphate
ISCs	Intestinal Stem Cells
iPS	Induced Pluripotent Stem Cells
kDa	Kilo Dalton
kg	kilogram
LG-DMEM	Low Glucose Dulbecco's Modified Eagle Medium
LIF	Leukemia Inhibitory Factor
M	Molar
MEF	Mouse Embryonic Fibroblast
MeOH	Methyl Alcohol
mg	milligram
MHC	Major Histocompatibility Complex
mL	Milliliter
mM	milliMolar
MSC	Mesenchymal Stem Cells
MRI	Nagnetic Resonans Imaging
NK	Natural Killer cell
nM	nanoMolar
NSCs	Neural Stem Cells
PBS	Phosphate Buffered Saline
PD	Population Doubling
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGE	Prostaglandin E

PVDF	Polyvinylidenedifluoride
Q-RT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
RNA	ribonucleic acid
RNA	Ribonucleic acid
Rpm	revolution per minute
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
rtTA	Reverse Tetracyclin Transactivator
SDS	Sodium Dodecyl Sulfate
TAE	Tris Acetate EDTA
TBS	Tris Buffered Saline
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
Tet	Tetracyclin
TGF	Transforming Growth Factor
Th	Helper T-cell
TNF	Tumor Necrosis Factor
TRAP	Telomeric Repeat Amplification Protocol
VEGF	Vascular Endothelial Growth Factor
µg	Microgram
µl	Microliter
µL	Microliter

CHAPTER 1

INTRODUCTION

Radiometric age dating indicates that the world is 4.5 billion years old and the origins of first human beings date back to 200.000 years ago. Throughout these 200.000 years, human beings have been facing with many problems. Majority of these problems have been caused by the humans themselves in the past and present such as pollution, war and feminine. In the last 50 years or so, humans have been facing another problem, aging. Aging is the result of the increased life quality due to the technological improvement to diagnose and treat diseases. According to World Health Organization's 2008 health report 85% of the world population is supposed to be over the age of 60 by 2050 while today the mean life expectancy in the world is over 75 years of age (http://www.who.int/whr/2008/whr08_en.pdf). In this chamber of globalization, urbanization and aging the incidence of chronic and non-infectious diseases now are the main health concerns of the population, like cancer, chronic heart diseases and diabetes. Moreover, together with the increase at life span more than 50% of the people above age 70 have more than one chronic disease. Thus, treatment of these chronic diseases is very important for a healthy future.

Stem cell research evolved as a new hope and has gained tremendous interest in the last two decades to develop new strategies for many of these

diseases. Applications involving the use of stem cells in humans that might have been considered “science fiction” fewer than 20 years ago are now being utilized with a great success rate (Akar *et al* 2006). In this thesis, our aim was to investigate the contribution of the mesenchymal stem cells (MSCs) in cellular therapies. We used two approaches to accomplish our aim:

i) directly by determining the induction timing of cardiomyocyte differentiation of mesenchymal stem cells and;
ii) indirectly by examining the *in vitro* effects of a telomerase antagonist, imetelstat (GRN163L) on mesenchymal stem cells.

Introduction section starts with a brief explanation of the stem cells. Embryonic stem cells, induced pluripotent stem cells and adult stem cells will be introduced. Among the adult stem cells, mesenchymal stem cells will be discussed in detail including their use in cellular therapy. Finally, the structural and functional features of telomerase enzyme will be analyzed.

1.1 Stem cells

Stem cells are group of cells that are able to self-renew and differentiate into several lineages to form specialized somatic cells (Morrison et al 1997, Weissman 2000; Till and McCulloch 1961). Stem cells are able to accomplish these tasks by undergoing symmetric and/or asymmetric cell division (Preston et al 2003). Symmetric division yields two daughter stem cells having the same properties of stemness which are undifferentiated and have the capacity to differentiate into other lineages. If there is a requirement for differentiated somatic cells, then a stem cell undergoes asymmetric division to yield a progenitor cell

and an undifferentiated copy of itself to maintain the stem cell pools of the body. Stem cells can be categorized based on their time of onset. Embryonic stem cells are present on in the very early part of the embryonic development, but germ line and adult stem cells become active later in the development and post nataly respectively. Better categorization of the stem cells can be made based on their potencies. Embryonic stem cells are pluripotent, which can form all the cell types from all body lineages where as adult stem cells are multipotent and able to produce multiple lineages.

1.1.1 Embryonic Stem Cells

After fertilization, zygote undergoes serial divisions during the embryonic development. At the blastula stage, cluster of cells form inner cell mass (ICM) where embryonic stem cells are obtained (Evans and Kaufman, 1981, Martin 1981, Thomson et al, 1998, Reubinoff et al 2000). Embryonic stem cells are pluripotent and can differentiate into any of the cells belonging to three germ layers, endoderm, mesoderm and ectoderm. (Figure 1.1.1.1). Furthermore, they can maintain their pluripotency for many passages without losing their genetic integrity (Burdon et al 2002, Smith 2001, Keller 2005).

ESCs form teratomas *in vivo*, which contains cell types from all germ layers (Wobus and Boheler 2005). X chromosome is active and ESCs do not undergo X inactivation until they are differentiated (Yang et al 2007). ESCs require a feeder layer composed of irradiated human or mouse embryonic fibroblast (MEF) cells. Feeder layers not only provide better attachment but also secrete growth factors for their maintanence (Amit et al 2003). In addition, mouse ESCs require leukemia inhibitory factor (LIF) to maintain their undifferentiated

state *in vitro* while human ESCs need FGF-2 (Odorico et al 2001, Pera and Trounson 2004, Keller 2005). When ESCs are removed from the feeder layers, some cells start differentiating spontaneously and some of them remain undifferentiated. These cells form clusters called embryoid bodies which are unique features of ESCs in suspension cultures. ESCs express transcription factors such as Oct4, Sox2 and Nanog which are important to keep them undifferentiated and pluripotent (Mitsui et al 2003, Strumpf et al 2005, Kuroda et al 2005, Rodda et al 2005). Human ESCs also express SSEA3 and 4 on their surfaces as markers of their undifferentiated state. They have active telomerase enzyme (Semb 2005, Hiyama and Hiyama 2007) that is rapidly reduced upon the differentiation (Armstrong et al 2005).

ESCs are important tools for studying embryogenesis, understanding the mechanisms of genetic diseases and for cellular therapies. There are severe ethical problems when human ESCs are considered despite the fact that these embryos are leftover embryos obtained from IVF clinics. In addition, the risk of tumor formation and immune rejection also the problems that need to be solved.

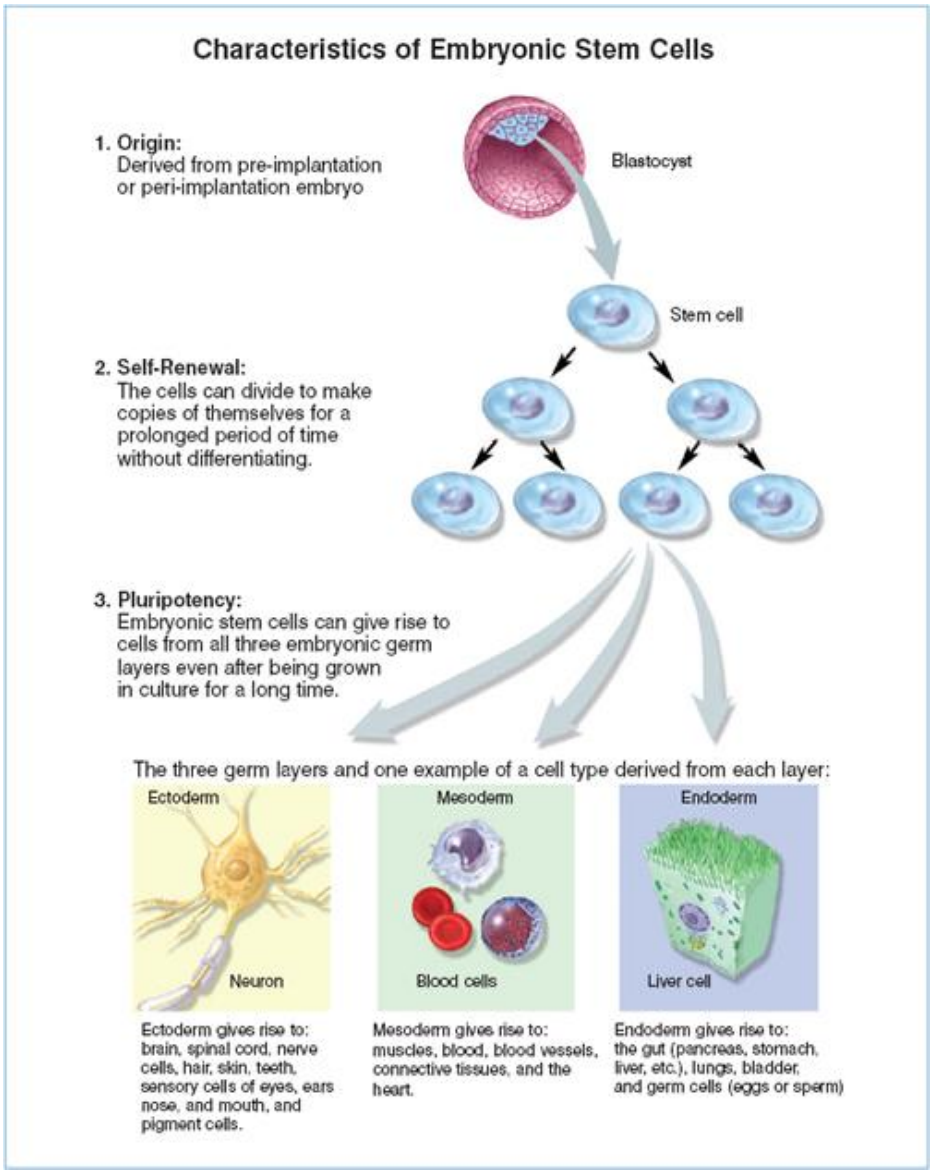


Figure 1.1.1 1 Characteristics of ESCs ©2006 Terese Winslow (http://stemcells.nih.gov/info/2006report/2006Chapter1.htm)

1.1.2 Induced Pluripotent Stem Cells

The problems that are associated with ESCs prompted the researchers to investigate new sources of stem cells that have the features of ESCs. Attempts

finally reached to a success in 2006 when two Japanese scientists, Takahashi and Yamanaka announced the induction of pluripotent cell from normal somatic cell. Their study revealed that, when mouse fibroblast cells were transfected with lentiviral vectors carrying Oct-4, Sox-2, Klf-4 and c-myc, the cells became pluripotent and this process was named as "Induced Pluripotent Cells (iPS)". These four factors are called as "Yamanaka Factors". In addition, iPS cells form embryoid bodies, regain their telomerase activity, elongate their telomeres and form teratomas *in vivo*, but more importantly they are capable of restarting the mouse developmental process after being injected into a blastocyst. When results of their study were first published, it opened a new era and raised hopes to obtain patient specific pluripotent stem cells. Afterwards studies towards better understanding the induced pluripotent stem (iPS) cells and their uses have gained tremendous interest. In 2008 iPS cells were developed by using human fibroblasts as a source (Park et al 2008, Takahashi et al 2008, Yu et al 2008). Several different cell types like pancreatic beta cells, dermal fibroblasts and keratinocytes (Lowry et al 2008, Stadtfeld et al 2008, Aasen et al 2008) were also used as a source with different transfection efficiencies. There are also some drawbacks in iPS cells. All these studies were employing retroviral vectors which might have mutagenic effects. In addition, introducing c-myc raised considerations against generation of tumors. As the main aim of the induced pluripotency was to generate patient specific cells for cell therapy, to improve the methods of iPS cell generation have become one of the fasted evolving areas of stem cell research. To tackle these problems drug induced systems, addition of N-myc instead of c-myc and using strategies without viral vectors (Wernig et al 2008, Nakagawa et al 2008, Okita et al 2008) have been utilized. A successful system employed piggyBac transposase to generate iPS cells. In this system, Yamanaka factors were inserted by a single cassette controlled by tet/rtTA. By this way the expression of the transgenes were conditionally controlled. After a successful

induction was generated, the genes inserted at the first transfection were removed with a second transposase transfection (Woltjen et al 2009). However at the end of all protocols the morphological, genetically and functional properties of iPS cells should be checked according to their similarities with ESCs. Recent studies also revealed that the cells which are reprogrammed into iPS cells retain their initial epigenetic codes that may affect their differentiation properties (Polo et al 2010, Kim et al 2010). Polo et al (2010) showed that continuous passaging reduced the differences between differentiation capacities into different lineages and the transcript identities between ESC and iPS cells derived from muscle, blood and fibroblast origins.

Although there are some drawbacks, using iPS cells in cellular therapies is one of the most promising options, at least in animal models. Alpio et al (2010) achieved to reduce high glucose levels in two different mouse models for diabetes mellitus by using differentiated stem cells obtained from iPS cells. In a mouse spinal cord injury model iPS cells were also used to induce neural differentiation and contributed to remyelination, formed functional neurons and astrocytes (Tsuji et al 2010). In addition, there are several studies that employ iPS cells successfully in the models of cardiovascular regeneration (Nelson et al 2009, Narazaki et al 2008, Zhang et al 2009) as well as Parkinson's disease (Wernig et al 2008).

1.1.3 Adult Stem Cells

Adult stem cells (ASCs), also known as somatic stem cells, are found in organs and tissues and maintain homeostasis. Compared to ESCs, they are more differentiated and their potential is more limited (multipotent vs pluripotent).

There are ASCs unique for almost all tissue types such as blood, bone, intestine, brain, liver and heart (Figure 1.1.3.1). Although tissue specific stem cells have their own specific markers, but they are not universal markers for all ASC types. Since, ASCs are quiescent cells, retention of DNA labeling dyes like BrdU or histone-GFP is used to overcome such identification problems (Tumbar et al 2004, Kiel et al 2007).

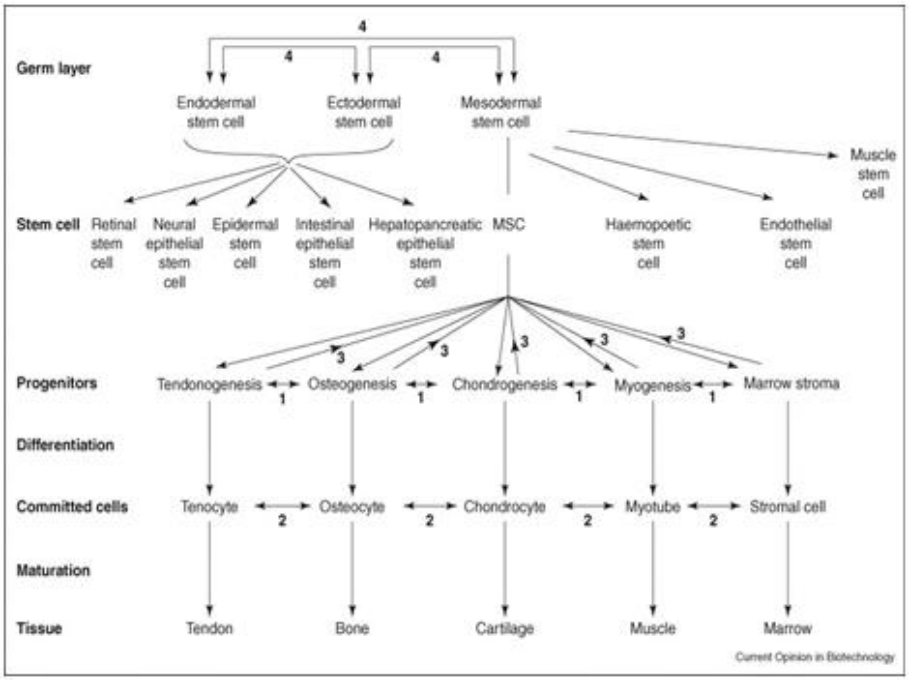


Figure 1.1.3 1 Adult Stem Cells (Raghunath et al 2005)

The first discovery of ASCs leads back to 1950s when irradiation studies were performed to find a cure after atomic bomb sent to Hiroshima and Nagasaki. Scientists discovered that, when mice were severely irradiated to deplete their bone marrow cells, they showed the same symptoms with the people survived after atomic bomb. If they were injected again with bone marrow of another mouse on the other hand, the whole blood system was regenerated (Lorenz et al 1951, Ford et al 1956, Nowell et al 1956, Gengozian et al 1957). These initial

studies lead to the discovery and characterization of the hematopoetic stem cells (HSCs) (Wu et al 1963, Sprangrude et al 1988).

HSCs are the most widely studied ASC type. They express CD34, CD117, Sca-1 as the surface markers (Kim et al 1998, Negrin et al 2000). They are able to differentiate into several cell types other than blood cells such as muscle cells, cardiomyocytes, neurons and hepatocytes (Ferrari et al 1998, Peterson et al 1999, Brazelton et al 2000, Jackson et al 2001). One of the main aims of bone marrow (BM) transplantation is actually the transplantation of HSCs. BM transplantation is considered as an effective treatment in many different hematopoetic diseases including malignancies. However HSC transplantation still has some draw backs mainly due to the immune rejection manifested as graft versus host disease (GVDH).

There are also tremendous amount of cell removal in the intestines. Therefore intestines are also another source of stem cells. Intestinal stem cells (ISCs) are localized in the crypts (Marshman et al 2002) and responsible for the maintenance of different cell types of the intestines. Their localization is believed to be at the +4 position according to the Paneth cells at the bottom of the crypt as shown by Potten (1974) and Marshman et al (2002). Several molecules were identified a intestinal stem cell marker like musashi-1, sFRP5, Dcamkl1, prominin1/CD133 and Lgr5 (Kayahara et al 2003, Asai et l 2005, Gregorieff et al 2005, Giannakis et al 2006, Barker et al 2007, Snippert et al 2009, Sato 2009). Among these markers Lgr5, which is an orphan G protein coupled receptor, is widely accepted as an intestinal stem cell marker since 2007 (Barker et al 2007).

Neural stem cells (NSCs) are found in the neural tissue generating neurons, astrocytes and oligodendrocytes and can be isolated from sub-ventricular

zone of the brain (Kokavay et al 2008). Although the clues about their presence were discovered in 1960s, the idea of brain can not regenerate was dominant until 1992. First clues about the functionality of NSCs were gathered when Reynods and Weiss (1992) discovered that the cells taken from adult mice brain could be stimulated *in vitro* with epidermal growth factor and they were able to proliferate and form neurons and astrocytes. Six years after, the proliferation of the cells in hippocampus and dentate gyrus of adult humans were demonstrated from the autopsy samples taken from the ex-patients treated with BrdU (Eriksson et al 1998). The mostly known markers of neural stem cells are Sox2, GFAP, Pax6, LeX and nestin (Kokavay et al 2008, Conti and Cattaneo 2010). Studies performed in several animal models of neurological diseases including Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis as well as spinal cord injury models revealed the success in cellular therapy models by using NSCs indicating their functional role in maintenance (Ronaghi et al 2010, Kim and Vellis 2009 and Taupin 2008).

In addition to ISCs, NSCs and HSCs there are several somatic stem cells as well and one of them is mesenchymal stem cells (MSCs) which are also localized mainly in BM like HSCs. In the next section MSCs will be described in detail.

1.1.4 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), also known as bone marrow stromal cells or mesenchymal stromal cells were discovered by the pioneering studies of Friedenstein in 1968. During the *in vitro* culturing of HSCs, it was discovered that there was a different type of cell in the bone marrow other than HSCs with fibroblastic morphology. They were able to attach to the culture plate were

forming colonies which were afterwards named as colony forming unit fibroblasts, CFU-F (Lanotte et al 1981). Moreover, they were also able to differentiate into bone, adipose, cartilage and muscle tissue (Figure 1.1.4.1). Later on, these adherent, fibroblastic and colony forming cells were first named by Owen as stromal stem cells (1988) which were then finally called as mesenchymal stem cells by Caplan (1991). First characterization study of MSCs was performed by Pittenger et al in 1999 obtained from human bone marrow aspirates. The BM aspirate was first separated according to the density gradient and plated afterwards. Attached cells were counted according to their colony formation capacities and when proportioned to the total cell number, only 0.001 to 0.01% of the nucleated cells formed colonies. Moreover, they have shown that MSCs were positive for CD29, CD90, CD71 and CD106 and negative for CD45, CD14 and CD34. Furthermore these cells were able to undergo 40 population doublings (PD) *in vitro* within 10 weeks.

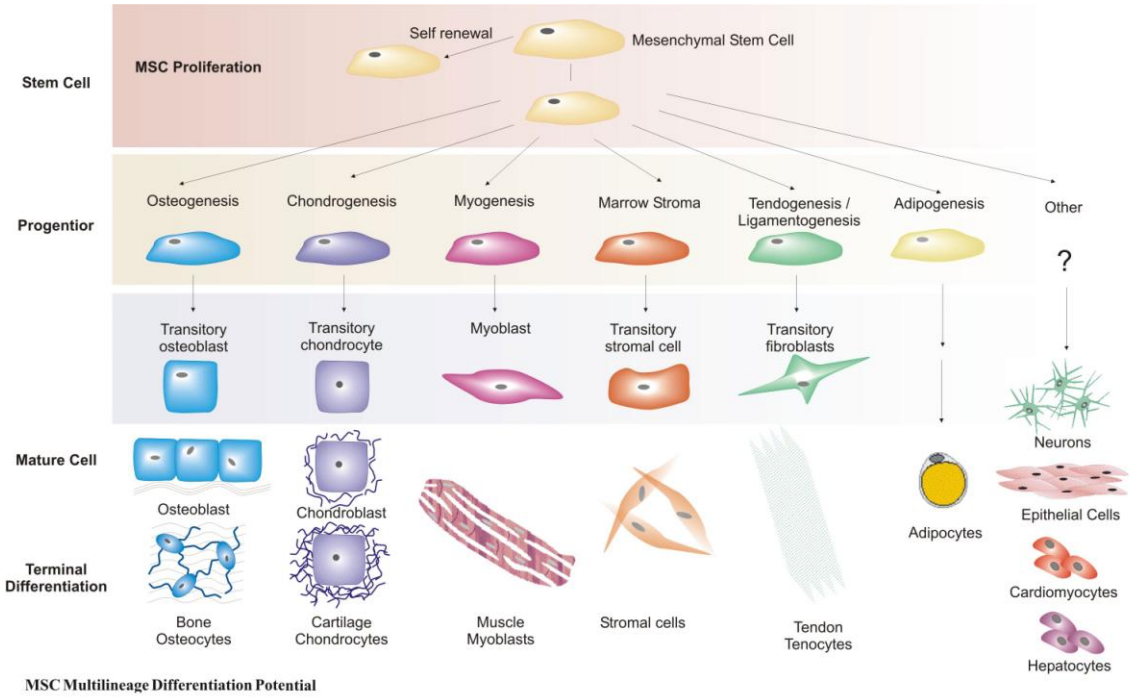


Figure 1.1.4 1 MSC multilineage differentiation potential (Caplan and Bruder 2001)

Other than bone marrow, MSCs were also isolated from many other sources such as adipose tissue, umbilical cord blood, placenta and even from dental pulp. However, studies revealed that MSCs from these sources had different CD marker expressions, phenotypes and PD. These results forced the scientific world to define certain criteria in identifying these cells. International Society for Cellular Therapy published a position paper by Dominici *et al* (2006) and set following features for MSCs. MSCs should be positive for CD73, CD90 and CD105, negative for CD19, CD34, CD45, CD11a and HLA-DR. In addition, MSCs should be isolated from fresh tissue and should attach to the plastic culture plates and differentiate into adipocytes, chondrocytes and osteocytes *in vitro*.

An important feature of MSCs is their homing capacity. Homing is defined as the migration of these cells to the site of injury. In the homing process of

MSCs, chemokines, cytokines and receptors on their surface were shown to be important (da Silva Meirelles *et al* 2008). When inflammation occurs at the site of injury, the gradient of cytokines and chemokines increase and the expression of chemokine receptors mediate the migration of MSCs to the injured tissue (Salem and Thiermann 2010). Especially CD44 was found to be important in homing of both mouse and human MSCs (Herrera *et al* 2004, Sackstein *et al* 2008). In addition, CXCR4 and VCAM-1 (CD106) had also a critical role in the migration of the MSCs (Segers *et al* 2006, Shi *et al* 2007, Hung *et al* 2007). It was also shown that MSCs can activate matrix metalloproteases to enter to the tissue from blood to localize the niche at the site of injury (De Becker *et al* 2007).

MSCs also have important antiapoptotic and immunomodulatory effects, which makes them non immunogenic. In animal models when the MSCs were injected to the scarred tissue, they were able to reduce the apoptotic rate of the surrounding cells, which were mediated by the secretion of several growth factors like VEGF, FGF2 and TGF-β especially in hypoxic conditions (Togel *et al* 2007, Parekkadan *et al* 2007, Block *et al* 2009). When immunomodulatory effects are considered, it was shown that the proliferation of the T-cells were inhibited during *in vitro* co-culturing (Di Nicola *et al* 2002, Krampera *et al* 2003, Le Blanc *et al* 2003). Furthermore, not only cytotoxic and helper T cells but also natural killer cells, B-cells and immature dendritic cells were found to be affected by MSCs. The immunomodulatory effects of MSCs on T-cells and NK cells were associated via secretion of molecules like TGF-β, PGE2 and IL10. Whereas their effect on B-cells was found to be indirect, via the modulation of plasma cells by leading the inhibition of immunoglobulin secretion (Sotiropoulou *et al* 2006, Nasef *et al* 2007, Rafei *et al* 2008, Nemeth *et al* 2009, da Silva Meirelles 2009).

MSCs are important source for cellular therapies for the following reasons:

i) Their well defined markers enable to obtain with high purity,

ii) They can easily be expanded *in vitro* with high numbers without losing their properties,

iii) They can differentiate into different cell types,

iv) They can migrate to the injured site *in vivo*,

v) They have immunosuppressive effects that make their use possible in allogeneic grafting and off-shelf use.

These special features of MSCs make them good candidates in regenerative medicine and cellular therapies.

1.1.5 Mesenchymal Stem Cells in Cellular Therapies

In the literature there are many studies investigating the regenerative capacities of MSCs in different disease models generated by employing different non-human animal species. Cardiac regeneration, liver regeneration, kidney regeneration, autoimmune diseases and graft versus host disease (GvHD), neurological diseases, pulmonary diseases, osteogenic diseases, and cartilage repair are the most widely studied conditions. Moreover MSCs are also being investigated extensively by clinical trials, mostly in United States, Europe and East Asia. Some of the clinical trials are studying their use in neurological, liver, bone, heart diseases, GvHD and some autoimmune diseases like diabetes and Chron's disease. In the following section, the application of MSCs in different cell based therapies will be discussed and a particular attention will be given to their roles in heart diseases.

1.1.5.1 MSCs in renal injuries

Kidney injuries if left untreated can progress to acute kidney failures followed by chronic kidney failures. At the end stage of renal disease dialysis or kidney transplantation is needed. Renal injuries could be due to immunological problems or to ischemia (Salem and Thiemermann 2010) suggesting the importance of restoring the microvasculature (Bussolati *et al* 2008). Treatment of kidney injuries requires either an immunomodulatory effect or replacement of the scared tissue. Therefore, MSCs are good candidates in curing kidney diseases. In the sheep model of ischemia reperfusion injury, it has been shown that the intraarterially injected MSCs differentiated into tubular and glomerular cells (Behr *et al* 2007). In a previous study performed with a rat model of ischemia reperfusion injury, the restoration of renal function was also observed after MSCs were introduced by intracarotid administration. However, this improvement was found to be a result of immunomodulatory action of MSCs as no differentiation was reported (Tögel *et al* 2005, Lange *et al* 2005). In recent studies performed with human umbilical cord derived MSCs similar result was obtained indicating the improvement of renal failure via immunomodulatory effects of MSCs (Chen *et al* 2010, Cao *et al* 2010). In this immunomodulatory activity homing of the MSCs to the injury site was found to be important. It has also been shown that the way of the administration of MSCs is also important for their immunomodulatory effect. In a rat model of injury better immunomodulatory effects were obtained when MSCs were introduced intraarterially than intravenously (Zonta *et al* 2010). Also in the homing of MSCs in renal injuries CXCR4 and CD44 were found to be important (Ji *et al* 2004, Herrera *et al* 2007).

1.1.5.2 MSCs in liver injuries

Although liver is able to regenerate upon injury, in the case of end stage liver injuries also require transplantation which may take very long time to find a donor. Therefore, cellular therapies are also important in liver injuries to replace the need for transplantation. Since isolation and culture of hepatocytes at efficient amounts is not possible *in vitro* (Serralta *et al* 2003, Serralta *et al* 2005), cellular therapies involving stem cells have an important role in curing liver diseases. Among the stem cells, MSCs receive special attention since their differentiation into hepatocyte like cells were reported (Chamberlain *et al* 2007, Sato *et al* 2005, Lee *et al* 2004).

Differentiation of MSCs into hepatocytes was reported both *in vitro* and *in vivo*. These hepatocytes were positive for the hepatocyte markers and found to be functional evidenced by their secretion of albumin and storing glycogen (Chamberlain *et al* 2007, Lee *et al* 2004, Schwartz *et al* 2002). Immunomodulatory effects of MSCs were also found to be important during liver regeneration. It was shown that engraftment of the MSCs into the liver, but not differentiation, stimulated proliferative and regenerative properties of the liver (Banas *et al* 2008, Parekkadan *et al* 2007, Caplan and Dennis 2006). In the murine injury models of carbontetrachloride treatment or partial hepatectomy the effect of MSCs on hepatic stellate cells were demonstrated, reducing fibrosis (Cavalho *et al* 2008, Abdel Aziz *et al* 2007, Zhao *et al* 2005, Sakaida *et al* 2004). Chamberlain *et al* (2007) also demonstrated that when the MSCs were administered by intrahepatic injection they formed hepatocytes more efficiently compared to intraperitoneal administration.

The cytokines that are responsible in the differentiation, homing and antifibrotic effects of MSCs were also investigated. FGF-4 and hepatocyte growth

factor (HGF) were found to be important in the differentiation of MSCs into hepatocytes (Dong *et al* 2010, Parekkadan *et al* 2007) while TNF-α and IL-6 were found to be important in antifibrotic effects (Pulavendran *et al* 2010, Parekkadan *et al* 2007). The expression of matrix metalloproteinases by MSCs was also found to be important in the reduction of fibrosis (Fang *et al* 2004, Sakaida *et al* 2004, Oyagi *et al* 2006). During the homing of MSCs to the injured liver it was demonstrated that CXCR4 and CCR9 were important molecules (Chen *et al* 2009).

1.1.5.3 MSCs in GvHD

GvHD is an inflammatory reaction caused by the T-cells of the host against the donor tissue HLAs after the transplantation which results in the rejection of the transplant (Flomenberg *et al* 2004, Goulmy *et al* 1996). Interestingly, MSCs were found to be very effective in suppressing the immune reaction during GvHD (Koç *et al* 2000, Frassoni *et al* 2002, Le Blanc *et al* 2004).

The inhibitory role of MSCs against GvHD depends on the effects of MSCs on dendritic cells, T cells, B cells and NK cells. The main inhibitory effect was found to be via dendritic cells, thereby preventing the antigen presentation (Reddy *et al* 2005). The interaction between antigen presenting cells and T-cells as well as the signaling mediated via co-stimulatory factors like CD28, ICOS and CD40 cause the initiation of immunological response against the graft (Blazar *et al* 2001, Blazar *et al* 1997). MSCs lacking co-stimulatory molecules like CD40 were not able to contact with the host immune cells (Tse *et al* 2003). When T-cells and MSCs were cultured together it was demonstrated that the release of IFN-γ from inflammatory Th1 cells decreased while IL-4 secretion from anti-inflammatory Th2 cells increased. Moreover, when MSCs were cocultured with activated T cells, PGE-2 secretion by MSCs was found to be increased and T-cell

inhibition was observerved. The same effect couldn't be observed when the MSCs were cocultured using PGE-2 inhibitors suggesting the importance of PGE-2 synthesis by MSCs (Aggarwal and Pittenger 2005).

In a baboon model of skin transplantation, it was shown that the MSCs suppress T-cells and enabled graft survival with a single intravenous administration (Bartholomew *et al* 2002). At murine heart transplantation model, *i.v.* injected MSCs prolonged the survival of the transplanted heart which was shown to be due to the regulatory effects of MSCs on Th1 and Th2 (Zhou *et al* 2006). Treg cells were shown to be induced by MSCs at another study using rat heart transplantation model mediating the graft survival (Casiraghi *et al* 2008).

The immunomodulatory effect of MSCs on immune system was also demonstrated in human cancer patients and GvDH patients after bone marrow transplant (Koç *et al* 2000, Frassoni *et al* 2002, Le Blanc *et al* 2004). Le Blanc *et al* (2004) also reported that the MSCs administered to the patient could be of any type of HLA antigens. MSCs were administered twice after transplantation and fully recovered within a year. A recent study reported the use of MSCs with the bone marrow transplant patients irresponsive to immunosuppressors and they have also demonstrated the regression of the disease after injection of MSCs twice (Lim *et al* 2010).

1.1.6 Mesenchymal Stem Cells in Cardiac repair

According to the World Health Statistics Report published by WHO in 2008, ischemic heart diseases have the highest mortality rate among all diseases today and 20 years later (http://www.who.int). Therefore, the treatment of heart

diseases is very important to provide a better living to the patients. Ischemic heart diseases are characterized by a shortage in the blood supply to the different regions of the heart. Cardiomyocytes in these regions go under necrosis and apoptosis which is called infarction. Although heart tissue renews itself very slowly by the help of cardiac stem cells, the regeneration rate is very slow to replace the damaged cardiac muscle with the healthy new cardiomyocytes (Oh *et al* 2003, Beltrami *et al* 2003). If left untreated at its end stage heart transplantation is the only thereupethic option.

Taylor and Jones (1979) reported the generation of cells, having mesenchymal phenotypes after several weeks, when 3T3 and 10T ½ cells were treated with 5-azacytidine which incorporates and leads the methylation of DNA reverting the cells to a more pluripotent state. Based on their findings in 1995, Wakitani *et al* published the first paper about the generation of cardiomyocytes *in vitro* from rat BM derived MSCs. Later on several studies were perfomed which reported the successful differentiation of MSCs into cardiomyocytes (Makino *et al* 1999, Bittira *et al* 2002, Xu *et al* 2004).

In vivo studies with different animal models were performed to reveal the effect of MSCs in cardiovascular diseases. Shake *et al* (2002) showed the differentiation of MSCs expanded from swine BM, into functional cardiomyocytes when injected into the infracted swine myocardium. In a canine model it was observed that the intracardialy injected MSCs were differentiated into smooth muscle cells and endothelial cells rather than cardiomyocytes however they have observed a better functionality at the infracted area (Silva *et al* 2005). In a myocardial infarct model it was demonstrated that when the rats were treated with MSCs, the infracted area get significantly smaller 4 weeks after the treatment and the MSCs that were labeled prior to the treatment were expressing

cardiac markers such as cardiac troponin and smooth muscle actin (Tang *et al* 2006). In a similar mouse model the *in vivo* effects of human MSCs obtained from the patient underwent ischemic heart disease were tested in which the infarct size and heart functions were assessed with MRI. According to the results of the study, MSCs were able to home to the scar tissue and improve the function of the left ventricule by differentiating into smooth muscle and endothelial cells as well as cardiomyocytes (Robert *et al* 2007). Administration ways and times were also investigated. Introducing MSCs by transendocardial electromechanical-guided delivery was more efficient than intracoronary delivery (Perin *et al* 2007) and it was demonstrated that if the MSCs were delivered to the heart 1 week after infarction better results were obtained in cardiac function and formation of blood vessels when compared to 1 hour and 2 weeks delivery (Jiang *et al* 2008). Moreover, there are studies which claim the regenerative effect of MSCs to their paracrine effects. MSCs that were genetically modified for the overexpression of Akt, exerted a better curing effect on the left ventricles of animal myocard infarction models (Lim *et al* 2006, Mangi *et al* 2003). Also, according to another study, the secretion of HGF from MSCs were triggered the migration of cardiac stem cells to the site of infarction and regeneration (Urbanek *et al* 2005).

Besides animal models, transplantation of MSCs had been performed to patients with myocardial infarction, who showed improved myocardial activity after the transplant (Katritsis *et al* 2005). The results of a clinical trial revealed that the function of left ventricle in myocardial infarction patient treated with autologous MSCs, was improved (Wollert *et al* 2004, Chen *et al* 2004). There are clinical trials going on that employ MSCs in the treatment of myocardial infarction. A recent trial to be carried on in France is still recruiting patients to investigate the administration of the MSCs intracardially (Phase1/2 study), yet another trial is investigating the intravenous administration (Phase 2 study)

(http://clinicaltrials.gov). Today there are 21 clinical trials either completed or recruiting patients that investigate the treatment capacities of MSCs in heart diseases. By the help of these *in vivo* and *in vitro* studies and clinical trials, the theurapeutic potential of MSCs in cardiac repair will be better understood.

1.2 Telomeres and Telomerase

Although the understanding of telomeres and telomerase goes back to 1880s, the first real evidence was discovered by Elizabeth Blackburn in 1978, who was awarded with Nobel Prize in 2009 with her colleagues for their studies on telomeres and telomerase.

Telomeres were identified in *Tetrahymena thermophila* with their unique sequence of TTGGGG (Blackburn and Gall 1978) which were present at the ends of the chromosomes and protect them from degradation. Later on, the telomeric sequence of *Tetrahymena* was ligated to the end of a budding yeast plasmid and it was observed that the sequence was replicated and also protected the plasmid ends. When the duplicated sequences were separated from the *Tetrahymena* sequences it was discovered that they were the yeast telomeric fragments (Szostak and Blackburn 1982). Further studies revealed the presence of a reverse transcriptase enzyme named telomerase which was composed of RNA and a protein subunit (Greider and Blackburn 1985, Greider and Blackburn 1987, Greider and Blackburn 1989) (Figure 1.2.1).

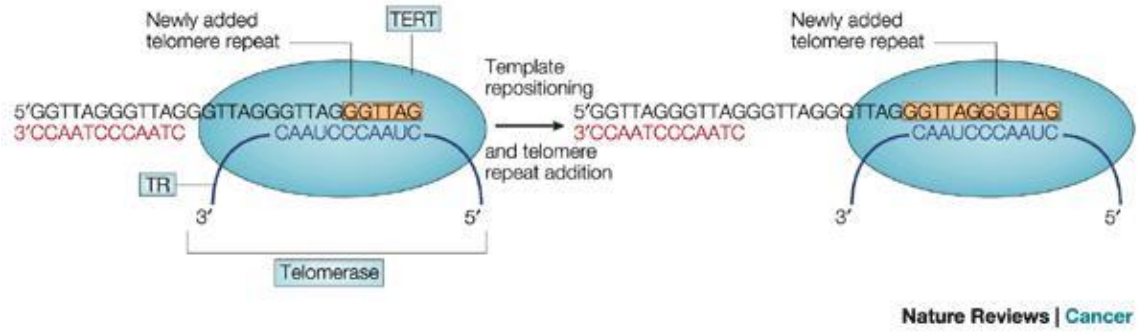


Figure 1.2 1 Elongation of telomeres by the enzyme telomerase (Manthon and Lloyd, 2001)

It is now known that mammals have the conserved telomeric sequence of TTAGGG (Moyzis *et al* 1988) and shortening of telomeres are associated with replicative senescence and aging of somatic cells (Lundblad and Szostak 1989, Harley *et al* 1990, Allsopp *et al* 1992) and replicative senescence can be overcame due to genomic instability and reexpression of telomerase enzyme (Hayflick 1965, Counter *et al* 1992, Kim *et al* 1994). The telomeres can be upto 15 kb in humans and at the end of the telomeric double stranded repeats a G-rich sequence is present which forms the T-loop that packages the chromosome ends and protects from 5'- 3'exonucleases activity together with the shelterin complex (Sullivan and Karlseder 2010, Gilson and Ségal-Bendirdjian 2010, Verdun and Karlseder 2007, de Lange 2005).

Shelterin complex that protects the chromosome ends is composed of six subunits (de Lange 2005). These factors are TRF1, TRF2, POT1, TIN2, TPP1 and Rap1 (Chong *et al* 1995, Bilaud *et al* 1996, Broccoli *et al* 1997, Kim *et al* 1999, Li et al 2000, Baumann and Cech 2001, Houghtaling *et al* 2004). These proteins

function together to form the T-loop and prevent telomerase from binding after adequate elongation of the telomeres (de Lange 2005).

Like shelterin complex that protects the telomeres, telomerase enzyme that elongates them is also a complex. Besides from its well known protein subunit telomerase reverse transcriptase (TERT) and telomerase RNA (TERC), it contains an RNA binding protein dyskerin (Cohen *et al* 2007). The three subunits of telomerase enzyme have to be packaged in the Cajal bodies which are then carried to the telomeres by telomerase cajal body protein1 (TCBP1) (Cristofari *et al* 2007, Venteicher *et al* 2009). At telomeric end binding of the proteins reptin and pontin provides the correct structure to the telomerase complex which then initiates the elongation of the telomeres (Venteicher *et al* 2008).

The elongation of telomeres is important for the maintenance of the cell. Therefore, to gain the telomerase enzyme activity provides a survival advantage to the cell. Stem cells undergo division and proliferation whenever their residing tissue requires regeneration. Therefore the presence of telomerase holoenzyme is also important for the maintenance of the stem cells pools and tissue regeneration.

1.2.1 Telomeres, Telomerase and Cancer

Hayflick *et al* (1961) showed that the somatic cells were able to replicate only for a limited number of passaging *in vitro,* which is called replicative senescence (Hayflick 1965). It was also shown that replicative senescence was due to the loss of telomeres which provide the genomic stability to the chromosomes. Today, it is known that somatic cells do not express telomerase which is essential for the maintenance of the telomeres and serves as a tumor

suppressor to prevent the proliferation of the cells having defects in their genomes (Li *et al* 2005). However when telomerase was introduced to senescent somatic cells it was observed that they could overcome replicative senescence and start dividing unlimitedly (Bodnar *et al* 1998). Moreover, *in vivo* studies performed with mouse models demonstrated that when telomerase was overexpressed, the mice formed more tumors compared to the wild types. If the mice were resistant to tumor formation, prolonged life span and increased stem cell activity were reported (Tomas-Loba *et al* 2009, Sarin *et al* 2005, Gonzalez-Suarez *et al* 2005, Canela *et al* 2004).

In many cancer types, the expression of telomerase is regained to maintain the telomere length (Harley 2008). However some cancer types maintain their telomere lengths by using the pathway called alternative lengthening of telomeres (ALT) (Bryan *et al* 1997, Bryan *et al* 1995). ALT is observed mostly in the tumorigenesis of the mesenchymal cell types (Cesare and Reddel 2010). ALT pathway employs homolog recombination for the elongation of telomeres (Lubland and Blackburn 1993, Bryan *et al* 1995, Bryan *et al* 1997). Different from the telomerase extended telomeres, the chromosome end of the cells contains mostly C-rich strands (Henson *et al* 2009) and it is proposed that the choice towards ALT can be due to the reduction in shelterin complex concentrations (Cesare and Reddel 2010).

Recent finding revealed that the epigenetic modifications of the subtelomeric repeats, which are highly methylated in normal cells, towards hypomethylation increased tumorigenesis and caused telomere elongation (Vera *et al* 2008). Moreover, telomeric repeat containing RNAs (TERRAs) were discovered which are transcribed from the telomeric repeats that are normally highly methylated. TERRAs are shown to be telomerase inhibitors and have low

TERRA transcript concentrations were observed in cancer (Azzalin *et al* 2007, Schoeftner and Blasco 2008, Luke and Lingner 2009, Redon *et al* 2010).

1.2.2 Telomerase and Stem Cells

Presence of telomerase enzyme was demonstrated in many stem cell types including ESC, HSCs and MSCs (Hiyama and Hiyama 2007). Among them, ESCs has the highest level of telomerase activity (Brumendov and Balabanov 2006, Izadpanah *et al* 2006). Embryonic stem cells which have limitless potential to proliferate, conserved the activity of telomerase enzyme after extensive passaging, that is more than 120 (Xie *et al* 2010). Moreover their differentiation potentials were demonstrated to depend on telomerase activity, the concentration of which is diminished after differentiation (Armstrong *et al* 2000).

In HSCs, it has been shown that the telomerase activity was not sufficient to maintain telomere length which was further proved by the overexpression studies of TERT (Allsopp *et al* 2001, Allsopp *et al* 2003). The regenerative capacities of the cells were also found to be determined by their niches (Conboy *et al* 2005). HSCs were shown to express more telomerase when they were cultured with cytokines *in vitro* and the enzyme levels were also found to be decreased after differentiation and extensive proliferation resulting in aging (Chiu *et al* 1996) although it prevents sudden telomere length decrease during regeneration which requires rapid proliferation (Allsopp *et al* 2003).

There are conflicting data in the presence of telomerase enzyme in MSCs. According to a study human MSCs *in vitro* could undergo 50 population doublings and have telomerase activity (da Silva and Nardi 2003), in contrast

another one indicates that MSCs do not express TERT and have life spans as long as somatic cells (Fehrer and Lepperdinger). Moreover another study states that without the expression of TERT, MSCs could maintain their telomere lengths (Yanada *et al* 2006). There are suggestions that maintenance of the telomeres in MSCs could be by ALT pathway (Serakinci *et al* 2008). It was demonstrated *in vitro* that when MSCs were isolated from telomerase knockout mice, they lost their potential to differentiate and proliferate in contrast with the wild type mice (Liu *et al* 2004).

1.2.3 Telomerase Antagonist GRN163L (Imetelstat)

During transformation of somatic cells into cancer cells telomerase is activated which make the cancer cells immortal, for this reason telomerase is a good target in the treatment of cancer as normal somatic cells do not express telomerase. There are several methods proposed to cure cancer by targeting telomerase. One of the approaches depends on the inhibition of telomerase by the use of small molecules to promote their efficient binding on the active site of the telomerase or telomerase RNA. Another approach employs the disruption of the telomeres and prevent the binding of telomerase enzyme. Furthermore there are strategies developed to inhibit the expression of the telomerase genes or for gene therapy and immunotherapy (Harley 2008).

GRN163L, Imetelstat, is an oligonucleotide that inhibits the activity of telomerase. It is a 13-mer oligonucleotide having the sequence of TAGGGTTAGACAA containing a N3'→ P5'-thio-phosphoramidate lipid conjugate which is palmitoyl. The palmitoyl serves in the entry of the GRN163L into the cell while thio-phosphoramidate provides the backbone a better

interaction with proteins and pH stability. When these properties are combined GRN163L can easily enter the cell without any requirements to liposomes and specifically binds to telomerase RNA forming a double strand that is durable to RNAse H degradation (Pongracz and Gryaznov 1999, Gryaznov *et al* 2001, Herbert *et al* 2005) (Figure 1.2.3.1).

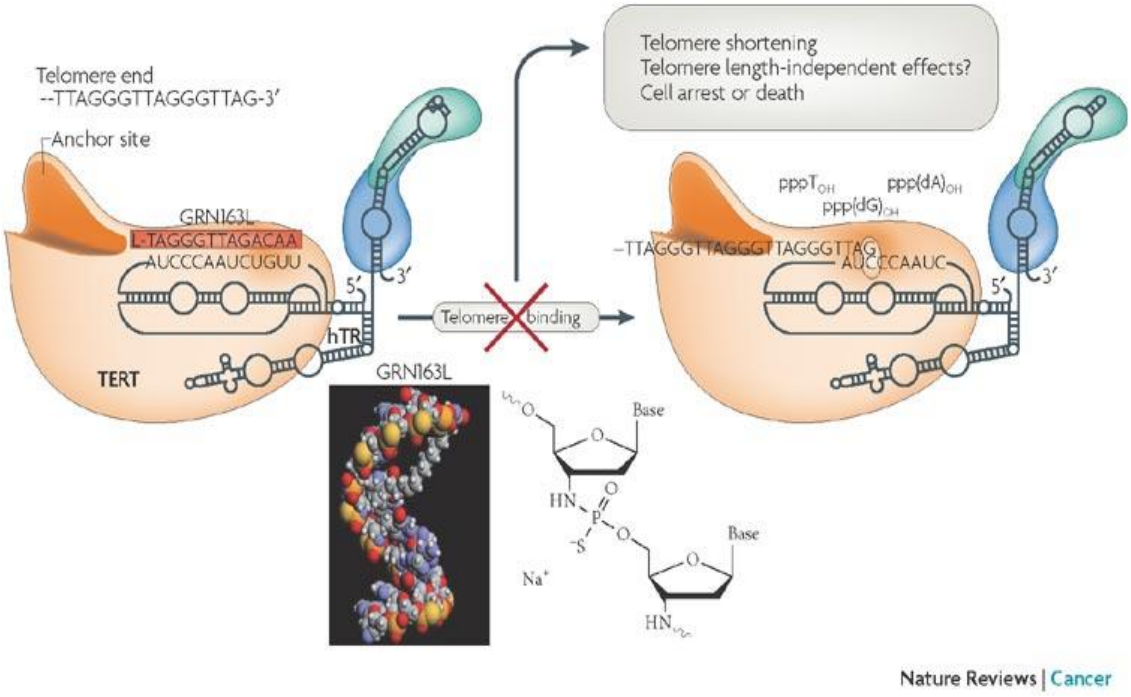


Figure 1.2.3 1 Action mechanism of GRN163L on telomerase (Harley 2008)

To date *in vivo* and *in vitro* properties of GRN163L is widely tested in animal models and several cancer cell lines. Dikmen et al (2005) demonstrated the effects of GRN163L on A549 luciferase (A549-Luc) cells *in vitro*, resulting in shortened telomeres, loose and less colonies. Moreover they successfully showed that immunodeficient mice have less metastasis when the A549-Luc cells were previously treated with GRN163L and intraperitoneal administration of GRN163L prevented the tumorigenesis of these cells *in vivo*. Djojosubroto *et al* (2005)

showed the effect of GRN163L on liver cancer cell lines Hep3B and Huh7. These cells became more sensitive to chemotherapeutics when previously treated with GRN163L and formation of tumors was prevented with intraperitoneal injections in nude mice. Similar results were also obtained when breast cancer cells were treated with GRN163L both *in vitro* and *in vivo* (Gellert *et al* 2006, Hoechreiter *et al* 2006). Moreover a telomerase independent effect of GRN163L was revealed by *in vivo* and *in vitro* studies performed with A549-Luc cells. The morphology of the cells were becoming round and they were detached from the plate surface loosing their adherent properties (Jackson *et al* 2007). There are also studies reporting the successful inhibition of tumorigenesis in glioblastomas, prostate cancer, breast cancer, bladder cancer and myelomas (Marian *et al* 2010, Calin *et al* 2009, Goldblatt *et al* 2009, Goldblatt *et al* 2009, Dikmen *et al* 2008, Shammas *et al* 2008, Gomez-Millan *et al* 2007, Gellert *et al* 2006, Hochreiter *et al* 2006).

GRN163L has ben in clinical trials since 2005 and today there are total of 7 clinical trials (http://clinicaltrials.gov/ct2/results?term=GRN163L). The aim of these trials is to reveal the effects of GRN163L in the treatment of cancer alone or as a combinatory treatment in lung cancer, myelomas and breast cancer. Although *in vivo* and *in vitro* effects of GRN163L is well described in cancer, its effects on the quiescent residers of the body, adult stem cells are not known as they also maintain telomerase.

CHAPTER 2

AIM OF THE STUDY

Mesenchymal stem cells are bone marrow derived stem cells that are able to differentiate into adipocytes, osteocytes, chondrocytes, cardiomyocytes and they are easy to isolate and manipulate. They can home to the injured tissue and have immunomodulatory effects. For these reasons they are important candidates in cellular therapies. MSCs can also be frozen to preserve them, and when they are thawed they can function apparently normally, thus allowing for future "off-the-shelf" therapy approaches. However, optimal timing of stem-cell delivery to acutely injured tissues or organs can be a major handicap. This issue is of paramount importance in cardiovascular medicine (Bartunek *et al* 2006). The assessment of donor MSC functional activity at the cellular level, which is the focus of several research groups, is also critically important. There is no available data from experimental studies or clinical trials that shows the exact timing of the induction of cardiomyocyte differentiation prior to MSC delivery to an injured heart or ischemic limb. In the first part of the study, we aimed to demonstrate the best timing to induce the differentiation of cardiomyocytes from rat bone marrow derived MSCs.

The presence of the telomerase activity in many different kinds of human tumors, but not in normal somatic cells has been known for years. Thus, inhibition

of telomerase activity is now an attractive tool on targeted cancer therapy. Telomerase inhibitor, GRN163L has been shown to have established effects on the treatments of the cancers. However this inhibition may lead to side effects since germline cells, proliferating stem and progenitor cells also exhibit telomerase activity. The effects of telomerase-targeting therapies must be well defined on MSCs. Therefore, we aimed to investigate *in vitro* effects of GRN163L on the self-renewal and differentiation processes of MSCs at the second part of this thesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

For all the experiments, adult female 9-week-old, 280–300 g Spraque Dawley rats were used. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled conditions at 22^{o} C with 12 hour light and 12 hour dark cycles. They were provided with unlimited access of food and water. The experimental procedures have been approved by Bilkent University Local Ethical Committee (BILHADYEK).

3.2 Isolation of the Cells from Rat Bone Marrow

After the rats were sacrificed by cervical dislocation, heterogeneous cell population was collected from the femurs and tibias by flushing with a 5 mL syringe containing 10% FBS (HyClone, Logan, USA) and 1% penicillin/streptomycin solution (Hyclone) in DMEM (HyClone). The mixed suspension was then centrifuged at 3000 rpm for 3 min. After the supernatant was removed, the cells were washed with 10 mL 1X PBS buffer once. The cells suspended in 1X PBS were centrifuged at 2500 rpm for 3 min and then the buffer was removed and cells were re-suspended in 10 mL 1X PBS buffer again. The

mixture was centrifuged at 2000 rpm for 3 min and then the supernatant was removed.10mL 1X PBS buffer was added to the cell pellet and the cells were re-suspended once more. The cell suspension was centrifuged at 1500 rpm for 3 min to get rid of the remaining impurities like fat, connective tissue and hair and then after the removal of supernatant, cell pellet was re-suspended in MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone) to prepare for tissue culturing.

3.3 Culturing of Mesenchymal Stem Cells (MSCs)

Cells were counted with hemocytometer and the cell number was calculated according to the below formula

Number of cells in the mixture = number of cells counted x 10^4 x *dilution factor*

After the cells were seeded to the plastic culture plates at equal numbers in MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone), they were cultured in a 5% CO_2 incubator at 37°C. The next day, the media of the tissue culture plates were changed and the nonadherent cells were removed. The media of the cells were changed every 3 days, after washing with sterile 1X PBS. The adherent cells on the 14^{th} day of the cell culture were mostly MSCs.

3.4 Treatment of the MSCs with Telomerase Template Antagonist GRN163L (GRN163L) and the Experimental Groups

After 14 days of culture, the cells were trypsinized (HyClone) and transferred to culture dishes. One day after the transfer, GRN163L (Geron Co., CA, USA) and its mismatch control oligonucleotide were added at a concentration of 1 μM to the cell plates. The media were changed every 3 days with the fresh GRN163L (hereafter stated as 163L group) and mismatch control oligonucleotide (hereafter stated as mismatch group) together with control MSCs with no treatment (hereafter stated as control group). 163L and mismatch cultures continued for 1 week. Finally, GRN163L was removed and MSCs were left for recovery for 1 week (hereafter stated as 163LR (recovery) group).

3.5 Colony Forming Unit (CFU) Assay

MSCs were washed with 1X PBS and were air dried. They were fixed by ice cold methanol (MeOH) for 5 minutes. After fixation MeOH was removed and the cells were washed with 1X PBS buffer. The fixed cells were then treated with giemsa staining reagent (Carlo Erba) for 5 min. To stop the reaction of giemsa staining, tap water was added and the cells were washed with tap water trice. The colonies stained in purple were counted under bright field light microscope.

3.6 Total RNA Isolation from Rat MSC

Total RNA from the MSCs were isolated at the 14^{th} day of the cell culture. The cells were first washed with 1X PBS buffer twice and were trypsinized at 37^{o}C for 3 min. Trypsinization was ended by the addition of DMEM (Hyclone)

containing 10% Fetal Bovine Serum (Hyclone) and 1% penicillin-streptomycin solution (Hyclone). Then the cell suspension was centrifuged at 1500 rpm for 5 min. After centrifugation the media was removed and the cell pellet was washed with 1X PBS buffer and centrifuged again at 1500 rpm for 5 min. Finally, the supernatant was removed and the total mRNA was isolated from the cell pellet by using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol.

3.7 cDNA Synthesis

The cDNAs were synthesized from the total RNA samples by using DyNAmo cDNA Synthesis Kit (Finnzymes, Finland) according to the manufacturer's protocol. 2 µg RNA was mixed with DEPC treated ddH_2O to a total volume of 10,5 µl and 1,5 µl of oligo(dT) primer was added and the mixture was centrifuged for 3 sec. The samples were incubated at 65°C for 5 minutes and then chilled on ice for 3 minutes. Then 15 µl of 2X RT buffer including dNTP mix and 10 mM $MgCl_2$ and 3 µl of M-MuLV RT RNase H^+ enzyme was added to the mixture, after a 3 sec centrifugation they were incubated at 25ºC for 10 min, 45ºC for 45 min, and 85ºC for 5 minutes, respectively.

3.8 Primer Design

Primer designs were performed by using the combination of Primer exe, Primer Blast (NCBI) and Primer Premier. The specificity of the primers and product sizes they would amplify were checked via Ensemble and NCBI databases publicly available through the internet. Primers for all the genes amplified were listed in Table 3.9.

Table 3.8 List of Primers and the product sizes they amplify

Name of the gene	Sequences of Forward (F) and Reverse (R) Primers	Size (bp)
TERT	(F) 5'- GCTACGCCATCCTGAAGGTC-3' (R) 5'-GGTAGCAGAGCCAACGTGTG-3'	99
CYCLINB1	(F) 5'- TCGATGTGGAGCAGCATACT-3' (R) 5'- GTCCATTCACCGTTGTCAAG-3'	142
CDK4	(F) 5'- GAAGACGACTGGCCTCGAGA-3' (R) 5'- ACTGCGCTCCAGATTCCTCC-3'	109
CDK6	(F) 5'- TTGTGACAGACATCGACGAG-3' (R) 5'- GACAGGTGAGAATGCAGGTT-3'	151
CDK2	(F) 5'- TGACCAACTCTTCCGGATCT-3' (R) 5'- ATAACAAGCTCCGTCCGTCT-3'	164
CDK1	(F) 5'- GTTGACATCTGGAGCATAGG-3' (R) 5'- CTCTACTTCTGGCCACACTT-3'	144
CYCLINA2	(F) 5'- CTGCCTTCCACTTAGCTCTC-3' (R) 5'- GAGGTAGGTCTGGTGAAGGT-3'	125
RB	(F) 5' – TTGAAAGAAGAGGCACTCCC -3' (R) 5' – CGCTACCTTAAATACCGCCT -3'	116
P53	(F) 5'- GTTCCGAGAGCTGAATGAGG -3' (R) 5'- AGACTGGCCCTTCTTGGTCT -3'	109
Bmi-1	(F) 5'- ATGTGTGTCCTGTGTGGAGG-3' (R) 5'-AGCCATTGGCAGCATCTGCT-3'	281
P21	(F) 5'-ATGTCCGATCCTGGTGATGTC-3' (R) 5'- CGGCTCAACTGCTCACTGTC- 3'	92
Cdc25a	(F) 5'-AGTGAAGAGAGCAGACCGAT-3' (R) 5'-GGAGAGAGACTGGTGTGGAA-3'	126
P16INK4a	(F) 5'- CTTCACCAAACGCCCCGAACA-3' (R) 5'- CGGGAGAGGGTGGTGGGGTC-3'	132
P19ARF	(F) 5'- GCAGAGCATGGGTCGCAGGTTC-3' (R)5'- CGGGAGAGGGTGGTGGGGTC-3'	295
CYCLIN D1	(F) 5'- AATGCCAGAGGCGGATGAGA- 3' (R) 5'- GCTTGTGCGGTAGCAGGAGA- 3'	189
CYCLIN E	(F) 5'- TTACTGATGGTGCTTGCTCC -3' (R) 5'- GTCGTTGACGTAGGCCACTT - 3'	136
Cyclophilin	(F) 5'-GGGAAGGTGAAAGAAGGCAT-3' (R) 5'-GAGAGCAGAGATTACAGGGT-3'	211
Cardiac Troponin I	(F) 5' – GCGAAGCAGGAGATGGAG – 3' (R) 5' – TGCCACGCAGGTCATAGA – 3'	250
Alpha Skeletal Actin	(F) 5' - ATCTCACGTTCAGCTGTGGTCA -3' (R) 5' - ACCACCGGCATCGTGTTGGAT - 3'	182

α-Sarcomeric Actin	(F) 5’ – CCAGGCACTGTGGAAAGG – 3’ (R) 5’ – CACGATGGATGGGAAGAC -3’	286
MEF2D	(F) 5’ – TGGGAATGGCTATGTCAGTG -3’ (R) 5’ – CTGGTAATCTGTGTTGTAGG – 3’	351
GAPDH	(F) 5’- CCTCCTCATTGACCTCAACTAC - 3’ (R) 5’ – CATGGTGGTGAAGACGCCAG – 3’	219
CD 90	(F) 5’- CCAGTCATCAGCATCACTCT-3’ (R) 5’- AGCTTGTCTCTGATCACATT- 3’	374
CD 34	(F) 5’- TGTCTGCTCCTTGAATCT -3’ (R) 5’- CCTGTGGGACTCCAACT- 3’	281
CD 71	(F) 5’- ATGGTTCGTACAGCAGCAGA-3’ (R) 5’- CGAGCAGAATACAGCCATTG-3’	182
CD 29	(F) 5’-ACTTCAGACTTCCGCATTGG -3’ (R) 5’- GCTGCTGACCAACAAGTTCA-3’	190
CD 45	(F) 5’- ATGTTATTGGGAGGGTGCAA-3’ (R) 5’- AAAATGTAACGCGCTTCAGG-3’	175

3.9 RT-PCR

cDNA amplification for *cardiac troponin I* (cTnI), *α-sarcomeric actin*, *MEF2D*, *GAPDH*, *CD90*, *CD71*, *CD45*, *CD34*, *CD29* and *cyclophilin* were performed by using DyNAzyme II (Finnzymes). PCR conditions for these genes are listed in Table 3.10.

Table 3.9 RT-PCR Conditions

Genes	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
CD 90	95°C, 5 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	30	72°C, 5 min
CD 34	95°C, 5 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	30	72°C, 5 min
CD 71	95°C, 5 min	94°C, 45 sec	66°C, 60 sec	72°C, 45 sec	35	72°C, 5 min
CD 29	95°C , 5 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	23	72°C, 5 min
CD 45	95°C, 5 min	94°C, 30 sec	60°C, 30	72°C, 30	23	72°C, 5 min

			sec	sec		
Troponin	95°C, 5 min	94°C, 45 sec	65°C, 30 sec	72°C, 40 sec	33	72°C, 5 min
α-Sarcomeric actin	95°C, 10 min	94°C, 45 sec	57°C, 60 sec	72°C, 60 sec	35	72°C, 10 min
MEF2D	95°C, 10 min	94°C, 30 sec	57°C, 60 sec	72°C, 50 sec	33	72°C, 5 min
GAPDH	95°C , 5 min	94°C, 30 sec	59°C, 30 sec	72°C, 30 sec	23	72°C, 5 min
Cyclophilin	95°C , 5 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	23	72°C, 5 min

3.10 Agarose Gel Electrophoresis

1% agarose gel was prepared with 1X TAE Buffer and 1 mg/ml ethidium bromide solution. Samples were prepared by addition of 4 µl 6X Agarose Gel Loading Dye to 25 µl of PCR product and loaded to the agarose gel at equal volumes. The gel was run at 85V for 45 minutes and visualized under transilluminator (Gel-Doc Bio-Rad, USA or Vilber Lourmat, France). Chemicapture (Vilber Lourmat, France) software was used to take photographs of the gels. The Gene Ruler DNA Ladder Mix (Fermentas, #SM0331) was used as a marker and 5 µl was loaded to each gel.

3.11 Semiquantitative Analysis of RT-PCR

Semiquantitative analysis was performed by using Bio1D (Vilber Lourmat, France) after the gel photos were taken.

3.12 Q-RT-PCR

DyNamo HS SybrGreen qPCR kit was used at all reactions at a reaction volume of 20 µl containing 10 µl DyNamo HS SybrGreen Taq mix 1 µl 10 pmol forward and 1 µl 10 pmol reverse primers, 7 µl ddH_2O and 1 µl cDNA. The primers used for Q- RT-PCR are shown in Table 1. Cyclophilin was used as a housekeeping reference gene. Before performing Q- RT-PCR reactions for experimental samples, the amplification efficiencies of all primers were calculated using a standard dilution series. Fold changes in the expression of the genes were estimated based on the comparative $2^{-\Delta\Delta Ct}$ method, using the normal MSCs as calibrator. The Q-RT-PCR amplification protocols for all investigated genes were listed in Table 3.13. Each protocol was followed by a melt curve to check the purity of the product amplified.

Table 3.12 Q-RT-PCR conditions

	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Cycles**	**Final extension**
TERT	95°C, 10 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CYCLINB1	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CDK4	95°C, 10 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CDK6	95°C , 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CDK2	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CDK1	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CYCLINA2	95°C, 10 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
RB	95°C , 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
P53	95°C, 10 min	94°C, 30 sec	65°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
Bmi-1	95°C, 10 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
P21	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
Cdc25a	95°C , 10 min	94°C, 30 sec	59°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
P16INK4a	95°C, 10 min	94°C, 30 sec	62°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
P19ARF	95°C, 10 min	94°C, 30 sec	68°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CYCLIN D1	95°C, 10 min	94°C, 30 sec	65°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
CYCLIN E	95°C , 10 min	94°C, 30 sec	59°C, 30 sec	72°C, 30 sec	45	72°C, 10 min
Cyclophilin	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	35	72°C, 10 min

3.13 Telomeric Repeat Amplification Protocol (TRAP)

$1x10^6$ MSCs were collected by trypsinization and a TRAP-eze Telomerase Detection Kit (Millipore, MA, USA) was used to detect telomerase activity. A nondenaturing polyacrylamide gel based assay was performed for visualization of the telomeric repeats after PCR, according to the manufacturer's protocol. Gels were stained with SYBR Green I (Sigma, MO, USA) at 1:10000 dilution in TAE and visualized with Vilber Lourmat ChemiCapture (Marne-La-Vallée Cedex, France).

3.14 Total Protein Isolation from MSCs

MSCs were scraped from the cell culture plates in 1X PBS after washing with 1X PBS twice. The cells were transferred to a falcon tube and centrifuged at 1500 rpm for 5 min at 4°C and the precipitate was treated with a lysis buffer for 30 min on ice, by vortexing with 5 min intervals for 30 sec. Then the lysate was centrifugated for 20 min at 13000 rpm at 4°C. The supernatants were transferred to a new eppendorf tube and the protein concentrations of supernatants were determined with Bradford protein assay as described by Bradford (1976).

3.15 Western Blotting

3.15.1 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The denaturing gel was prepared with 4-6% stacking gel and 8-10% separating gel, the former pore size was used for the proteins bigger than 45 kDa and the latter one was used for the protein smaller than that size. The 30 μg of

protein was mixed with 10 µl of 2% cracking buffer which contains 5% b-mercaptoethanol and heated at 95°C for 5 min. After denaturation step they were held on ice for 5 min. After the gel was soaked into the 1X running buffer that completely covers the wells and the electrodes, the proteins were loaded to the gel with 5 µl of the prestained protein molecular weight marker (SM0671, MBI Fermentas, Ontario, Canada) and run at 60V with 20V increments after every 45 min. The gel was run until the prestained marker was separated enough to detect the required range of kDa.

3.15.2 Semi-dry transfer of proteins to a PVDF membrane

After the gel was run and removed from the gel apparatus the stacking part was cut off and separating part was soaked in semi-dry transfer buffer at most 5 min. Beforehand, PVDF membrane and 4 wattmann papers were cut to be at the same size with the separating part of the gel. PVDF membrane was activated by soaking with MeOH for 30 sec. The excess MeOH was removed by washing with ddH_2O for 2 min by vigorous shaking. Then it was soaked in semi-dry transfer buffer at the same time with the gel in different containers. Wattmann papers were soaked in semi-dry transfer buffer for 1 min. The transfer sandwich was prepared at the order of; 2 wattmann papers, membrane, gel, 2 wattmann papers. At the first 3 steps and last step the air bubbles were removed. The transfer was performed at a current to be 3,5 mA/cm^2 for 45 min. After the transfer is completed the gel was stained with coomassiee brilliant blue for 30 min and destained overnight to check complete transfer at the required range of kDa.

3.15.3 Immunological detection of Immobilized Proteins

After the transfer membrane was equilibrated in 1X TBS buffer for 5 min and then blocked with a blocking solution in 1X TBS-(0.1%)T at least for 1h at

RT on an orbital shaker (Thermolyne). The membrane is then cut by two with a sterile blade two perform the detection of housekeeping protein at the same blotting. Primary antibodies were added at the concentration of 1:200 for Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, Cyclin E, 1:1000 for Cdk1, Cdk2, Cyclin B1, p53, 1:2000 for Cdk6, α-Tubulin and left o/n at 4^{o}C. The following day the membrane was washed with 1X TBS-T four times at intervals indicated; 5 min, 10 min, 10 min, 5 min. They were incubated with the related horseradish peroxidase linked secondary antibody (anti-rabbit IgG HRP (Cell Signaling Technology, USA) 1:2500 and anti-mouse IgG HRP (Santa Cruz, CA, USA) 1:2000) in blocking solution for 1 h at RT on an orbital shaker. After 1 h membranes were washed again as indicated previously. Finally, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL, USA) was applied to the membrane for 5 min and after draining the excess solution covered with stretch film on glass and placed in an X-ray film cassette and developed. (Anti-Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, and Cyclin E antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Cdk1, Cdk2, Cyclin B1 and p53 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- α-Tubulin antibody was purchased from Calbiochem (Darmstadt, Germany).

3.16 Immunocytochemistry

Cells in the cover slips were fixed for 7 min in ice cold MeOH after removing the medium and washing with 1X PBS. The cells were then treated with 3% H_2O_2 to quench endogenous peroxidase activity. After washing 3 times with PBS, After washing three times with 1X PBS, the cells were left in blocking solution which contains 1% BSA and 0.5% Tween20 in 1XPBS, for 30 min at RT. Primary antibodies of CD90 and CD34 (Chemicon, Temecula, Canada) were applied at a concentration of 1:500 dilution in blocking solution and kept at 4-8ºC

overnight. After washing 3 times with 1X PBS, cells were incubated for 15 min with universal biotinylated link (DakoCytomation, Glostrup, Denmark) then biotinylated link is washed and the cells were treated with streptavidinHRP (DakoCytomation) for 15 min at RT. After washing with 1X PBS, color developments were achieved by incubation for 5 min with liquid DAB+ (DakoCytomation). Excess was washed by ddH_2O and counterstaining was performed with hematoxylin by staining for 30sec at RT then the cells on coverslips were mounted with mounting medium (DakoCytomation).

3.17 Differentiation of MSCs

3.17.1 Differentiation of MSCs into Cardiomyocytes

Two different time points were used for the cardiomyocyte differentiation of MSCs. In the first group, differentiation was induced on day 9 of the culture (hereafter stated as the 9th day) and in the second group, differentiation was induced on day 14 (hereafter stated as the 14th day). The induction of cardiomyocyte differentiation was performed by incubating cells with 10mmol/L 5-azacytidine (Sigma, Steinheim, Germany) for 24 h. The cells were washed and expanded for another 14 days with low-glucose DMEM (Invitrogen) containing 20% FBS (HyClone), and 1% penicillin-streptomycin (HyClone).

3.17.2 Differentiation of MSCs into Adipocytes

An adipogenic induction medium was prepared by freshly adding 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), 100 μM indomethacin (Sigma) and 0.5 mM IBMX to LG-DMEM (HyClone), containing 1% penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for

21 days, changing the medium every 2 days with freshly prepared medium. After 21 days the cells were fixed with 4% paraformaldehyde for 30 min. Fixative was washed with 1X PBS twice and 3 times with ddH_2O and the cells were covered with Oil Red O Solution (Sigma, Steinheim, Germany) and incubated for 50 min at RT. The cells then were washed with ddH_2O 3 times and counterstained with hematoxylin for 30 sec at RT. The reaction was ended by washing with tap water 3 times. The cells were kept in ddH_2O to prevent from drying for observation.

3.17.3 Differentiation of MSCs into Osteocytes

An osteogenic induction medium was prepared by freshly adding 0.1 μM dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma) and 10 mM glycerol-2- phosphate (Sigma) to LG-DMEM (HyClone), containing 1% penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days by freshly prepared medium. After 21 days the cells were fixed with 70% ethanol for 1h at RT and rinsed twice with ddH_2O and stained with Alizarin Red S (Sigma) for 30 min at RT. The cells were washed with ddH_2O for 3 times and kept in ddH_2O to prevent from drying for observation.

3.18 Intracellular Ca^{+2} measurement

Cells were grown on round cover slips in 6-well plates and loaded with fura-2 AM at RT for 50 min. Cells were then washed before the experiments with Hepes-buffered physiologic NaCl bathing solution containing 1.2 mmol/L Ca^{+2} and 1 mmol/L Mg^{+2}. Cells were challenged either with caffeine to stimulate ryanodine receptors or with ATP to stimulate P2Y purinergic receptors. In addition, high-KCl bathing solutions were applied and intracellular calcium

concentrations were monitored to see whether depolarization could induce any intracellular Ca^{+2} transients. Cover slips were fixed to a Teflon chamber and mounted on an inverted fluorescent microscope (Nikon TE-300, Japan). Fluorescence ratios were obtained by exciting cells at 340 and 380 nm with a microscope-based spectrofluorimeter with a temporal resolution of one ratio every 0.5 s. Emission signals were collected at 510 nm with an IC-300 ICCD (intensified charge-coupled device) camera with Delta Ram, (Photon Technology International, USA). Data was collected and analyzed by Image Master software (Photon Technology International).

3.19 Statistical Analysis

All data are expressed as means ± SD. Data were analyzed by performing a paired t- test using Minitab Statistical Software ® (State College, Pennsylvania, USA). A value of $p<0.05$ was considered to be statistically significant.

3.20 Buffers and Solutions

The preparation of the buffers and solutions are listed in Appendix A

CHAPTER 4

RESULTS

4.1 Timing of Induction of Cardiomyocyte Differentiation for *in vitro* Cultured Mesenchymal Stem Cells

According to the World Health Organization (WHO) 2008 health report, ischemic heart diseases are the number one life threatening diseases followed by several types of cancers. Moreover, this ranking is expected to be the same in the fore-coming 20 years.

Mesenchymal stem cells (MSCs) have important features for cellular therapies as they can differentiate into different lineages like cartilage, bone, fat and muscle. They can also differentiate into cardiomyocytes which is very well established in the literature together with its cellular applications in murine animals (Friedenstein *et al* 1970, da Silva *et al* 2008). However, for clinical applications the timing of the cellular therapy can be life saving.

According to our established MSC culturing protocol, it takes 14 days to generate fully mature MSCs. According to our previous studies, also on the 9^{th} day of the culture there are, although less, mature MSCs in the cell culture plate (Terzioğlu E, 2006). However, their differentiation capacities into any lineages were not checked as early as day 9. We hypothesized that if the cells could

differentiate into cardiomyocytes as early as day 9, it would save 5 days for cellular therapies.

To determine the timing of induction into cardiomyoctes we used primary bone marrow of adult female rats and all the experiments were performed on both 9^{th} and 14^{th} days of *in vitro* cell culture. All the experiments were performed with 3 different sets of animals for significancy.

4.1.1 Characterization of Mesenchymal Stem Cells (MSCs) on 9^{th} and 14^{th} Days of Cell Culture

We first investigated the characteristics of MSCs on the 9^{th} and 14^{th} days of the culture by their CD90 expressions and CFU-F activities. We performed CD90 immunocytochemistry analysis on day 9 and 14. Both on the 9^{th} and 14^{th} day of the culture, the cells were positive for CD90 (Figure 4.1.1.1A and 4.1.1.2A).

Another distinguishing feature of MSCs is their colony forming capacity. Giemsa staining is performed to determine their CFU-F. It was observed that both on 9^{th} and 14^{th} days the cells were forming colonies (Figure 4.1.1.1C and 4.1.1.2C). When we omitted primary antibody as negative control, we did not observe any staining (Figure 4.1.1.1B and 4.1.1.2B).

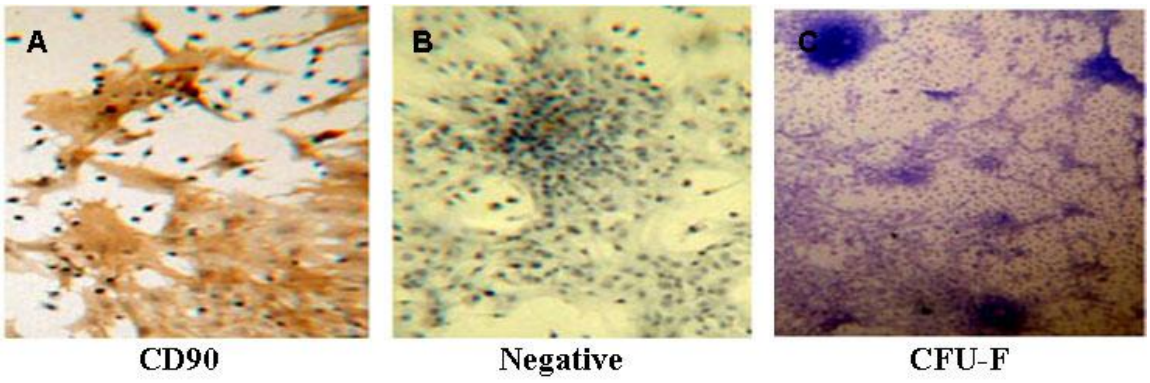


Figure 4.1.1 1 Characterization of MSCs on the 9th day of cell culture (magnification 20x)

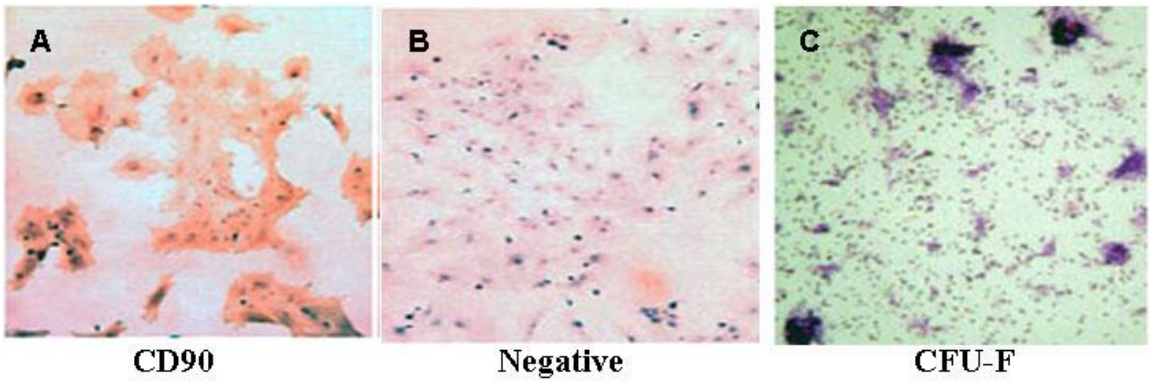


Figure 4.1.1 2 Characterization of MSCs on the 14th day of cell culture (magnification 20x)

Cardiomyocytes contract in synchrony to enable the contraction of the heart. For this purpose a fully differentiated cardiomyocyte must respond to depolarizations and must enable the spreading of action potentials which is performed by the increase in cytoplasmic Ca^{+2} levels. Therefore, we examined the levels of Ca^{+2} by using fura-2AM staining.

Before differentiation of MSCs into cardiomyocytes, their depolarization properties were determined as a negative control. For this purpose, cytoplasmic Ca^{+2} levels of MSCs in response to extracellular KCl and caffeine were examined.

Upon treatment with 45-60 mmol/L extracellular KCl and 10 mmol/L caffeine, at neither 9th day nor 14th day of cell culture increase at cytosolic Ca^{+2} levels were observed (Figure 4.1.1.3 and 4.1.1.4 respectively). On the other hand, they responded to ($0.5x10^{-4}$ M) extracellular ATP application with a clear Ca^{+2} transient (Figure 4.1.1.3 and 4.1.1.4 respectively).

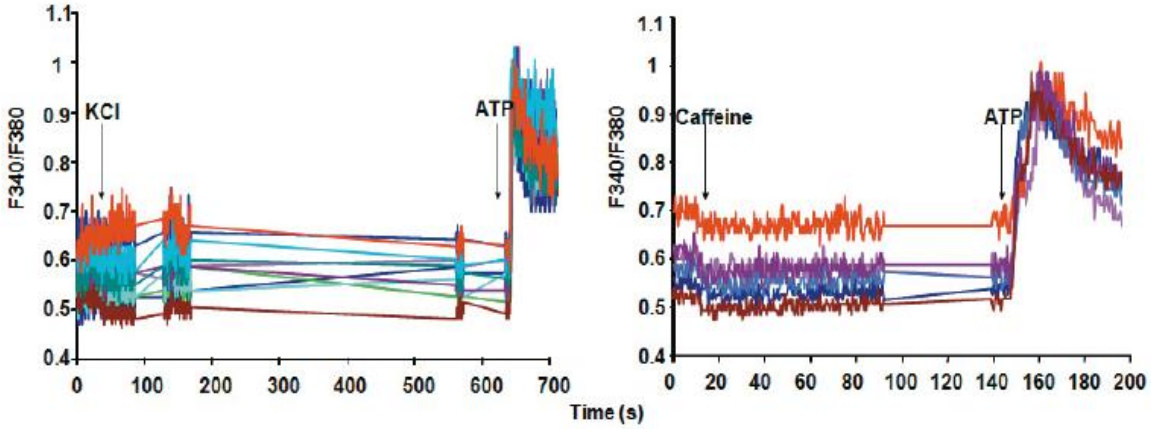


Figure 4.1.1 3 Cytoplasmic calcium levels of MSCs in response to KCl and caffeine measured with fura-2AM on the 9th day of the culture

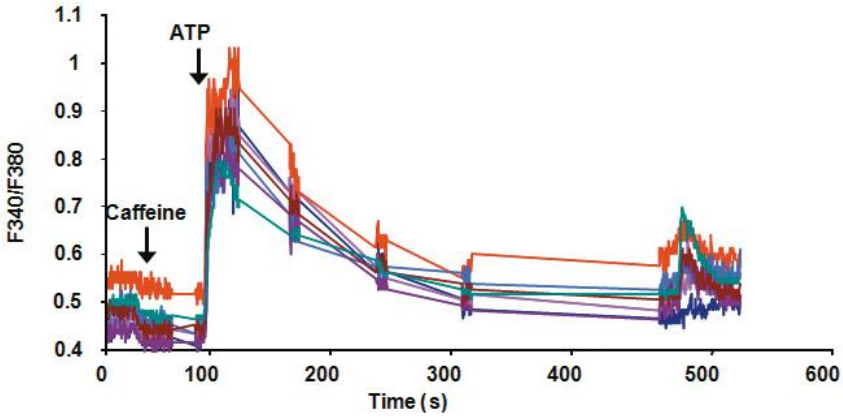


Figure 4.1.1 4 Cytoplasmic calcium levels of MSCs in response to caffeine measured with fura-2AM on the 14th day of the culture

Immunocytochemistry and CFU-F results confirmed that the cells that were obtained both on the 9^{th} and 14^{th} days of the culture were MSCs and further analysis of intracellular Ca^{+2} levels revealed that the cells were undifferentiated as they did not respond to depolarization.

4.1.2 Validation of the Cardiomyocyte Differentiation

As indicated before, cardiomyocytes contract in synchrony to enable the contraction of the heart. For this purpose a fully differentiated cardiomyocyte must respond to depolarizations and must enable the spreading of action potentials which is performed by the increase in cytoplasmic Ca^{+2} levels.

MSCs from the 9^{th} and 14^{th} days of the culture were treated with 5-Azacytidine to induce cardiomyocyte differentiation. Fourteen days after this treatment, the cells were checked for their responses upon 45-60 mmol/L KCl and 10 mmol/L caffeine incubation. We checked their cytosolic Ca^{+2} levels with fura-2AM to validate their contractility. It has been observed that when the cells were treated with 10 mmol/L caffeine neither the cells from day 9 nor day 14 showed increase in cytoplasmic Ca^{+2} levels (4.1.2.1A and 4.1.2.2A respectively). However, when the cells were treated with 45-60 mmol/L extracellular KCl, an increase at cytoplasmic Ca^{+2} levels were observed at both time points of induction (Figure 4.1.2.1B and 4.1.2.2B respectively).

As caffeine increases cytosolic Ca^{+2} levels via ryanodine receptors, it is possible that the functional receptors have not been formed yet. The responses to KCl, however show apparently that the voltage gated Ca^{+2} channels became active after induction of cardiomyocytes both on 9^{th} and 14^{th} days of cell culture. All cells responded to ATP ($0.5x10^{-4}$ M) application with a sudden cytoplasmic Ca^{+2} increase.

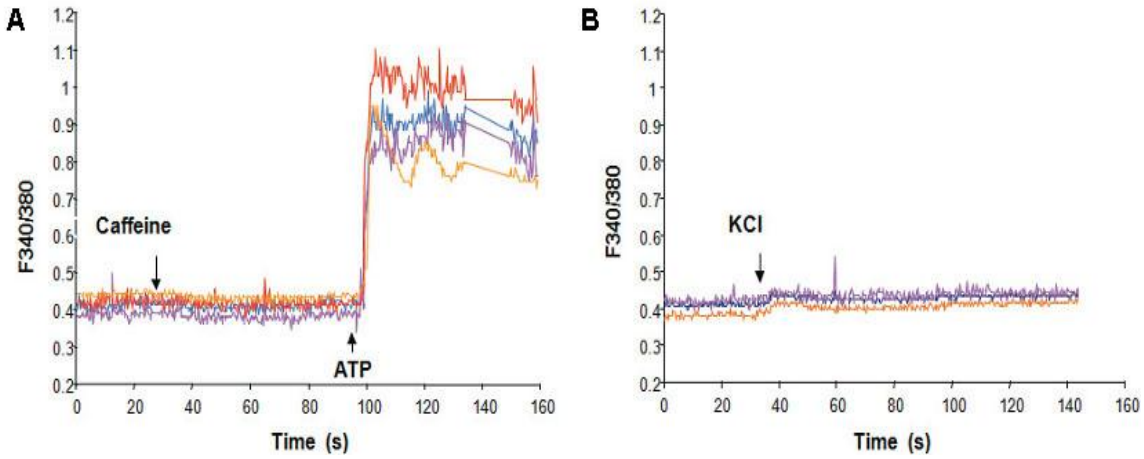


Figure 4.1.2 1 Cytoplasmic calcium levels of cardiomyocytes, differentiated from the MSCs on 9th day of the culture, in response to KCl and caffeine measured with fura-2AM.

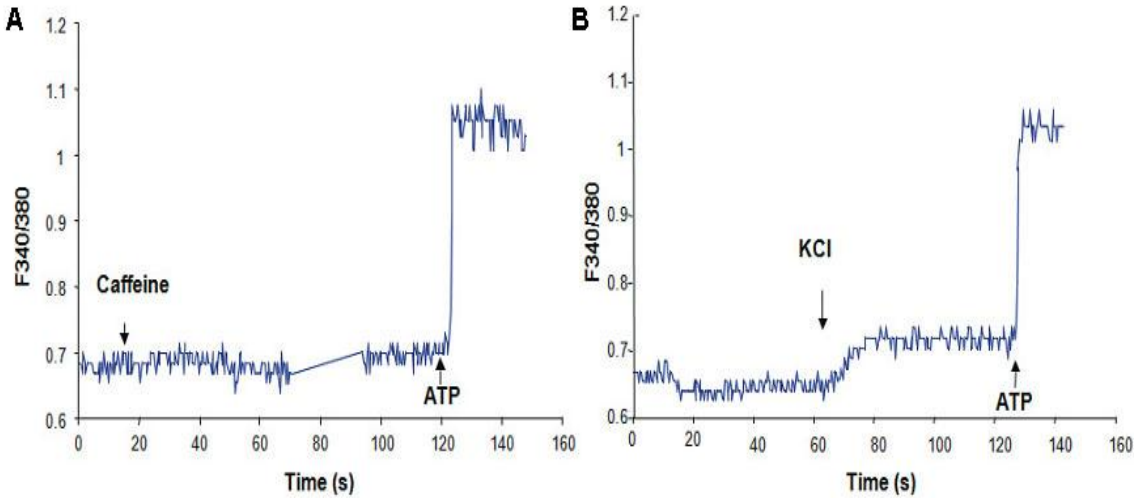


Figure 4.1.2 2 Cytoplasmic calcium levels of cardiomyocytes, differentiated from the MSCs on 14th day of the culture, in response to KCl and caffeine measured with fura-2AM.

CD90 immunocytochemistry assay was also performed after induction of differentiation to check whether there were undifferentiated MSCs. After cardiomyocyte differentiation, the cells which were induced to differentiate on 9th day and 14th day of the culture found to be negative for CD90 protein expression (Figure 4.1.2 3A and Figure 4.1.2 3B).

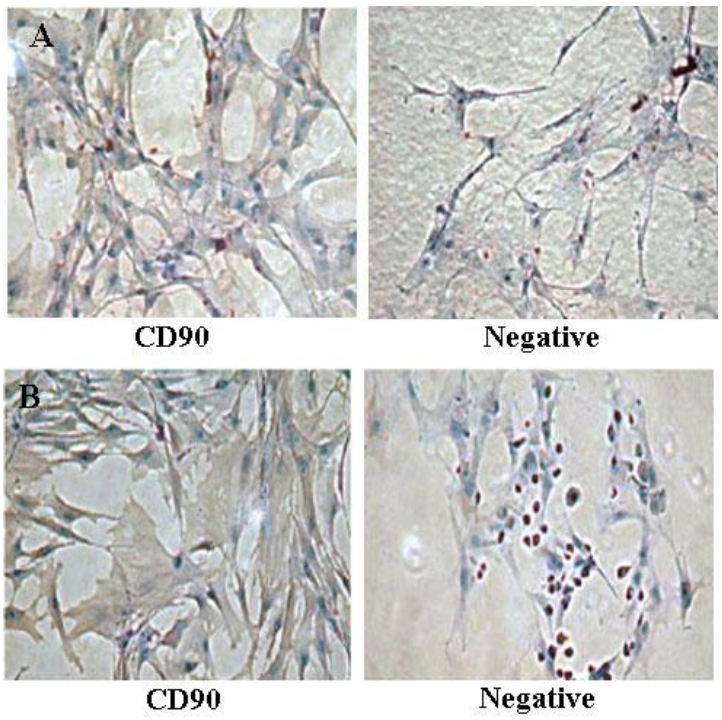


Figure 4.1.2 3 CD90 immunocytochemistry after induction of differentiation (A) on 9th day (B) on 14th day (magnification 20x)

Differentiation of MSCs into cardiomyocytes was further investigated by analyzing the mRNA expressions of cardiac-specific early markers such as troponin I, α-sarcomeric actin and MEF2D. The expressions of these marker genes were semiquantitatively analyzed by RT-PCR relative to GAPDH expressions by using cells grown in control media as calibrator. The expression of these genes were found to increase slightly at both induction time points and

expressions of troponin I (p = 0.04) and α-sarcomeric actin (p = 0.01) were significant at the cells induced to differentiate on 9th day of the culture (Figure 4.1.2 4).

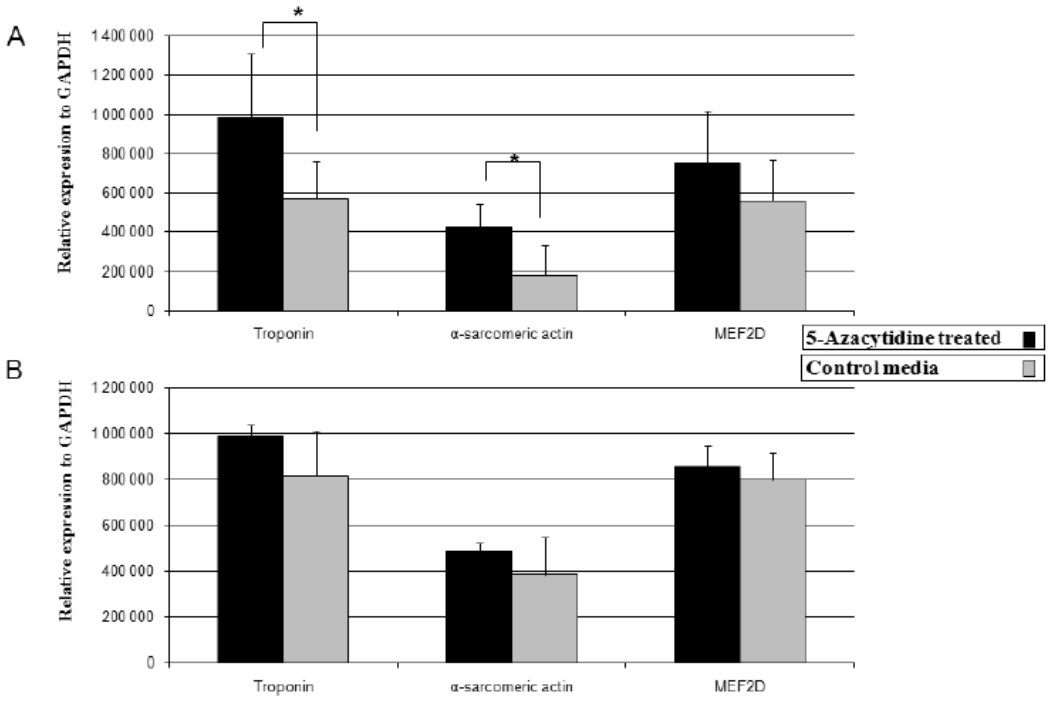


Figure 4.1.2 4 Expression profile of cardiac-specific markers in 5-azacytidine-treated MSCs on the (A) 9th and (B) 14th day of culture (* indicates p<0.05).

4.2 Effects of GRN163L on MSCs

GRN163L is a telomerase antagonist which is used in cancer treatment. It reduces the telomeric length by inhibiting telomerase activity and moreover it reduces the attachment of the cells on the plate surface which is referred as the rounding effect. In consequent treatment the combination of these effects kills the cancer cells (Gellert *et al* 2006, Dikmen *et al* 2005, Wang *et al* 2004).

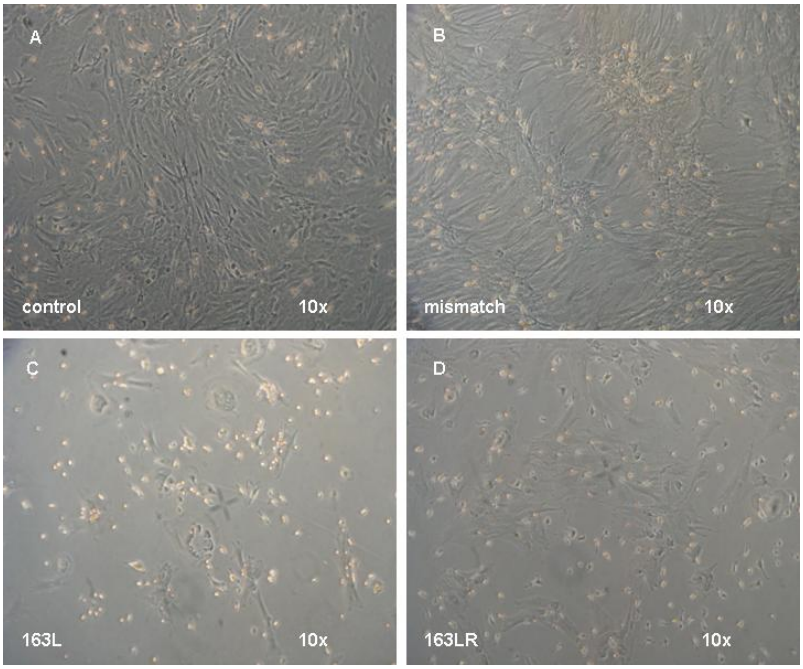
Stem cells also have telomerase activity to maintain their telomere length which is kept stable to provide longevity to these cells. MSCs, one of the adult stem cells in the body, are also shown to have telomerase activity at early passages *in vitro* (Sethe *et al* 2006). The effect of telomerase inhibition on adult stem cells is known neither *in vitro* nor *in vivo*.

To determine the effect of GRN163L on MSCs, BM from 3 sets of adult female rats were used and all the experiments were performed 3 times for significancy. Four experimental groups were involved as previously described in section 3.4. Normal MSCs which received no treatment, hereafter stated as control group; MSCs treated with the mismatch oligonucleotide of GRN163L, hereafter stated as mismatch group; normal MSCs treated with 1 μM GRN163L for 1 week, hereafter stated as 163L group; normal MSCs treated with 1 μM GRN163L for 1 week and left for recovery for 1 week hereafter stated as 163LR (recovery) group.

4.2.1 Phenotypic Effects of GRN163L on MSCs

GRN163L is known to affect the phenotype of the cells by causing a rounding effect, detaching the cells from culture surface, at first this phenotypic effect of GRN163L on MSCs was examined. After 14^{th} day of the culture when the MSCs were mature, the cells were trypsinized and counted to be $1x10^6$ cells and reseeded to 6-well plates. GRN163L was added into two wells at the same time with seeding at a concentration of 1 μM. As the oligonucleotide loses its effect in 3 days *in vitro*, fresh oligonucleotide was added into the medium every 3 days. While changing the media of GRN163L treated samples, extensive care was taken as the cells were floating. In order to not to loose cells the media were centrifuged and the cells were added to the culture plate again.

During the treatment, the rounding effect was observed at the 163L treated group, the cells detached from the plate surface and the cells ceased growing (Figure 4.2.1 1 C). MSCs in the control and mismatch treated group, were growing normally and had fibroblastic phenotypes as expected (Figure 4.2.1 1 A and B). in the treatment group, during treatment with GRN163L, rounding effect and cessation of growth were observed, however it was noticed that after the cells left for recovery (163LR group), so that the drug was no more administered, the cells reattach to plate surface and start dividing again (Figure 4.2.1 1 D).



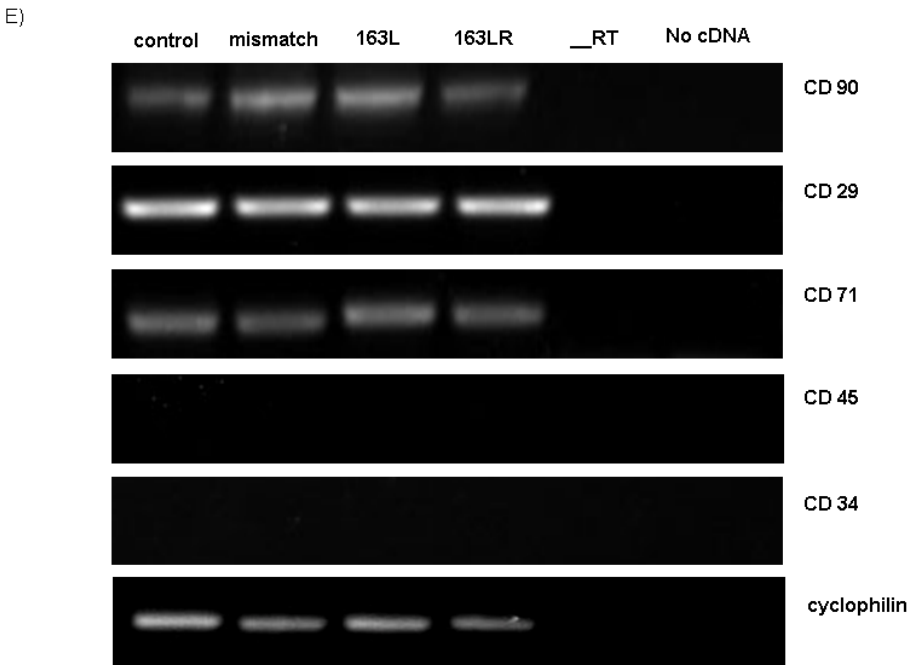


Figure 4.2.1 1 Effect of GRN163L (a-d: phenotypic effects, **A.** untreated MSCs (control), **B.** mismatch oligonucleotide treated MSCs (mismatch) **C.** 1μM GRN163L treated MSCs (163L) **D.** MSCs treated with GRN163L and then left for recovery (163LR) **E.** expression of CD markers of MSCs.

As GRN163L ceased the growth of the cells and detached them from the plate culture *in vitro*, it was important to check the surface markers to assure that the cells were still MSCs. CD90, CD29 and CD71 which are the important CD markers of MSCs were checked together with the hematopoetic markers CD34 and CD45 by RT-PCR. All the groups were expressing the positive markers CD90, CD29 and CD71 and no mRNA expression of the negative markers CD34 and CD45 were observed as expected (Figure 4.2.1 1 E). The results showed that although the cells were treated with 163L they kept their MSC markers both at the time of treatment and after they recovered.

4.2.2 Effects of GRN163L on the Differentiation capacities of MSCs

One of the most important properties of MSCs is their differentiation capacities into different lineages therefore, adipogenic and osteogenic differentiation capacities of the MSCs were checked after treatment with 163L *in vitro*. As the cells couldn't attach to plate surface during 163L treatment they couldn't be induced to differentiate. However it is important to keep their ability to differentiate after recovering from 163L treatment. For this purpose 163LR group was employed together with the control group in differentiation protocols.

To determine adipogenic differentiation the cells were induced in adipogenic medium for 21 days and then stained with Oil Red O staining to visualize the lipid droplets. It was observed that in adipogenic differentiation there were lipid droplets in control and in 163LR group (Figure 4.2.2 1 A and C) whereas no droplets were seen at the negative control samples (Figure 4.2.2 1 B and D). Osteogenic differentiation was performed by incubating the control MSCs and 163LR group in osteogenic induction media and the calcium deposits were determined by Alizarin Red staining. At both control and 163LR group the calcium deposits were observable after 21 days of induction (Figure 4.2.2 2 A and C) and no staining was observed at negative controls (Figure 4.2.2 2 B and D).

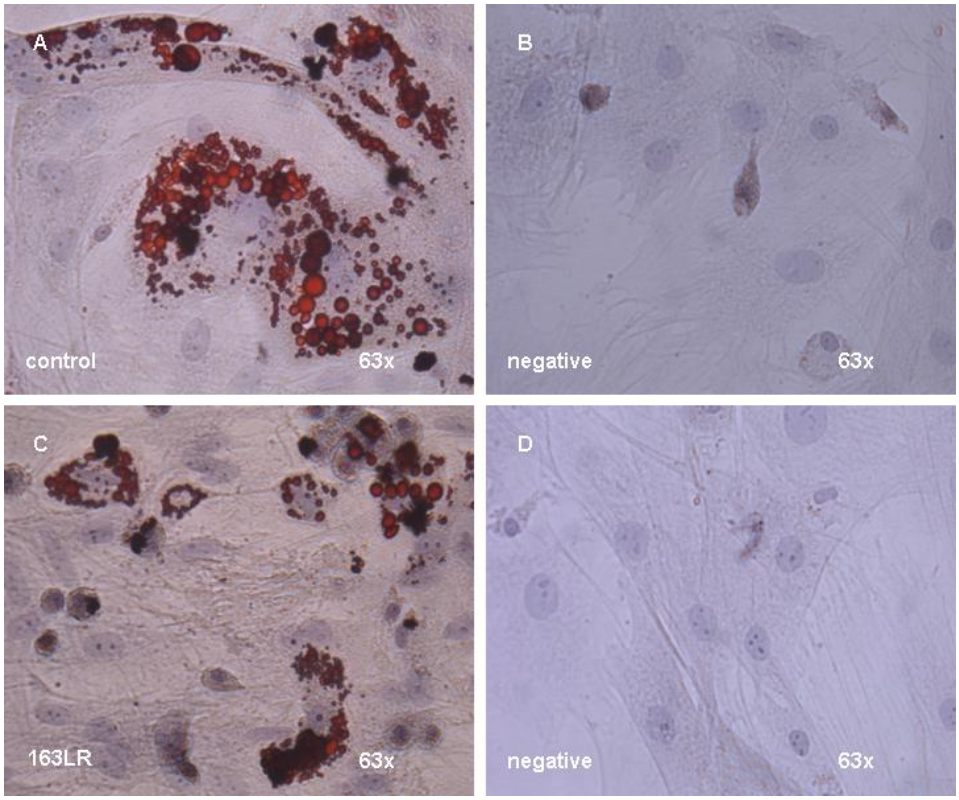


Figure 4.2.2 1 Adipogenic differentiation of MSCs determined by Oil Red O staining

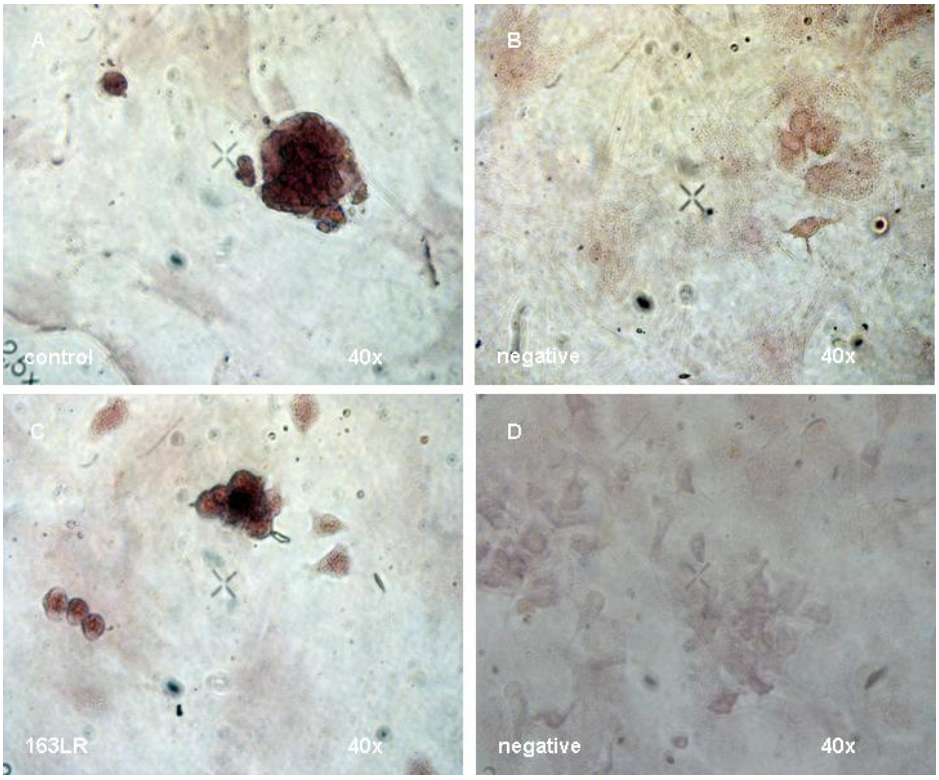


Figure 4.2.2 2 Osteogenic differentiation of MSCs determined by Alizarin Red staining

4.2.3 Effects of GRN163L on Telomerase of MSCs

The telomerase enzyme activity was checked by TRAP assay. In this assay A549 non small cell lung cancer cell line was used as a positive control as it was previously shown to have telomerase activity (Dikmen *et al* 2005). Heat treated samples and lysis buffer were negative controls.

TRAP assay results revealed that MSCs have telomerase activity and when they were treated with 163L their telomerase activity was inhibited as no telomeric ladder was observed (Figure 4.2.3 1). However such an inhibition was not observed at the mismatch treated group. Moreover after recovery, at 163LR group, the telomerase activity was regained.

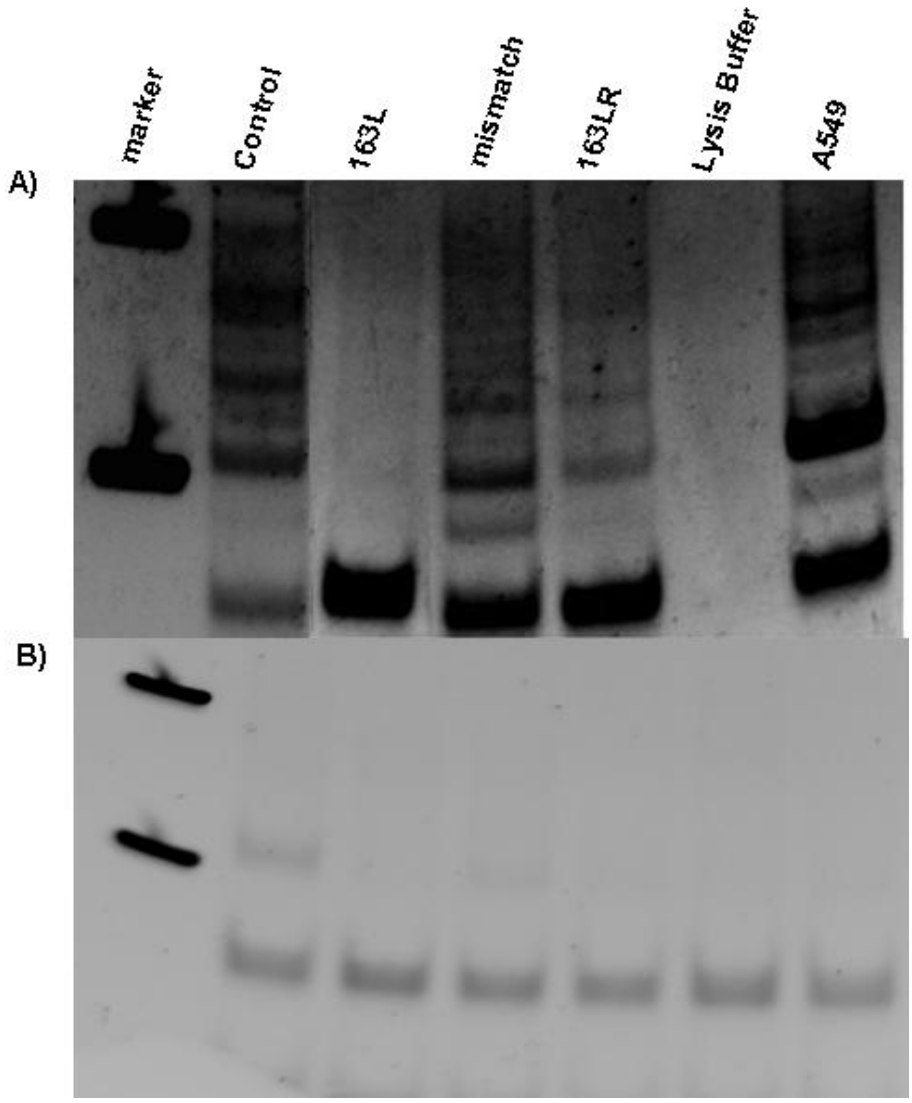


Figure 4.2.3 1 TRAP assay of MSCs to determine telomerase activity **A.** normal samples **B.** heat inactivated samples

4.2.4 Effects of GRN163L on the Cell Cycle of MSCs

As mentioned before, it was observed that the cells ceased growing during treatment with GRN163L. Cell cycle genes were investigated both at mRNA and protein level in order to reveal which genes were affected. To investigate the mRNA expressions of the cell cycle genes q-RT-PCR was employed by using

SyberGreen I detection system. For each primer a run with dilution series of ½, involving 6 dilution samples, was performed and amplification efficiencies were determined by plotting standard curves according to the slope of the trend line. The efficiency values used are listed in (Table 4.2.4).

Table 4.2.4 Efficiency values for the primers used in q-RT-PCR.

Name of the gene	Efficiency
TERT	1.78
CYCLINB1	1.88
CDK4	1.78
CDK6	1.94
CDK2	1.94
CDK1	1.94
CYCLINA2	1.74
RB	2.0
P53	1.94
Bmi-1	1.99
P21	1.72
Cdc25a	1.94
P16INK4a	1.90
P19ARF	1.81
CYCLIN D1	1.78
CYCLIN E	1.78
Cyclophilin	1.94

The mRNA expressions of all the genes listed were investigated by using control MSC sample as a calibrator. Gene expressions of untreated control MSCs, 163L, 163LR and mismatch groups were examined. After the analysis of q-RT-PCR results, mRNA expressions of all the genes were observed to decrease when treated with 163L, whereas among them the decrease at *cdc25A*, *cyclinB1*, *cyclinD1*, *cyclinE*, *cdk6*, *p21*, *p19* and *Bmi-1* were significant with $p<0.05$ (Figure 4.2.4 1). The expression levels of all the genes were getting back to the same level or slightly higher except *cyclinB1* and *RB* that have slightly lower expressions. When the mismatch treated group is considered it was observed that all the expressions were higher than 163L treated group.

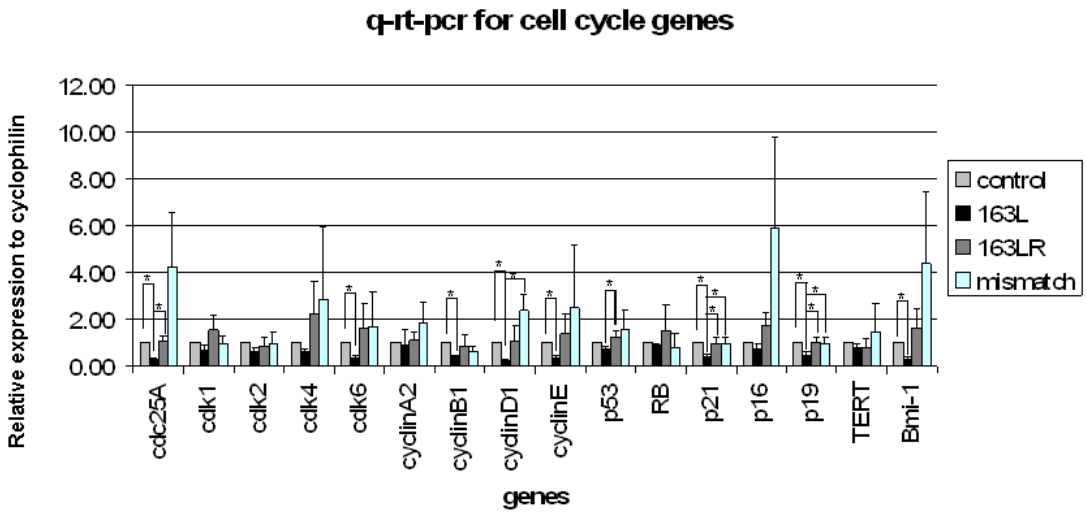


Figure 4.2.4 1 mRNA expressions of cell cycle genes in MSCs from control, 163L, 163LR and mismatch groups, determined with Q-RT-PCR. * indicates $p<0.05$.

The protein expressions were checked with Western blotting. The protein amounts of cdk2, cdk4, cyclinD1 were found to decrease at 163L treated groups

when compared with control and restored to the same level with control when 163L is removed and cells were left for recovery (Figure 4.2.4 2). The expression of cdc25A were absent at control and 163L groups but increases after removal of 163L while other proteins tested showed a similar pattern with tubulin alpha, which is used as a loading control

The experiments were performed with 3 different sets of animals for more than once and visually the clearest results were presented here. At all sets and runs, the results were the same.

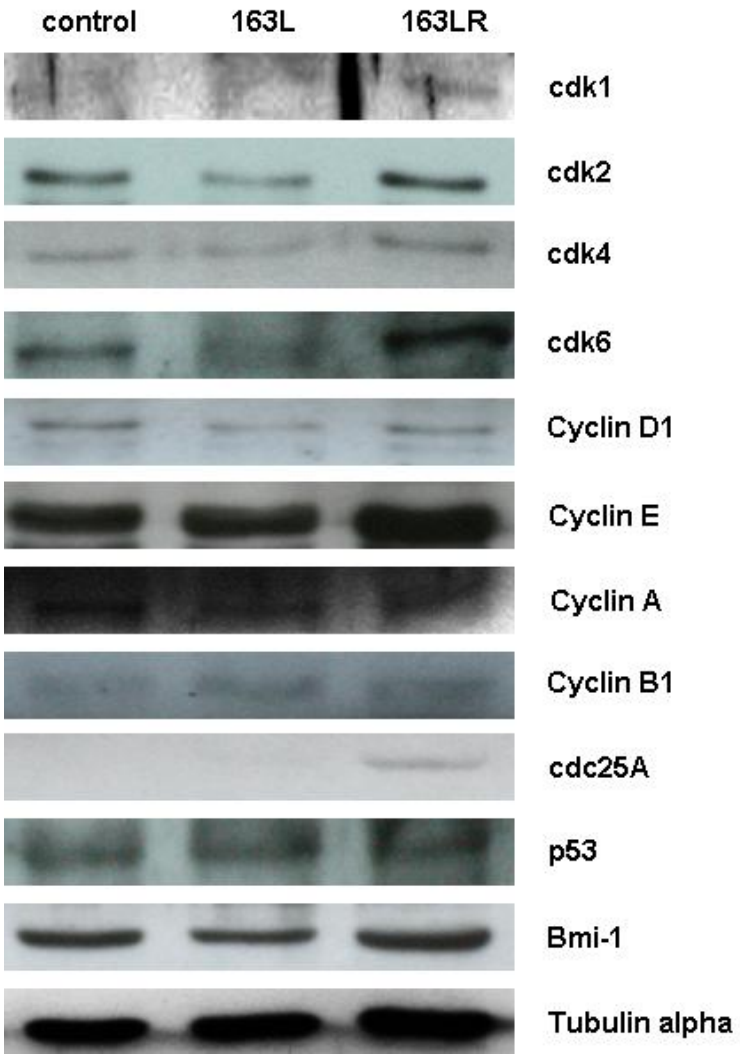


Figure 4.2.4 2 Protein expressions of cell cycle genes in MSCs from control, 163L and 163LR groups determined with Western blotting

CHAPTER 5

DISCUSSION

MSCs are ASCs that have self-renewal and differentiation capacity. These two properties are important both in regeneration of the damaged tissues and maintenance of the body stem cell pools. However, there are superior capacities of MSCs compared to the other types of ASCs. They have the homing capacity where they can migrate to the injured sites by sensing the chemokine gradient changes. Moreover when regeneration capacities of MSCs are considered, they can differentiate into the cells of mesenchymal lineage as well as other lineages like endodermal and ectodermal lineages such as pancreatic beta cells and nerve cells. MSCs can also mediate the differentiation of the other stem cells at the niche they migrate by their immunomodulatory effects. The immunomodulatory effects that MSCs exert on the cells of the damaged tissue have positive effects on the curing of the injury by inhibiting the T-cell, B-cell, DC and NK cell responses. Therefore use of MSCs is a valid option in the cure of autoimmune diseases and can be used as a supplementary treatment in transplantations. Furthermore, their regenerative capacities are well established in the treatment of ischemic heart diseases. Despite the fact that they are successful candidates in cardiovascular diseases their expansion in the culture in high amounts is an issue of concern and

limiting factor. Another important property of MSCs is their telomerase activity. Telomerase activity is known to have important effects on the survival and differentiation properties of MSCs and it is also important for their self-renewal. On the other hand telomerase activity is also present in cancer cells and there are effective cancer therapies targeting telomerase activity to demolish cancer cells without damaging the somatic cells. MSCs are one of the targets when these agents are administered since they possess telomerase activity. GRN163L is one of these agents, which prevents tumorigenesis in several cancer types, both *in vivo* and *in vitro*. The effects of GRN163L on stem cells have not yet been shown. In this study we aimed to show at first the best timing of the induction of cardiomyocyte differentiation *in vitro* by using MSCs and then the *in vitro* effects of GRN163L on MSCs.

MSCs are promising agents for cellbased therapies however, there is no available data from experimental studies or clinical trials that shows the exact timing of the induction of cardiomyocyte differentiation prior to MSC delivery to an injured heart or ischemic limb. At the first part of this thesis we aimed to demonstrate the exact timing of the cardiomyocyte induction *in vitro* by using MSCs. For this purpose we first investigated the present conditions of the MSCs that are isolated from the bone marrow of the Sprauge-Dawley rats as a mixed culture. The undifferentiated state of the MSCs was proven by the presence of CD90 immunostaining and colony forming units at the 9^{th} and 14^{th} days of cultures. Further proof is provided by the measurement of the intracellular Ca^{+2} levels. At 9^{th} and 14^{th} days of the culture before induction with 5-azacytidine, MSCs were treated with 10 mmol/L caffeine and 45-60 mmol/L KCl. Caffeine is known to exert its effects via ryanodine receptors that induce the increase in intracellular Ca^{+2} levels via IP3 pathway and KCl by depolarization. MSCs neither on the 9^{th} day nor on the 14^{th} day responded to any of the treatments

except ATP treatment which was applied as a positive control. As a result, we concluded that the cells do not show the properties of the differentiated cells in the culture prior to induction and they do not respond to depolarization and do not possess any ryanodin receptors.

After proving the undifferentiated states of the cells, induction of cardiomyocyes was performed by 5-azacytidine treatment both on 9^{th} and 14^{th} days of the culture and 14 days after the treatment the cells were checked for their differentiation status. At first the MSC marker CD90 expression was checked. If the cells were differentiated, they should no more express CD90 as a surface marker. The immunocytochemistry results demonstrated the expected result and the expression of CD90 was negative at 9^{th} and 14^{th} day groups. The contractility of the cells was then checked by determining the intracellular Ca^{+2} levels. The cells were treated with caffeine and KCl at the same amounts indicated before. Caffeine evoked no response neither on the cells induced at 9^{th} day nor at the 14^{th} days of the culture. The ryanodine receptors were still not present on the differentiated cells as there was no increase in their intracellular Ca^{+2} levels measured by fura-2AM. Another reason for not observing evoke in intracellular Ca^{+2} levels via ryanodine receptors could be their continuous activation due to extensive phosphorylation, which could occur during the differentiation process. For a fully functional cardiomyocyte the proper functioning of ryanodine receptors should be achieved as ryanodine receptors are the main receptors for the release of Ca^{+2} from the inrtacellular Ca^{+2} pools. However when the cells were treated with KCl, the response of the cells were observable to depolarization. The increase in Ca^{+2} levels was clearly observed with the cells induced to differentiate into cardiomyocytes at day 9 and even more at day 14 indicating that the cells started to express voltage gated Ca^{+2} channels.

After checking the contractility of the cells specific for the cardiac muscle cells, expression of the cardiac troponin I, a-smooth muscle actin and MEF2D (myocyte specific enhancer factor 2D) that are markers specific to the cardiac cells were checked. MEF2D is a transcription factor that is important for the development of the cardiomyocytes and muscle cells. Cardiac troponin is specific to the cardiac cells and as well known, mediates the interaction between actin and myosin while a-sarcomeric actin is also important in contractility. According to the semi-quantitative RT-PCR results, the expression of the genes were higher than the control cells that were cultured in the control medium without 5-azcytidine. Especially the increase at expression of the troponin I and sarcomeric actin were significant ($p<0.05$) on 9^{th} day, however on day 14 the expression was also present, but not significant.

As a result, we were able to show that the MSCs were successfully able to differentiate into cardiomyocytes as early as day 9. This time is 5 days earlier than the normal culturing time of the MSCs. Shortening the culture period of MSCs could be useful especially for the patients waiting for urgent therapies that are suffering from ischemic heart diseases and may be very important as a cure for these diseases.

The other life threatening disease is cancer as indicated by WHO and the targeted treatment of tumors is an issue of concern because the chemotherapeutics do not only kill the cancerous cells but also toxic to somatic cells. GRN163L is an oligonucleotide, a specific telomerase template antagonist, which binds to the telomerase RNA subunit and therefore inhibits the elongation of telomeres. Telomere maintenance is important for the survival of the cancer cells and when inhibited by GRN163L which also has a detachment effect on the cells, the drug effectively inhibits the proliferation of the telomerase expressing cancer cells, yet

MSCs also have telomerase activity and this activity is important for the maintenance of the stem cell pools and differentiation potentials.

The effect of telomerase antagonist GRN163L on any adult stem cell is not demonstrated since its discovery in 2005. Treatment with a telomerase inhibitor could have some potential side effects on MSCs which are expected to be mild because stem cell populations have longer telomeres than tumor cells, and as they divide rarely, their telomeres should shorten at a much slower rate than proliferating cancer cells (Shay and Wright 2007). Therefore we aimed to reveal its effects on rat bone marrow derived MSCs when administered both *in vivo* and *in vitro*. For this purpose, we first investigated the phenotypic effects of GRN163L on MSCs. After the generation of the MSCs, the cells were seeded together in the presence of GRN163L and its mismatch oligonucleotide. When the treated cells were compared with the untreated MSCs that are control cells, it was observed that the treated cells were detached from the plate surface and get rounded instead of their attached spindle-like phenotype. Moreover although all the samples were seeded at the same number, control cells were growing continuously but the cells treated with the telomerase antagonist ceased growing. On the other hand the cells treated with mismatch oligonucleotide were behaving like the control cells in all manners. To check whether these effects on phenotype are reversible *in vitro*, we treated the cells for 3 days and then removed the drug and left the cells for recovery. Most of the cells recovered and gained their attachment and proliferative capacities and their fibroblastic shapes as well. These phenotypic effects of GRN163L are however supposed to be due to its properties of oligonucleotide backbone instead of the specific action on telomerase RNA subunit. On the other hand, its effects may be due to the inhibition of telomerase enzyme as telomerase independent from telomeres have other non-canonical roles in the cells. It was demonstrated that telomerase is important in the growth and

proliferation of the cells, by inducing the synthesis of growth factors (Smith *et al* 2003), moreover it induces the mobility and differentiation of the stem cells and acts on the genes responsible in adhesion (Gilson and Ségal-Bendirdjian 2010).

After determining the reversible effect on phenotype, we checked the MSC marker expressions of the treated, untreated, mismatch treated and recovered cells. CD90, CD29 and CD71 expressions were positive at all stages while CD34 and CD45 expressions were negative as determined by RT-PCR indicating that although the cells were treated with oligonucleotides either mismatch or GRN163L they do not differentiate or loose and decrease their marker compositions. Further investigation on telomerase activity is performed by using TRAP assay. The telomerase enzyme activity of the GRN163L treated cells was observed to have no telomeric ladders while the other cells, untreated, mismatch treated and recovered cells had telomeric ladders. Therefore we concluded that GRN163L like it does on cancer cells blocks the activity of telomerase in MSCs however when the cells are recovered the activity of the enzyme was regained and mismatch oligonucleotide had no effect on the enzymatic activity of telomerase. Our study independent from the effects of GRN163L on MSCs has also proven the telomerase activity of MSCs which is controversial in the literature as some studies indicate that MSCs do not possess telomerase but use ALT pathway instead and some of the reports indicate that MSCs show low level of telomerase expression and telomerase levels decrease with the increased passage. The latter is possible as unlike ESCs, MSCs can be passed upto 50 PDs and our study was performed with the cells at the first passage.

The differentiation properties of MSCs were also checked after recovery. The reason to check the differentiation properties of the recovered cells depends on the fact that when the cells are treated *in vivo* they should maintain their

differentiation capacities to regenerate the damaged tissues. Also differentiation of the stem cells is an important signature of stemness. Furthermore the expression of telomerase in stem cells is important for their differentiation capacities. As GRN163L is active on telomerase it was important to determine the differentiation capacity to reveal whether any permanent effects were present on the telomerase activity. The adipogenic and osteogenic differentiation capacities of the control cells and recovered cells were checked and both cells were differentiated into osteocytes and adipocytes successfully as demonstrated with Alizarin Red S staining and Oil Red O staining respectively. So, we demonstrated that after the short term administration of GRN163L, when the cells were recovered from the effects of the drug, they still maintain their differentiation potentials *in vitro*. As telomerase is active on the differentiation potentials of the cells it was important to show the reversible nature of the telomerase inhibition.

As indicated above the cells ceased growing when incubated with 1 μM GRN163L for 1 week. For this reason we also investigated the cell cycle genes to demonstrate at which step GRN163L was acting. The mRNA and protein expressions of the cells were checked by qRT-PCR and Western blotting respectively. In the combination of these results it was demonstrated that the cells were kept at G1 state of the cell cycle which is also reversible *in vitro*. Our results support the findings of Smith *et al* (2003), who showed the growth inducing effects of telomerase.

MSCs are important candidates in cellular therapies they can be lifesaving in diseases that require urgent treatments however although our results revealed the reversible effects of GRN163L *in vitro*, extensive care must be taken to optimize the beneficial effects of the curative agents that can also affect MSC pools.

CHAPTER 6

FUTURE PERSPECTIVES

In this thesis study the differentiation of cardiomyocytes as early as day 9 in the culture medium was achieved which was proven by the CD90 immunocytochemistry, intracellular Ca^{+2} levels and the expression of cardiomyocyte specific markers. The functioning of voltage gated Ca^{+2} channels were seem to be achieved during the differentiation process, which was evidenced by elevation of intracellular Ca^{+2} levels after high extracellular KCl treatment. On the other hand, as caffeine treatment had no effect over the cells, we concluded that the ryanodine receptors, that have important role in the release of Ca^{+2} from intracellular Ca^{+2} pools, were not functioning. For this reason the formation and the functioning of the ryanodine receptors should be investigated with further studies from MSCs.

The reversible activity of imetelstat on MSCs was revealed in this study as well. The reversible effects were proven by q-RT-PCR, immunobloting, differentiation protocols and TRAP assay. However all these procedures were conducted in vitro. GRN163L is an anticancer agent which is administered in combination with other antitherapeutics in clinical trials. Although all the effects of GRN163L on MSCs are reversible in vitro, the in vivo effects could be different. Therefore, the in vivo

effects of imetelstat should be determined in vivo with further studies to have a better understanding about the effect of GRN163L on MSC pools.

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APPENDIX

STANDARD SOLUTIONS AND BUFFERS

DEPC-Treated ddH_2O
1ml DEPC
1lt ddH_2O
Stirred in a hood for 1 hour, autoclaved in order to inactivate the DEPC

10x PBS
80 g NaCl
2 g KCl
8,01g $Na_2HPO_4.2H_2O$
2g KH_2PO_4
1 liter ddH_2O pH: 7,2
Working solution (1XPBS) was prepared by diluting 10XPBS by 10 times

50X TAE Buffer
2M Tris Base (242 g)
57,1 ml Glacial Acetic Acid
50mM EDTA
Add to 1 lt by ddH_2O
Working solution (1XTAE) prepared by diluting 50XTAE.

10 X Agarose Gel Loading Dye
0,009g BFB
0,009g XC
2,8mL ddH2O
1,2ml 0,5M EDTA.
Total volume brought to 15ml by adding glycerol, dilute 1:10 in sample prior to loading to electrophoresis gel

10%SDS
100g SDS
1lt ddH_2O

1M Tris
60.55g Tris
300ml ddH2O

21ml 37% HCl
pH adjusted to 8.0 with HCl, total volume brought to 500ml by adding ddH_2O.

0.5M EDTA
93.05g EDTA
300ml ddH2O
pH adjusted to 8.0 with NaOH, total volume brought to 500ml by adding ddH_2O.

30% Acrylamide Mix
145g Acrylamide
5g bis-acrylamide
500ml ddH_2O
Filtered, stored in dark at 4^0C.

SDS-PAGE Running Buffer (5X stock solution)
15g Tris
73,2g Glycine
5g SDS
1lt ddH2O
1X working solution prepared by diluting the 5X SDS-PAGE running buffer 5 times, stored at 4^0C

Bradford Reagent
100 mg Coomassie Brilliant Blue G-250
100 ml 85%phosphoric acid
50 ml 95%EtOH
850 ml ddH_2O
Filtered through Whatman no:1

Lysis Buffer
2M NaCl
1M Tris pH:8.2
0.9% Igepal CA-630 (Sigma, Germany)
10x Protease Inhibitor Cocktail (Roche, Germany)
778.5µl ddH_2O

10X TBS
12.19 g Tris
87,76 g NaCl
pH adjusted to 8, total volume brought to 1lt by adding ddH_2O

1X TBS-T 0.1%
50 ml 10X TBS
450 ml ddH_2O
1ml Tween-20
Total volume brought to 500ml by adding ddH_2O

Blocking Solution for Western Blot (5%)
2,5 g milk powder
50mL 1X TBS-T(0.1%)

5% BSA Blocking Solution
0.5g BSA
10mL 1X TBS-T(0.1%)

Blocking Solution for Immunohistochemistry and Immunoflourescence Staining
4 µl 2% BSA
4 µl 1X PBS
20 µl Tween-20

1XPBS-T (0.1%)
500ml 1XPBS
0.5ml Tween-20

Cracking Buffer (2X Protein Loading Buffer)
50mM Tris HCl pH: 6,8
2mM EDTA pH: 6,8
1% SDS
20% Glycerol
0,02% BFB
Add 1% ß-mercaptoethanol prior to use

Coomassie Blue Staining Solution:
0,25 g coomassie brilliant blue
45 ml Methanol
45 ml ddH_2O
10 ml glacial acetic acid

5X Running Buffer
15 g Tris base

72 g Glycine
5 g SDS
1 liter ddH_2O
Diluted to 1X prior to use

Semi-dry Transfer Buffer
2,5 g glycine
5,8 g Tris base
0,37 g SDS
200 ml methanol
800 ml ddH_2O

Destaining Solution
100 ml methanol
35 ml acetic acid
365 ml ddH_2O

Cold 70% Ethanol
70 ml Ethanol
30 ml ddH_2O
Dissolved and keep at 4°C

4% PFA
4g PFA
100ml 1XPBS
Dissolved by heating, solution prepared freshly

Timing of induction of cardiomyocyte differentiation for in vitro cultured mesenchymal stem cells: a perspective for emergencies[1]

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Abstract: Mesenchymal stem cells (MSCs) have the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and cardiomyocytes. Several established methods are presently available for in vitro isolation of MSCs from bone marrow. However, the duration necessary to culture them can be a major handicap to cell-based therapies needed for such urgent cardiovascular conditions as acute myocardial infarction and acute hindlimb ischemia. The best timing of cardiomyocyte differentiation induction after MCS isolation and expansion is still an unresolved issue. Our goal was to investigate the possibility of obtaining functional cardiomyocytes from rat MSC within a shorter time period. We examined MSCs' colony-forming capacity, CD90 and CD34 immunoreactivity during the 14 days of culturing. Cardiomyocyte differentiation was induced by 5-azacytidine. Immunohistochemic staining, together with intracellular Ca^{2+} measurement experiments, revealed that MSCs do not differentiate into any specific cell lineage but show the characteristics of MSCs on both the 9th and 14th days of the culture. To check the potential for differentiation into cardiomyocytes, experiments with caffeine application and depolarization with KCl were performed. The cells possessed some of the specific biochemical features of contracting cells, with slightly higher capacities on the 14th day. Cells from 9th and 14th days of the culture that were treated with 5-azacytidine had a higher expression of cardiac-specific markers such as troponin I, α-sarcomeric actin, and MEF2D compared with the control groups. This study illustrates that it is possible to get functional cardiomyocytes from in vitro MSC culture in a shorter time period than previously achieved. This reduction in time may provide emergency cases with access to cell-based therapies that may have previously been unavailable.

Key words: mesenchymal stem cells, cardiomyocytes, differentiation, rat, in vitro, gene expression.

Résumé : Les cellules souches mésenchymateuses (CSM) ont la capacité de se différencier en ostéoblastes, chondrocytes, adipocytes, myocytes et cardiomyocytes. Plusieurs méthodes éprouvées permettent d'isoler in vitro les CSM de la moelle osseuse. Toutefois, la durée requise pour leur culture pourrait être un désavantage majeur pour la thérapie cellulaire d'urgences cardiovasculaires comme l'infarctus du myocarde et l'ischémie des membres inférieurs. Le meilleur moment de l'induction de la différenciation des cardiomyocytes après l'isolement et l'expansion des CSM n'a toujours pas été déterminé. Nous avons étudié la possibilité d'obtenir des cardiomyocytes fonctionnels de CSM de rats sur une courte période de temps. Nous avons examiné la capacité de formation de colonies des CSM et l'immunoréactivité à CD90 et CD34 durant les 14 jours de culture. La différenciation en cardiomyocytes a été induite par la 5-azacytidine. La coloration immunohistochimique ainsi que des mesures du Ca^{2+} intracellulaire ont révélé que les CSM ne se différencient pas en une lignée cellulaire spécifique, mais montrent les caractéristiques des CSM lors des 9^{e} et 14^{e} jours de culture. Pour vérifier le potentiel de différenciation en cardiomyocytes, nous avons fait des expériences avec application de caféine et dépolarisation au KCl. Les cellules possédaient certaines des caractéristiques biochimiques spécifiques des cellules pouvant se contracter, avec des capacités légèrement supérieures le quatorzième jour. Les cellules des 9^{e} et 14^{e} jours de culture traitées avec la 5-azacytidine ont montré une plus grande expression de marqueurs spécifiques du cœur, tels que la troponine I, l'actine α-sarcomérique et MEF2D, comparativement à celles des groupes témoins. Cette étude montre qu'il est maintenant possible d'obtenir des cardiomyocytes fonctionnels d'une culture in vitro de CSM dans une courte période de temps. Cette réduction de temps pourrait fournir aux cas d'urgence un accès à des thérapies cellulaires jusqu'alors inaccessibles.

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Mots-clés : cellules souches mésenchymateuses, cardiomyocytes, différenciation, rat, in vitro, expression génique.

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Introduction

Stem cell research has gained tremendous interest in the past two decades because it provides opportunities to develop new strategies for debilitating diseases. Stem, or progenitor, cells have the potential to provide unique sources for tissue restoration in regenerative medicine and tissue engineering (Ivanova et al. 2002). Although self-renewal and differentiation are their common features, as a result of cell-to-cell communication, stem cells vary in their potential to differentiate, in their duration and pathways of self-renewal, in the places where they are mostly found, and in their divisional characteristics (Morrison et al. 1997).

Autologous stromal mesenchymal stem cells (MSCs) from bone marrow, first identified by Friedenstein in the 1970s (Friedenstein et al. 1974, 1976), may offer the possibility of a renewable source for the replacement of injured cells or tissues without raising ethical concerns or the possibility of immunologic reactions. MSCs are a very small fraction of the nucleated cells in the bone marrow, only 0.01%–0.1% of the total population (Pittenger et al. 1999). They have fibroblastic morphology and they are adherent spindle-shaped cells. MSCs adhere to the culture plate, leading to the formation of colonies. Usually, basal mediums with serum support are used to expand MSCs in tissue culture, and growth factors are added in order for MSCs to differentiate (Barry and Murphy 2004). In fact, their most prominent feature is their ability to differentiate into osteoblasts, chondrocytes, adipocytes, hepatocytes, neurons, and myocytes under defined conditions. They are also thought to differentiate into certain types of ectodermal and endodermal tissues, such as neurons and endothelial cells, including skeletal myocytes (Jackson et al. 2001), central and peripheral neurons (Mezey et al. 2000; Brazelton et al. 2000), and hepatic cells (Petersen et al. 1999). The master switches regulating the transitions from stem cells to differentiated cells, and the genes active in specific differentiation patterns are still not known. The reason for the difficulty in finding these regulatory genes is because of their temporal activity, the response given to inductive molecules, and the varying differentiation pathways between organisms (Baksh et al. 2004; Caplan and Bruder 2001).

Bone marrow-derived cardiac myocytes have been shown to populate rodent heart tissue after myocardial infarction (Jackson et al. 2001) or cardiac transplantation (Edelberg et al. 2002). Moreover, preclinical studies have shown that the injection of bone marrow cells directly into the myocardium enables these cells to differentiate into cardiac myocytes, smooth muscle cells, and endothelial cells. These transformations lead to the regeneration of the myocardium and improvement of cardiac function (Orlic et al. 2001; Tomita et al. 2002).

A major roadblock in the use of MSCs, however, is that MSCs are rare and the long duration of their culture limits their potential use in urgent cell-based therapies. In addition, the assessment of donor MSC functional activity at the cellular level, which is the focus of several research groups, is critically important. To our knowledge, no study has looked at the best timing to induce cardiomyocyte differentiation after MSC isolation and expansion. Therefore, we aimed to shorten the time period required for MSC culture before cardiomyocyte differentiation.

Materials and methods

Cell culture

MSCs were obtained from female, 9-week-old, 280–300 g Sprague–Dawley rats. Bone marrow heterogeneous cell population was collected from the femur and tibia by flushing with a 5-millilitre syringe containing 10% FBS (HyClone, Logan, USA) in DMEM (Invitrogen, Paisley, UK) after the rats were sacrificed by cervical dislocation. Negatively selected supernatant from CD34-labeled paramagnetic beads (Dynal, Oslo, Norway) was cultured in plastic cell-culture dishes with MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone) in a 5% CO_2 incubator at 37 °C. The next day, the media of the tissue culture plates were changed and the nonadherent cells were removed. The media of the cells were changed every 4 days, after washing with sterile 1× PBS before the change.

Our experimental study protocol was approved by the Animal Ethics Committee of Bilkent University (Bil-AEC). All the animals received care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science, and this study protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Colony-forming assay

MSCs were washed in 1× PBS and then air dried. Methanol was used for fixation and left for 5 min. The cells were washed again in a 1× PBS buffer and a giemsa-staining reagent (Carlo Erba, Milano, Italy) was added and left for 5 min. The reaction of the giemsa staining was stopped by the addition of tap water.

Induction of cell differentiation

Two different time points were used for the cardiomyocyte differentiation of MSCs. In the first group, differentiation was induced on day 9 of the culture (hereafter stated as the 9th day) and in the second group, differentiation was induced on day 14 (hereafter stated as the 14th day). The induction of cardiomyocyte differentiation was performed by

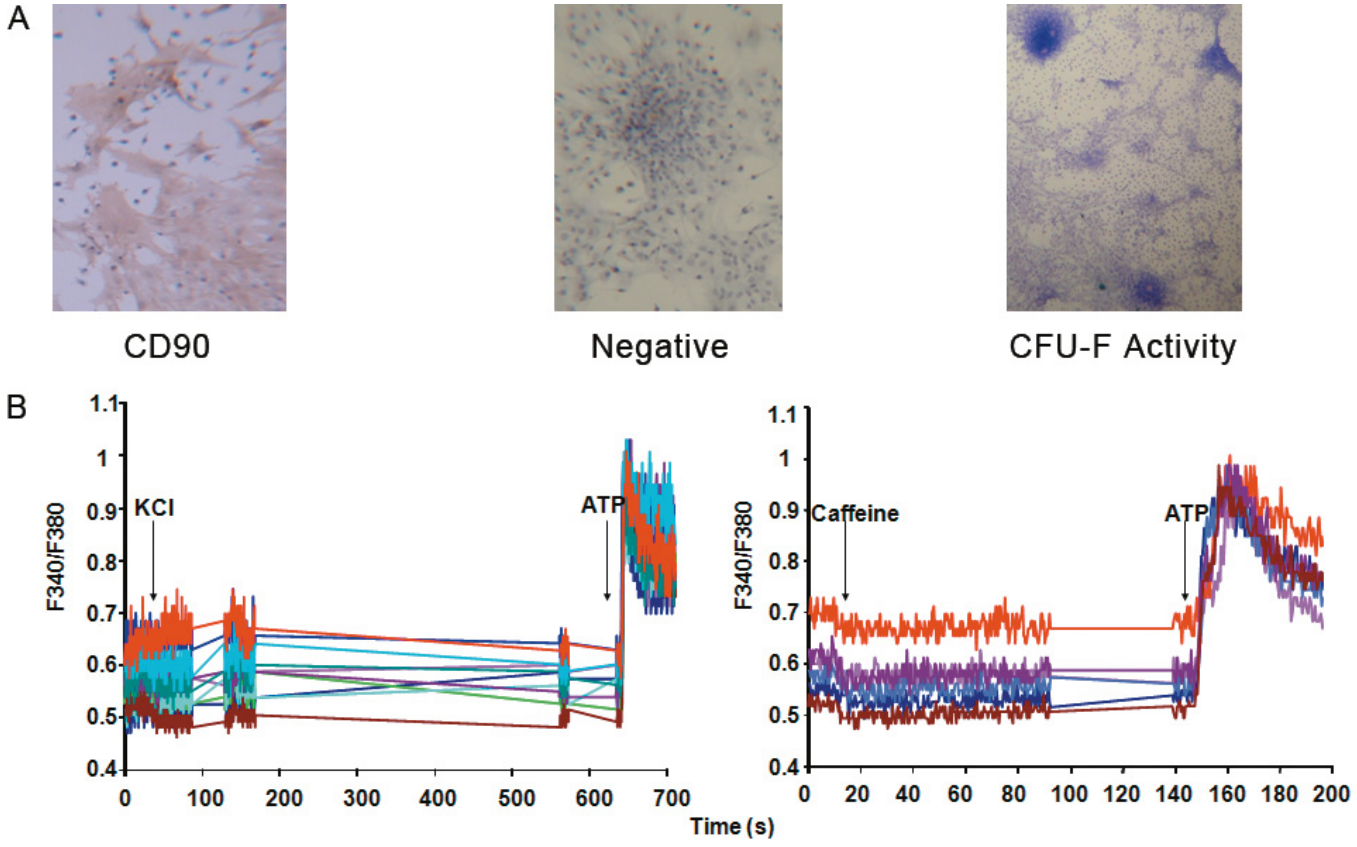
Table 1. Primers and the product size of the cardiac-specific gene markers for cDNA amplification.

Gene	Primers	Product size
Troponin I	Forward: 5′–GCGAAGCAGGAGATGGAG–3′ Reverse: 5′–TGCCACGCAGGTCATAGA–3′	250 bp
α-Sarcomeric actin	Forward: 5′–CCAGGCACTGTGGAAAGG–3′ Reverse: 5′–CACGATGGATGGGAAGAC–3′	115 bp
MEF2D	Forward: 5′–TGGGAATGGCTATGTCAGTG–3′ Reverse: 5′–CTGGTAATCTGTGTTGTAGG–3′	351 bp
GAPDH	Forward: 5′–CCTCCTCATTGACCTCAACTAC–3′ Reverse: 5′–CATGGTGGTGAAGACGCCAG–3′	219 bp

Table 2. PCR conditions for the genes.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
Troponin I	95 °C, 5 min	94 °C, 45 s	65 °C, 30 s	72 °C, 40 s	33	72 °C, 5 min
α-Sarcomeric actin	95 °C, 10 min	94 °C, 45 s	57 °C, 60 s	72 °C, 60 s	35	72 °C, 10 min
MEF2D	95 °C, 10 min	94 °C, 30 s	57 °C, 60 s	72 °C, 50 s	33	72 °C, 5 min
GAPDH	95 °C, 5 min	94 °C, 30 s	59 °C, 30 s	72 °C, 30 s	23	72 °C, 5 min

Fig. 1. Characteristics of rat mesenchymal stem cells on the 9th day of culture. (A) CD90 immunoreactivity and colony-forming capacity. No staining is observed in the negative control. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. No response was observed with either stimulation. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.

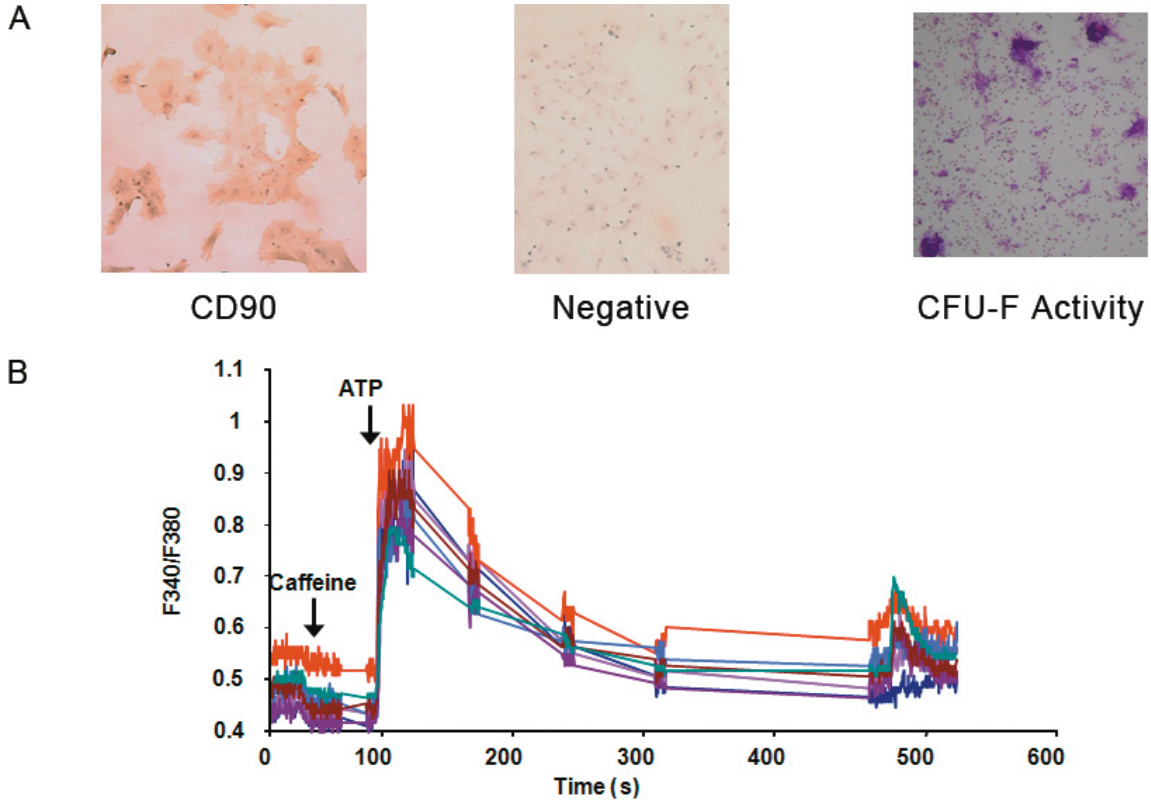


incubating cells with 10 μmol/L 5-azacytidine (Sigma, Steinheim, Germany) for 24 h. The cells were washed and expanded for another 14 days with low-glucose DMEM (Invitrogen), 20% FBS (HyClone), and 1% penicillin-streptomycin (HyClone).

Total RNA isolation and RT-PCR analysis

On the 9th and 14th day of the MSC culture and 14 days after the 5-azacytidine treatment of each group, cells were trypsinized and removed. Total cellular RNA was isolated from the precipitate by using the RNeasy Mini kit (Qiagen,

Fig. 2. Characteristics of rat mesenchymal stem cells on the 14th day of culture. (A) CD90 immunoreactivity and colony-forming capacity. No staining is observed in the negative control. (B) Cytoplasmic calcium levels in response to caffeine (or KCl, data not shown) measured with fura-2. No response was observed with either stimulation. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



Hilden, Germany) according to the manufacturer's protocol. The cDNAs were synthesized from the total RNA samples with the RevertAid First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's protocol. cDNA amplifications were performed by using oligonucleotide primers for the following genes: cardiac troponin I (cTnI), α-sarcomeric actin, MEF2D, and GAPDH (Table 1). PCR conditions of these genes are listed in Table 2.

Immunohistochemistry

Cells in the cover slips were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After washing 3 times with PBS for 10 min each, slides were incubated with preblocking serum (normal goat serum 1.5%, bovine serum albumin 2%, Triton X-100 0.1%) for 1 h at room temperature. Primary antibodies of CD90 and CD34 (Chemicon, Temecula, Canada) were applied at a concentration of 1:500 dilution in preblocking solution and kept at 4 °C overnight. After washing 3 times with PBS, tissue sections were incubated for 15 min each with biotinylated anti-mouse and anti-rabbit Ig (DakoCytomation, Glostrup, Denmark) and streptavidin–horseradish peroxidase (HRP) (DakoCytomation) at room temperature. After washing for 10 min with PBS, slides were rinsed in a 0.5% solution of Triton X-100 and PBS for 30 s. Color developments were achieved by incubation with liquid DAB+ (DakoCytomation). The slides were then counterstained with hematoxylin and mounted with Faramount aqueous mounting medium (DakoCytomation).

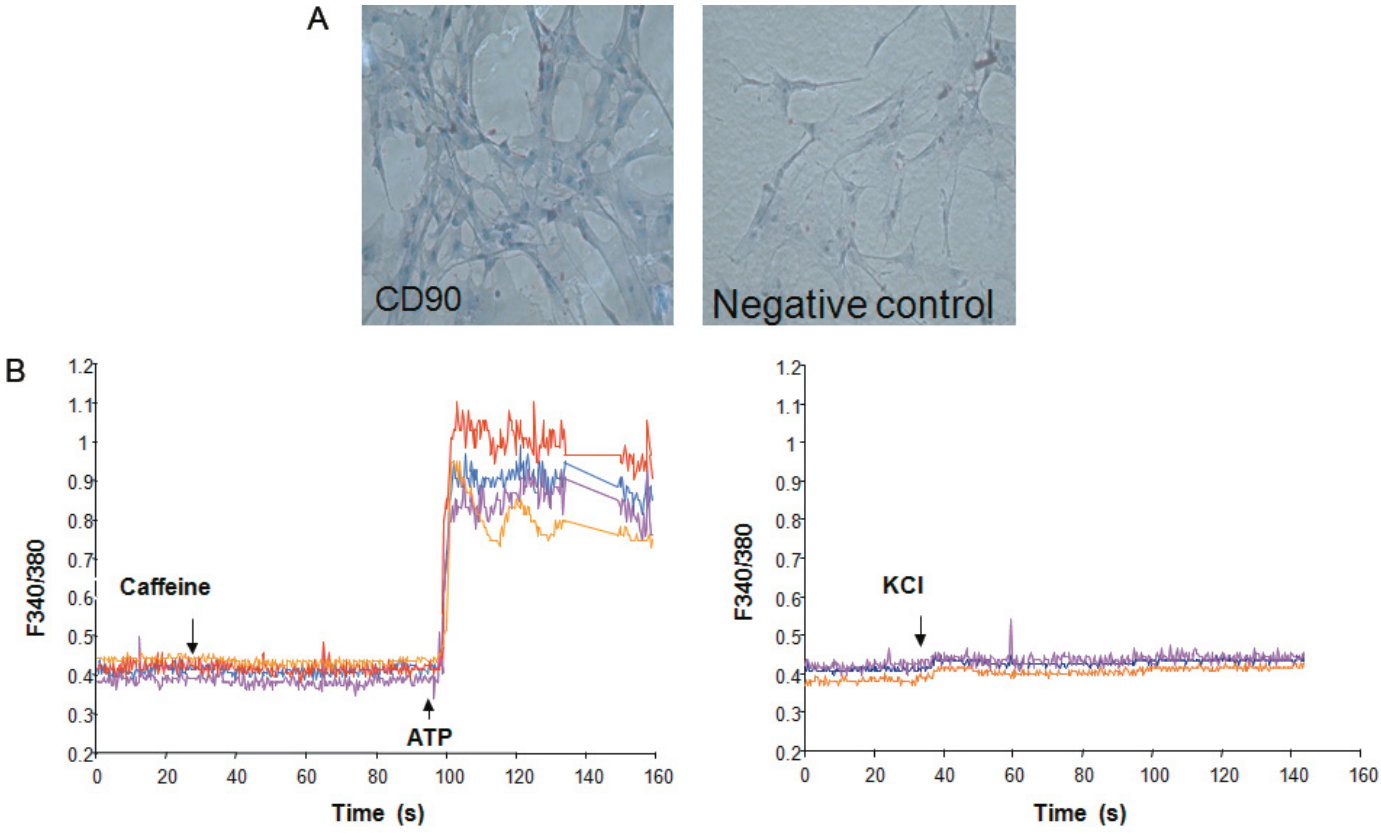
Intracellular Ca^{2+} measurement

Cells were grown on round cover slips in 6-well plates and loaded with fura-2 AM at room temperature (21–24 °C) for 50 min. Cells were then washed before the experiments with Hepes-buffered physiologic NaCl bathing solution containing 1.2 mmol/L Ca^{2+} and 1 mmol/L Mg^{2+}. Cells were challenged either with caffeine to stimulate ryanodine receptors or with ATP to stimulate P2Y purinergic receptors. In addition, we applied high-KCl bathing solutions and monitored intracellular calcium concentrations to see whether depolarization could induce any intracellular Ca^{2+} transients. Cover slips were fixed to a Teflon chamber and mounted on an inverted fluorescent microscope (Nikon TE-300, Japan). Fluorescence ratios were obtained by exciting cells at 340 and 380 nm with a microscope-based spectrofluorimeter with a temporal resolution of one ratio every 0.5 s. Emission signals were collected at 510 nm with an IC-300 ICCD (intensified charge-coupled device) camera with Delta Ram, (Photon Technology International, USA). Data was collected and analyzed by Image Master software (Photon Technology International).

Statistical analysis

All data were expressed as means ± SD. Data were ana-

Fig. 3. Differentiation into cardiomyocytes was induced by applying 5-azacytidine to MSCs on the 9th day of culture. (A) No CD90 immunoreactivity is present after induction. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



lyzed by performing paired *t* test using Minitab Statistical Software (State College, USA). A value of $p < 0.05$ was considered to be statistically significant.

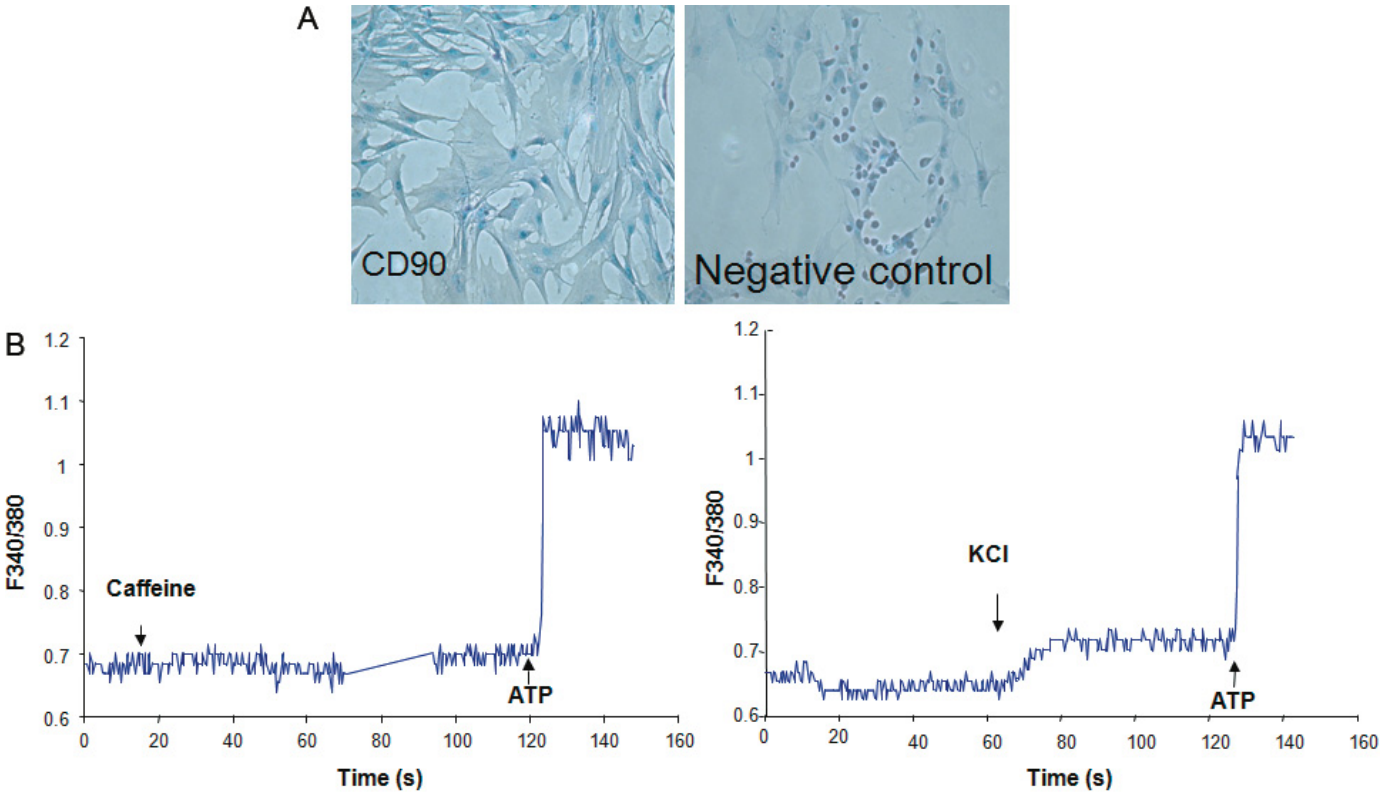
Results

We first investigated the characteristics of MSCs on the 9th and 14th days of the culture by their CD90 expressions and CFU-F (colony-forming unit – fibroblast) activities (Figs. 1A and 2A). We also compared their free intracellular Ca^{2+} concentrations (Figs. 1B and 2B). On the 9th day of the culture, rat MSCs were stained positive for CD90 (Fig. 1A), started to form colonies (Fig. 1A), and did not evoke any Ca^{2+} response upon application of caffeine and depolarization with KCl (Fig. 1B). These results were similar to those on the 14th day of the culture, confirming that both cells were undifferentiated (Figs. 2A and 2B). On the other hand, they responded to (0.5×10^{-4} mol/L) extracellular ATP application with a clear Ca^{2+} transient (Figs. 1B and 2B). These cells stained negative with the CD34 antibody (data not shown).

Next, MSCs from the 9th and 14th days of the culture were treated with 5-azacytidine to induce cardiomyocyte differentiation. Fourteen days after this treatment, we checked their CD 90 expressions (Figs. 3A and 4A) and their responses to extracellular KCl and caffeine as an increase in cytosolic Ca^{2+} was noted after the cells were loaded with fura-2 (Figs. 3B and 4B). We applied 10 mmol/L caffeine and 45–60 mmol/L extracellular KCl to the cells. Cells treated with 5-azacytidine on the 9th and 14th days of the culture did not respond to 10 mmol/L caffeine. On the other hand, cells treated with 5-azacytidine on the 14th day of the culture responded to the KCl application with a small increase of cytosolic Ca^{2+}. Cells treated with 5-azacytidine on the 9th day also responded with a very small Ca^{2+} increase. These results may suggest that voltage-activated Ca^{2+} channel expression is starting in both groups of cells 14 days after the 5-azacytidine treatment; however, there is no indication of the presence of functional ryanodin receptors. All cells responded to ATP (0.5×10^{-4} mol/L) application with a sudden cytoplasmic Ca^{2+} increase.

To illustrate the cardiomyocyte commitment of these cells, we performed RT-PCR analysis to investigate the expression pattern of cardiac-specific markers before and after the induction with 5-azacytidine. RT-PCR experiments showed that cardiac-specific troponin1, α-sarcomeric actin, and MEF2D expression increased after the 5-azacytidine induction compared with that of the cells that were cultured with the control media both on the 9th day (Fig. 5A) and the 14th day of the culture (Fig. 5B). The expression of cardiac-specific troponin1 and α-sarcomeric actin were statistically significant on the 9th day compared with that of the

Fig. 4. Differentiation into cardiomyocytes was induced by applying 5-azacytidine to MSCs on the14th day of culture. (A) No CD90 immunoreactivity is present after induction. (B) Cytoplasmic calcium levels in response to KCl and caffeine measured with fura-2. ATP stimulation is used as a positive control. Fura-2 signal is given as a ratio of fluorescence at 340 and 380 nm.



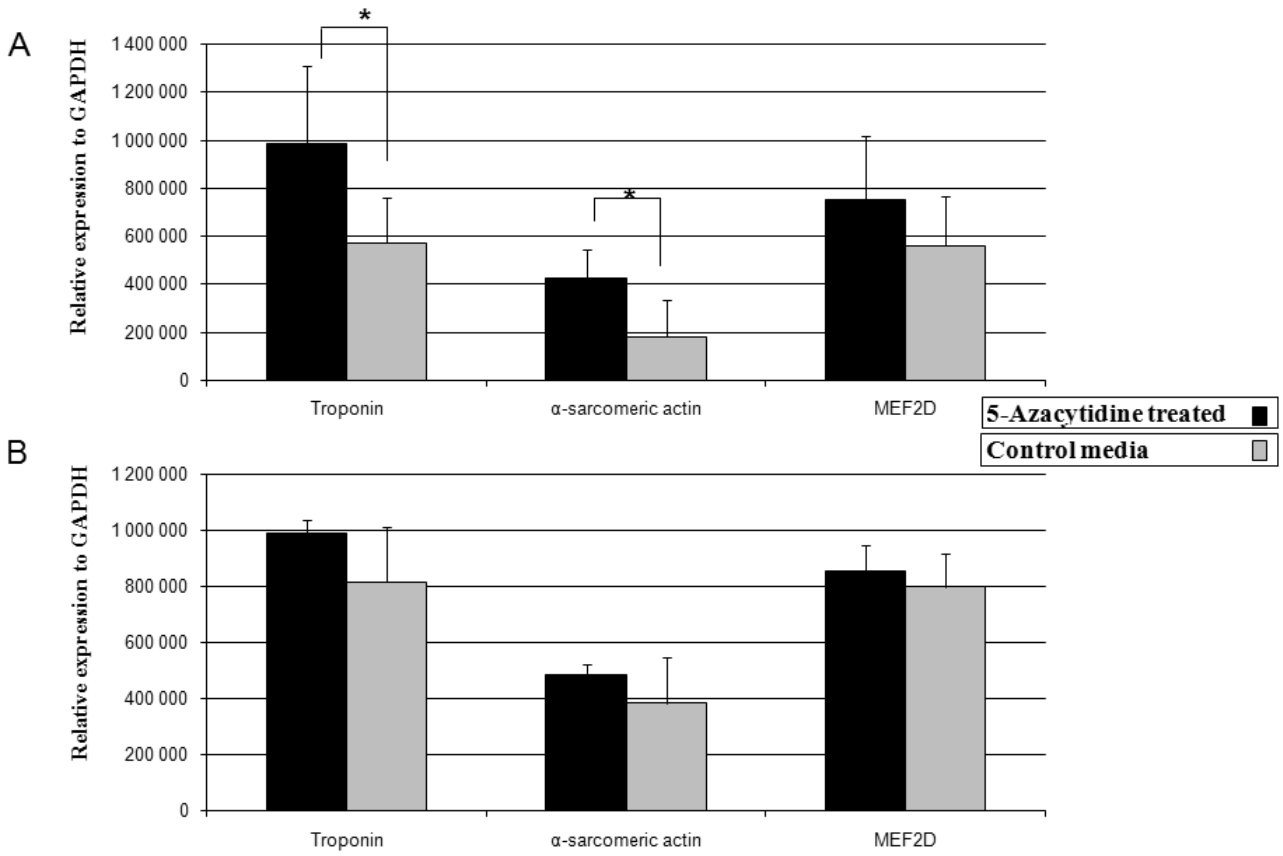
control group. The values shown in Figs. 5A and 5B were obtained by normalizing the expression values to that of the loading control, GAPDH.

Discussion

The possibility of using stem cell-based therapies has opened a new therapeutic era for cardiovascular diseases, which claim roughly as many lives as cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, influenza, and pneumonia combined (1999–2002 National Health and Nutrition Examination Survey, available from www.americanheart.org, "Heart disease and stroke statistics, 2005 update"). Applications involving the use of stem cells in humans, which might have been considered 'science fiction' fewer than 20 years ago, are now being utilized with a great success rate (Akar et al. 2006). Different types of stem or progenitor cells have been used to target cardiac regeneration, including MSCs (Laflamme and Murry 2005; Tousoulis et al. 2008; Wang and Li 2007; Atsma et al. 2007; Ohnishi and Nagaya 2007). Aside from their differentiation potential, there are other aspects that make MSCs valuable for new therapeutic strategies. MSCs actively inhibit T-cell proliferation and as a result are considered nonimmunogenic or hypoimmunogenic, which is important for a host response to allogenic MSC therapy. MSCs can also be frozen to preserve them, and when they are thawed they function apparently normally, thus allowing for future 'off-the-shelf' therapy approaches. However, the time required for isolation, expansion, and differentiation of specific cell types can be a major handicap to the optimal timing of stem-cell delivery to acutely injured tissues or organs. This issue is of paramount importance in cardiovascular medicine (Bartunek et al. 2006). The assessment of donor MSC functional activity at the cellular level, which is the focus of several research groups, is also critically important. There are no available data from experimental studies or clinical trials that show the exact timing of the induction of cardiomyocyte differentiation before MSC delivery to an injured heart or ischemic limb.

Our study demonstrated that rat MSCs did not differentiate but exhibited characteristics of MSCs by the 9th day of the culture similar to those on the 14th day of the culture. As expected, these cells started to form colonies on day 7 (data not shown) and these colonies became positive for CD90 staining by day 9. We observed that caffeine (10 mmol/L) application and depolarization with KCl (45–60 mmol/L) did not evoke any Ca^{2+} responses in MSCs on the 9th and 14th days of the culture, which confirmed that they were still undifferentiated cells. On the other hand, they responded to extracellular ATP (0.5×10^{-4} mol/L) application with clear Ca^{2+} transients. These results indicate the absence of any functional ryanodine receptor in rat MSCs and also demonstrate that these cells do not differentiate and clearly exhibit characteristics of MSCs by the 9th day of the culture. Therefore, 9 days of in vitro isolation and expansion of MSCs can be adequate for the application of any differentiating agents to these cells. To our knowledge, this is the shortest time yet that obtains functional MSCs in rat models. Shortening the culture time by nearly

Fig. 5. Expression profile of cardiac-specific markers in 5-azacytidine-treated MSCs on the (A) 9th and (B) 14th day of culture. Expression of the genes was normalized by using GAPDH as the loading control and comparing with the cells cultured in the control media. The increase in the expression of cardiac-specific troponin1 ($p = 0.04$) and α-sarcomeric actin ($p = 0.01$) on the 9th day was statistically significant. Black bars, 5-azacytidine-treated; grey bars, control media.



5 days could be a very useful development for patients awaiting urgent cell-based therapies.

In this study we also demonstrated that MSCs could be differentiated into cardiomyocyte-like cells by applying 5-azacytidine in vitro (Makino et al. 1999) and in vivo (Toma et al. 2002; Shake et al. 2002), as shown previously. However, the exact mechanism of MSCs' differentiation into cardiomyocyte-like cells is not clear. Several groups have reported that in vitro differentiation of MSCs into cardiomyocytes depends on different factors, including the number of passages or the combination of certain growth factors or molecules (Zhang et al. 2005; Antonitsis et al. 2007; Muscari et al. 2008).

To further characterize the cardiomyocyte differentiation, MSC cultures from day 9 and day 14 were treated with 10 μmol/L 5-azacytidine to induce cardiomyocyte differentiation (Figs. 3 and 4). Fourteen days after the 5-azacytidine treatment, we observed that these cells, both on the 9th (Fig. 3A) and 14th (Fig. 4A) days of the culture, did not express the mesenchymal stem cell marker CD90. Both groups responded, albeit slightly, to extracellular KCl with an increase in cytosolic Ca^{2+}; however, this response appeared to be more evident on the 14th day of the culture. On the other hand, cells from both cultures did not respond to 10 mmol/L caffeine application, but responded to ATP (0.5×10^{-4} mol/L) application with a sudden cytoplasmic Ca^{2+} increase (Figs. 3B and 4B), probably due to the stimulation of endogenous P2Y receptors. These results may suggest that voltage-activated Ca^{2+} channel expression starts 14 days after the 5-azacytidine treatment on the 9th and 14th days of the culture, but there is no indication of the presence of functional ryanodin receptors. Further experiments are needed to verify this point. It is noteworthy that compared with the controls, both the 9th- and 14th-day 5-azacytidine-treated cells expressed increased levels of cardiomyocyte-specific genes (Figs. 5A and 5B). The increase in the expression of cardiac-specific troponin1 ($p = 0.039$) and α-sarcomeric actin ($p = 0.012$) on the 9th day was statistically significant. These findings suggest that MSCs from day 9 may be used for the in vitro differentiation into cardiomyocytes.

In conclusion, our study demonstrated that MSCs from adult rat bone marrow are able to differentiate into cardiomyocyte-like cells as early as 9 days after isolation and expansion after being induced by 5-azacytidine, evidenced by expressing cardiomyocyte-specific genes and losing the expression of mesenchymal stem cell marker genes. These results indicate that the culture time for MSCs can be shortened by nearly 5 days, which could be useful for patients awaiting urgent cell-based therapies.

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The Effect of Telomerase Template Antagonist GRN163L on Bone-Marrow-Derived Rat Mesenchymal Stem Cells is Reversible and Associated with Altered Expression of Cyclin d1, cdk4 and cdk6

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Abstract Telomerase activity is essential for the continued growth and survival of malignant cells, therefore inhibition of this activity presents an attractive target for anti-cancer therapy. The telomerase inhibitor GRN163L, was shown to inhibit the growth of cancer cells both in vitro and in vivo. Mesenchymal stem cells (MSCs) also show telomerase activity in maintaining their self-renewal; therefore the effects of telomerase inhibitors on MSCs may be an issue of concern. MSCs are multipotent cells and are important for the homeostasis of the organism. In this study, we sought to demonstrate in vitro effects of GRN163L on rat MSCs. When MSCs were treated with 1 μM GRN163L, their phenotype changed from spindle-shaped cells to rounded ones and detached from the plate surface, similar to cancer cells. Quantitative-RT-PCR and immunoblotting results revealed that GRN163L holds MSCs at the G1 state of the cell cycle, with a drastic decrease in mRNA and protein levels of cyclin D1 and its cdk counterparts, cdk4 and cdk6. This effect was not observed when MSCs were treated with a mismatch control oligonucleotide. One week after GRN163L was removed, mRNA and protein expressions of the genes, as well as the phenotype of MSCs returned to those of untreated cells. Therefore, we concluded that GRN163L does not interfere with the self-renewal and differentiation of MSCs under short term in vitro culture conditions. Our study provides additional support for treating cancers by administrating GRN163L without depleting the body's stem cell pools.

Keywords Mesenchymal stem cells · Telomerase · Cyclin D1 · cdk4 · cdk6 · GRN163L

SM Grazynov is an employee of Geron Corporation and performed many studies to show the use of telomerase inhibitors in different type cancers. He received equity interest, patent rights, or corporate affiliations, including consultantships, for GRN163L.

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Introduction

Telomerase is a ribonucleoprotein complex consisting of a reverse transcriptase catalytic subunit (hTERT) and an RNA moiety (hTR or hTERC). The template region of hTR is complementary to telomeric DNA and together with hTERT elongates telomeres by adding TTAGGG repeats to the end of the chromosome. This leads to stabilization of telomere lengths, prevention of t-loop and overhang loss, and continued cell division [1, 2]. Telomerase activity is reduced or absent in majority of normal tissues, which leads to a progressive shortening of the telomere with each cell division. In contrast, tumor cells and self-renewable tissues express telomerase to sustain their growth. The presence of this activity in many different kinds of human tumors, but

not in normal somatic cells, makes inhibition of telomerase activity an attractive tool on targeted cancer therapy [3–5].

Experimental studies based on anti-telomerase cancer therapies, such as immunotherapy, gene therapy, small molecule inhibitors and oligonucleotide-based therapeutics [3–5] have been making progress in recent years. A novel human telomerase RNA (hTR) antagonist GRN163L (Imetelstat) is a lipid-modified N3′→P5′ thio-phosphoramidate that is complementary to the template region of hTR, preventing it from binding to the telomeric repeats. The GRN163L sequence (5′-Palm-TAGGGTTAGACAA-3′) is apparently unique in the human transcriptome, and shows greatly enhanced stability as well as specific and high-affinity binding to telomerase. It has been shown that GRN163L causes telomerase inhibition and subsequent telomere shortening in many cell types, including in breast cancer, lung cancer, multiple myeloma and non-Hodgkin's lymphoma cell lines [6–8]. GRN163L has recently entered into Phase I/II clinical trials in patients with chronic lymphocytic leukaemia and some solid tumors such as in lung and breast cancer. Despite being a promising therapeutic agent, inhibition of telomerase activity may lead to side effects since germline cells, proliferating stem and progenitor cells also exhibit telomerase activity. Thus inhibition of telomerase could potentially cause a decline or defect in regenerative capacity and organ homeostasis [9].

Mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal and differentiating into multiple lineages such as osteocytes, adipocytes, chondrocytes, myoblasts, cardiomyocytes [10–12]. MSCs are also promising agents for cell-based therapies due to their ability to migrate in vivo to promote regeneration of damaged tissue, treat inflammation, and promote angiogenesis without inducing immune reaction in addition to the lack of ethical concerns [13, 14]. Additionally, their high self-renewal potential makes them strong candidates for stem cell gene therapy treating acquired and inherited disorders and restoring organ system functions, since tissue repair and regeneration is made possible by the presence of adult stem cells [15]. For the above reasons, the effects of telomerase-targeting therapies on MSCs must be well defined. The present study was undertaken to investigate the in vitro effects of GRN163L on the self-renewal and differentiation processes of MSCs.

Materials and Methods

Isolation and Culture of MSCs

MSCs were obtained from female, 9-week-old, 280–300 g Sprague–Dawley rats. After the rats were sacrificed by cervical dislocation, bone marrow a heterogeneous cell population was collected from the femurs and tibias by flushing with a 5 mL syringe containing 10% FBS (HyClone, Logan, USA) in DMEM (HyClone). The cells were cultured in plastic cell-culture dishes with MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone) in a 5% CO2 incubator at 37°C. The next day, the media of the tissue culture plates were changed and the nonadherent cells were removed. The media of the cells were changed every 3 days, after washing with sterile 1x PBS. Our experimental study protocol was approved by the Animal Ethics Committee of Bilkent University (BILHADYEK). All the animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science of the USA, and this study protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Experimental Groups

After 14 days of culture, the cells were trypsinized (HyClone) and transferred to culture dishes for RNA and protein isolation as well as for differentiation and telomerase activity assays. One day after the transfer, GRN163L (Geron Co., CA, USA) and its mismatch control oligo were added at a concentration of 1 μM to the cell plate. The media were changed every 3 days with the fresh GRN163L (hereafter stated as 163 L group) and mismatch control oligonucleotide (hereafter stated as mismatch group) together with control MSCs with no treatment (hereafter stated as control group). 163 L and mismatch cultures continued for 1 week. Finally, MSCs were left for recovery for 1 week after GRN163L was removed (hereafter stated as 163LR (recovery) group).

Total RNA Isolation and Reverse Transcription

MSCs were trypsinized and the total cellular RNA was isolated from the precipitate by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with additional DNase treatment. The cDNAs were synthesized from the total RNA samples with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol.

RT-PCR

cDNA amplification for *CD90, CD71, CD45, CD34, CD29* and *cyclophilin* were performed by using DyNAzyme II (Finnzymes). The primers are listed in Table 1. The initial

Table 1 Primer sequences for Q-RT-PCR and RT-PCR and product size

Gene	Sequence	Product size (bp)
TERT	(F) 5′-GCTACGCCATCCTGAAGGTC-3′ (R) 5′-GGTAGCAGAGCCAACGTGTG-3′	99
CYCLIN B1	(F) 5′-TCGATGTGGAGCAGCATACT-3′ (R) 5′-GTCCATTCACCGTTGTCAAG-3′	142
CDK4	(F) 5′-GAAGACGACTGGCCTCGAGA-3′ (R) 5′-ACTGCGCTCCAGATTCCTCC-3′	109
CDK6	(F) 5′-TTGTGACAGACATCGACGAG-3′ (R) 5′-GACAGGTGAGAATGCAGGTT-3′	151
CDK2	(F) 5′-TGACCAACTCTTCCGGATCT-3′ (R) 5′-ATAACAAGCTCCGTCCGTCT-3′	164
CDK1	(F) 5′-GTTGACATCTGGAGCATAGG-3′ (R) 5′-CTCTACTTCTGGCCACACTT-3′	144
CYCLIN A2	(F) 5′-CTGCCTTCCACTTAGCTCTC-3′ (R) 5′-GAGGTAGGTCTGGTGAAGGT-3′	125
RB	(F) 5′-TTGAAAGAAGAGGCACTCCC-3′ (R) 5′-CGCTACCTTAAATACCGCCT-3′	116
P53	(F) 5′-GTTCCGAGAGCTGAATGAGG-3′ (R) 5′-AGACTGGCCCTTCTTGGTCT-3′	109
Bmi-1	(F) 5′-ATGTGTGTCCTGTGTGGAGG-3′ (R) 5′-AGCCATTGGCAGCATCTGCT-3′	281
P21	(F) 5′-ATGTCCGATCCTGGTGATGTC-3′ (R) 5′-CGGCTCAACTGCTCACTGTC-3′	92
Cdc25a	(F) 5′-AGTGAAGAGAGCAGACCGAT-3′ (R) 5′-GGAGAGAGACTGGTGTGGAA-3′	126
P16INK4a	(F) 5′-CTTCACCAAACGCCCCGAACA-3′ (R) 5′-CGGGAGAGGGTGGTGGGGTC-3′	132
P19ARF	(F) 5′-GCAGAGCATGGGTCGCAGGTTC-3′ (R)5′-CGGGAGAGGGTGGTGGGGTC-3′	295
CYCLIN D1	(F) 5′-AATGCCAGAGGCGGATGAGA-3′ (R) 5′-GCTTGTGCGGTAGCAGGAGA-3′	189
CYCLIN E	(F) 5′-TTACTGATGGTGCTTGCTCC-3′ (R) 5′-GTCGTTGACGTAGGCCACTT-3′	136
Cyclophilin	(F) 5′-GGGAAGGTGAAAGAAGGCAT-3′ (R) 5′-GAGAGCAGAGATTACAGGGT-3′	211
CD 90	(F) 5′-CCAGTCATCAGCATCACTCT-3′ (R) 5′-AGCTTGTCTCTGATCACATT-3′	374
CD 34	(F) 5′-TGTCTGCTCCTTGAATCT-3′ (R) 5′-CCTGTGGGACTCCAACT-3′	281
CD 71	(F) 5′-ATGGTTCGTACAGCAGCAGA-3′ (R) 5′-CGAGCAGAATACAGCCATTG-3′	182
CD 29	(F) 5′-ACTTCAGACTTCCGCATTGG-3′ (R) 5′-GCTGCTGACCAACAAGTTCA-3′	190
CD 45	(F) 5′-ATGTTATTGGGAGGGTGCAA-3′ (R) 5′-AAAATGTAACGCGCTTCAGG-3′	175

denaturation step was at 95°C for 5 min, followed by 30 (for *CD90* and *CD34*), 35 (for *CD 71*), 26 (for *CD29* and *CD45*) and 23 (for *cyclophilin*) cycles of denaturation for 30 s for all genes at 94°C, annealing for 30 s at 55°C (for *CD90, CD34* and *cyclophilin*), 60 s at 66°C (for *CD71*) and 30 s at 60°C (for *CD29* and *CD45*), followed by extension for 30 s (for *CD90, CD34, CD29, CD45* and *cyclophilin*) and 45 s (for *CD71*) at 72°C. A final extension at 72°C for 5 min was applied to all the reactions.

Q-RT-PCR

The primers used for Q- RT-PCR are shown in Table 1. Before performing Q- RT-PCR reactions for experimental samples, the amplification efficiencies of all primers were calculated using a standard dilution series. Fold changes in the expression of the genes were estimated based on the comparative $Ct\left(2^{-\Delta\Delta Ct}\right)$ method, using the normal MSCs as calibrator. The Q- RT-PCR conditions for all

investigated genes have an initial denaturation 95°C, 10 min followed by 45 cycles (for all target genes) and 35 cycles (for housekeeping *cyclophilin*) of denaturation for 30 s at 94°C, annealing for 30 s at 60°C (for *TERT, cdk4, cyclin A* and *Bmi-1*), at 55°C (for *cyclin B1, cdk6, cdk2, cdk1, RB, p21* and *cyclophilin*), at 65°C (for *cyclin D1* and *p53*), at 59°C (for *cdc25A* and *cyclin E*), at 62°C (for $p16^{INK4a}$), at 68°C (for $p19^{ARF}$), followed by extension of 30 s at 72°C. Final extension at 72°C for 5 min was applied to all the reactions.

Protein Isolation and Quantification

MSCs were scraped from the cell culture plates in 1x PBS and the precipitate was treated with a lysis buffer containing 0.05 M Tris HCl, 1x protease inhibitor, 0.25 M NaCl and 1% (v/v) IGEPAL for 30 min on ice. Then the lysate was centrifugated for 20 min at 13,000 rpm at 4°C. Protein concentrations of supernatants were determined with Bradford protein assay as described [16].

Western Blotting

The proteins were separated on 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with blocking solution for 1 h at room temperature and was incubated in antibody solution at the concentration of 1:200 (for Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, Cyclin E), 1:1000 (for Cdk1, Cdk2, Cyclin B1, p53), 1:2000 (for Cdk6, α-Tubulin) for o/n at 4°C. Then horseradish peroxidase-linked secondary antibodies were applied for 1 h in blocking solution. Finally, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL, USA) was applied to the membrane for 5 min and placed in an X-ray film cassette and developed. Anti-Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, and Cyclin E antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Cdk1, Cdk2, Cyclin B1 and p53 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- α-Tubulin antibody was purchased from Calbiochem (Darmstadt, Germany).

Estimation of Telomerase Activity Using Telomeric Repeat Amplification Protocol Assay (TRAP)

1×10^6 MSCs were collected by trypsinization and a telomerase assay was performed by using TRAP-eze Telomerase Detection Kit (Millipore, MA, USA). A gel-based assay was performed for visualization of the telomeric repeats after PCR, according to the manufacturer's protocol. Gels were stained with SYBR Green I (Sigma, MO, USA) at 1:10000 dilution in TAE and visualized with Vilber Lourmat ChemiCapture (Marne-La-Vallée Cedex, France).

Adipogenic Differentiation

An adipogenic induction medium was prepared by freshly adding 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), 100 μM indomethacin (Sigma) and 0.5 mM IBMX to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days with freshly prepared medium. After 21 days the cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Sigma).

Osteogenic Differentiation

An osteogenic induction medium was prepared by freshly adding 0.1 μM dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma) and 10 mM glycerol-2-phosphate (Sigma) to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days for freshly prepared medium. After 21 days the cells were fixed with 70% ethanol and stained with Alizarin Red S (Sigma).

Statistical Analysis

All data are expressed as means ± SD. Data were analyzed by performing a paired *t*- test using Minitab Statistical Software ® (State College, Pennsylvania, USA). A value of $P< 0.05$ was considered to be statistically significant.

Results

We first investigated the phenotypic effect of GRN163L on MSCs (Fig. 1). Untreated control MSCs (Fig. 1a) and mismatch oligonucleotide treated MSCs (Fig. 1b) showed normal phenotypes with fibroblastic morphology and spindle-shaped cells. At the same time, when MSCs were plated in the presence of single dose of 1 μM GRN163L (163L), we observed phenotypic effects as early as 3 days after the treatment, such as the cells losing their fibroblastic shapes, rounding up, and appearing to detach from the plate surface (Fig. 1c). However, when the drug was removed and the cells were given time to recover (163LR), GRN163L-treated MSCs reattached to the plate surface and regained their normal phenotype within 1 week (Fig. 1d). By using RT-PCR, we showed that all of the experimental groups were positive for mesenchymal stem cell markers (*CD29, CD71* and *CD90*) and negative for

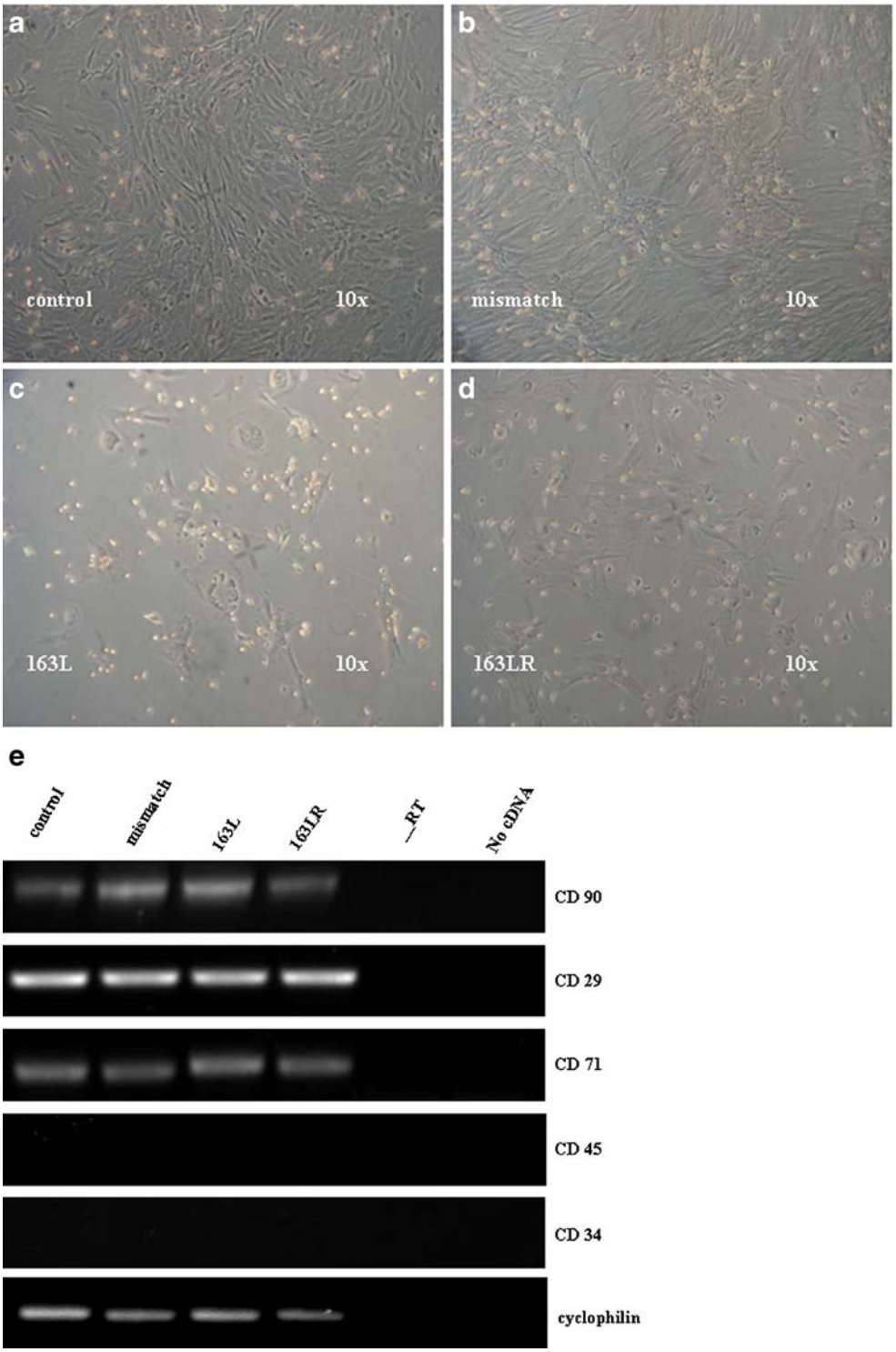

Fig. 1 The effect of GRN163L a Untreated control MSCs (*control*); **b** Mismatch oligonucleotide treated MSCs (*mismatch*); **c** 1 μM GRN163L treated MSCs (*163L*); **d** MSCs that were treated, and then left for 1 week without the ligand (*163LR*); **e** The expression of the markers of MSCs (CD90, CD29 and CD71) and hematopoietic cells (CD34, CD45)

hematopoietic cell markers (*CD34, CD45*) (Fig. 1e). We also examined telomerase activity of MSCs from control, 163L, mismatch and 163LR groups by the TRAP assay (Fig. 2). The telomerase activity of the MSCs in the 163L group was completely inhibited, whereas inhibition was not observed in the mismatched, 163LR or control MSCs. The effect of GRN163L on telomerase activity was reversible because the telomeric repeats were also observed in the 163LR group with a similar pattern as in the untreated control MSCs (Fig. 2a). No telomeric repeats were present when cell extracts were heat inactivated (Fig. 2b).

To illustrate if after the removal of the ligand whether MSCs regain their differentiation properties, we induced lipidogenic (Fig. 3) and osteogenic (Fig. 4) differentiation

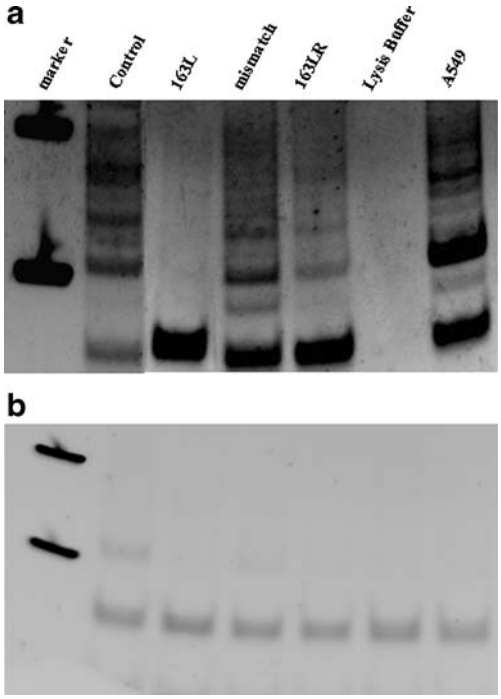

Fig. 2 TRAP assay of MSCs from control, mismatched, 163L and 163LR groups. A549 lung cancer cell line was used as a positive control to determine the telomerase inhibition by 163L. Cell extracts were prepared from **a** normal and **b** heat inactivated samples

in 163LR and compared them to that of control group. These results show that MSCs are able to differentiate into adipocytes (Fig. 3c) and osteocytes (Fig. 4c) after they recovered from the GRN163L treatment, similar to the MSCs from the control group (Figs. 3a and 4a). We did not observe differentiation of MSCs from control and 163LR groups into adipocytes or osteocytes when these cells were cultured in non-inducing media (Figs. 3b, d and 4b, d respectively).

As the MSCs cease growing in the presence of GRN163L and then regain their growth abilities when the drug was removed, the expression of cell cycle genes (*cyclin A, B, D* and *E* and their counterparts, *cdk 1, 2, 4* and *6)* were checked both at the mRNA with qRT-PCR (Fig. 5) and protein level with Western blotting (Fig. 6). In addition, *cdc25A, p16, p19, p21, p53, RB, TERT* and *Bmi-1* levels were also investigated. The expression of all the tested genes were found to decrease upon 163L treatment, compared to the control group at the mRNA level. Among those genes, the decrease was statistically significant in the expression of *cyclin D1, cdk6, cyclin E, cyclin B1, cdc25A, Bmi1, p21*and *p19* (Fig. 5; control vs 163L). Interestingly, the expression of all the genes increased in the 163LR group where the ligand was removed and the MSCs recovered. Among these genes the increase was significant in the expression of *cdc25A, p53, p21* and *p19* (Fig. 5; 163L vs 163LR). The effect of the ligand appears telomerase

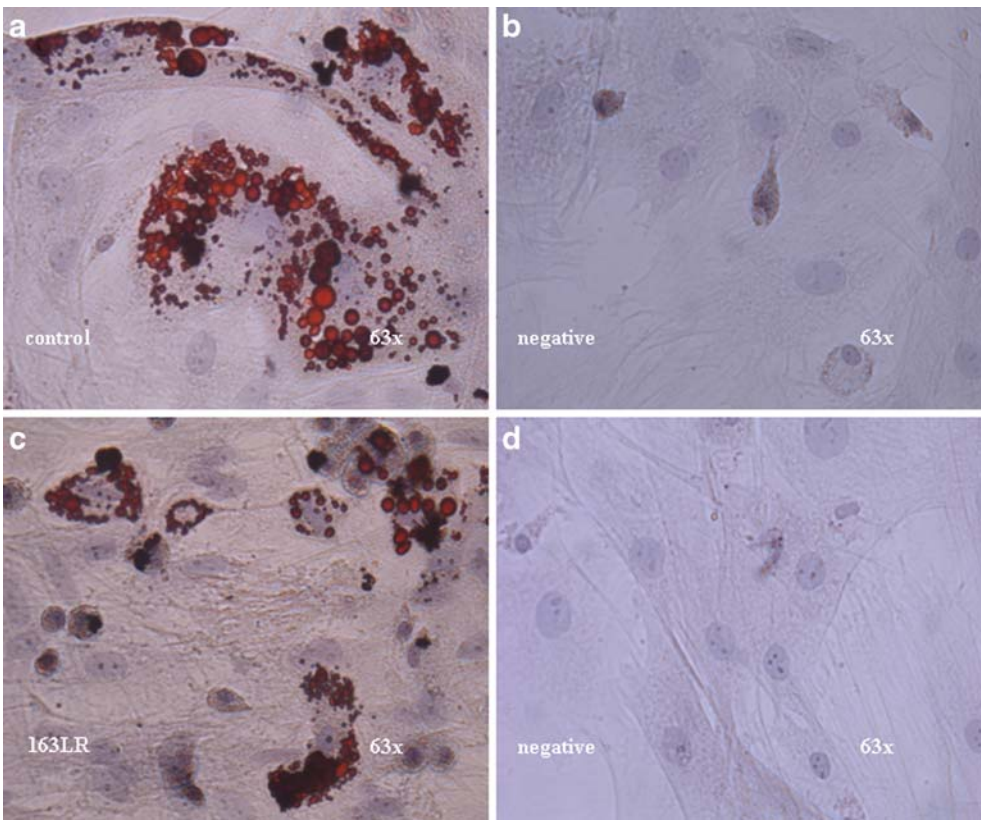

Fig. 3 Oil Red O staining to determine adipogenic differentiation of MSCs. **a** control and **c** 163LR group by using induction media. MSCs from **b** control group and **d** 163LR groups did not induce adipogenic differentiation when non-induction media was used

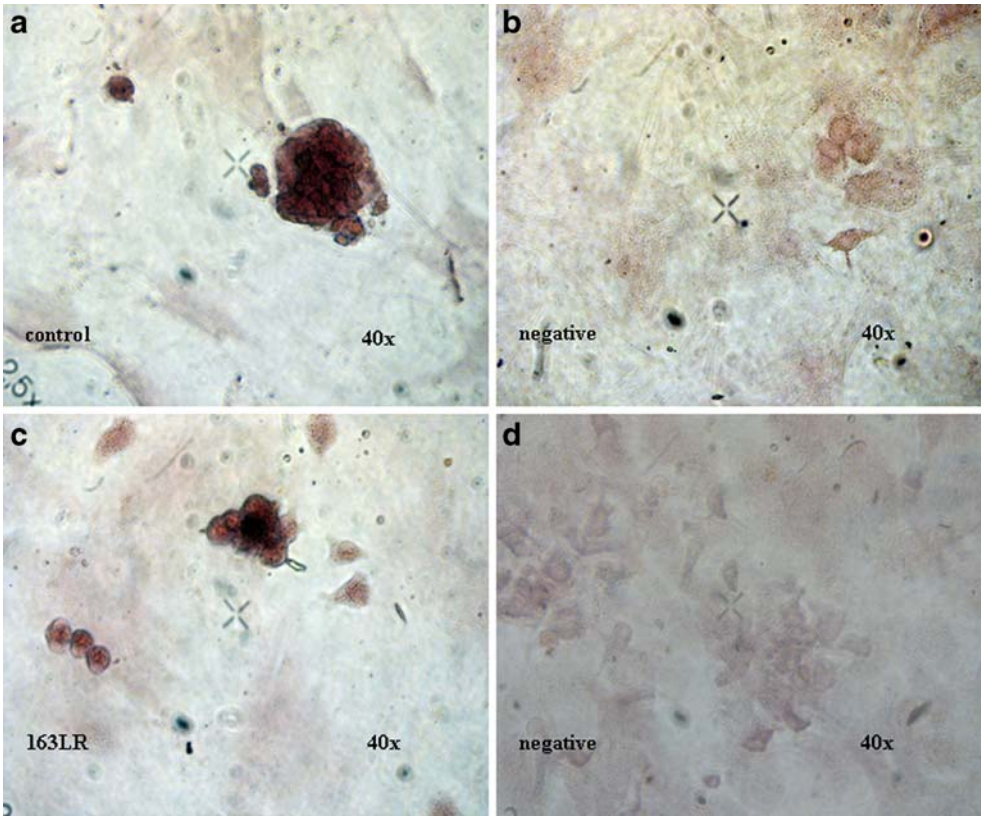

Fig. 4 Alizarin Red S staining to determine osteogenic differentiation of MSCs. **a** control and **c** 163LR group by using induction media. MSCs from **b** control group and **d** 163LR groups did not induce osteogenic differentiation when non-induction media was used

inhibition specific, since the expression of these genes in the mismatch group did not decrease (Fig. 5; 163L vs mismatch). The expression of all the genes, except *cyclin B1* and *RB*, which were higher than the control group (Fig. 5; control vs mismatch). A similar pattern of expression, that is, to decrease upon the 163L treatment and to increase 1 week after the removal of 163L was also observed in the expression of some of these genes at the protein level (Fig. 6). Our Western blot results showed that the expression of Cdk2, Cdk4, Cdk6, Cyclin D1, and Bmi-1 decreased in the 163L group compared to the control group (Fig. 6; control vs 163L) and increased 1 week after the removal of 163L (Fig. 6; 163L vs 163LR). In contrast, the expression of Cyclin E and Cdc25A were similar in control and 163L groups, however, they increased after 163L was removed (Fig. 6; 163L vs 163LR). Other proteins that were tested did not show any difference between the groups.

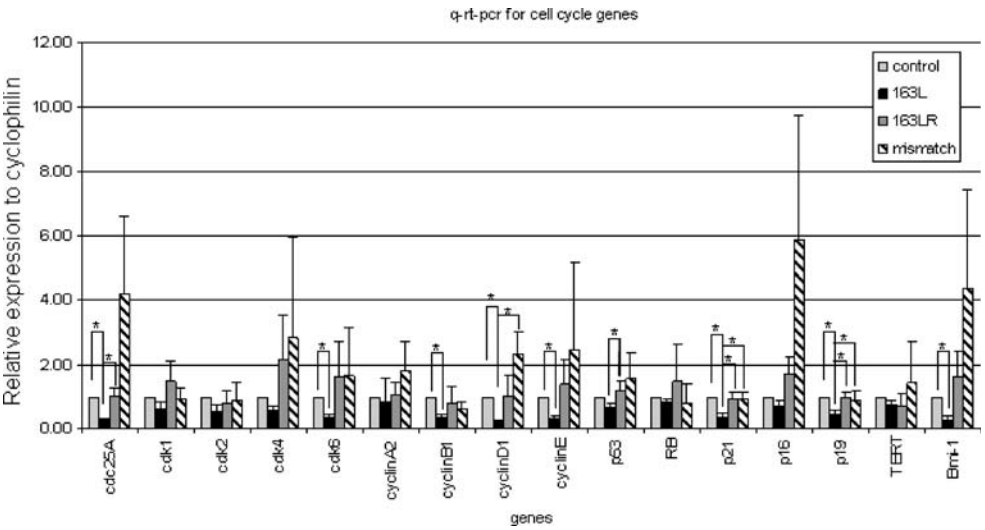

Fig. 5 mRNA expression of cell cycle genes in MSCs from control, mismatch, 163L and 163LR groups determined with qRT-PCR. * indicates $p \leq 0.05$

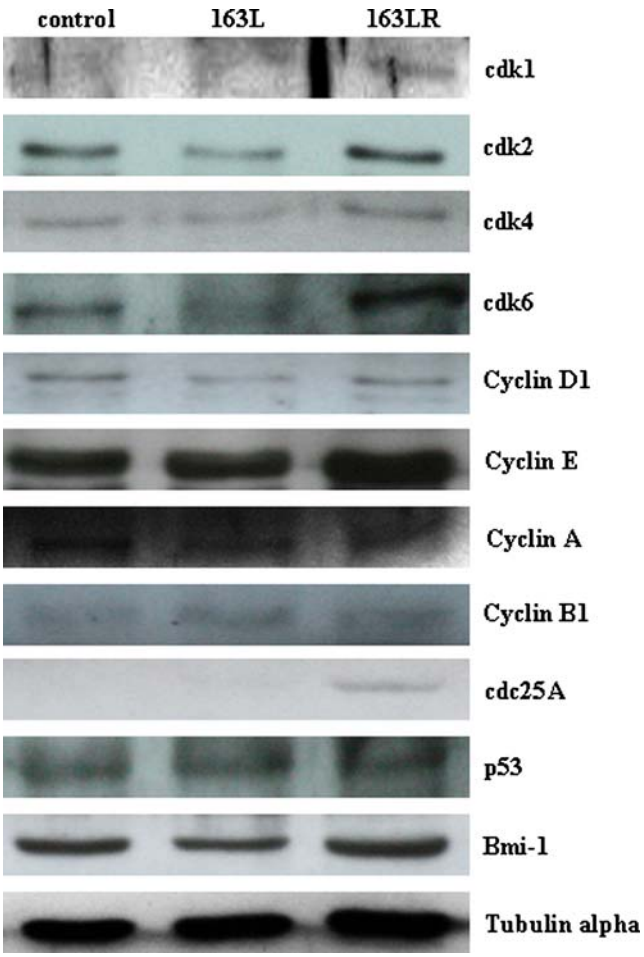

Fig. 6 Protein expression of cell cycle genes in MSCs from control, 163L and 163LR groups determined by Western blot

Discussion

The use of anti-telomerase agents as cancer therapeutic agents is still in an early stage for complete analyses of clinical outcomes, efficacy, tolerance and toxicity on normal tissue [17]. Potential effects of the inhibition of telomerase activity on stem cells are important because different stem cell compartments have different levels of telomerase activity [18]. Embryonic stem cells express high levels of telomerase activity [19] while several adult stem cell compartments, such as hematopoietic stem cells, epidermal stem cells, neural stem cells and mesenchymal stem cells also maintain telomerase activity but with low levels [9, 18, 20].

Among these stem cells, the effect of telomerase inhibitor GRN163L on mesenchymal stem cells is an issue of potential concern when applied to tumor tissue in vivo because MSCs emerge as an important player of cell-based therapies in a wide variety of pathologies, including cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, cartilage and bone injury, Crohn's disease and graft-versus-host disease (GVHD) during bone marrow transplantation [15, 21]. In vivo bone formation potential of telomerase-over expressing hMSCs was highly enhanced suggesting telomerase is required for both cell replication and differentiation [22]. Moreover, mouse MSCs with their telomerase activity knocked-down completely failed to differentiate into adipocytes or chondrocytes [23]. Thus, one could speculate that hMSCs may express at least a low level of telomerase activity to carry out the regenerative capacity as well as the differentiation potential [18]. Treatment with a telomerase inhibitor could have unwanted side effects on MSCs. While the potential side effects are unknown, they are expected to be mild because stem cell populations have longer telomeres than tumor cells, and as they divide rarely, their telomeres should shorten at a much slower rate than proliferating cancer cells [24].

The present study was undertaken to investigate the in vitro effects of GRN163L on the self-renewal and differentiation processes of MSCs. First, we evaluated the morphological changes of MSCs upon treatment of GRN163L. Previous studies have shown that rapid morphological changes occur within 24 h in cancer cells when plated in the presence of GRN163L and cells become rounded up, loosely attached to the surface, cease growing and die [25, 26]. These morphological changes were independent of telomerase inhibitory effect of GRN163L via hTR antagonism, but related to the lipidation of the compound, the nitrogen-phosphorous-sulfur–containing backbone chemistry (NPS linkage) and the presence of triple-G motif. It has also been suggested that antimetastatic effects of GRN163L may be related with the anti-cell adhesive effects of this novel cancer therapeutic agent [25]. In our study, similar to the data reported for cancer cell lines, we observed similar morphological changes when MSCs were plated in the presence of GRN163L, suggesting a similar mechanism of action of GRN163L both in MSCs and cancer cells. The cells' rounding effects were observed within 24 h after GRN163L treatment (Fig. 1c), whereas mismatch oligonucleotide-treated MSCs became tightly attached to the cell culture plate, similar to the untreated control cells (Fig. 1b and a, respectively). Moreover, "rounding effect" was not observed when GRN163L was withdrawn (163LR group) and they regained the original mesenchymal stem cell morphology as the unreated control group (Fig. 1d).

MSCs play an important role in the homeostasis of organisms therefore GRN163L would not be predicted to cause any alteration in the long term irreversible differentiation potential of MSCs. To address this issue, we treated MSCs with GRN163L, let them recover for 1 week and then evaluated their osteogenic and adipogenic differ-

entiations (Figs. 3 and 4, respectively). Our results showed that there was no noticeable difference in the potential of these MSCs to differentiate into adipocytes and osteocytes when compared with the untreated control cells. Observing of no measurable effects on the differentiation of MSCs upon GRN163L treatment is of particular clinical importance, mainly for older patients. Aging deteriorates the differentiation potential of MSCs [27, 28]; therefore to use GRN163L for anti-cancer treatment of the elderly would seem not further decrease the potential.

Several studies have examined telomerase activity in MSCs, providing somewhat differing results. As discussed in Sethe et al. 2006, some studies find no telomerase activity in MSCs, whereas others have detected telomerase activity [29]. Our results clearly showed that MSCs do have telomerase activity, and the activity is inhibited by GRN163L (Fig. 2). Importantly, telomerase activity recovers upon withdrawal of GRN163L (Fig. 2; 163LR group). The reason for this disparity could be explained by the presence of the heterogeneous group of MSCs (telomerase positive and negative) and it clearly warrants further research [30, 31]. The difference could also be related to the passage number of MSCs used. It has been recently shown that telomerase activity is present at 10 days but negative at 30 days [32]. The MSCs that were used in this study derived from the first passage and were physiologically more relevant.

As the "rounded" MSCs were not able to proliferate as much as the untreated cells, several cyclins and cdks that regulate cell cycle were analyzed to determine whether telomerase inhibition leads to cell cycle arrest. Our real-time PCR and Western blot data clearly showed that inhibiting telomerase activity with GRN163L does leads to cell cycle arrest at the G1 phase, and that importantly, these effects were reversible. Therefore, it is possible to conclude that the MSCs will regain their proliferation and self-renewal capacity following the cessation of anti-telomerase therapy. Additionally, the mismatch oligonucleotide had no effect on the proliferation status of these cells, indicating high specificity of this compound.

It is also important to note that the in vitro effects of GRN163L on MSCs may not necessarily reflect their possible in vivo effects. When the telomerase activity was knocked down, mouse MSCs failed to differentiate into adipocytes or chondrocytes [23]. On the other hand telomerase-over expressing hMSCs had been shown to have a high osteogenic differentiation potential [22]. Systemic effects of telomerase inhibition on bone marrow-derived MSCs might be different than in vitro reversible effects. Therefore, new studies are warranted to investigate the role of GRN163L on in vivo conditions.

In summary, we have investigated the potential effects of GRN163L on MSCs. Our results suggest that the inhibitory effect of GRN163L on mesenchymal stem cells is reversible in under short term in vitro cell culture conditions. We conclude that hTR antagonist GRN163L does not interfere with the self-renewal and differentiation of MSCs and can be used without apparent toxic side effects. Nevertheless, in clinical settings anti-telomerase cancer treatment should be stopped before telomere depletion in stem cells, so that telomerase activity and telomeres in reproductive and stem cells can be restored. Our study provides additional support for treating cancers by administrating GRN163L without depleting the body's stem cell pools.

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