IMMORTALIZATION OF BONE MARROW AND PLACENTAL DERIVED MESENCHYMAL STEM CELLS BY TRANSFECTION

M.Sc. Thesis

2016 AD

Submitted To

CENTRAL DEPARTMENT OF BIOTECHNOLOGY,

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for degree in Master of Science in Biotechnology

Uddhab Karki

Exam roll no: BT123-069

T.U. Regd. No.: 5-2-37-903-2008

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Uddhab Karki

ABBREVIATIONS

B2M	Beta-2-Microglobulin
BM	Bone marrow
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxy Ribonucleic Acid
CFU	Colony Forming Units
CDK	Cyclin Dependent Kinase
DAPI	4, 6-diamidino-2-phenylindole
DEPC	Di-Ethyl Pyro Carbonate
DMSO	Dimethyl Sulphoxide
DNA	Deoxy-Ribonucleic Acid
DTT	Di-Thiothreitol
EDTA	Ethylene Diamine Tetra Acetate
FACS	Fluorescent Assisted Cell Sorting
FBS	Fetal Bovine Serum
GFP	Green Fluorescence Protein
HEK cells	Human Embryonic Kidney cells
HSC	Hematopoitic Stem Cell
hTERC	Telomerase RNA Component
hTR	Human Telomerase RNA
hTERT	Human Telomerase Reverse Transcriptase
IgG	Immunoglobin G

IMDM	Iscoves Modified Dulbecco's Medium
iPSCs	Induced pleuripotent Stem Cells
mg	Miligram
µg	Microgram
mM	Milli Molar
µl	Microlitre
ml	Mililitre
MSC	Mesenchymal Stem Cell
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NTC	Non Template Control
PBS	Phosphate Buffer Saline
pM	Pico Molar
RNA	Ribonucleic Acid
rpm	Rotation Per Minute
RT-PCR	Real Time Polymerase Chain Reaction
RT	Room Temperature
SV40	Simian Virus 40
SVT	SV40 plus TERT
TBP	TATA-binding protein
TERT	Telomerase Reverse Transcriptase
UC	Umbilical Cord
UV	Ultraviolet

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Abstract

Human Mesenchymal Stem Cells are multipotent cells that can differentiate into a variety of cell types and can be isolated from different sources such as human placenta, bone marrow, umbilical cord, adipose tissue etc. Multipotent Mesenchymal Stem Cells (MSC) are widely used as seed cells in studies of tissue engineering and regenerative medicine but their clinical application is limited due to replicative senescence. To overcome vulnerability of replicative senescence and crisis of cells, immortalization of MSCs is one option. In this study, human bone marrow MSCs were isolated from marrow aspirates and placental MSC were isolated from human placenta after informed consent. The cells were grown, transfected with TERT, SV40 and SV40+TERT combination and the cells were selected by using Hygromycin B. The SV40+TERT transfected bone marrow MSCs and TERT transfected Placental MSCs formed prominent colonies in Hygromycine B antibody selection media but normal non-transfected MSCs couldn't even survive. The morphological difference analysis, population doubling time calculation in each passage was done for the selected cells. For the selected cells, comparative analysis with normal non-transfected cells was carried out using immunostaining technique, cell cycle analysis by FACS, Colonization assay, relative gene expression of TERT, hTR and p21 of early and late passage by Real Time PCR, population doubling time calculation, morphological difference etc. The SV40+TERT transfected bone marrow MSCs and TERT transfected placental MSCs showed good results as compared to SV40 transfected MSCs, GFP transfected MSCs (as a positive control) cells and normal non-transfected MSCs. The SV40+TERT transfected bone marrow MSCs were characterized as conditionally immortal which was confirmed by morphology, colony forming capacity of single cell, colony formed in Hygromycine B selection media, more DNA contents in M2 and M3 phase of cell cycle during FACS, higher relative gene expression of proliferation marker TERT (36 times more) and hTR (3 times more) than normal control and decline the expression of senescence marker p21 level in late passage during Real Time PCR and prominent expression of proliferation marker(Ki67) and decline senescence marker (p21) in immunostaining.

Key words: Transfection, Mesenchymal stem cells, immortality, Immunostaining, Flow cytometry

1. INTRODUCTION

1.1. Background

A stem cell is an undifferentiated cell of a multicellular organism that is capable of giving rise to indefinitely more cells of the same type and from which certain other kinds of cell arise by differentiation. Stem cells are the cells having the ability to differentiate along different lineages and the ability to self-renew. Stem cells are of basically three types:first, embryonic Stem Cells (ESC) which is Pluripotent stem cells isolated from the inner cell mass during the blastocyst stage of an embryo. Second adult (Somatic) Stem Cells which are undifferentiated cell found amongst differentiated cells in a tissue or organ. Its primary role is to differentiate and produce the specialized cell type of the tissue. Third, Induced Pluripotent Stem Cells (iPSCs)which are adult cells that are genetically modified to an embryonic stem cell like state by being forced to express genes and factors important for maintaining properties of ESC. Although the therapeutic potential of both ESCs and iPSCs is enormous due to their auto reproducibility and pluripotentiality, there are still some limitations to their practical use i.e. cellular regulation of teratoma formation, ethical considerations and immune concerns. Stem cells can be found in many different places in the body, including bone marrow, the brain, infant cord blood, the placenta, adipose tissue, the lungs, intestines, and even hair follicles (Kevin Au, 2015).

Present days promising bioengineering technologies are using interdisciplinary approach involving physicians, engineers and scientists in the research. It may provide novel tools for reconstructive surgery. A number of conditions such as organ failure, tissue loss, cancer and congenital structural anomalies can be treated by current clinical procedures including organ transplantation, autologous tissue transfer and the use of artificial materials. But these treatments have potential limitations including organ shortages, damage to healthy parts of the body during treatment, allergic reactions and immune rejection. So the developments in the emerging field of stem cell science, stem cell associated growth factors and regenerative medicine may allow the use of stem cells to repair tissue damage and eventually to replace organs (Mizuno H.*et.al*, 2012).

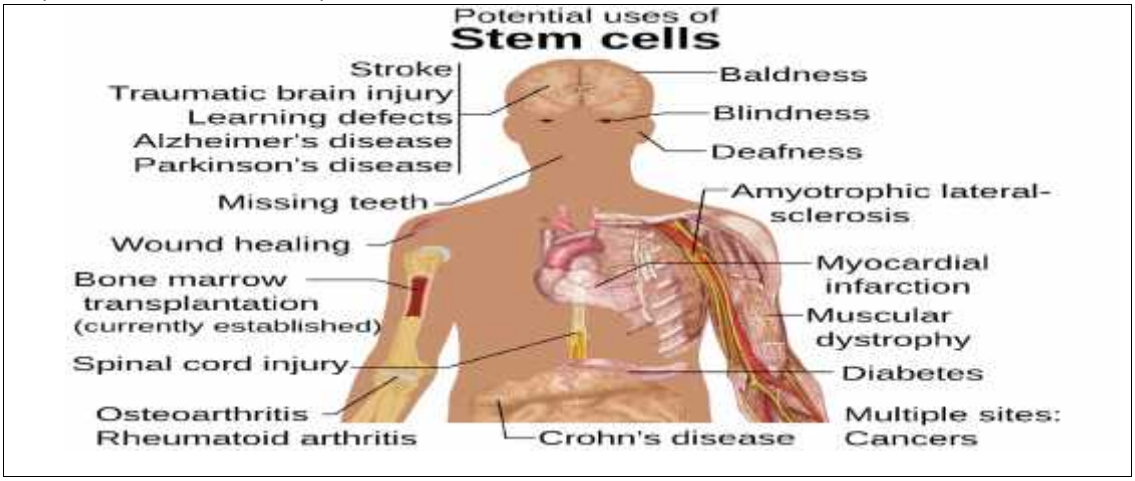


Figure 1.1: potential uses of stem cells for the human defects and disease (source: Kevin Au, 2015/atrium.lib.uoguelph.ca)

There is still some debate and ethical issues for the proper utilization and clinical application of stem cells. The scientific community, bureaucratic agencies, and universities views stem cell usage as absolutely necessary in order to further improve medical care and scientific discoveries. But certain religious groups view the usage of stem cells as immoral and forces people to choose between the prevention of suffering or the respect for human life.According to certain groups, it has been seen that the 14^{th} day after fertilization, the embryo is considered a person. This moment is when the central nervous system first appears and it is also the last chance for the embryo to split in order to form twins. Those who support the usage of stem cells argue that an embryo should not be given the status of person before this time. (Nisbet M.C, 2005)

1.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells that were initially isolated from bone marrow and noted for their ability to differentiate into bone cells (osteoblasts), cartilage cells (chondrocytes), and fat cells (Crisan M, *et al.,*2008). This property renders them interesting as a model system for studies of differentiation pathways and potentially useful for cell and gene therapy. MSCs were first characterized in bone marrow, but a wealth of studies have demonstrated the presence of uncommitted MSCs progenitor cells in the connective tissue of several organs including muscle, adipose tissue and trabecular bone(Pittenger*et.al*.,1999). Basically MSCs are defined by three major characteristics; First, MSC must be plastic adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 orCD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro (Dominici *et al.,* 2006).

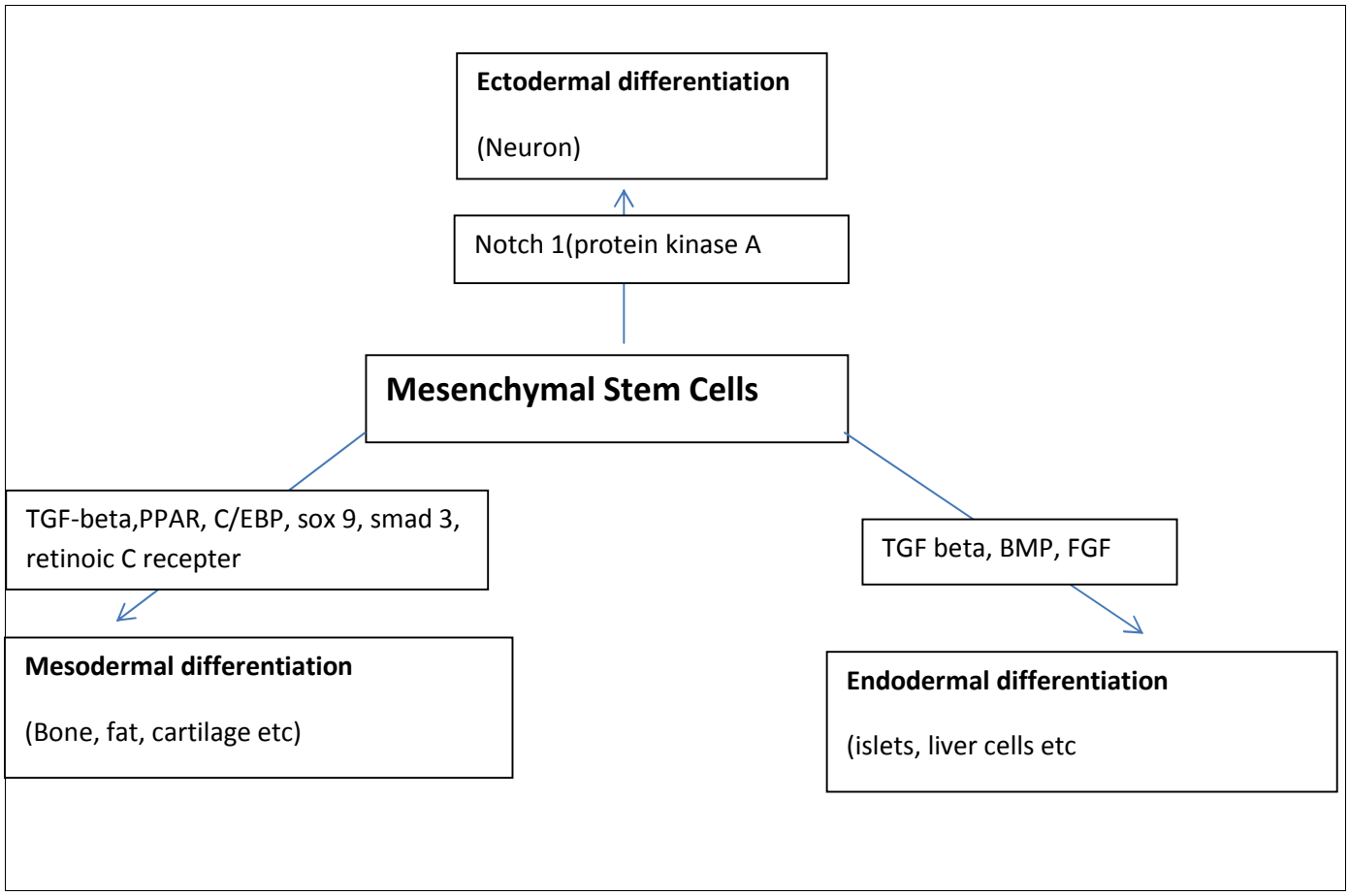


Figure 1.2: Effects of various pathways on MSCs differentiation to form various lineages (Source: Ding D.C, 2011)

MSCs can differentiate to form various lineages through different cytokines and pathways, TGF, transforming growth factor; BMP, bone morphogenetic protein; FGF, fibroblast growth factors (Ding D.C., 2011).

1.3. Immortalization and Lenti Viral Vector

Cell immortalization is the characteristic of continuous proliferation of the cells (Satija*et al*. 2007). Cell immortalization is a very complicated cellular process and the exact biological mechanisms are still largely not well understood. With years of experience in cell immortalization, scientistshave developed a comprehensive cell immortalization product line that is comprised of retroviral, lentiviral and adenoviral vectors for hTERT, p53, RB, siRNA and SV40 T antigens.

Lentiviruses are a subgroup of the retrovirus family which can integrate into the host cell genome to allow stable, long-term expression (Anson, 2004). In contrast to other retroviruses, lentiviruses are more versatile tools as they use an active nuclear import pathway to transduce

non-dividing, terminally differentiated cell populations such as neuronal and hematopoietic cells.

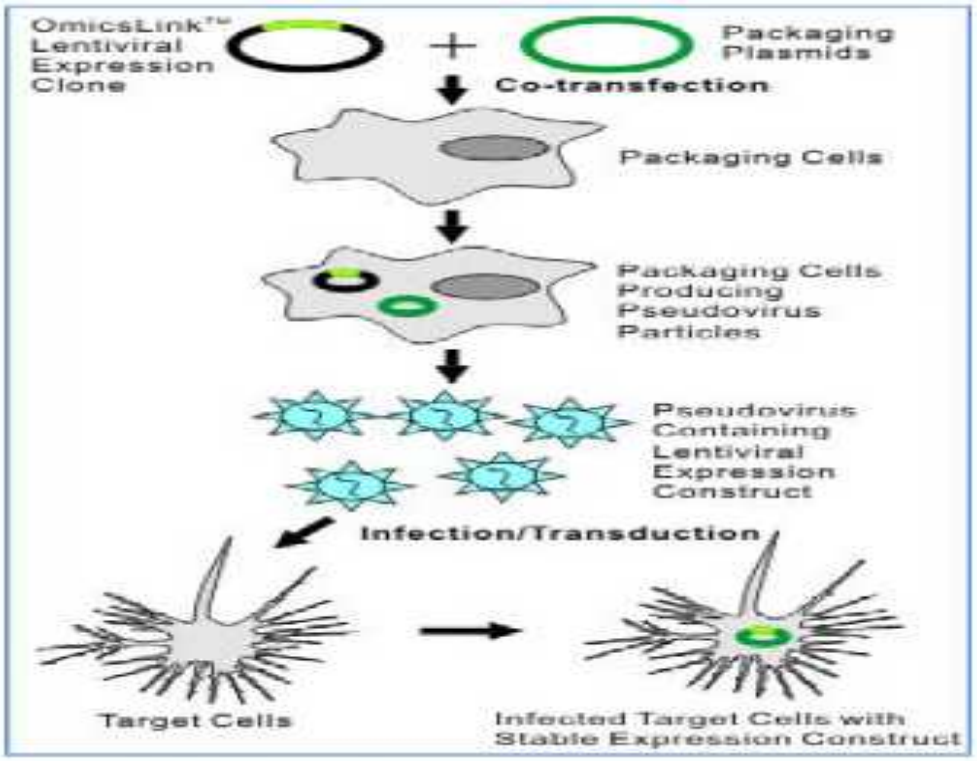


Figure1.3: Schematics of Lentivirus production and infection of target cells (source:http://www.genecopoeia.com/product/lentivirus-system/)

1.3.1. SV40 viral gene

The Simian Virus 40 large T antigen (SVLT) regulates viral genome replication and expression in the host cell upon infection. When expressed alone, the SVLT functions as a proto-oncogene capable of inducing cellular transformation through direct actions on the retinoblastoma and p53 genes (MahonJ.M., 2011).

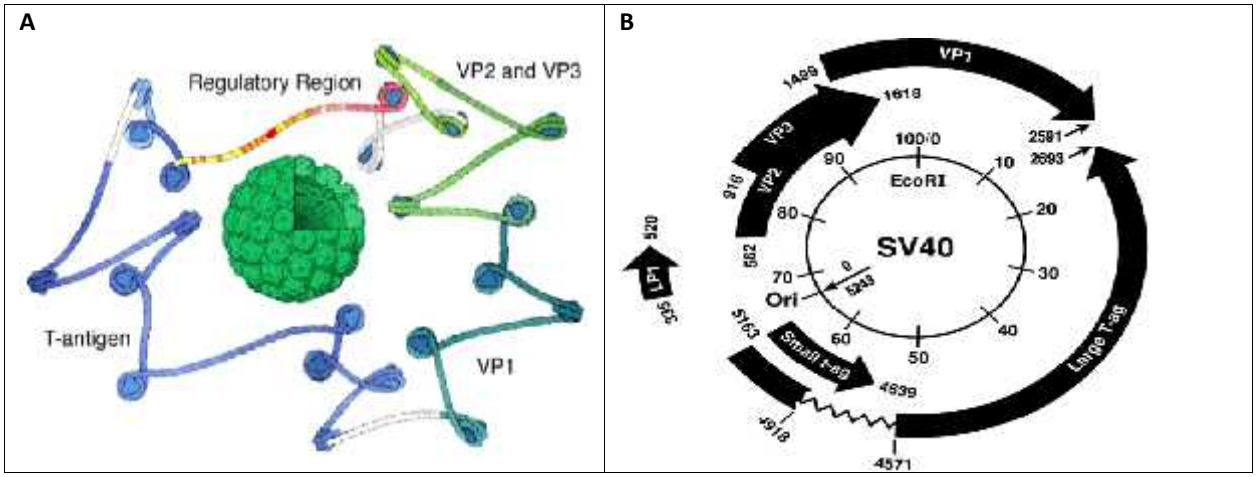


Figure 1.4: (A) SV40 viral components (Source: GoodsellD., 2003) and (B)Genetic map of SV40 with the unique EcoRI site (Source: Regis A. Vilchezl,Clinical Microbiology Reviews, July 2004, p. 495–508)

1.3.2 Human Telomerase Reverse Transcriptase (hTERT) gene

Telomerase is a ribonucleoprotein enzyme which contains two essential subunits-telomerase reverse transcriptase (hTERT) and telomerase RNA (hTR), which acts as a template for the synthesis of TTAGGG repeats by TERT and its associated proteins. It is well known that TERT is the rate limiting determinant of telomerase activity. Its activity can be regulated at various levels such as the transcriptional control and post translational modifications including phosphorylation (Yan Huang *et.al.* 2015).

1.4 Immortality and telomerase

Immortalization of human cells is often associated with reactivation of telomerase, a ribonucleoprotein enzyme that adds TTAGGG repeats onto telomeres and compensates for their shortening.Telomeres are the specialized structures found at the ends of eukaryotic chromosomes which protect the chromosome ends against degradation and fusion with other ends. Telomeric DNA is highly conserved throughout evolution and consists of a large number of tandem repeats of short G rich sequences, TTAGGG in humans and other vertebrates, slime molds and trypanosomes (Blackburn, 1991).Telomerase activity is not the only factor determining telomere length. Telomerasemediated telomere elongation and maintenance depend on telomere structure, which is regulated by epigenetic modifications at telomeres and by telomere binding proteins.Telomere binding proteins are also mediators of telomere length that may inhibit or facilitate the binding of telomerase to telomeric DNA (Marion R.M., 2010).

1.5 Research plan and design

1.5.1 Research hypothesis

Null hypothesis: When the bone marrow and placental derived Mesenchymal Stem Cell are transfected with SV40, TERT or co-transfection of SV40+TERT, the cells become immortalized.

Alternative hypothesis: When the bone marrow and placental derived Mesenchymal Stem Cells are transfected with SV40, TERT or co-transfection with SVT, the cells become elongated and die faster than normal non transfected cells.

1.5.2 Research Objectives

General objectives

To immortalize the placental and bone marrow derived Mesenchymal stem cells by SV40 and hTERT gene using viral vectors.

Specific objectives

1. Transfection of bone marrow and placental derived Mesenchymal stem cells with SV40, TERT and SV40+TERT gene and Selection of transfected cells by Hygromycine B.
2. Count the cells in every passage; calculate the population doubling time and Colonization Assay with Giemsa Staining of transfected cells with control.
3. Isolation of RNA, converting to cDNA and Real time PCR for analyzing gene expression level of the transfected cells with reference gene and control.
4. Immunostaining by using specific Ki67 antibody (proliferation marker) and P21 antibody (senescence marker).
5. Cell cycle analysis of the transfected cells by using FACS analyzer.

1.5.3 Design of research plan

For the immortalization of BM and PL derived Mesenchymal Stem Cells by using longevity genes(SV40 and TERT), the following research plan was designed:

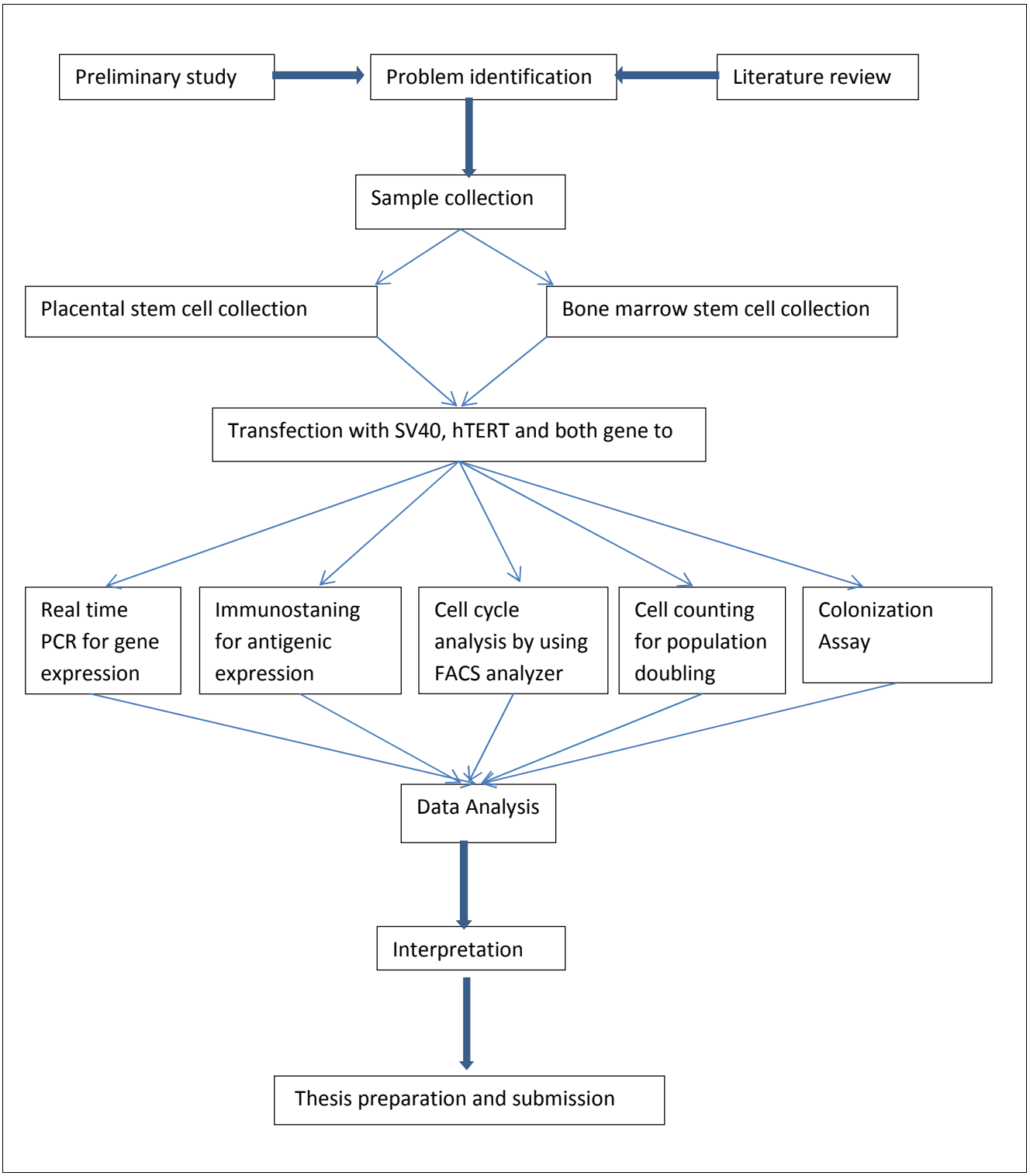


Figure 1.6: Schematics representation of design of research plan

1.6 Rationale of the study

Recent pre-clinical and clinical studies have shown that stem cell based therapies hold tremendous promise for the treatment of human disease. Numerous pre-clinical and clinical

studies suggest that Mesenchymal stem cells, also known as multipotentMesenchymal Stromal Cells (MSCs), may improve pathologic conditions involving different organs. However, MSCs have a finite capacity to replicate *in vitro* and eventually enter a state of irreversible growth arrest for about 15-20 generations and then enter into senescence phase. These cells were the important primary cells in tissue engineering and can be differentiated into many kinds of mature cells in suitable conditions *in vivo* or *in vitro*, such as cartilage, nerves, muscles, cutaneous covering, liver and bone. But due to the life period of MSCs is limited and it's hard for further research and its application. So the establishment of human normal Mesenchymal stem cell lines is the premise to implement the application.

2.1. Mesenchymal Stem Cell

Mesenchymal stem cells (MSCs) must be plastic adherent when maintained in standard culture conditions using tissue culture flasks. About 95% of MSC population must express CD105, CD73

and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 as measured by flow cytometer. MSC cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions (Dominici*et al*., 2006).

Mesenchymal stem cells (MSCs) are adult stem cells present in many tissues, such as bone marrow, adipose tissue and peripheral blood. They are able to differentiate into multiple mesodermal lineage cells, such as osteocytes, chondrocytes and adipocytes (Jiang Y*. et al*., 2002, Pittenger M.F. *et al*., 1999). However, recent studies have demonstrated that MSCs have the ability to Trans differentiate across embryonic boundaries and be induced to differentiate into non mesodermal cells such as hepatocytes, endothelial cells and neuronal cells under appropriate environmental conditions both in vitro and in vivo. There are so many studies have clearly demonstrated that MSCs can be induced to differentiate into neuronal cells and used to improve neurological function in animal models of hypoxic ischemic brain damage (HIBD) by cell transplantation therapy.

Human mesenchymal stem cells (MSC), which are first isolated from bone marrow and subsequently isolated from other tissues such as adipose tissue, cutaneous tissue, fetal hepatic and pulmonary tissue which are pluripotent progenitors for a variety of tissues including bone, cartilage, tendon, fat, and muscle (Pittinger M *et al.,1999).* It has been approved that having the capability to support expansion of hematopoietic stem cells (HSC) through expressing cytokines and reconstructing hematopoietic microenvironment (Haynesworth SE *et al, 2004*, Majumdar MK *et al*.,2000).

Preliminary investigation has revealed that MSC are not immunogenic although weakly expressing class II major histocompatibility complex (MHC-II). MSC also exhibit immune regulatory properties, which have a direct immunosuppressive effect on T cell proliferation in vitro, as demonstrated by their ability to suppress the mixed lymphocyte reaction (MLR) (Bartholomew A *et al*, Di Nicola M *et al*.)

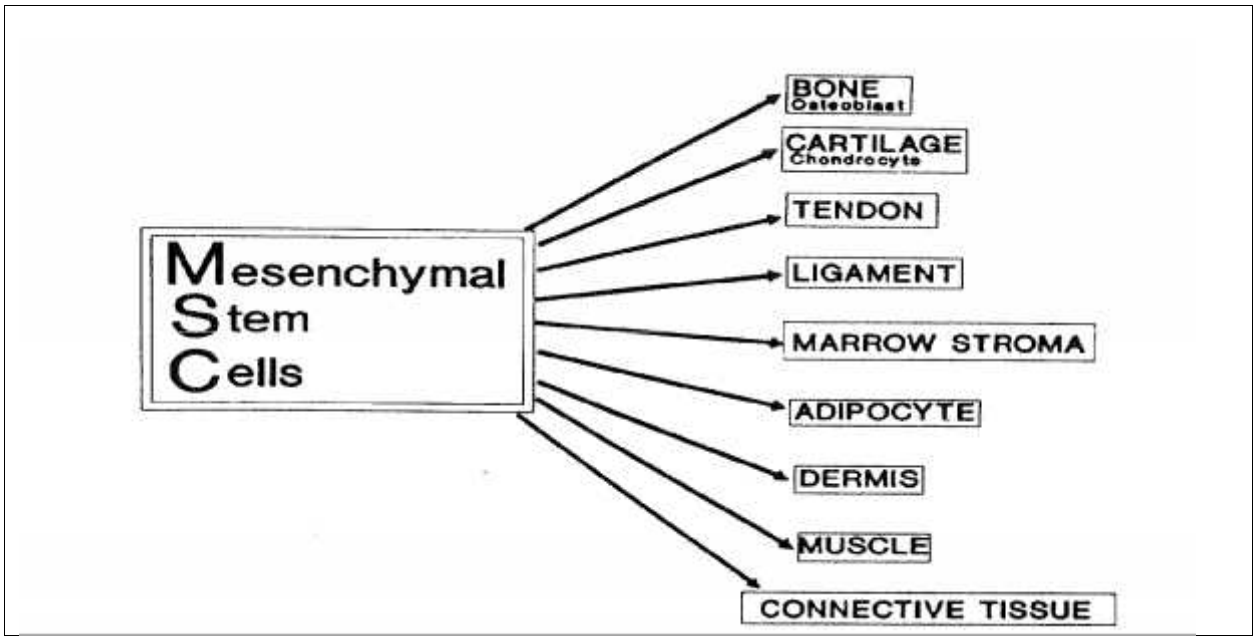


Figure 2.1: Mesenchymal stem cells, theoretically capable differentiating through a series of separate and unique lineage transitions into a variety of end stage phenotypes (Caplan A I., 1991).

2.2 Immortality and Aging of MSCs

Cell immortalization refers to the characteristic of continuous proliferation of the cells (Satija*et al*. 2007). MSCs have a finite capacity to replicate in vitro and eventually enter a state of irreversible growth arrest for about 20 generations, and then become aging. These cells are the important primary cells in tissue engineering and can be differentiated into many kinds of mature cells in suitable conditions in vivo or in vitro, such as cartilage, nerves, muscles, cutaneous covering, liver and bone (Jiang Y. *et al*., 2002). However, the life period of MSCs is limited and it's hard for further research and the application to MSCs. The establishment of human normal marrow mesenchymal stem cell lines is the premise to implement the application. In recent years, some methods have been used to induce the cells to immortalization, through virus, radioactive factors, oncogenes as well as proto-oncogene, and telomerase. Recently, hTERT and simian virus40 large T-antigen (SV40L Tag) has been used to immortalize the mammalian cells (Bodnar A.G. *et al.1998*, Kirchhoff C. *et al*., 2004).The single use of hTERT or SV40Tag is of low efficiency. It can be tried to combine the hTERT and SV40 Tag genes together to transform cells, so as to establish a reliable and lasting cell lines.

Aging has been defined as ''the sum of primary restrictions in regenerative mechanisms of multicellular organisms (Sames K.S., Stolzing S.A., 2000). Cellular senescence is a complex phenotype that entails changes in both function and replicative capacity. Because of its finite replicate capacity, MSCs will become senescent after multiple passages. The morphology and multilineage differentiation capacity of senescent MSCs are different from young MSCs. Since a tremendous number of cells are needed for a clinical application, MSCs must be expanded by

consecutive passages to provide enough cell sources. A significant increase in the fraction of flattened and multinucleated MSCs over consecutive passages seriously affects its application value and therapeutic effects for cell based regenerative medicine and tissue engineering. Simian virus 40 large T (SV40T) antigens which are derived from polyomavirus SV40 is capable of perturbing the retinoblastoma (pRB) and p53 tumor suppressor proteins and interfering with one or more specific cyclins. It causes the cells to leave G1 phase and enter into S phase, which promotes DNA replication and cell proliferation. SV40T can be used as a prototypical immortalizing gene (Westerman and Leboulch 2004). During the propagation in monolayer culture in vitro, cells show limited proliferation capacities and undergo irreversible senescence or apoptosis. Proliferation of normal cultured cells reaches a period, termed M1 stage, in which cells cease to grow. However, if cells are transfected with viruses (SV40) at M1 stage, they can continue to proliferate for a longer time. Ultimately, the cells will reach a crisis point (M2 stage), in which more cells die and chromosomes are abnormal.

Simian virus 40 (SV40), involved in virus induced tumorigenesis, consists of three structural proteins VP1, VP2, VP3 and the large and small tumor antigens. The large tumor antigen (large T antigen) is able to activate ATPase and DNA helicase, induce transcription of host cells, and up regulate relevant enzymes for DNA synthesis (Peden K.W.C. *et al.*, 1992, Pipas J.M. *et al*., *2009*). Moreover, large T antigens can induce cellular transformation with multiple targets, including the member of the retinoblastoma tumor suppressor family (pRB) and the tumor suppressor (p53) (Chen W. *et al*, 2003, Spence S.L. *et al.*, 1994). At present, SV40 Tag has been used extensively in the experiments in vitro to study the underlying mechanisms of the cell life cycle, apoptosis and immortalization.

2.2.1. hTERT Gene mediated Immortality of MSCs

In the present study, human telomerase reverse transcriptase (hTERT) was transfected into hMSCs, which could activate the activity of telomerase and delay the aging process of hMSCs. Thus, hTERT transfected hMSCs become immortalized and remain the potential of multi-directional differentiation. These cells provide basis for subsequent basic and clinical studies on biological engineering (Teng, Y. *et al*., 2010).

In several studies, exogenous virus, or oncogene has been introduced to target the cells to construct the immortalized cells (Kelekar and Cole 1987; Arimura*et al*. 2007; Wu *et al*. 2007), in which the integration of target gene was random and expression of target gene might have interfered with the intracellular physiological processes, which could result in unexpected changes such as loss of differentiation characteristic and lack of control of check point. The cells treated with virus, or oncogene belong to transformed cells but not the normal cells, and thus they are different partially, or completely from the normal cells in the transformation features

such as changes in cell morphology, karyotype and tumorigenicity, as well as loss of suspended growth and contact inhibition (Gipson *et al*. 2007).

The hTERT gene is an immortalization gene. If the exogenous hTERT could be transfected into the target cells and expressed stably, the reverse transcription activity of telomerase would increase to promote the reverse transcription of telomerase RNA. In addition, hTERT can also protect, or stabilize the telomerase RNA to prolong the half-life of telomerase RNA (Petersen and Niklason, 2007). The hTERT gene is an immortalization gene and exogenous hTERT can activate the activity of telomerase in target cells and maintain the length of telomere. So cells can pass the senescence phase (M1 phase) and crisis phase (M2 phase), leading to immortalization (Petersen and Niklason, 2007). Transient expression of the hTERT in normal diploid cells could activate the telomerase activity and prolong the cell survival (Bodnar*et al*., 1998).The hTERT has been successfully introduced to human periodontal ligament cells, osteoblasts, mammary epithelial cells and Spinal arachnoid cell line (Xiaoxue*et al*. 2004; Zhao *et al.* 2010; Hasegawa *et al*. 2010; Janson*et al*. 2011). Different from the traditional immortalization gene, the process that TERT activates telomerase to induce the immortalization is similar to the physiological process in germline stem cells. Thus, hTERT transfected cells with immortalization preserve more biological characteristics of normal cells. It has been confirmed that the transfection of exogenous hTERT not only led to cell immortalization, but had no influence on normal functions of the cell, which were critical in tissue engineering (Shay and Wright, 2000).

2.2.2. SV40 Tag mediated Immortality of MSCs

SV40 large T antigen (Simian Vacuolating Virus 40 TAg) is a hexamer protein that is a proto-oncogene derived from the polyomavirus SV40 which is capable of transforming a variety of cell types. The transforming activity of T-Ag is due in large part to its perturbation of the retinoblastoma (pRB) and p53 tumor suppressor proteins. In addition, T Ag binds to several other cellular factors, including the transcriptional co-activators p300 and CBP, which may contribute to its transformation function.SV40 DNA replication is initiated by binding of large T-antigen to the origin region of the genome (WANG J. *et al*, 2009). The function of T-antigen is controlled by phosphorylation, which attenuates the binding to the SV40 origin. Protein-protein interactions between T-antigen and DNA polymerase-alpha directly stimulate replication of the virus genome.After entering into the cell, the viral genes are transcribed by host cell RNA polymerase II to produce early mRNAs. Because of the relative simplicity of the genome, polyomaviruses are heavily dependent on the cell for transcription and genome replication. The cis-acting regulatory element surrounding the origin of replication directs transcription, and T-antigen directs transcription and replication.

Human diploid cells have a limited life span, ending in replicative senescence, in contrast to cell lines derived from tumors, which show an indefinite life span and are immortal, suggesting that replicative senescence is a tumor suppression mechanism. We have utilized introduction of SV40 sequences to develop matched sets of non-immortal and immortal cell lines to help dissect the mechanism of immortalization and have found that it has multiple facets, involving both SV40 dependent and independent aspects. These studies have led to the identification of a novel growth suppressor gene (SEN6) as well as providing a model system for the study of cellular aging, apoptosis, and telomere stabilization among other things. It is anticipated that SV40 transformed cells will continue to provide a very useful experimental system leading to insights into the behavior of cells with altered expression of oncogenes and growth suppressor gene products.

2.2.3. Immortality of MSCs by Co-Expression of SV40 and hTERT

The enzyme telomerase is expressed in most cancer cells and immortalized tumors cell lines (Kim *et al*., 1994). In contrast, normal human somatic cells do not express telomerase and have a tightly regulated replicative lifespan. These observations led to the proposal that telomerase activity is responsible for the immortal phenotype of cancer cells. Telomerase is a ribonuclear protein complex that has a reverse transcriptase (hTERT) as a catalytic domain and an RNA component that functions as a template for transcription. Telomerase synthesizes TTAGGG DNA repeats at chromosomal termini, which are referred to as telomeres (Moyzis*et al*., 1988). In the absence of telomerase activity, telomeres in normal somatic cells shorten with each cell division (Harley *et al*., 1990). When telomeres become critically short, normal cells undergo an irreversible growth arrest, referred to as senescence.

Over expression of hTERT reconstitutes telomerase activity in vivo, elongates telomeres and thereby enables primary human fibroblasts and retinal epithelial cells to proliferate beyond senescence (Bodnar*et al*., 1998; MacKenzie*et al*., 2000). Transformation with viral oncogenes, such as SV40 T antigen (SV40T) also enables primary human cells to proliferate beyond senescence (Girardi*et al*., 1965). Expression of SV40 Large T antigen inactivates p53 and RB pathways and thereby enables telomerase negative cells to bypass senescence and proliferate for an additional 20-40 population doublings (Shay *et al*., 1991). However, cells that bypass senescence in this way are still subject to telomeric shortening, continue to age, accumulate chromosomal abnormalities and eventually succumb at a second mortality check point, referred to as crisis (Counter *et al*., 1992; Girardi*et al*., 1965). As a very rare event, a clone may escape crisis and proliferate indefinitely. Recent studies have demonstrated that induction of telomerase is critical at crisis cells that spontaneously escaped crisis were shown to express telomerase (Klingelhutz*et al*., 1994) and ectopic expression of hTERT overcome crisis and immortalized virally transformed fibroblasts and epithelial cells (Counter *et al*., 1998; Halvorsen*et al*., 1999; Zhu *et al*., 1999).

2.3. Lentiviral Vector

Lenti viruses are a subgroup of retroviruses that can infect non dividing cells owing to the karyophilic properties of their pre integration complex, which allow for its active import through the nucleopore. They can be used for non-dividing or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, and muscle and liver cells. HIV is a very effective lentiviral vector because it has evolved to infect and express its genes in human helper T cells and other macrophages (Amado and Chen, 1999). Lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration and long term expression of transgenes into non mitotic cells both in vitro and in vivo (Naldini*et al*., 1996; Naldini*et al*., 1996; Blomer*et al*., 1997). HIV based vectors can efficiently transduce human CD34+ hematopoietic cells in the absence of cytokine stimulation (Akkina*et al*., 1996; Sutton *et al*., 1998).

The vector itself is the only genetic material transferred to the target cells. It typically comprises the transgene cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription, nuclear import and integration. Lentiviral vectors have been made that are "self-inactivating" in that they lose the transcriptional capacity of the viral long terminal repeat (LTR) once transferred to target cells (Zufferey*et al*. 1998). This modification further reduces the risk of emergence of replication competent recombinants (RCR) and avoids problems linked to promoter interference.

In the retroviral genome, a single RNA molecule that also contains all the necessary cis-acting elements carries all the coding sequences. Vector production system is best achieved by distributing the sequences encoding its various components over as many independent units as possible, to maximize the number of crossovers that would be required to re-create an RCR. Lentivector particles are generated by co-expressing the virion packaging elements and the vector genome in host producer cells, e.g. 293 human embryonic kidney cells. HIV-1-based vectors, the core and enzymatic components of the virion come from HIV-1, while the envelope protein is derived from a heterologous virus, most often VSV due to the high stability and broad tropism of its G protein. The genomic complexity of HIV, where a whole set of genes encodes virulence factors essential for pathogenesis but dispensable for transferring the virus genetic cargo, substantially aids the development of clinically acceptable vector systems. Multiply attenuated packaging systems typically comprise only three of the nine genes of HIV-1: gag, encoding the virion main structural proteins, pol, responsible for the retrovirus specific enzymes, and rev, which encodes a post transcriptional regulator necessary for efficient gag and pol expression (Dull *et al*., 1998). From such an extensively deleted packaging system, the parental virus cannot be reconstituted, since some 60% of its genome has been completely eliminated.

HIV type 1 (HIV-1) based vector particles may be generated by co expressing the virion packaging elements and the vector genome in a so called producer cell, e.g. 293T human embryonic kidney cells. These cells may be transiently transfected with a number of plasmids. Typically from three to four plasmids are employed, but the number may be greater depending upon the degree to which the lentiviral components are broken up into separate units. Generally, one plasmid encodes the core and enzymatic components of the virion, derived from HIV-1. This plasmid is termed the packaging plasmid. Because of its high stability and broad tropism, the next plasmid encodes the envelope protein most commonly the G protein of vesicular stomatitis virus (VSV G) termed the envelope expression plasmid and another plasmid encodes the genome to be transferred to the target cell for the vector itself and is called the transfer vector

2.4. Telomere, Telomerase and Immortality

2.4.1. Telomere

Telomere, a complex of guanine rich repeat sequences and associated proteins, caps and protects every eukaryotic chromosome end against chromosomal fusion, recombination and terminal DNA degradation (Blackburn, 2001). Telomeric DNA consists of short guanine rich repeat sequences in all eukaryotes with linear chromosomes and its length in human somatic cells is remarkably heterogeneous among individuals ranging from 5 to 20 kb, according to age, organ, and the proliferative history of each cell (Wright and Shay, 2005).During a process of DNA synthesis and cell division, telomeres shorten as a result of the incomplete replication of linear chromosomes called 'end replication problem'. This progressive telomere shortening is one of the molecular mechanisms underlying ageing. As critically short telomeres trigger chromosome senescence and loss of cell viability (Collins and Mitchell, 2002; Blasco, 2005; Wright and Shay, 2005). To prevent degradation by exonucleases or processing as damaged DNA, the telomere 30 single strand overhang folds back into the D loop of duplex telomeric DNA to form a protective 'T-loop', which is reinforced with TRF2 and other telomeric DNA-binding proteins named Shelterin (de Lange, 2005).

Inside the nucleus of virtually all of human cells are 46 chromosomes, the thread like packages that carry the genes. At the tips of these chromosomes, like the hard ends of shoelaces, are structures called telomeres. While they do not contain genes, telomeres are important for replication or duplication of the chromosomes during cell division. They are made up of approximately 1,000 to 2,500 copies of a repeated DNA sequence, TTAGGG. During the time of birth don't have every cell the bodies will ever need. As it grows, it needs new skin, bone, blood, and many other kinds of cells. Even as adults, it needs to make new cells i.e. skin cells and those cells that line our intestines are constantly replaced. All of these reproducing cells need their telomeres for cell division. Without their telomeres, cells would be unable to reproduce at all.

Telomeres also play an important protective role in our cells. Their presence prevents important genetic material from being lost during cell division. They also serve as a "cap" on the ends of chromosomes, protecting chromosome ends from appearing broken. This is an important function, because broken chromosomes trigger unwanted biological responses. The human reverse transcriptase, telomerase, has been studied extensively for the last 20 years for its vital role in aging, stem cells and cancer (Daniali L. *et al*., 2013).

Telomerase is a ribonucleoprotein complex that consists of two core components: a protein component (telomerase reverse transcriptase, hTERT), and a template RNA component (hTERC) that act together to add hexameric 5'TTAGGG repeats to the ends of linear chromosomes. The maintenance of linear chromosome ends is important for cellular survival. The ends of linear chromosomes are similar to deoxyribonucleic acid (DNA) double strand breaks and thus these ends must be masked otherwise they would form end to end fusions and chromosome bridge breakage cycles. The telomere reaches critically shortened and uncapped length due to the end replication problems (Zou Y *et al*., 2004). Due to that DNA damage signal has been produced and senescence will be induced. Telomerase can delay or prevent telomere length induced senescence. The enzymatic activity of telomerase has been widely studied in normal proliferating stem cells and in cancer cells (Ludlow A.T. *et al*., 2014**)**.

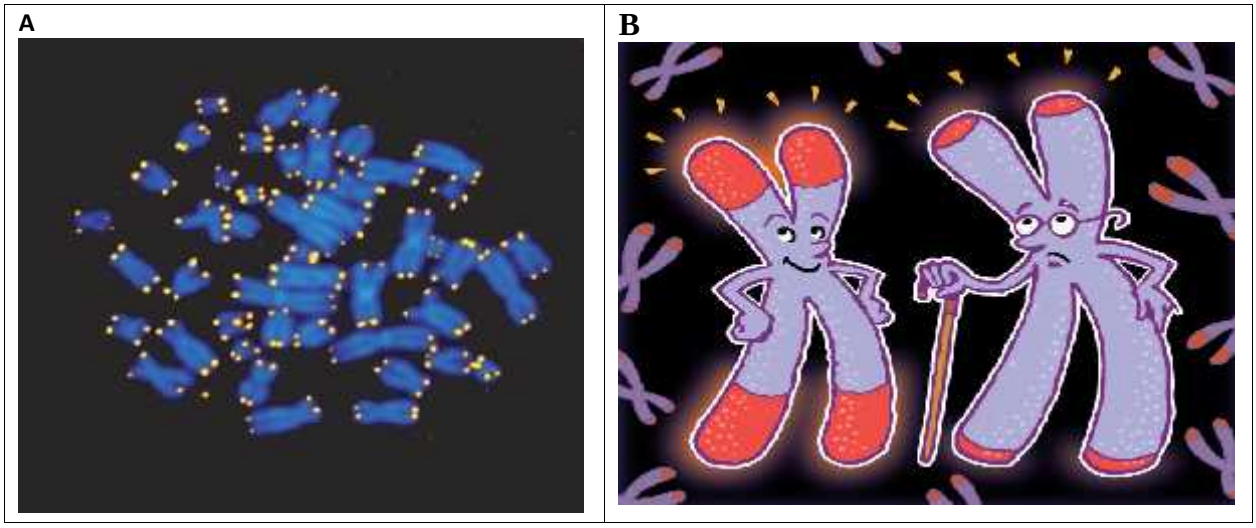


Figure 2.2: (A) Telomeres are shown in yellow, whereas the DNA of chromosomes, counterstained with DAPI, is shown in blue. (B) Representation of telomere length on young and old cell (source: Walter Sorochan, 2014.)

Telomeres are composed of double strands of deoxyribonucleic acid (DNA), except for the very ends called telomere overhangs, which have single strands. Many telomeres, including those from humans, appear to form t-loops special folded structures where the single stranded tail of the telomere is tucked into the more internal double stranded part. T-loops are thought to be important for the protective capping function of telomeres. Research published in the January

21, 2003, issue of the Proceedings of the National Academy of Sciences has been suggested that the end of a cell's reproductive life may actually be triggered when this loop unravels, either due to DNA damage or to telomeres that have become excessively short. Telomeres tend to get shorter over time. Two researchers, Alexei Olovnikov and James D. Watson, independently recognized that DNA replication machinery cannot copy chromosome ends completely. Watson named this the "end replication problem." Each time a normal cell divides, the ends don't get completely copied, and the telomeres become just a bit shorter. Eventually, telomeres are so short that the chromosome reaches a critical length, and no further cell division can occur. This cellular aging phenomenon is known as replicative senescence or the Halyflick limit (Hayflick L., 1968)

1.4.2. Telomerase and Immortality

Telomerase is a complex of a reverse transcriptase protein encoded by the TERT (telomerase reverse transcriptase) gene and a template RNA TERC (telomerase RNA component). Telomerase can add telomeric repeats onto the chromosome ends, and prevents the replication dependent loss of telomere and cellular senescence in highly proliferative cells of the germ line and in the majority of cancers (Blasco, 2005). Thus, telomerase activity and telomere maintenance are associated with the immortality of cancer cells, germ-line cells, and embryonic stem (ES) cells.

In most human somatic cells except for stem cells and lymphocytes, telomerase activity is diminished after birth so that telomere length shortens with each cell division. A critical length of telomere repeats is required to ensure proper telomere function and avoid the activation of DNA damage pathways that result in replicative senescence or cell death. As stem cells have elongated proliferative capacity, they should have a mechanism that maintains telomere length through many cell divisions. In fact, low levels of telomerase activity have been found in human adult stem cells including haematopoietic and non haematopoietic stem cells such as neuronal, skin, intestinal crypt, mammary epithelial, pancreas, adrenal cortex, kidney, and mesenchymal stem cells (MSCs). The telomere and telomerase status in stem cell populations is different between humans and mice (Harrington, 2004). The level of telomerase is low in the majority of human stem cells, whereas it is up regulated in cells that undergo rapid expansion, such as committed haematopoietic progenitor cells, activated lymphocytes, or keratinocytes, even within tissues with a low cell turnover such as the brain (Haik*et al.*, 2000).

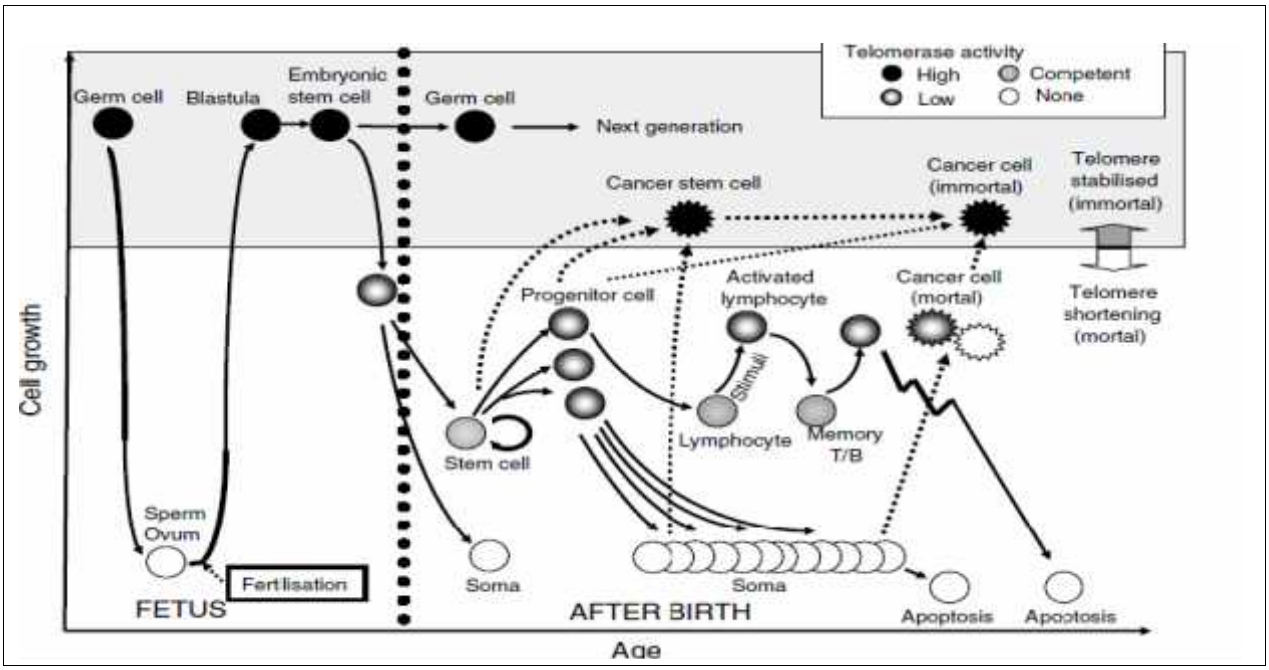


Figure 2.3: Telomere and telomerase dynamics in human stem cells. Germ cells have high levels of telomerase activity during rapid proliferation (Source: E Hiyama,British Journal of Cancer, 2007)

2.4.3. Telomerase and stem cells

Telomerase activity and telomere maintenance have been associated with immortality in tumor and embryonic stem cells. Whereas most normal somatic cells are telomerase negative, low levels of this enzyme have been found in adult stem cells from the skin, gut and the hematopoietic system (S. Zimmermann *et al,* 2004). It has been established that the proliferative capacity of human cells is causally linked to the functional maintenance of their telomeres, which constitute the final ends of chromosomes. Telomeres consist of TTAGGG repeats and associated proteins in human cells (Collins K *et al*.,2002). In most normal somatic cells, telomere sequences are lost during replication in vitro and in vivo, limiting their proliferative capacity (Harley CB *et al.*, 1990). In contrast, immortal cells such as tumor and embryonic stem cells are capable of maintaining telomere function, in general, by activation of the reverse transcriptase telomerase (Kim NW *et al*., 1994). Unlike most normal somatic cells that are telomerase negative, low to moderate levels of the enzyme have been described in adult stem cells from skin, gut, and from the hematopoietic system (Bachor H.C.,1998). The telomerase activity in hematopoietic cells has been associated with a capacity for self-renewal (Zimmermann S. *et al.,* 2003).

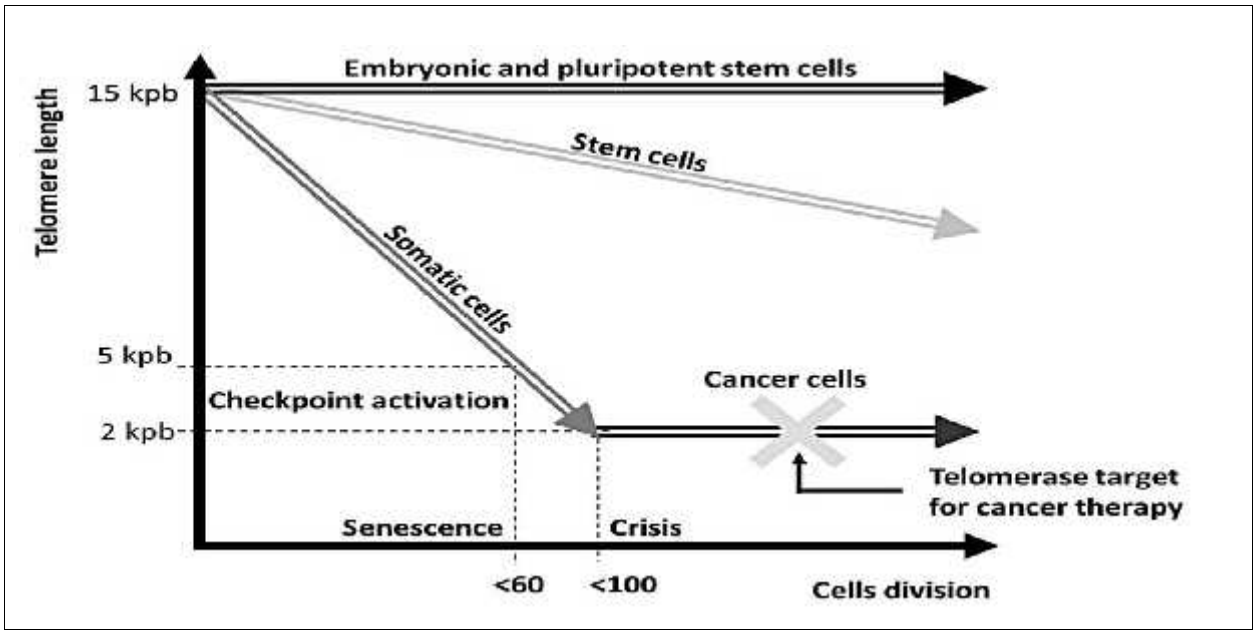


Figure 2.4: Telomere lengths vs. cell divisions of different types of cell (Source: Diego L., international journal of oncology, 2013)

The cells that have a constitutive telomerase activity (embryonic and pluripotent stem cells) may completely maintain telomere lengths. Stem cells transiently express telomerase or express moderate amounts of telomerase maintaining partially telomeric length, with increased age and cell divisions, telomeres continue to shorten in these cells. Normal somatic cells that do not express telomerase have their telomeres shorten with each cell division. Cancer cells which reactivate or up regulate telomerase fully maintain telomeres but generally at reduced lengths. This feature has made telomerase a promising target for cancer therapy (Diego L. Mengual Gómez, 2013).

The activation of telomerase, the ribonucleoprotein responsible for extending telomere length at the end of chromosomes, can prevent telomere erosion and inhibit replicative senescence in vitro. It seems to be expressed by highly proliferating germ line cells, hematopoeitic stem cells, and various types of cancer cells (Broccoli, D. *et al*. 2004). Telomerase contains an RNA component, which provides the template for the synthesis of TTAGGG repeats, and protein components including the catalytic subunit hTERT, which provide the reverse transcriptase activity. It has been suggested that telomere associated cellular senescence may contribute to various age related disorders (Rudolph, K.L. *et al.* 2006). Recent studies reported that the introduction of hTERT into osteoblasts isolated from human trabeculae induced telomerase activity and extended the life span of these cells (Yudoh, K., Matsuno H., *et al*. 2005). However, the role of telomerase in bone formation, particularly with respect to maintenance of the osteogenic precursor cell population, is largely unknown.

One of the best known cell intrinsic events associated with aging is the progressive shortening of telomeres, the natural ends of chromosomes. The speed at which telomeres shorten with aging seems to vary between men and women and can be influenced by factors considered to accelerate aging and to be a risk of premature death, such as perceived stress, smoking and obesity, all of which have been proposed to negatively impact on telomere length (Klatt P. *et al*., 2006, Valdes A.M. *et al.,* 2003)

3.1. Sample collection and processing

Mesenchymal stem cells were isolated from bone marrow and placenta of human (the details of isolation procedure is in 3.1.1 and 3.1.2). For obtaining pure population of MSCs, these samples were processed. During the processing, sample was washed with PBS.PBS acts as a physiological buffer which maintains pH and osmotic balance with providing water and essential ions. So it gave the artificial environment to the cells. After that these cells were treated with enzymes to disrupt the cell-cell and cell-matrix interactions. The enzymes i.e. trypsin, DNase, dispase and collagenase were used which played a role in disruption of the peptide bonds presents between the cells and matrices.

3.1.1. Collection and processing of placental MSC

Placenta was collected in aseptic conditions from hospitals after delivery, with informed consent of concerned donors and their family. The collected placenta was cut into small tissue pieces from in and around the umbilical cord area and well minced using scalpel. To the minced tissue PBS was added along with the enzyme trypsin (0.1%), DNase + Dispase (1 mg/ml). The tissue pieces with enzymes mixture were mixed continuously using a magnetic stirrer for 40-45 minutes at 37˚C. After that the mixture was filtered into separate 50 ml falcon tubes and layered over a layer of FicollHypaque. Then the tubes were centrifuged at 600g for 20 minutes at 4˚C. Due to gradient centrifugation caused the formation of different layers, among the layers the buffy coat containing lymphocytes was isolated into fresh tubes. Again the buffy coat was suspended in PBS and centrifuge at 400g for 10 minutes. After that the pellet was collected and re-suspended in 1.5ml of PBS. Finally from which cells were used as initial seeding of culture plate (100µL of sample) in IMDM media.

This FicollHypaque method was used for placental sample processing which enriched the stem cell contained in MNC subpopulation found predominantly in the buffy coat obtained by gradient centrifugation.

3.1.1.1. Reagents and Instruments used

The reagents and instruments used for the collection and processing for the placental MSCs are as follow:

a. Phosphate buffer saline solution
b. Enzymes (trypsin, DNase, Dispase, collagenase Type 1)
c. FicollHypaque
d. Falcon tubes (15 ml and 50 ml)
e. Pipettes (5ml and 10 ml)
f. Syringes(10ml)
g. Sterilized and autoclaved forceps, scalpels and filters
h. Magnetic beads and stirrer

i. CO2 incubator at 37˚C

3.1.2. Collection and processing of Bone Marrow MSC

Bone marrow was collected in aseptic condition from femur bone of dead fetus obtained from hospital after the informed consent. After that the bone marrow tissue was flushed using PBS at sterilized laminar hood. The flushed out contents were plated into appropriate dishes with IMDM media.

3.2. Media Preparation for Cell Culture

3.2.1. Materials and Reagents for media preparation

The reagents and instruments used for the media preparation are as follow:

a. Iscoves Modified Dulbecco's Media (IMDM) from Invitrogen.
b. Fetal bovine serum from Gibco
c. 2-Mercaptoethanol
d. Hydrocortisone
e. Antibiotics
f. Milli Q water
g. Sterile filter and syringes

3.2.2. Iscove's Modified Dulbecco's Media(IMDM)

Iscove's Modified Dulbecco's Media is a modification of Dulbecco's Modified Eagles Medium (DMEM) containing selenium, sodium pyruvates, HEPES buffer, additional amino acids and vitamins and potassium nitrate instead of ferric nitrate. This media would support murine B lymphocytes, hemopoietic tissue from bone marrow, T lymphocytes and different types of hybrid cells. IMDM is highly enriched so it is useful for rapidly proliferating, high density cell cultures.

3.2.3. MSC Fetal Bovine Serum(FBS)

Fetal bovine serum is the mostly used serum supplements for the in vitro cell culture of eukaryotic cells which are collected by removing of RBC using centrifugation of bovine blood coagulants. FBS provides so many growth factors to the cells which help to survive grow and divide rapidly. MSC FBS was obtained from Gibco.

3.2.4. Mercaptoethanol

2- Mercaptoethanol is hybrid of ethylene glycol which is used to reduce disulphide bonds. It is mainly used in cell culture to prevent oxygen radical's toxicity because it is potential reducing agents. It acts as biological antioxidants.

3.2.5. Hydrocortisone

Hydrocortisone is steroid hormone which acts as suppressor of immune system and growth of macrophages in cultured cell.

3.2.6. Antibiotics

During the Mesenchymal Stem Cell culture, combination of penicillin and streptomycin were used in cell culture to destroy the growth of bacteria. Penicillin acted by inhibiting in cell wall synthesis and crosslinking of peptidoglycan strands of bacterial cell. Streptomycin acted by binding to 30S subunit of bacterial 70S ribosome.

3.2.7. Preparation of stock solution

3.2.7.1.Iscoves Modified Dulbecco's Medium (IMDM)

For the preparation of 1X stock of IMDM, 17.67 grams of media was weighed with additional 3.024 grams of NaHCO3. Then the volume was adjusted up to 850 ml using milli Q water it is because in normal working IMDM solution contains 15 % of serum. Then it was filtered and stored in refrigerator.

3.2.7.2. Hydrocortisone

For the preparation of 20 ml of 0.1 M Hydrocortisone stock, 0.72 grams of Hydrocortisone was weighed and dissolved in 1 ml DMSO then the volume was adjusted up to 20 ml by adding alcohol.

3.2.7.3. Antibiotics

For the preparation of 100 ml antibiotics solution 0.6 grams of penicillin and 0.5 grams of streptomycin were weighed and dissolved in 100ml of 1X PBS and filtered it and stored in refrigerator.

3.2.8. Preparation of working media

To prepare the IMDM working solution of 100 ml, 84 ml(84%) of media(IMDM), 15ml (15%)of FBS and 1ml(1%) Antibiotics were mixed. Then to this solution 0.1% of Hydrocortisone and 0.1% of 2- Mercaptoethanol were also added. After that it was filtered and used.

3.3. Passaging of cells

When the adherent MSC cells in culture dishes reached up to 85% confluence, the disc was sub cultured for reduction of cell density. IMDM of the culture disc was aspirated out and washed with PBS. Then 0.1% Trypsin EDTA was added (1 ml for 90 mm disc) and kept for 5-7 minutes into CO2 incubator for enzymatic action. Then the disc was checked for complete detachment from the surface. After that the enzyme was inactivated by adding 2 ml of media and the clumps were broken. Then the cells were counted and plated 75000 cells in 90 mm disc by adding fresh IMDM working media. The remaining cells were cryopreserved by using Recovery

Media. During the passaging of cells the population doubling was estimated by using following formula:

Population doubling (P.D) = (log N – log n)/ log 2

Where, N= No of cells harvested

n= No of cells seeded

Population doubling time= P.D/ Number of days

3.4. Transfection

3.4.1 Preparation of Recombinant Lentivirus

The recombinant lentivirus was prepared (description not included due patent of CCMB) and given for the transduction to the cells.

3.4.2 Transduction by Recombinant Lentivirus

The bone marrow and placental (zero passage) cells were plated and waited for 50-60% confluence. After that the recombinant lentiviraltitre was inoculated to the cell and it was incubated for 48 hours. The recombinant lentiviruses were prepared by transfecting HEK cells with respective plasmid and viral packaging reagents. After that the fresh IMDM media was added by removing viral titer containing media.

3.4.3 Selection of transfected cells using Hygromycin B

The bone marrow and placental transfected cells were selected by using Hygromycin B (100µg/ml) containing media. When the transfected cells were 60-65% confluent, the Hygromycin B (100µg/ml) containing media was added and incubated for 7 days. The transfected cells formed colonies and non-transfected cells didn't form the colonies. The selected colonies were subcultured with normal 15% serum containing IMDM media. Then the conformational tests were done i.e. immunostaining, Real time PCR, FACS,colonization Assay, population doubling by growth curve and morphology of cell in late passage.

3.5. Immunostaining for P21 and Ki67 expression

3.5.1. Materials and Reagents for immunostaining

The reagents and instruments used for the immunostaining are as following:

a. 70% Chilled Methanol
b. 1X PBS
c. 3% BSA (3ml in 100 ml PBS)
d. Primary antibody (1:500 dilution in 1% BSA)

- Mouse monoclonal to P21 (500 µL IgG fraction abcam ab-3929-500)
- Goat polyclonal to Ki67 (200µg/mL IgG Santa cruz SC 7844)

e. Secondary antibody (1:1000 dilution in 1% BSA)

- Anti-mouse monoclonal to p21
- Anti-goat polyclonal to Ki67

f. 1X DAPI(4,6-diamidino-2-phenylindole) with glycerol
g. Glass slides and coverslips
h. Fluorescence Microscope (Axio imager)

For the immunostaining the different types of MSC cells i.e. SV40. hTERT, SVT,GFP transfected and normal control cells were grown on the cover slips. After the 60-70% of confluence the culture media were aspirated and washed with PBS. Then the cells were fixed with 70% chilled Methanol for 10 minutes at room temperature (RT). Then the Methanol was aspirated out and washed with PBS 10 minutes. After that, 200 µl of blocking solution (3% BSA) was added and left for 40 minutes. Then the primary antibody of P21 and Ki67 (1:500 dilution) was added to completely cover the cells and incubated over night at 4˚C. Then the slides were washed three times by 1X PBS and Fluorochrome conjugated secondary antibody i.e. anti-mouse monoclonal P21 and anti-goat polyclonal Ki67 (1:1000 dilution) was added and incubated at room temperature(RT) for 1 hour in the dark place because Fluorochrome is light sensitive. Then the coverslips were washed three times with 1X PBS. The coverslips were then picked up by using forceps and mounted on a glass slides containing glycerol with 1X DAPI. The mounted slides were left for sometimes for fixation and captured the image by using Fluorescence Microscope.

3.6. Isolation of total RNA from MSCs by TRIzol method

3.6.1. Reagents and Materials for RNA isolation

The reagents and instruments used for the RNA isolation are as following:

a. 1 X PBS
b. TRIzol reagents (Invitrogen)
c. Chloroform
d. Isopropanol
e. 70% ethanol in DEPC water
f. Cell scraper

TRIzol maintained RNA integrity during tissue homogenization and disruption of cell and cell components. Phenol: Chloroform caused precipitation of RNA into aqueous supernatants. RNA extraction was done by 5 basic steps as following:

Homogenization: hTERT, SV40, SVT, GFP transfected and non-transfected placental and bone marrow MSCs were collected, media was aspirated out and rinsed with 1x PBS in order to remove the IMDM media. Then the cells were lysed by adding 1mL of TRIzol in 90 mm culture disc and cells were scrapped out by using sterile scrapper. Then the lysate was transferred to a fresh microfuge tube and incubated at -80˚C for 2 hours to overnight.

Phase separation: The cells were taken out from the -80˚C, thawed on ice, added 200µL Chloroform (1/5th volume of TRIzol), mixed well by short vortexing and incubated on ice for 10 minutes. Then it was centrifuged at 12000 rpm for 15 minute at 4˚C. The aqueous phase was carefully transferred to next fresh microfuge tube.

RNA precipitation: The obtained RNA from the aqueous layer was precipitated by adding equal volume of chilled isopropanol and incubated on ice for 10 minutes. The samples were then centrifuged at 12000 rpm for 12 minutes at 4˚C. The gel like pellet on the bottom of tube was obtained.

RNA wash: The precipitate was washed by 70% ethanol in DEPC water by dislodging the pellet by gently tapping and centrifuged at 7500 rpm for 5 minutes at 4˚C. Then the supernatant was discarded and made the tube dry.

Dissolve of RNA: The air dried pellet was dissolved in 20µL of DEPC water.It was kept into water bath for 10 minutes at 55˚C in order to remove trace amount of ethanol and isopropanol and complete dissolution of RNA on DEPC water. Then the isolated RNA samples were quantified and checked the purity by using Nano drop spectrophotometer (Thermo fisher) and integrity of the RNA was screened by running 0.8% Agarose gel at 60 V for 45-55 minutes.

3.7. Synthesis of cDNA from RNA

3.7.1. Reagents and Materials for cDNA synthesis

The reagents and instruments used for the cDNA synthesis are as following:

a. RNA template
b. dNTPs(10mM)
c. OligodT primer
d. 5X first strand buffer (TaKaRa)
e. 0.1M DTT(TaKaRa)
f. RNase Inhibitor(TaKaRa)
g. Superscript Reverse Transcriptase (TaKaRa)
h. Milli Q

The isolated, quantified and screened RNA from late and early passage of transfected and normal MSCs was taken into microfuge tube. Then the mixture was prepared where 4µg of RNA was maintained in each tube and following components were added.

Table 3.1: Reaction parameter of cDNA synthesis for the real time PCR

Components	**Quantity per tube**
RNA	Based on concentration of RNA, 4µg of RNA was taken
DNTPs(10Mm)	1µL
OligodT(0.5µg/µl)	1µL
Milli Q	Volume based on RNA
TOTAL	**10µL**

After mixing the above reactions, tubes were incubated at 65˚C for 5 minutes in order to break the secondary structure formed in RNA. Then the tubes were set by addition of following components for synthesis of cDNA.

Table3.2: Final reaction mixture for cDNA synthesis for the Real Time PCR

Components	**Quantity per tube**
5X first strand buffer	4µL
0.1M DTT	1µL
RNase inhibitor (40U/µL)	0.5µL
Superscript III reverse transcriptase(200U/µL)	1µL
RNA sample	10µL
Milli Q	3.5µL
TOTAL	**20µL**

The components were mixed well by pipette and following PCR cycle was set up:

Table3.3: Thermo cyclic condition for preparation of cDNA for real time expression of gene using real time PCR

Step 1	50˚C for 50 minutes
Step 2	72˚C for 15 minutes
Step 3	10˚C for consistency
Step 4	END

The synthesized cDNA was screened using 0.8% agarose gel and stored at -20˚C for further use.

3.8. Relative quantification of hTR, TERT and P21 genes using Real Time PCR

Gene expression level was quantified by using Real Time PCR. The relative quantification technique was used for the comparison of gene expression relative to the reference gene. TBP and B2M gene were used as reference gene or housekeeping gene for relative quantification of

hTR, TERT and P21 gene expression of hTERT, SV40, SVT gene transfected and normal cell. The relative quantification was done by using two steps process.

3.8.1. Optimization of primers by Real Time PCR

The stock primers were diluted to 6 different concentrations ranging from 2.5 pM to 15 pM and 36 different combinations of forward and reverse primers were made as following:

Table 3.4: Matrix for NTC-optimization of primers for determination of suitable combination of forward and reverse primer for real time PCR

Forward Primer(F)/Reverse Primer (R)	2.5Pm	5Pm	7.5Pm	10Pm	12.5pM	15pM
2.5pM	2.5F 2.5R	5F 2.5R	7.5F 2.5R	10F 2.5R	12.5F 2.5R	15F 2.5R
5pM	2.5F 5R	5F 5R	7.5F 5R	10F 5R	12.5F 5R	15F 5R
7.5pM	2.5F 7.5R	5F 7.5R	7.5F 7.5R	10F 7.5R	12.5F 7.5R	15F 7.5R
10pM	2.5F 10R	5F 10R	7.5F 10R	10F 10R	12.5F 10R	15F 10R
12.5pM	2.5F 12.5R	5F 12.5R	7.5F 12.5R	10F 12.5R	12.5F 12.5R	15F 12.5R
15pM	2.5F 15R	5F 15R	7.5F 15R	10F 15R	12.5F 15R	15F 15R

On the basis of NTC matrix, different parameters like background noise/contamination, primer dimers formation etc. were checked using ABI Real time PCR instrument. The primer concentrations which showed undetermined values were selected as optimized primer concentrations. The optimized sets for forward and reverse primers for each gene are given below:

Table 3.5: Optimized primer concentrations for test and reference genes

S.N	Test and Reference genes	Forward primer(pM)	Reverse primer (pM)
1	TERT	5	2.5
2	p21	2.5	5
3	HTR	2.5	7.5
4	TBP	5	7.5
5	B2M	2.5	2.5

3.8.1.1. Reagents and materials for optimization of primers for Real Time PCR

The reagents and instruments used for the optimization of primers for Real Time PCR are as following:

a. hTR forward and reverse primers(100pM stock)

b. TERT forward and reverse primers (100pM stock)
c. P21 forward and reverse primers (100pM stock)
d. SYBR green dye (Applied bio system) which contains AmpliTaq gold DNA polymerase, dNTPS, SYBR I green dye optimized buffers and ROX, passive reference dye
e. Micro Amp optical 384 wells PCR plate and optical clear film
f. Real time PCR machine(Applied Biosystem- SDS 2.3 software)

3.8.2. Quantification of gene expression of hTR, TERT and P21

For the relative quantification of hTR, TERT and P21 gene present in the transfected cells and control cells, the master mix was prepared for triplicate reaction by using optimized primers combination from the NTC results. The reaction was prepared as following:

Table 3.6: Reaction Mixture for determination of real time expression of gene using Real Time PCR

Components	**Volume/ Concentration**
cDNA template	According to RNA concentration/ 100ng
Forward primer	1µL
Reverse primer	1µL
SYBR green dye	5µL
Milli Q	Based on cDNA volume
Total	10µL for 1 reaction (triplicates were used)

From the master mix, 10µL was pipetted into each well of a 384 well plate and each sample was pipetted as triplicates to optimize the result. Then a clear optical film was used to cover the plate and sealed the content after loading the all samples. The plate was centrifuged at 500 rpm for short time to mix the sample. The sample was run using ABI Real Time PCR by using the software SDS 2.3 version Real time including NTC and reference gene primers i.e.B2M, TBP. The cycling parameters were set as following:

Table 3.7: Thermo cyclic condition for real time expression of gene using Real-Time PCR

Stage 1	Stage 2	Stage 3	Stage 4
50˚C 02:00	95˚C 10 min.	95˚C 15 sec. 30 sec. 60˚C 72˚C 30 sec.	95˚C 10 min. 60˚C 95˚C 10 min. 30 sec.
		Repeats 40 times	Dissociation stage

After completion of the run the data were saved in a folder and analysed the data to check Ct value in NTC, standard used and in the sample template. The level of gene expression was determined by computing ΔΔCt as following:

ΔCt= Ct Test sample – Ct Reference

ΔΔCt= Ct Test sample – Ct control

3.8.2.1 Reagents and Materials for Real Time PCR

The reagents and instruments used for the Real Time PCR are as following:

a. TERT,hTR, P21 forward and reverse primers (100pM stock)
b. cDNA
c. Housekeeping genes i.e.18S rRNA, TBP, B2M
d. SYBR green dye (Invitrogen SYBR green qPCRsupermix) containing AmpliTaq gold DNA polymerase, dNTPs, SYBR green optimized buffer.
e. Micro Amp optical 384 wells PCR plate and optical clear film
f. Real time PCR machine(Applied Biosystem-SDS 2.3 software) and Milli Q

The sequences of primer (invitrogene) and housekeeping genes are as following:

Table 3.8: The primers used for quantification of genetic expression and their sequences

S.N	Primer name	Primer Sequence(5'-3')
1.	TERT Forward	**CGTGGTTTCTGTGTGGTGTC**(20bp)
2	TERT Reverse	**CTTGTCGCCTGAGGAGTAG**(19bp)
3	hTR Forward	**GCGCCGTGCTTTTGCT**(16bp)
4	hTR Reverse	**TTTTCCGCCCGCTGAA**(16bp)
5	P21 Forward	**TTAGCAGCGGAACAAGGAGT**(20bp)
6	P21Reverse	**AGCCGAGAGAAAACAGTCCA**(20bp)
7	TBP Forward	**TGCACAGGAGCCAAGAGTGAA**(21bp)
8	TBP Reverse	**CACATCACAGCTCCCCACCA**(20bp)
9	B2M Forward	**GGCTATCCAGCGTACTCCAA**(20bp)
10	B2M Reverse	**GATGAAACCCAGACACATAGCA**(22bp)

3.9. Cell Cycle Analysis by using FACS

3.9.1 Reagents and Materials for FACS

The reagents and instruments used for the cell cycle analysis by using FACS are as following:

a. Propidium iodide (50mg/mL stock), RNase A, 70 % chilled Methanol
b. FACS tube with filter

c. BD FACS Array bio analyzer

In 35 mm dish, 50,000 cells of SV40, hTERT, SVT, GFP gene transfected and normal control cells were plated and incubated for 24 hours. Then the cells were harvested by trypsinization and washed with PBS. Then the cells were fixed with chilled 70% methanol for at least 30 minutes (it can be kept up to one week at 4˚C). Then it was centrifuged and discarded the methanol carefully and washed with PBS. The 500µL PBS suspended cells were stained with 1µg/µL Propidium iodide as a final concentration at dark with 1 µL RNase. Then the cell cycle was analyzed on the BD FACS Array bioanalyzer.

3.10 Colony forming Assay

3.10.1. Reagents and Materials for colony forming assay

The reagents and instruments used for the colony forming assay are as following:

a.70% chilled Methanol

b.0.1% Giemsa stain

c.Distilled water

In 90 mm dish, 500 cells of transfected and normal (non-transfected) placental and bone marrow MSCs were plated. After 10 days of incubation, there was formation of colony by the cell. Then the plate was washed with 1X PBS twice and fixed the cells by chilled 70% Methanol for 10-15 minutes and stained with 0.1% Giemsa dissolved in 100% Methanol. After that it was washed with distilled water twice and observed, counted by marking the culture dish with marker and measured the diameter of larger 10 colonies using normal scale the colony.

3.11 Morphological Difference

The shape, size and granularity of cells were observed in each passage under the Microscope(100X). The morphology of transfected and normal cell in early(2^{nd}Passage) and late passage (12^{th}Passage) was analyzed. The microscopic appearance was captured by camera and detailed analysis was done.

The different gene (SV40, TERT and SV40+TERT) transfected cells and normal (non- transfected) cells were selected by Hygromycine B selection procedure. The SV40+TERT (SVT) transfected Bone Marrow(BM) derived Mesenchymal Stem Cells (MSCs) and TERT transfected Placental derived Mesenchymal Stem Cells formed good colony in Hygromycine B.All non-transfected cells did not form colony and die out all cells after 7 days of incubation. the immortality of selected cells were confirmed by conformational tools i.e. Real Time PCR, Immunostaining, FACS, Colonization assay, Growth curve analysis, Morphological difference.

4.1 Growth curve and population doubling

The placental and bone marrow derived MSCs were transfected with SV40-Tag gene, hTERT gene and SVT gene. Then the transfected cells were grown on Hygromycin B (100µg/mL)containing IMDM media for seven days without changing the media for selection. After that the transfected cells with normal control and the GFP transfected cells as positive control were grown on IMDM media containing 15% serum. Total numbers of cells were counted by using Heamocytometer after they attained 90-95% confluence and the number of days taken to reach that level was noted for different types of cells. Population doubling and population doubling time were calculated on each passaging.

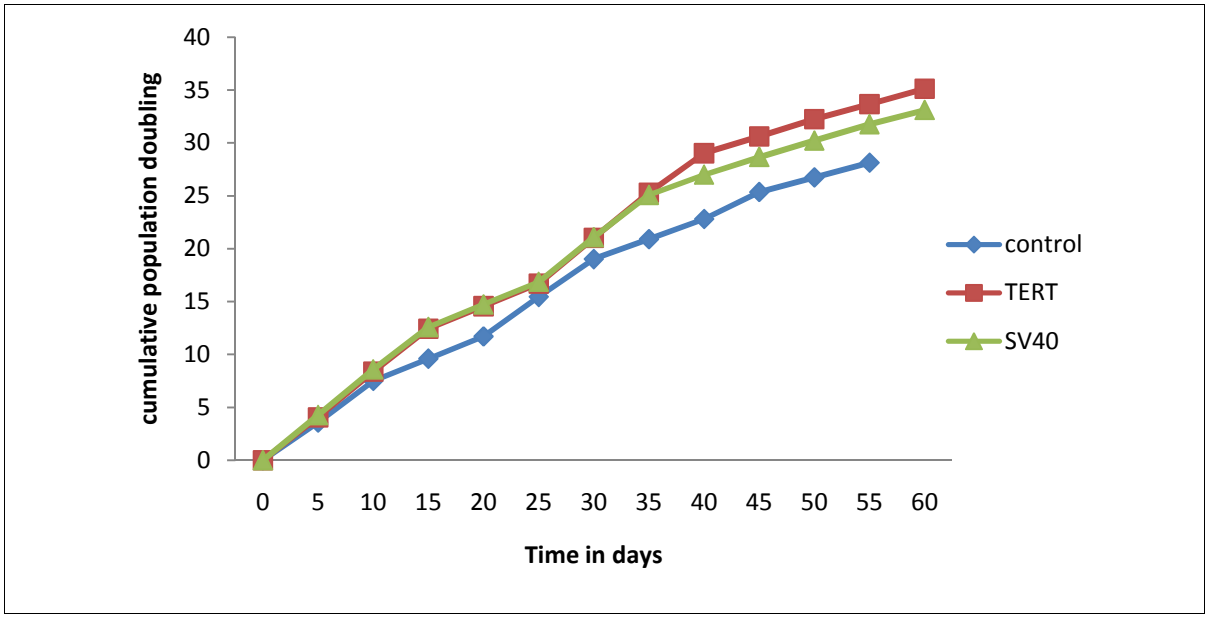


Figure 4.1: Growth curve of normal, TERT transfected and SV40 transfected placental derived MSCs in normal growth media

According to population doubling curve, hTERT transfected placental cells had more cumulative population doubling on same time as compared to control and SV40 transfected cells. The hTERT transfected cells were grown faster as compared to other cells.

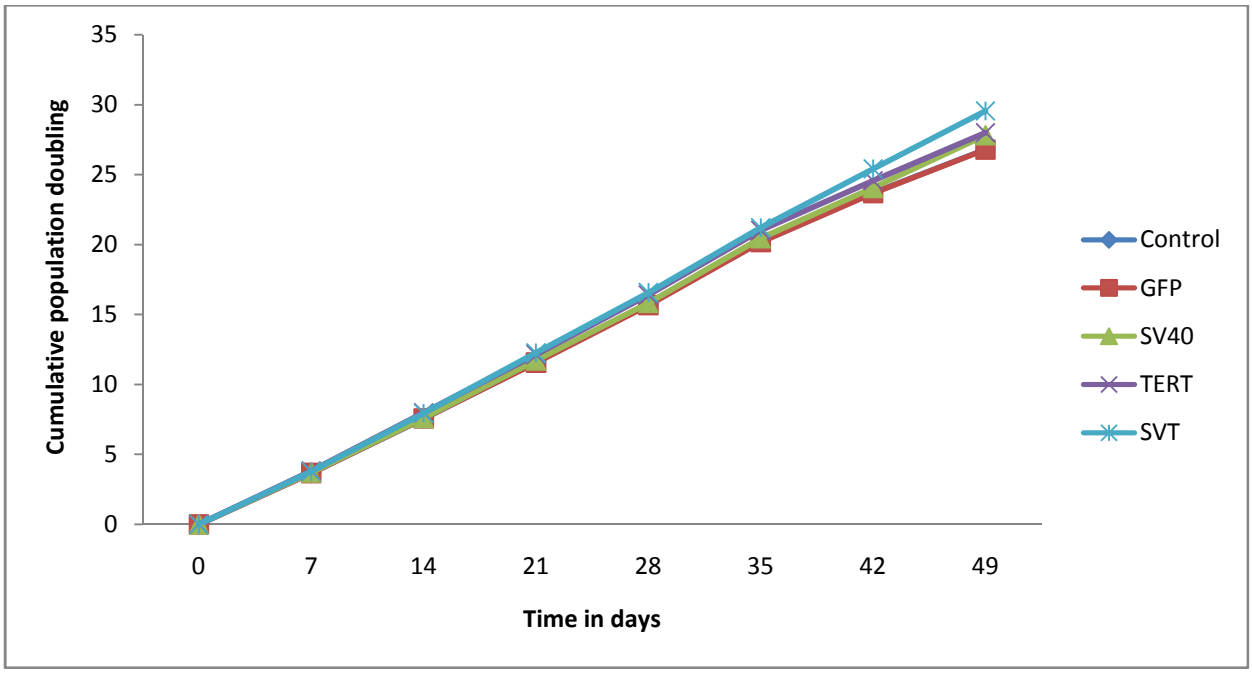


Figure 4.2: Growth curve of bone marrow normal, GFP transfected, SV40 transfected, TERT transfected and SVT transfected MSCs in normal growth media

By the analysis of growth curve of transfected and normal bone marrow MSCs cells, it was found that the SVT transfected and TERT transfected cells had better and constant growth as compared to control bone marrow MSCs till late passage. The SVT transfected and TERT transfected cells were proliferating in late passage also. Among all transfected and normal MSCs, SVT gene transfected cells showed better proliferation from early passage to late passage. This cell was continuously proliferating after 30 populations doubling as well. Normal bone marrow MSCs cannot proliferate in this manner. Normal bone marrow cannot proliferate after 30 population doubling. So the SVT gene transfected BM MSCs was characterized as proliferating cells till late passage i.e. after 30 populations doubling as well.

4.2 Morphology of transfected and normal MSCs Cells

Morphological appearance is one of the main criteria of aging studies of cells. In this study different transfected cells and normal control cells were grown in normal IMDM media containing 15 % serum. During the late non mitotic phase, the cells were changed in size and morphology, become granular in appearance and accumulated debris. The senescent cells displayed an enlarged, flattened morphology. These cells were characterized as an irreversible G1 growth arrest.

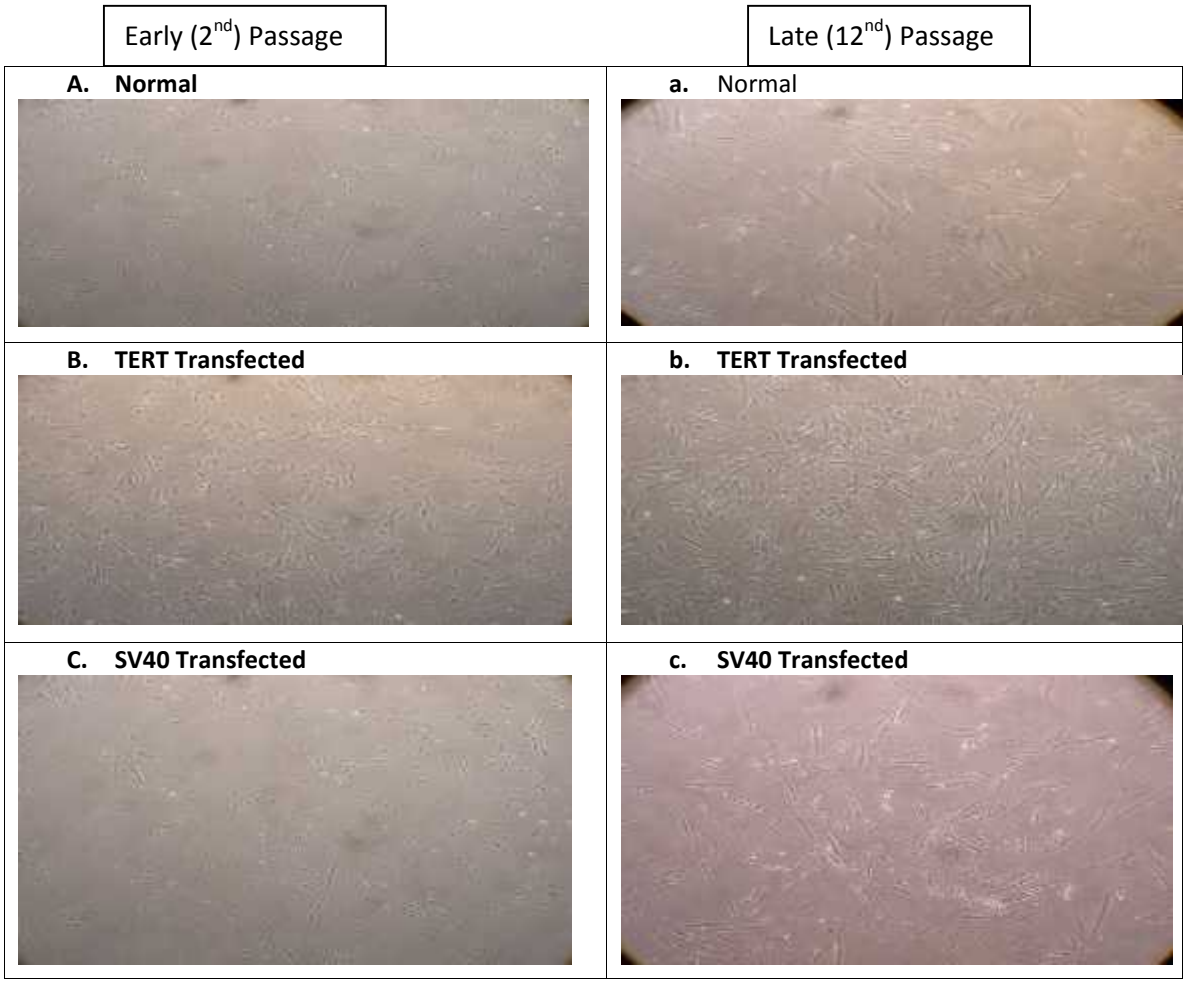


Figure 4.3: morphological changes in placental derived normal (A, a), TERT transfected (B, b) and SV40 (C, c) transfected MSCs in early and late passage. Early and late passages are represented by capital letter and small letters respectively.

As compared to normal control and SV40 transfected PL MSCs, the hTERT transfected cells showed less elongation with few death cells and debris in same late passage. So the TERT transfected cells were characterized as more proliferating cells as compared to normal placental cells by morphological analysis.

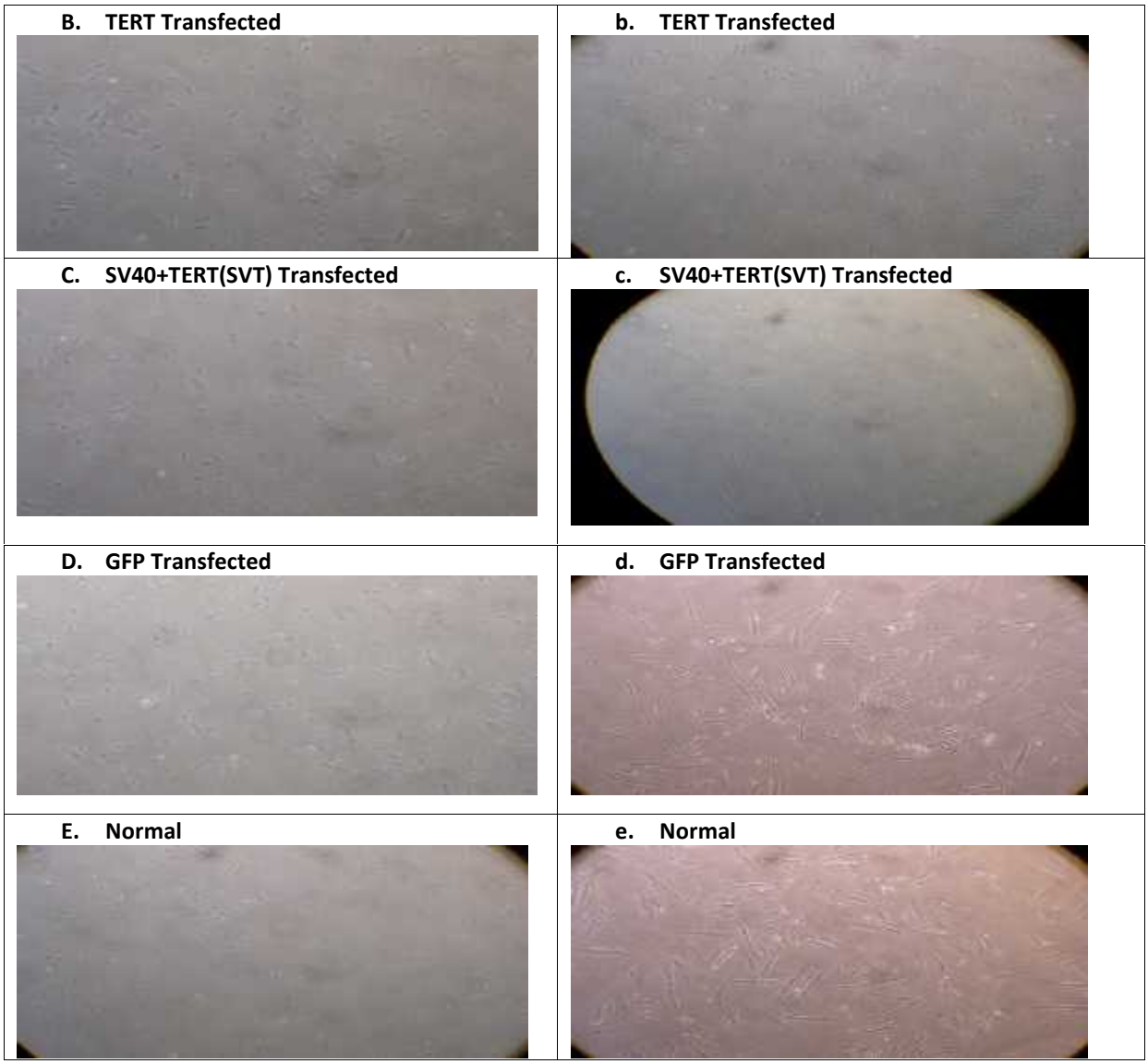


Figure 4.4: Morphological changes in bone marrow derived marrow cells of SV40 transfected (A, a), TERT transfected (B, b) and SVT transfected (C, c), GFP transfected (D, d) and normal (E, e) in early and late passage. Early and late passages are represented by capital letters and small letters respectively.

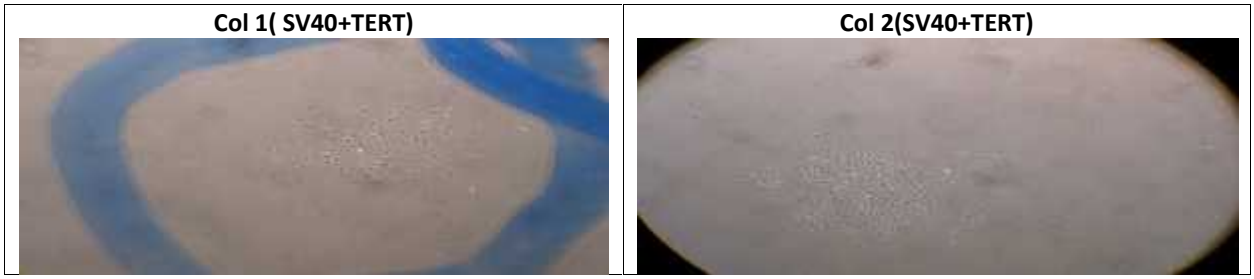


Figure 4.5: Morphology of Colony of SV40 +TERT (SVT) transfected bone marrow derived MSCs selected after 8 days of **Hygromycin B** selection media. Two colonies were selected named as col 1 and col 2 respectively.

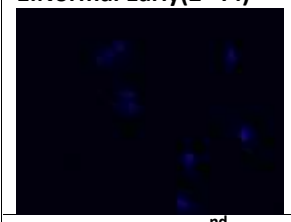
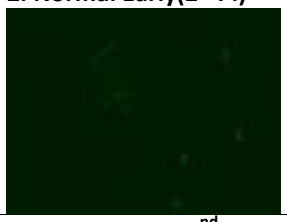
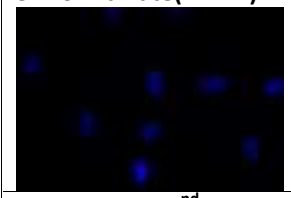
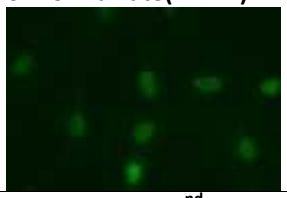
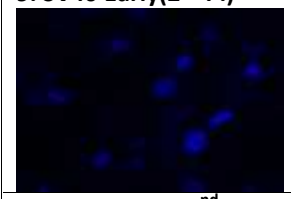
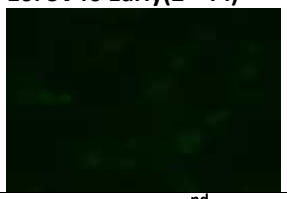
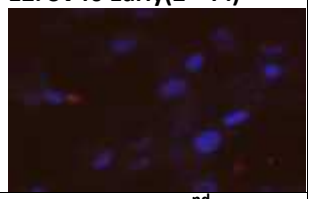
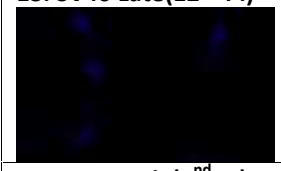
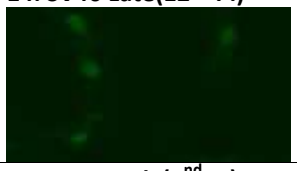
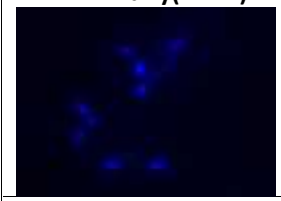
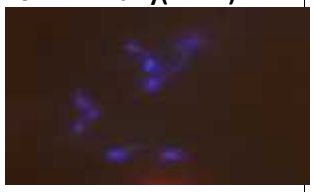
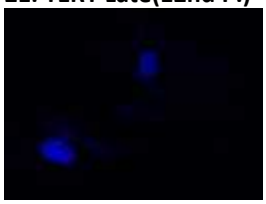
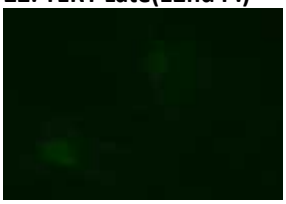
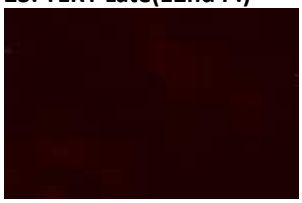
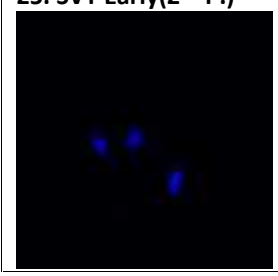
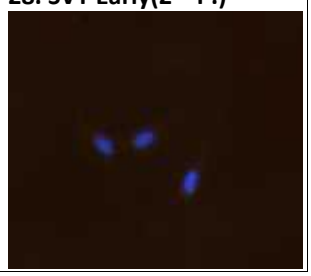
As compared to normal BM, GFP transfected, SV40 gene transfected and TERT transfected BM MSCs, the SVT transfected cells showed better growth with no debris and slight elongation with healthy appearance till very late passage. So the SVT transfected BM MSCs was characterized as healthy till late passage in morphological analysis. The selected colonies of SVT transfected BM MSCs had also distinct appearance as other normal BM MSCs.

4.3 Immunostaining for proliferation marker (Ki67 protein) and senescence marker (P21 protein)

The proliferation marker Ki67 and senescence marker p21 of different transfected and normal bone marrow and placental MSCs were analyzed by using immunostaining technique. The Ki67 protein is a cellular marker for proliferation. It is strictly associated with cell proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Ki67 proteins are present in all active phases of cell cycle except G0 phase.P21 is a potent cyclin dependent kinase inhibitor. The p21 protein binds and inhibits the activity of cyclin CDK2, CDK1 and CDK4/6 complexes which can mediate cellular senescence. The ApoTome fluorescent microscope was used for observation after the staining. The filter of microscope for the visualization was selected according to flourochrome conjugated secondary antibody. The ds red alexa 594 filter was used for Ki67 and GFP was used for p21. So the intensity of red colour signified its proliferation and green fluorescence signified the senescent.

As compared to normal control and GFP transfected positive control of bone marrow MSCs, TERT, SV40 and SVT gene transfected bone marrow MSCs showed more detachable level of Ki67. In the early passage, expression of Ki67 (ds red) was higher in all cells and expression of p21 (GFP) was lower. In the late passage, the normal control of bone marrow MSCs shows prominent expression of p21 (Green) and decreased or almost undetectable expression of Ki67 (ds red). But the transfected cells showed detectable constant level of Ki67 expression. Among the transfected cells TERT+SV40 gene transfected cells showed more detectable expression of Ki67 and less expression of p21 (green) was observed in late passage as well.

In placental MSCs cells, normal placental MSCs showed more expression of p21 (green) and less expression of Ki67 (ds red) in late passage. But TERT and SV40 transfected cells showed constant expression of Ki67 (ds red) and less expression of p21 (green) as compared to normal control. Among the transfected cells TERT transfected cells showed more prominent expression of Ki67 and less p21 in late passage as well.

DAPI	p21	ki67	Merged image
1.Normal Early(2nd P.)	2. Normal Early(2nd P.)	3. Normal Early(2nd P.)	4. Normal Early(2nd P.)
			
5. Normal late(12nd P.)	6. Normal late(12nd P.)	7. Normal late(12nd P.)	8. Normal late(12nd P.)
			
9. SV40 Early(2nd P.)	10. SV40 Early(2nd P.)	11. SV40 Early(2nd P.)	12. SV40 Early(2nd P.)
			
13. SV40 Late(12nd P.)	14. SV40 Late(12nd P.)	15. SV40 Late(12nd P.)	16. SV40 Late(12nd P.)
			
17. TERT Early(2nd P.)	18. TERT Early(2nd P.)	19. TERT Early(2nd P.)	20. TERT Early(2nd P.)
			
21. TERT Late(12nd P.)	22. TERT Late(12nd P.)	23. TERT Late(12nd P.)	24. TERT Late(12nd P.)
			
25. SVT Early(2nd P.)	26. SVT Early(2nd P.)	27. SVT Early(2nd P.)	28. SVT Early(2nd P.)
			

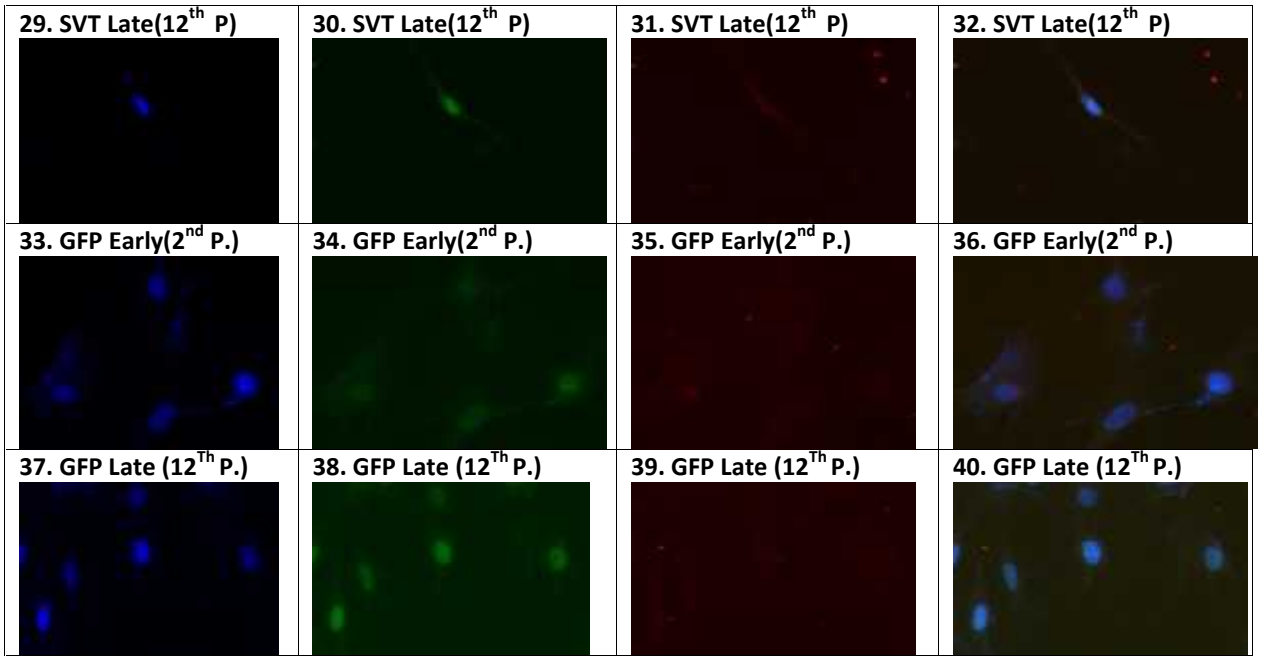
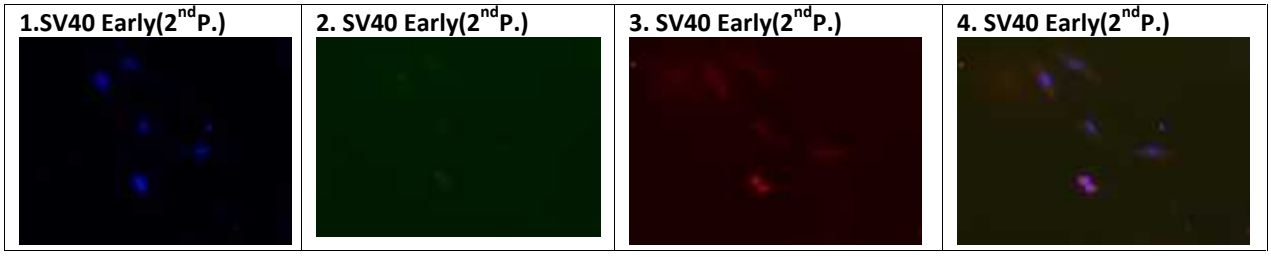


Figure 4.6: Immunostaining of non-transfecting and different transfected BM MSCs. Figure no 1 to 4 represents DAPI stained, p21 stained, ki67 stained and merged image of normal early(2^{nd}) passage. Figure 5 to 8 represents DAPI stained, p21 stained, ki67 stained and merged image of normal late (12^{th}) passage. Figure 9 to 12 represents DAPI stained, p21 stained, ki67 stained and merged image of early($2^{nd)}$) passage of SV40 transfected early(2^{nd}) passage. Figure 13 to 16 represents DAPI stained, p21 stained, Ki67 stained and merged image of SV40 transfected late (12^{th}) passage. Figure 17 to20 represents DAPI, p21, Ki67 stained and merged image of TERT transfected early (2^{nd}) passage. Figure 21 to 24 represents DAPI, p21, Ki67 stained and merged image of TERT transfected late (12^{th}) passage. Figure 25 to28 represents DAPI, p21, Ki67 stained and merged image of SVT transfected early (2^{nd}) passage. Figure 29 to 32 represents DAPI, p21, Ki67 stained and mixed image of SVT transfected late (12^{th}) passage. Figure 33 to 36 represents DAPI, p21, Ki67 stained and mixed image of GFP transfected early (2^{nd}) passage. Figure 37 to 40 represents DAPI, p21, Ki67 stained and mixed image of GFP transfected late (12^{th}) passage of BM derived MSCs**.**

DAPI **p21** **ki67** **Merged image**



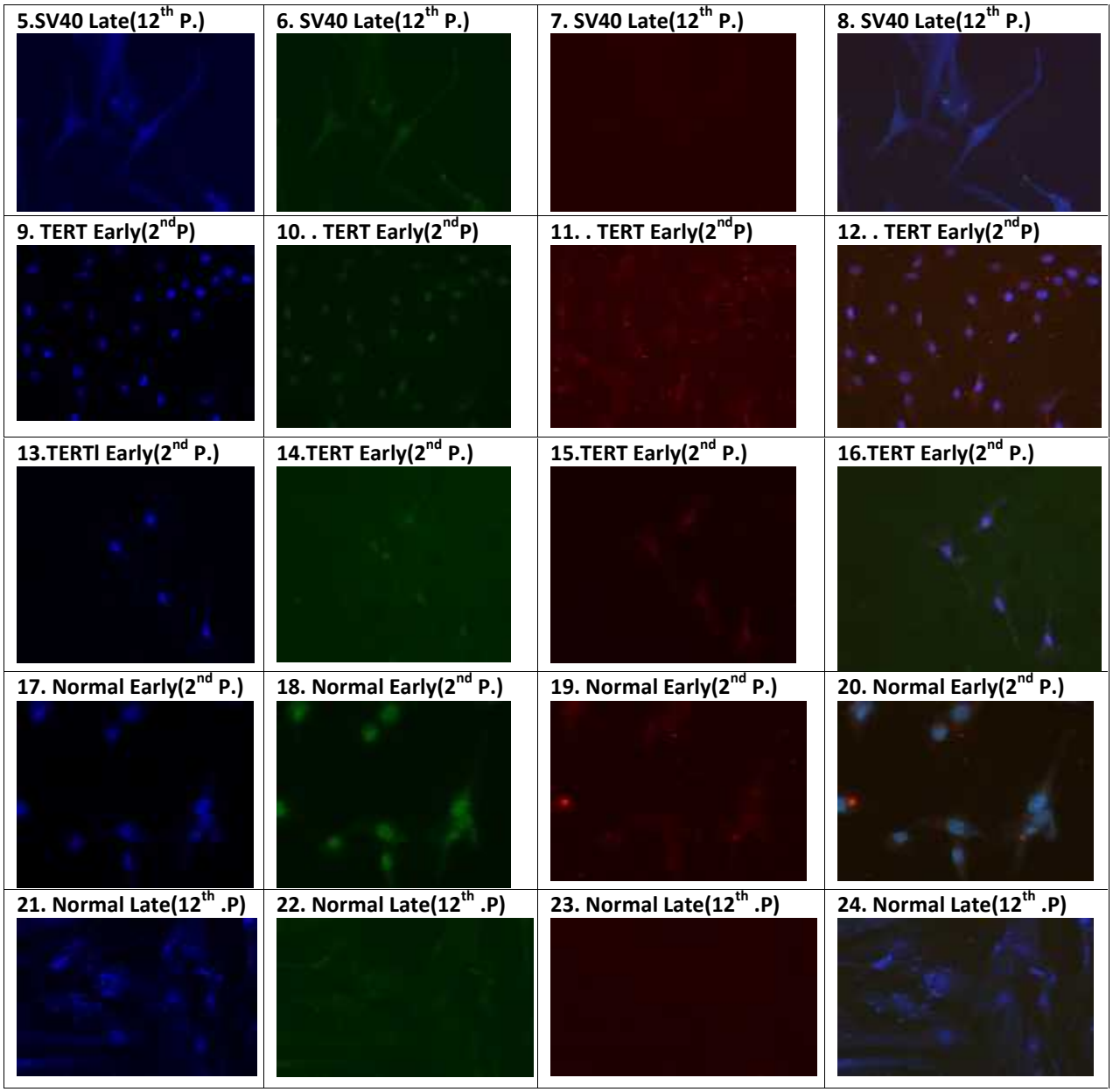
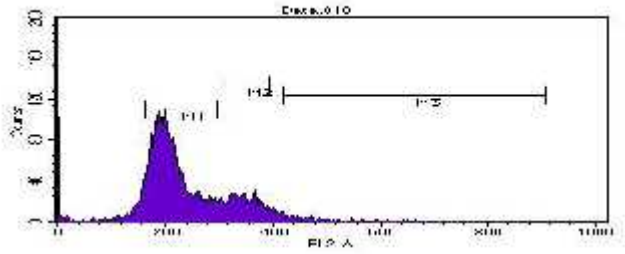


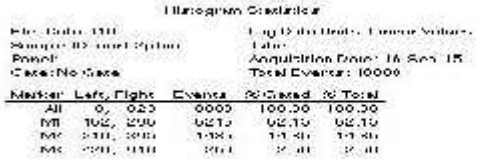
Figure 4.7 : Immunostaining of non-transfecting and different transfected PL MSCs. Figure no 1 to 4 represents DAPI stained, p21 stained, Ki67 stained and merged image of SV40 transfected early(2^{nd}) passage. Figure 5 to 8 represents DAPI stained, p21 stained, Ki67 stained and merged image of SV40 transfected late (12^{th}) passage. Figure 9 to 12 represents DAPI stained, p21 stained, Ki67 stained and merged image of TERT transfected early (2^{nd}) passage. Figure 13 to 16 represents DAPI stained, p21 stained, ki67 stained and merged image of TERT transfected late (12^{th}) passage. Figure 17 to20 represents DAPI, p21, Ki67 stained and merged image of non- transfected early (2^{nd}) passage. Figure 21 to 24 represents DAPI stained, p21 stained, Ki67 stained and merged image of non-transfected normal late (12^{th}) Passage of placental derived MSCs.

4.4 FACS for cell cycle analysis

Flow cytometry, the methodology that provided a way to measure physical and chemical attributes of individual cells rapidly and with high accuracy. This technique is widely used in cell cycle analysis. The most common assays are: Univariate analysis of cellular deoxyribonucleic acid (DNA) content discloses frequencies of cells in $G_{0/1}$, S and G_2/M phases and bivariate analysis of DNA versus ribonucleic acid (RNA) content reveals frequencies of G_0, G_1, S and G_2M cells, of DNA content versus histone H3 phosphorylated on Ser10 defines frequencies of $G_{0/1}$, S, G_2 and M cells. Univariate analysis was done for the detection of cellular DNA content frequency in different phases.

BM control 2P





BM TERT 2P and BM SV40 2P

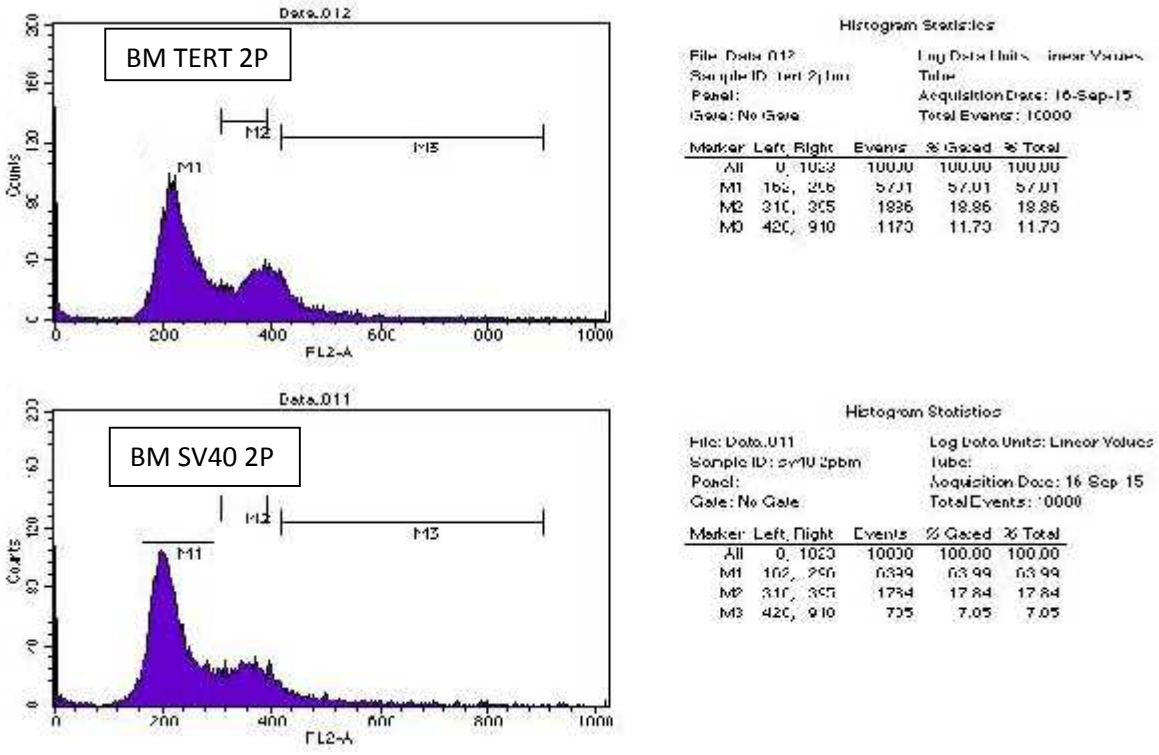
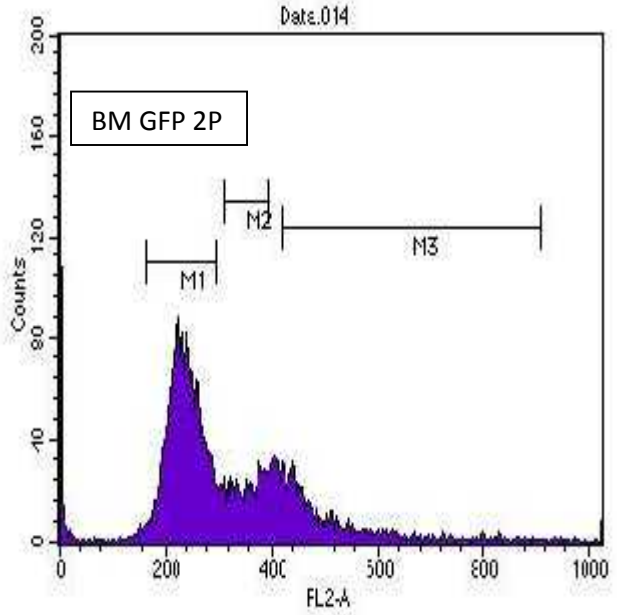


Figure 4.8: Histogram showing phases of cell cycle of BM derived normal non transfected normal MSCs (2P),TERT transfected BM MSCs (2P), SV40 transfected BM MSCs (2P)

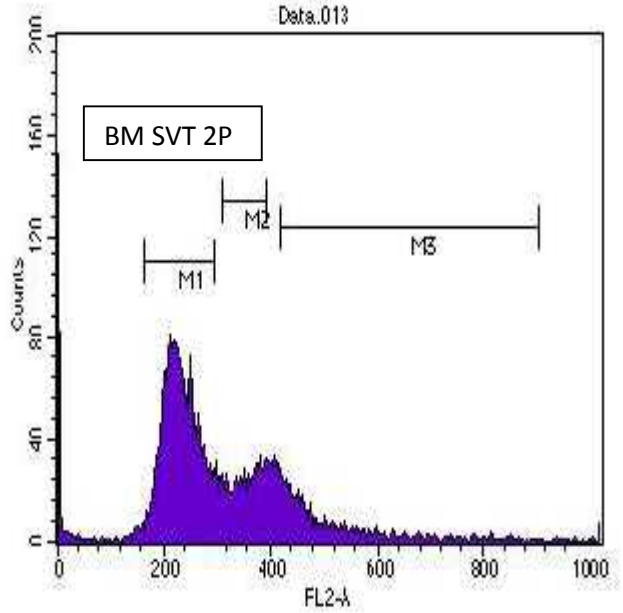
BM GFP 2P and BM SVT 2P



Histogram Statistics

File: Data.014 Log Data Units: Linear Values
Sample ID: gfp 2pbm Tube:
Pane: Acquisition Date: 16-Sep-15
Gate No Gate Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	10000	100.00	100.00
M1	162, 296	5376	53.76	53.76
M2	310, 395	1529	15.29	15.29
M3	420, 910	1788	17.88	17.88



Histogram Statistics

File: Data.013 Log Data Units: Linear Values
Sample ID: tert+40 2pbm Tube:
Panel: Acquisition Date: 16-Sep-15
Gate: No Gate Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	10000	100.00	100.00
M1	162, 296	5422	54.22	54.22
M2	310, 395	1800	18.00	18.00
M3	420, 910	1503	15.03	15.03

Figure 4.9: Histogram showing phases of cell cycle of BM derived SVT transfected BM MSCs (2P) and GFP transfected BM MSCs (2P) early passage.

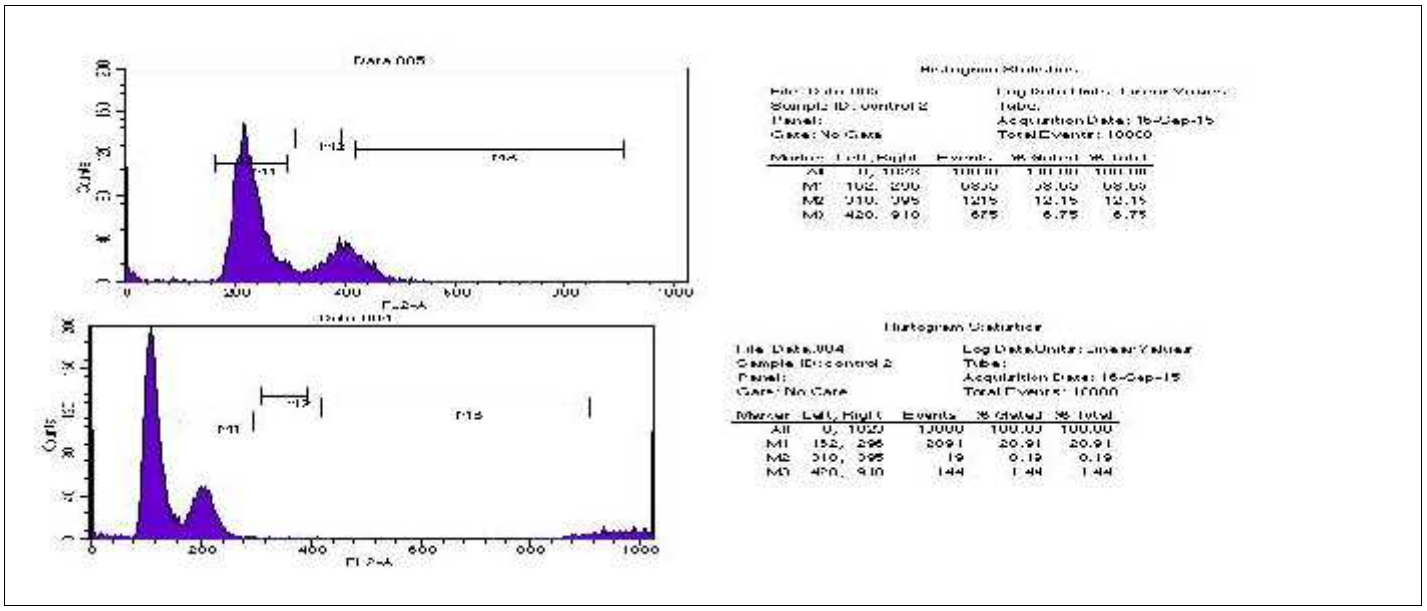


Figure 4.10: Histogram showing different phases of cell cycle of late passage (7P) of non-transfected normal BM derived MSCs.

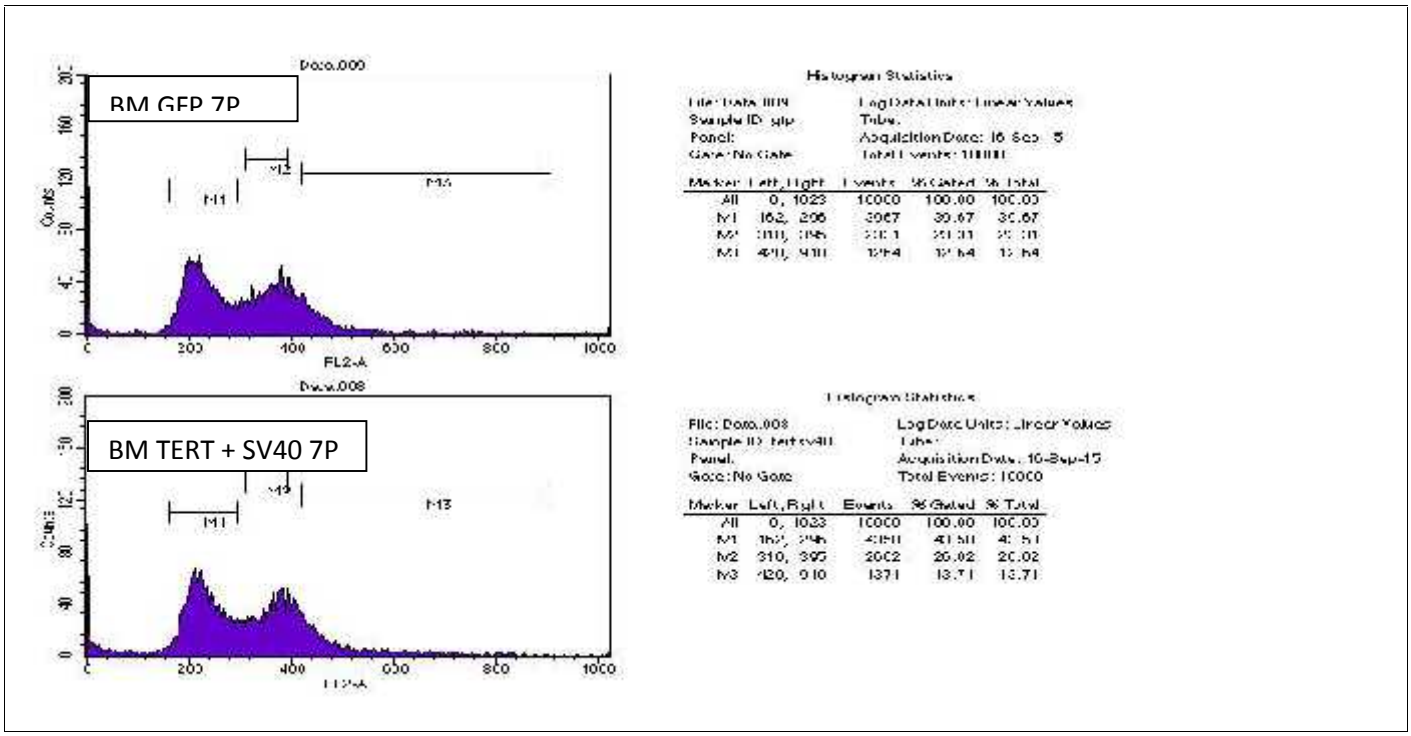


Figure 4.11: Histogram showing different phases of cell cycle of GFP transfected (7P) and SV40+TERT transfected (7P) of late passage of BM derived MSCs

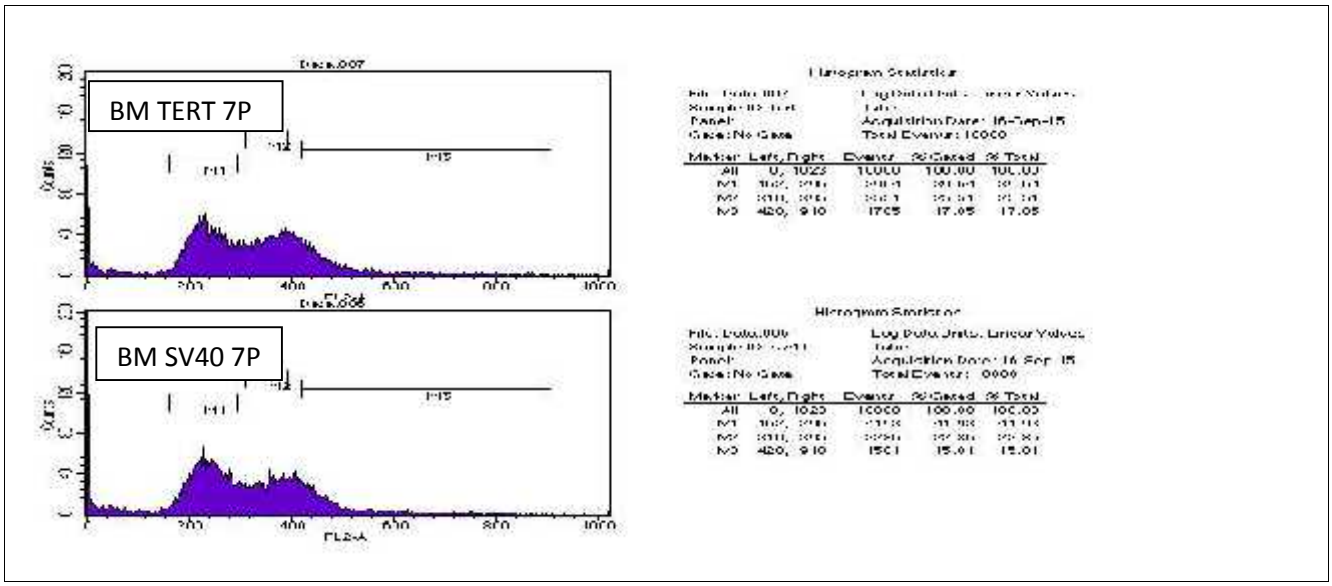


Figure 4.12: Histogram showing different phases of cell cycle of late passage of TERT transfected (7P) and SV40 transfected (7P) bone marrow derived MSCs

The above histogram showed the distribution of cells according to their DNA content. M1 represents cells in G1 phase of cell cycle, M2 represents cells in S phase of cell cycle and M3 represents the cells in G2+M phase. If more numbers of DNA contents of the cells are in S and G2+M phase, the cells are in proliferating and are in healthy state. But if more numbers of cells are in G1 phase, their growth retards and start to arrest.

According to the cell cycle analysis of normal and transfected bone marrow MSCs, more DNA contents of normal control cells were in G1 phase as compared to transfected cells in early and late passage. The SVT transfected bone marrow MSCs cells, more contents of DNA are in S and G2+M phase as compared to other cells. The analysis showed that SV40, TERT and SVT transfected cells were more proliferating and healthy as compared to normal BM MSCs. Among the transfected cells, the DNA contents of SVT gene transfected cells were in M2 and M3 phase. So the SVT transfected cells were characterized as more proliferating during FACS analysis.

4.5 Colony Forming Unit (CFU) Assay

Proliferating stem cells can form colony by the individual cells as well. After counting the cells by Haemocytometer, 500 cells were plated in 90 mm culture dish for 10 days. After 10 days, colonies were detected with Gimsa staining. The number of colonies and size of colonies were varied from normal control to transfected cells. In bone marrow MSCs, SVT gene transfected cells showed more colonies and bigger in size as compared to other cells. But in late passage there were no more colonies numerically as in early passage. But the size of colonies in SVT gene transfected bone marrow MSCs was more as compared to normal bone marrow MSCs in late passage as well.

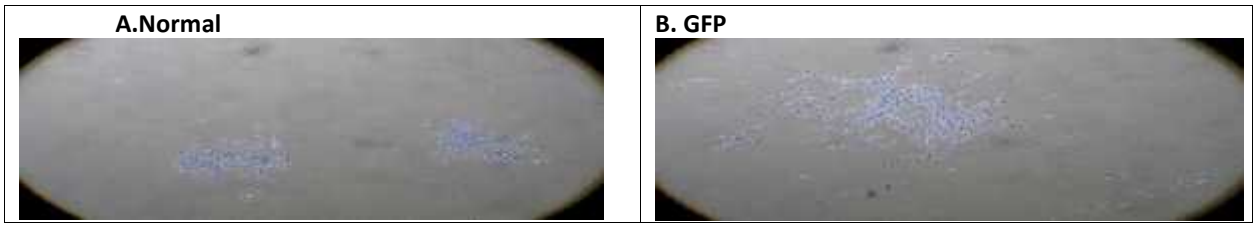


Figure 4.13: Microscopic observation of colony formed by normal control (A) and GFP transfected (B) bone marrow (BM) MSCs.

Figure 4.14: Microscopic observation of colony formed by TERT transfected BM derived MSCs(**C**), SV40 transfected BM derived MSCs **(D**) and SV40+TERT transfected BM derived MSCs (**E**) after the Giemsa staining (50x).

Figure 4.15: Microscopic observation of colony formed by placental derived non-transfected normal MSCs **(F**), placental derived TERT transfected MSCs (**G**) and placental derived SV40 transfected MSCs (**H**) after the Giemsa staining (50x).

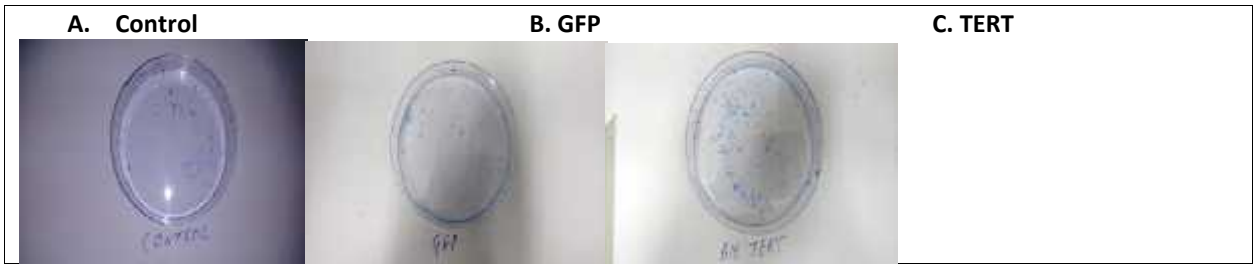


Figure 4.16: The colony seen in non-transfected control BM derived MSCs (A), GFP transfected BM derived MSCs (B), and TERT transfected BM derived MSCs (C) after plating with same number of cells in 10 days with Giemsa staining.

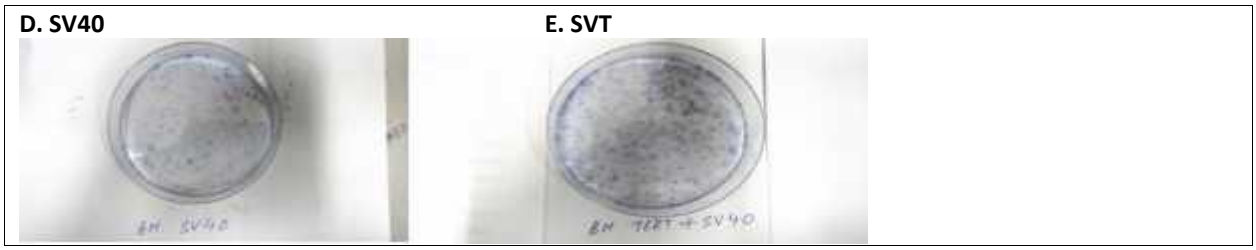


Figure 4.17: The colony seen in SV40 transfected BM derived MSCs (D) and SV40+TERT (E) transfected BM derived MSCs with Giemsa staining after plating same number of cells for 10 days of incubation in each dish.

The number of colonies formed and their average diameter of the selected 10 large colonies were as following:

SN	Name of the cells	Number of colonies in 90 mm plate	Average diameter of 10 larger colonies
1	Bone marrow normal control	267(2P) 90(7P)	3.055mm (2P) 2.46mm(7P)
2	Bone marrow GFP transfected	221(2P) 85(7P)	3.31mm(2P) 2.19mm(7P)
3	Bone marrow SV40 transfected	384(2P) 95(7P)	4.25mm(2P) 3.38mm(7P)
4	Bone marrow TERT transfected	398(2P) 102(7P)	3.38mm(2P) 3.75mm(7P)
5	Bone marrow SV40 plus TERT transfected	477(2P) 133(7P)	4.44mm(2P) 3.80mm(7P)
6	Placental SV40 transfected	255(2P) 49(8P)	3.23mm(2P) 2.42mm(8P)
7	Placental TERT transfected	386(2P) 82(8P)	4.10mm(2P) 3.12mm(8P)
8	Placental normal control	248(2P) 43(8P)	3.21mm(2P) 2.36mm(8P)

Table 4.1: Number of colony formed and their average diameter by the non-transfected and transfected BM and PL derived MSCs after the incubation of same number of cells for 10 days of incubation with Giemsa staining.

The numbers of colonies were counted and marked larger 10 colonies after the Giemsa staining for normal and transfected placenta derived MSCs as well. The TERT transfected placenta derived MSCs showed better proliferation in late passage as compared to normal and SV40 transfected cells.

4.6 Real time quantification of Gene expression

4.6.1 RNA isolation and Reverse Transcription

The 90% confluent cell in 90 mm culture flask was removed from incubator and scrapped by new autoclaved scrapper by adding 1 ml of TRizol solution and RNA was isolated by TRizol methods. The isolated RNA was checked its integrity by running 1% Agarose gel and observed by UV transilluminator. The fine three bands of RNA were obtained from normal and different transfected BM MSCs.

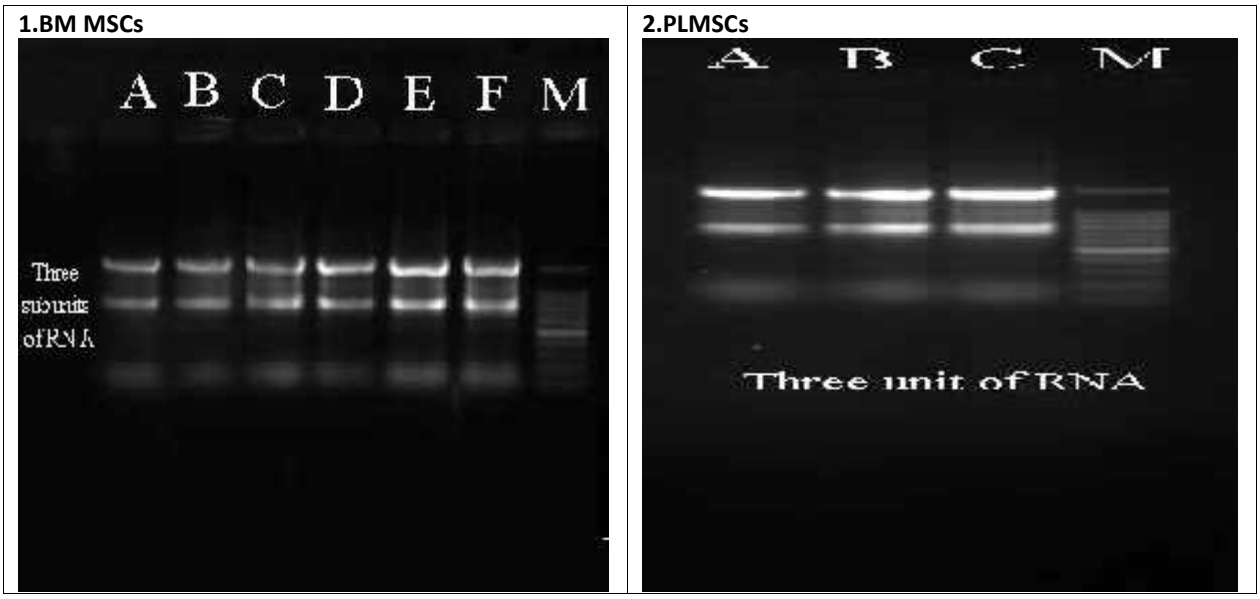


Figure 4.18: The three sub unit of RNA(28S, 18S and 5S) isolated from normal and transfected1.BM MSCs, Normal non transfected BM(A), GFP transfected BM MSCs (B), SV40 transfected BM MSCs (C), hTERT transfected BM MSCS (D), SVT transfected BM MSCS (E and F) and 100 bp marker(M). RNA bands obtained from 2.placental derived MSCs, Normal non transfected PL MSCs (A), SV40 transfected PL MSCs (B), Htert transfected PL MSCs (C) and marker 100bp (M)

The three bands of RNA were obtained from placental derived MSCs. The integrity of RNA was good enough for reverse transcription and Real Time Gene expression studies.The isolated RNA was quantified and reversed transcribed by using cDNA synthesis Kits.

4.6.2 Real time PCR and Gene expression

Real time PCR was done for the quantification of level of expression of hTR, TERT and p21 gene in different gene transfected cells and control cells in both early and late passage. For this study RNA was isolated by TRizol method, quantified and Reverse transcribed using cDNA synthesis Kit. For the immortality testing, two proliferating marker (i.e. hTR and TERT) and a senescence marker (i.e. p21) were used with equal concentration of all early and late passage cDNA.

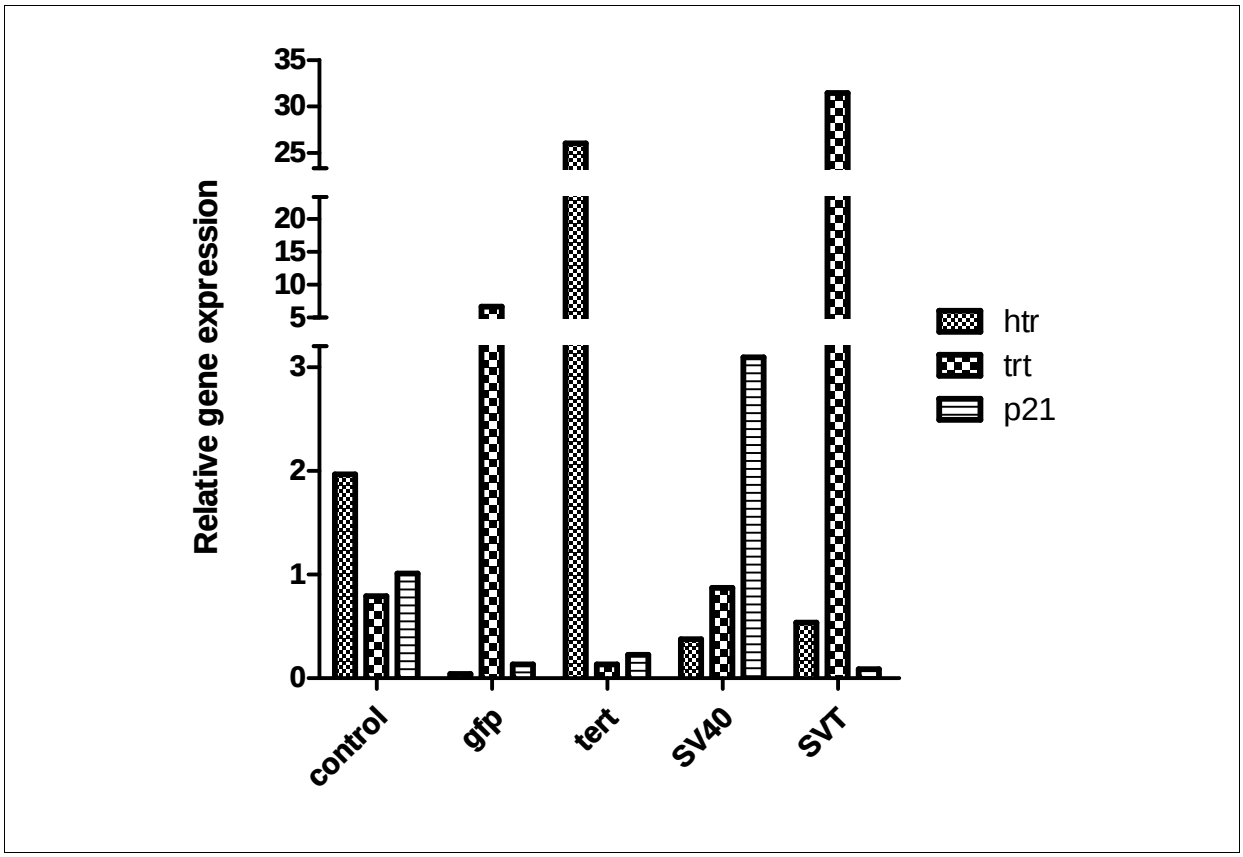


Figure 4.19: Relative expression of TERT, hTR and p21 gene in non-transfected normal, GFP transfected, TERT transfected, SV40 transfected and SVT transfected BM derived MSCs during the Real Time PCR.

The real time PCR data showed the variation of expression of proliferating gene hTR, TERT and senescence gene p21 in normal control and different transfected bone marrow derived MSCs. The SVT gene transfected bone marrow MSCs showed higher level of TERT and very less expression of p21 gene as compared to normal cells and other transfected cells. This data showed that the SVT gene transfected cells were in proliferating phase in late passage as well. These cells showed less expression of senescence marker in late phase signified that they were not aged till 30 population doubling. So, the SVT gene transfected BM MSCs was characterized as proliferating till late passage as compared to normal non-transfected, SV40, GFP and TERT gene transfected BM MSCs.

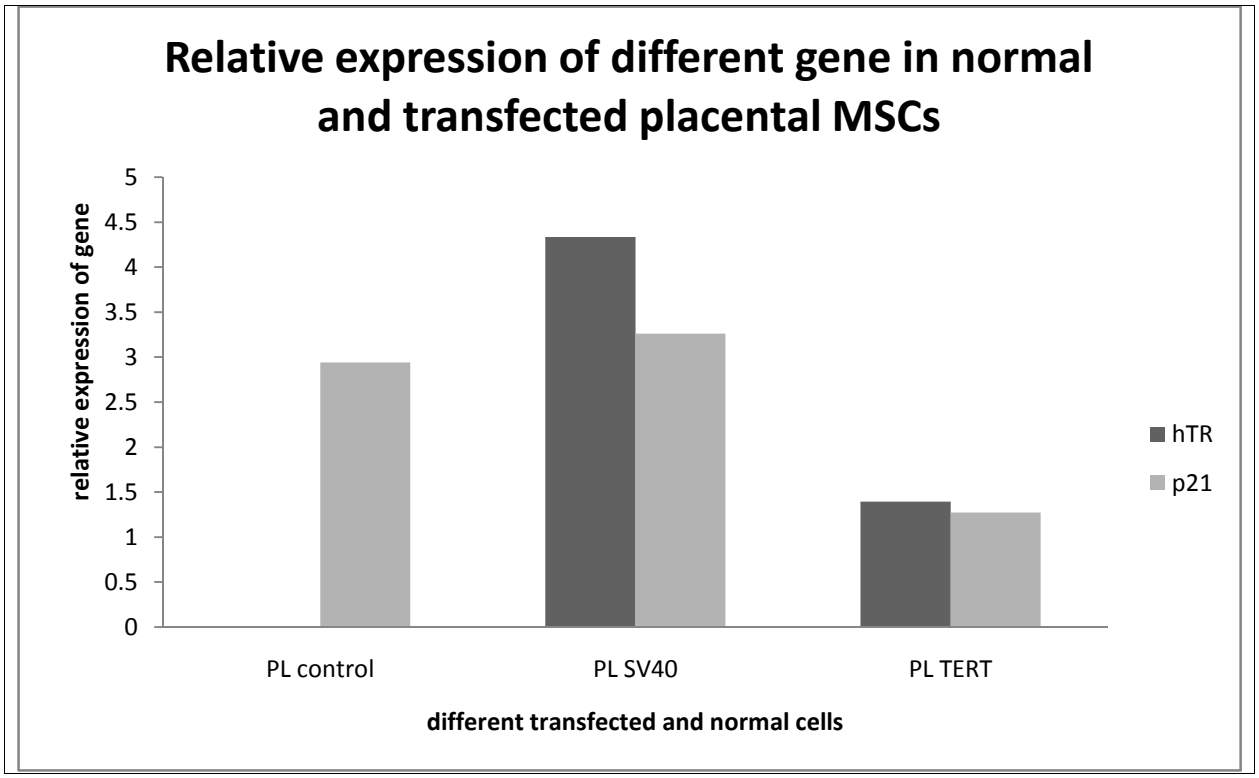


Figure 4.20: Relative expression of hTR and p21 gene in non-transfected normal, SV40 transfected and TERT transfected placental derived MSCs in Real Time PCR

The SV40 and TERT transfected placental MSCs showed good expression of hTR gene as compared to control. The control cells showed more senescence marker gene p21 and negligible expression of hTR gene during Real time estimation by using Real Time PCR. Due to technical difficulties, the TERT primer to PL MSCs, the Ct value showed undetermined. So we couldn't calculate its relative expression.

5.1 Isolation and plating of placental and bone marrow derived MSCs

Human MSC (hMSC) are becoming a basis of cell therapies because they can be readily isolated from patients, able to be expanded rapidly invitro, amenable to being genetically engineered to introduce genes of interest for therapy, not tumorigenic unless subject to stress or expanded extensively in culture. They also act as a part of natural repair system. MSCs have several mechanisms to reach the injured tissue and repair them. These mechanisms include differentiation into tissue-specific phenotypes, secretion of chemokine to enhance repair of damaged cells and stimulate proliferation of tissue endogenous stem cells (Wolfe M. *et al.,* 2008). Though the MSC have great therapeutic potential, primary cells reach senescence after a limited number of population doublings so researchers frequently need to re-establish fresh cultures from explanted tissue. It's a tedious process which can also add significant variation from one preparation to another. In order to have consistent material throughout a research project, researchers need primary cells with an extended replicative capacity or immortalized cells. Mesenchymal stem cells (MSCs) provide a source for fascinating models of differentiation cell therapy, and regenerative medicine. There are increasing reports that MSCs can be isolated from various adult mesenchymal tissues such as synovium, periosteum, skeletal muscle, and adipose tissue in addition to bone marrow. These MSCs have been assumed to be similar irrespective of their original tissue source since they all have self-renewal and multidifferentiation potential with common surface epitopes. However, the properties of MSCs can be affected by their preparations, which have not been properly controlled for in some studies (Sakaguchi Y.*et.al,* 2005).

5.2Transfection, Growth curve and morphological analysis

To overcome the limitations of replicative senescence, we tried to make the MSCs immortalized by using longevity genes. For immortalization, the cells were transfected with SV40, TERT and their combination and tested them by using various techniques. After the transfection and selection of the bone marrow and placenta derived MSCs cells, the population doubling was calculated for each passage during the cell culture. The normal and transfected cells showed variation in their cell proliferation capacity but yet aged. The SV40 plus TERT gene transfected bone marrow derived MSCs showed good proliferation as compared to normal cells and other transfected cells. This was confirmed by healthily growing non granular morphology of transfected cells till late passage. The population doubling and growth curve showed higher cell count and lesser population doubling time in SVT transfected bone marrow and TERT transfected placental MSCs. Generally, bone marrow derived MSCs has maximum 30 population doubling capacity (Banfi*et al*., 2000). But the SV40 plus TERT transfected bone marrow showed good proliferation results beyond the 30 population doubling as well. In placenta derived MSCs, the TERT gene transfected cells showed good proliferation capacity as compared to control and other transfected cells. Primarily cultured human cells have a finite

proliferative lifespan before they enter the Stage of permanent growth arrest, which is known as replicative senescence. However, on rare occasions a clone of cells could escape from the replicative senescence and give rise to an immortalized cell line. In order to maintain a stable biological property of cultured cells in vitro, many kinds of gene transfer methods have been developed to achieve the immortalization of cells, and SV40Tag transfection is a good alternative(WANG J.*et al.,* 2009).

5.3 Immunostaining of proliferation and ageing related markers

For the further confirmation of proliferation capacity of the cells was done by immunostaining using two protein markers: ki67 as a proliferation marker and p21 as a senescence marker. The Ki67 is good marker for the detection of proliferation of cells and p21 negatively regulates the cell progression by inhibiting the activity of cyclin/cdk complexes (Gardes*et al*., 1984, Sherr*et al.*, 2000). All early passage cells showed the higher expression of ki67 and lesser expression of p21. But in the late passage cells, the transfected cells showed better proliferating proteins ki67 as compared to normal control of bone marrow derived MSCs. Among the transfected cells, SV40 plus TERT transfected cells showed better ki67 and less p21 expression as compared to other transfected cells and normal cells. The TERT transfected placental MSCs showed good expression proliferation marker ki67 and less expression of p21 in late passage as compared to normal cells. This finding suggested that there was significant variation of expression of ageing related marker and proliferation related marker among transfected and normal MSCs during early and late passage. In recent years, MSC-based cell therapy and gene therapy have merged, by which the MSCs are genetically engineered with different vectors, including retrovirus (Chan *et al*., 2005; Marx *et al*., 1999), adenovirus (Gazit*et al*., 1999; Tsuda*et al*., 2003), and lentivirus (Lee *et al*., 2001). The engineered MSCs can be transplanted into animal models and the therapeutic gene products (e.g., BMP-2) expressed by these vectors in vivo potentially stimulate tissue repair/regeneration (Gazit*et al*., 1999; Tsuda*et al*., 2003). Although sustained expression can be achieved by these vectors, extinction of transgene expression often occurs as a result of vector degradation or gene silencing (e.g., DNA methylation). Besides MSCs, the MSC-derived osteogenic and adipogenic progenitors have been shown susceptible to virus-mediated genetic modification (Blum *et al*., 2004; Hung *et al*., 2004).

5.4 Cell cycle analysis by FACS and colony forming unit assay

For the further confirmation, cell cycle analysis and colony forming unit assay was done. The cell cycle analysis was done by staining DNA of 24 hours cultured cells by using Propidium iodide. In bone marrow derived MSCs, all cells had proportional distribution of DNA in M1, M2 and M3 phase of cell cycle in early passage. But in late passage cells, the distribution showed significance variation among different transfected cells and normal control cells. Among the transfected cells, SVTtransfected cells showed more numbers in synthetic phase and G2+M phase as compared to other transfected cells. TERT transfected cells also showed more

synthetic and proliferating stage of cell cycle as compared to control. In normal control bone marrow MSCs, more numbers of cells were in M1 phase and least numbers of cells in M2 and M3 phase. This variation showed that the SVT gene transfected cells showed proliferating and synthetic nature in late passage as well. But most of the cells were arrested in M1 phase in normal control cells; this concluded that these cells entered in senescence phase during late passage.

Colony forming unit assay is a good technique for the confirmation of proliferation capacity of single cell. In this study, the transfected bone marrow derived MSCs showed more proliferation than normal control cells. Among the transfected cells, more colonies with greater size were found in SV40 +TERT transfected cells. This variation showed that the SVT gene transfected cells had higher proliferating capacity. In placenta derived MSC, the TERT transfected cells showed more numbers of colonies with greater size as compared to normal and other transfected cells. But early cessation of transgene expression by these cells may occur before repair/regeneration becomes irreversible. Hence transduction of partially differentiated progenitors followed by implantation may extend the in vivo transgene expression to accelerate tissue regeneration (Kalajzic*et al*., 2001). In this regard, partially differentiated progenitors hold promise as a cell source alternative to MSCs for genebased tissue engineering (Lieberman *et al.,* 1999).

5.5 Real Time PCR analyses

The Real Time PCR was employed for the calculation and analysis of relative expression of proliferation specific marker i.e. TERT and hTR and senescence specific marker p21 in early and late passaged normal and transfected MSCs. In this investigation real time PCR result showed the higher expression of TERT and very less expression of p21 in SVT transfected bone marrow derived MSCs but in other normal MSCs showed higher relative expression of senescence associated marker p21 and lesser expression of proliferation associated marker TERT and hTR. This signified that the SVT transfected cells were highly proliferating and healthily growing as compared to normal and transfected cells. The TERT transfected cells also showed good relative expression of hTR and TERT gene as compared to control cells. The GFP transfected cells showed higher expression of hTR but negligible expression of TERT. For the real time quantification of gene good quality of RNA showing clear three bands was isolated and RT PCR was done for cDNA preparation. The quality of RNA and cDNA preparation also affects the quantification. Real Time PCR helps simultaneous measurements of gene expression in many different samples (Fink *et al*. 1998).

We have been able to select three clones with high proliferative capacity in SVT transfected cells when subjected these to antibody selection. These colonies show higher TERT expression levels with decline in p21. It needs to be tested further for longer periods of time to prove its immortality.

6. Summary

Human Mesenchymal Stem Cells are multipotent cells that can differentiate into multiple cell types and can be isolated from different sources such as human placenta, bone marrow, umbilical cord, adipose tissue etc. Though there are multiple uses of mesenchymalStem cells as seed cells in studies of tissue engineering and regenerative medicine, their clinical application is limited due to replicative senescence. So for the research purpose, the mesenchymal stem cells should be isolated frequently from different source because they have also limited proliferation capacity.To overcome vulnerability of replicative senescence and crisis of cells, immortalization of MSCs is one option. Cell immortalization is a very complicated cellular process and the exact biological mechanisms are still largely not well understood. Immortalization of human cells is often associated with reactivation of telomerase, a ribonucleoprotein enzyme that adds TTAGGG repeats onto telomeres and compensates for their shortening.

In this study, placental and bone marrow derived MSCs were used. By analysis of growth curve of transfected and normal bone marrow MSCs cells, the SVT transfected and TERT transfected cells showed better and constant growth as compared to control bone marrow MSCs till late passage. Among the transfected cells SVT gene transfected BM MSCs cells and TERT transfected placental MSCs showed more detectable expression of Ki67 and less expression of p21 (green) in late passage was observed during immunostaining. More numbers of BM MSCs cells which were transfected with SV40+TERT were in S and G2+M phase than G1 phase as compared to other cells at late passage during cell cycle analysis using FACS. The TERT transfected placental derived MSCs and SVT transfected BM MSCs showed bigger and more numbers of colonies as compared to other transfected and normal cells in colonization assay. The real time PCR data showed that the SV40+TERT transfected bone marrow MSCs showed higher level of TERT and very less expression of p21 gene as compared to normal cells and other transfected cells. This data showed that the SVT gene transfected cells were in proliferating phase in late passage as well. The TERT transfected placental MSCs showed higher level of hTR and less expression of p21 in real time PCR data. We have been able to select three clones with high proliferative capacity in SVT gene transfected cells when subjected these to antibody selection. These colonies show higher TERT expression levels with decline in p21. It needs to be tested further for longer periods of time to prove its immortality.

7. CONCLUSION

Mesenchymal stem cells (MSCs) are adult stem cells present in many tissues, such as bone marrow, adipose tissue and peripheral blood which are self- renewing, clonal precursors of non hematopoitic tissue able to differentiate into multiple mesodermal lineage cells, such as osteocytes, chondrocytes and adipocytes (Jiang Y. et al., 2002, Pittenger M.F. et al., 1999). For the research purpose, stem cell should be isolated frequently from different source because they have also limited proliferation capacity. After certain passaging it also become aged which make difficulties for tissue engineering. So for the remedy of the limitation we tried to make the MSCs immortal. We got three clones from the bone marrow derived MSCs and one clones of placenta derived MSCs. The relative expression level of proliferation marker HTR, TERT and senescence marker p21 gene in real time PCR of normal and different transfected cells confirmed the variation of expression level in normal and SVT transfected bone marrow and TERT transfected placenta derived MSCs. The antigenic expression of proliferation marker Ki67 and senescence marker p21 in normal and the selected clones in immunostaining showed additional support for confirmation. The SVT transfected BM MSCs showed higher expression of proliferation marker and lesser expression of senescence marker in late passage as compared to normal BM MSCs. The high peak of S phase and G2+M phase of SVT transfected BM MSCs in FACS analysis confirmed the cells were in healthy and highly dividing during late passage as well. The morphological appearance of SVT transfected BM and TERT transfected PL MSCs were healthier and less granular in late passage as well but normal MSCs became granular and elongated. This showed that the clones produce telomerase continuously that prevent the cells undergoing senescence. The colony forming unit of transfected cells with normal control gave supportive confirmation of immortality of SVT transfected clones of BM MSCs and TERT transfected PL MSCs. It can be illustrated that the lentiviral vector containing TERT and SV40 gene was integrated to the telomere complex of the MSCs during the transfection which deregulates the control mechanism of telomere complex due to which continuous production of telomerase enzymes which prevents the shortening of linear chromosome. The cells which had no integration were selected during Hygromycin selection process. The selected cells after selection were clone which was confirmed by various techniques i.e. Real Time PCR, immunostaing, FACS, colony forming unit, morphology, growth curve and population doubling in late passage etc.

Thus we have been able to select three clones with high proliferative capacity in SVT gene transfected cells when subjected these to antibody selection. These colonies show higher TERT expression levels with decline in p21. It needs to be tested further for longer periods of time to prove its immortality.

Recommendations

The research is preliminary work on cellular and molecular biology for the longevity of cellular life span. It initiated the strategies of cellular immortality and act as a baseline work for further research. Based on the outcome of this investigation following recommendation are suggested:

1) This investigation was done in six months duration so the immortal cells should be further confirmed by continuity of the cell up to very late passage with simultaneous selection.
2) The present study was done in vitro only. In vivo studies should be done in mice or other animal model to investigate and analysis of the real scenario and functional aspects of the cells.
3) The cell which was obtained in our investigation should be used for various clinical researches, drug testing and various other stem cell therapies in different models which help to confirm human application the cells in various deformities.

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Appendix 1: components of FBS (source: Price and Gregory, 1982)

Components	Concentration	Range
Total protein	3.8 g/100ml	3.2-7.0
Albumin	2.3 g/ 100 ml	2.0- 3.6
Endotoxin	0.36 ng/ ml	0.01-10.0
Haemoglobin	11.3 mg/ 100 ml	2.4-18.1
Cholesterol	31 mg/ 100 ml	12-63
Fatty acids	……..	……
Phospholipids	…….	…….
Triglycerides	……..	……
Glucose	125 mg/ 100 ml	85-247
Insulin	10 U/ml	6-14
Cortisone	0.5 g/ 100 ml	0.1-2.3
Trijodo-thyronine	119 ng/ 100 ml	56- 223
Thyroxine	12.1 ng/ 100 ml	7.8- 15.6
PTH	1718 pg/ ml	85-6180
PGE	5.91 ng/ ml	0.5-30.5
PGF	12.33 ng/ ml	3.8-42.0

Appendix 2: population doubling time of PL MSCs

Name of cell	Cumulative population doubling	Time
Pl Control	3.57	6
Pl Control	7.51	12
Pl Control	11.7	20
Pl Control	15.44	26
Pl Control	19.01	33
Pl Control	22.58	40
Pl Control	25.35	47
PL TERT	4.05	5
PL TERT	8.37	11
PL TERT	12.43	17
PL TERT	16.7	24
PL TERT	21.02	31
PL TERT	25.29	36
PL TERT	29.02	43
PL TERT	32.24	51
PL SV40	4.27	5
PL SV40	8.58	11
PL SV40	12.59	17
PL SV40	16.87	24
PL SV40	21.01	31
PL SV40	25.08	38
PL SV40	28.68	46
PL SV40	31.77	56

PL SV40	34.5	66

Appendix 3: population doubling time of BM MSCs

Name of cell	Cumulative P.D.	Time in days
BM MSCs control	0	0
BM MSCs control	3.67	7
BM MSCs control	7.55	14
BM MSCs control	11.58	21
BM MSCs control	15.75	28
BM MSCs control	20.24	35
BM MSCs control	23.7	42
BM MSCs control	26.8	49
BM MSCs GFP	0	0
BM MSCs GFP	3.67	7
BM MSCs GFP	7.57	14
BM MSCs GFP	11.56	21
BM MSCs GFP	15.67	28
BM MSCs GFP	20.17	35
BM MSCs GFP	32.68	42
BM MSCs GFP	26.78	49
BM MSCs SV40	0	0
BM MSCs SV40	3.7	7
BM MSCs SV40	7.6	14
BM MSCs SV40	11.71	21
BM MSCs SV40	15.82	28
BM MSCs SV40	20.44	35
BM MSCs SV40	24.04	42
BM MSCs SV40	27.8	49
BM MSCs TERT	0	0
BM MSCs TERT	3.82	7
BM MSCs TERT	7.97	14
BM MSCs TERT	12.13	21
BM MSCs TERT	16.4	28
BM MSCs TERT	21.01	35
BM MSCs TERT	24.56	42
BM MSCs TERT	27.99	49
BM MSCs SVT	0	0
BM MSCs SVT	3.77	7
BM MSCs SVT	7.93	14
BM MSCs SVT	12.25	21
BM MSCs SVT	16.56	28
BM MSCs SVT	21.21	35

BM MSCs SVT	25.41	42
BM MSCs SVT	29.7	49