STUDIES ON CYTOSKELETON, DIFFERENTIATION AND PHENOTYPIC PROPERTIES OF HUMAN MESENCHYMAL STEM CELLS

A Thesis

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by

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DECLARATION

I hereby declare that the contents of the research work described in the thesis entitled ***"Studies on cytoskeleton, differentiation and phenotypic properties of human mesenchymal stem cells"***, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, India, under the supervision of **Dr. Bithiah Grace Jaganathan.**

Whatever contributions from others, ideas, techniques, quotations or any other materials are involved, every effort has been made to indicate those clearly with due reference to literature. All collaborative work, discussions and help during experiments are duly acknowledged. Mr. Atul Kumar is hereby acknowledged for performing the phosphorylation studies on differentiated MSC.

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CERTIFICATE

This is to certify that the work described in the thesis entitled ***"Studies on cytoskeleton, differentiation and phenotypic properties of human mesenchymal stem cells"***, submitted by Himangshu Sonowal (Roll no: 09610617) to Indian Institute of Technology, Guwahati, India, for the award of the Degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, India.

The present thesis or any part thereof has not been submitted elsewhere for award of any other degree or diploma.

February, 2015

Dr. Bithiah Grace Jaganathan

Thesis supervisor

List of Tables

List of Figures

List of Abbreviations

ALL: Acute lymphoblastic leukemia

ALP: Alkaline phosphatase

ALS: Amyotrophic Lateral Sclerosis

AML: Acute myeloid leukemia

bFGF: Basic fibroblast growth factor

BM: Bone marrow

BMMSC: Bone marrow mesenchymal stem cells

BMP-2: Bone morphogenetic protein-2

CAF: Carcinoma-associated fibroblasts

Cbfa-1: Core binding factor-1

CCL5: Chemokine (C-C motif) ligand 5

CD: Cluster of differentiation

cDNA: Complementary DNA

CFU-E: Colony forming unit-erythroid

CFU-F: Colony forming unit-fibroblasts

CLL: Chronic lymphocytic leukemia

CML: Chronic myeloid leukemia

CXCL-12: Chemokine (C-X-C motif) ligand 12

CXCR4: Chemokine (C-X-C motif) receptor 4

CYD: Cytochalasin-D

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EGF: Epidermal growth factor

ERK: Extracellular-signal-regulated kinases

FAK: Focal adhesion kinases

FITC: Fluorescein-isothiocyanate

Flt-3: FMS-like tyrosine kinase-3

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GM- CSF: Granulocyte macrophage colony-stimulating factor

GvHD: Graft-versus-host disease

HLA: Human leucocyte antigen

HSC: Hematopoietic stem cell

IBMX: 3-Isobutyl-1-methylxanthine

ICAM-1: Intercellular adhesion molecule 1

IFN-β: Interferon beta

IGF-1: Insulin-like growth factor 1

IL-2: Interleukin-2

IL-6: Interleukin-6

IL-8: Interleukin-8

LSC: Leukemic stem cell

LTC-IC: Long term culture initiating cells

M-CSF: Macrophage colony-stimulating factor

MDS: Myelodysplastic syndrome

MFI: Mean fluorescence intensity

MLD: Metachromatic Leukodystrophy

MM: Multiple myeloma

MSC: Mesenchymal stem cell

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NGF Receptor: Nerve growth factor receptor

NHL: Non-Hodgkin's Lymphoma

p38MAPK: p38 mitogen-activated protein kinases

PBS: Phosphate buffered saline

PE: Phycoerythrin

PKCα: Protein kinase C alpha

PPAR gamma: Peroxisome proliferator-activated receptor

RBC: Red blood cell

RNA: Ribonucleic acid

ROCK: Rho associated protein kinases

RPMI-1640: Roswell Park Memorial Institute-1640

RUNX2: Runt-related transcription factor 2

Sca-1: Stem cell antigen-1

SCF: Stem cell factor

SDF-1: Stromal cell-derived factor1

SEM: Scanning electron microscope

SLAM: Signaling lymphocytic activation molecule

SSEA-4: Stage-specific embryonic antigen-4

TGF-β1: Transforming growth factor beta-1

TNF-α: Tumor necrosis factor alpha

TRITC: Tetramethyl rhodamine isothiocyanate

US-FDA: US-Food and Drug Administration

VCAM: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

Abstract

Mesenchymal Stem Cells (MSC) have the potential for use in various clinical and therapeutic applications. Properties like multilineage differentiation potential, secretion of various cytokines etc. are being utilized for use of MSC in various therapeutic applications. In the present study, the differentiation and phenotypic properties of MSC isolated from bone marrow (BM) was studied. In the first part of the thesis, the role of actin cytoskeleton during adipogenic and osteogenic differentiation of MSC was elucidated. Differential actin polymerization was observed during adipogenic and osteogenic differentiation of MSC, which was found to be a very early event during differentiation. Inhibition of actin polymerization during osteogenic differentiation led to decreased osteogenesis and inhibition of actin polymerization during adipogenesis led to enhanced adipogenesis. It was observed that actin polymerization might regulate osteogenic differentiation of MSC by signaling through p38MAPK pathway.

In the second part of the thesis, the differentiation and phenotypic properties of MSC isolated from BM of patients with hematologic malignancies (HM) was studied. Decrease in osteogenic differentiation and CD90 surface expression was observed in MSC isolated from patients diagnosed for Acute Lymphocytic Leukemia (ALL), Acute Myeloid Leukemia (AML) and Multiple Myeloma (MM). MSC form an important component of the hematopoietic stem cell niche in the BM. Signals from the niche regulate the properties of hematopoietic stem cells (HSC) and maintain a balance between self-renewal and differentiation of HSC. The changes observed in differentiation and phenotype of MSC might be due to alterations in properties of MSC in the BM during HM. An interesting observation in MSC isolated from Acute Lymphocytic Leukemia (ALL) patients undergoing therapy was that, CD90 surface expression was found to be of comparable levels to MSC from normal samples. *In vitro* co-culturing of MSC isolated from normal BM with leukemic cell lines HL-60 and THP-1 resulted in a decrease in CD90 expression and osteogenic differentiation of MSC. The decrease in osteogenic differentiation and CD90 expression in MSC from BM of HM might have important implications in the BM microenvironment during normal and disease conditions like leukemia, which has to be further investigated.

Synopsis

Hematopoiesis is the process by which different blood cells are produced by the hematopoietic stem cells (HSC) present in the bone marrow. Bone marrow is the major site of hematopoiesis wherein all the blood cells are produced in the adult body. The hematopoietic stem cells are found in a homeostatic microenvironment in the bone marrow termed the "hematopoietic stem cell niche". The hematopoietic stem cell niche is a complex network of cells, extracellular matrix proteins, cytokines and growth factors. Various cell types such as adipocytes, osteoblasts, vascular endothelial cells, osteoclasts, mesenchymal stem cells (MSC) etc. form important cellular component of the hematopoietic stem cell niche. Signals from the niche regulate the balance between hematopoietic stem cell self-renewal and differentiation.

The non-hematopoietic stromal compartment in the bone marrow is composed mainly of mesenchymal stem cells and their derivatives. Mesenchymal stem cells are multipotent stem cells first isolated from the bone marrow. Apart from the bone marrow these cells can also be isolated from cord blood, cartilage, adipose tissue, synovial fluid, dental pulp, muscles, tendons etc. The adipocytes and osteocytes derived from mesenchymal stem cells play an important role in maintaining a homeostatic niche in the bone marrow. Disrupting the balance between stromal populations in the bone marrow disrupts the balance in hematopoiesis in the body.

MSC were being used for various clinical applications like treatment of skeletal defects, facilitating hematopoietic recovery during bone marrow transplantation after ablative chemotherapy, facilitating allogenic engraftment etc. In various clinical complications like Hurler's Syndrome, Amyotrophic Lateral Sclerosis (ALS), Metachromatic Leukodystrophy etc., patients infused with MSC showed significant progress in disease recovery. MSC were shown to migrate to sites of injury and facilitated wound healing and tissue recovery by directly differentiating into respective tissue types or modulating the microenvironment around the tissue to hasten the repair process. On the contrary, some reports also show that MSC facilitated tumor growth and transplanted MSC themselves gave rise to tumors after engraftment.

The properties of mesenchymal stem cells *in vivo* were affected by various factors. Extracellular matrix proteins and signals from the microenvironment have been

found to control the differentiation of mesenchymal stem cells. Understanding the factors responsible for or directing MSC differentiation is important to use these cells efficiently for therapy.

Mesenchymal stem cells from the bone marrow have multilineage differentiation potential and express the cell surface markers CD13, CD29, CD73, CD90, CD105, CD146, CD166 etc. and are negative for hematopoietic and endothelial lineage markers such as CD11, CD14, CD31, CD33, CD34, CD45 and CD133.

In the current study, the initial events that control the adipogenic and osteogenic differentiation of mesenchymal stem cells were analyzed. MSC underwent significant changes in morphology during adipogenic and osteogenic differentiation. Undifferentiated MSC were spindle shaped but after 14 days of induction into adipogenic or osteogenic lineages, adipo-differentiated MSC became globular and oil droplets could be seen inside the cell and osteo-differentiated MSC attained an angular shape with increased cell extensions.

In addition to morphological changes, there were significant differential changes in adipo-differentiated and osteo-differentiated MSC. In undifferentiated cells, actin filaments appeared parallel with filaments running from one end of the cell to the other. This pattern of cytoskeleton organization remained unaltered during different passages in culture. When MSC were differentiated into osteocytes, these parallel filaments reorganized to from peri-nuclear filaments containing increased stress fibers. On the other hand, during adipogenic differentiation, the actin filaments become discontinuous and formed a criss-cross network and when oil droplets were formed inside the cell, actin filaments formed a disrupted network around the oil droplets. The results showed that actin modification was a very early event during differentiation. Actin cytoskeleton modifications occurred as early as 12 hours after addition of induction media, much before the differentiation genes were expressed by the cells (undocumented observation). Differentiation genes such as *OSTEOCALCIN* for osteocytes and *ADIPONECTIN* for adipocytes were expressed only after 48 hours of addition of induction media.

To determine whether actin modifications have any active role to play during adipogenic or osteogenic differentiation of MSC, actin polymerization was inhibited by addition of Cytochalasin-D (CYD). CYD inhibits actin polymerization by preventing

formation of F-actin polymers. The effect of CYD on actin polymerization was reversible and the effect could be reverted on withdrawal of CYD from the media. Interestingly, inhibition of actin polymerization during differentiation led to decrease in osteogenic but increase in adipogenic differentiation of MSC. Treatment with CYD for 7 days during osteogenic differentiation led to ~55% decrease in osteogenic differentiation as determined by alkaline phosphatase staining (59.4%±5.4% differentiation during normal osteogenic induction versus 4.4%±2.9% differentiation in induction media containing CYD). This decrease in osteogenic differentiation was further evident when cells were treated for 14 days with CYD during osteogenic differentiation with 100%±18.5% osteogenic differentiation during normal differentiation versus 9.5%±1.5% differentiation on treatment with CYD. There was little or no actin polymerization in the samples treated with CYD during osteogenic differentiation. Even 24 hours treatment of CYD during osteogenic differentiation during 3 days induction period led to 50% decrease in osteogenic differentiation. However, when the cells were allowed to recover for 48 hours without CYD in the induction media, it was sufficient to allow the polymerization of actin and polymerized F-actin filaments were seen in the differentiating cells. Furthermore when the cells were treated with CYD for 3 days and allowed to recover for 4 days in induction media without CYD, there was a 3-fold reduction in osteogenic differentiation potential whereas actin cytoskeleton rearrangement appeared normal.

In contrast, when the cells were treated with CYD during adipogenic differentiation, there was a significant increase in Oil-red-O positive adipocytes. Three days of initial CYD treatment during 7 days of adipogenic induction was sufficient to increase adipogenic differentiation by ~30%. During the recovery period without CYD in the induction media, the actin cytoskeleton reverted back to its cross linked form as seen in normal adipocytes. Treatment with CYD for 7 days, i.e., throughout the adipogenic induction period led to 3-fold increase in adipogenic differentiation compared to untreated controls. Further treatment with CYD for 14 days during adipogenic induction resulted in a 2.8-fold increase in adipogenic differentiation of MSC compared to control 14 days induction without CYD. Lack of actin polymerization was seen in the cells treated with CYD for 7 days or 14 days after staining with Phalloidin TRITC. Consistent with the increase in Oil-red-O positive cells, subsequent increase in mRNA expression

levels of *ADIPONECTIN* and *PPAR gamma* was also observed in the CYD treated cells during adipogenic differentiation.

Hence, from the above observations it was clear that actin modifications was an early event during adipogenic and osteogenic differentiation. This was further confirmed by inhibition of actin polymerization with CYD prior to induction of differentiation and thereafter differentiating the cells into adipocytes and osteocytes. CYD pre-treatment alone was sufficient to increase the expression of *PPAR gamma* and decrease *OSTEOCALCIN* expression levels in MSC in normal growth media in the absence of induction media. Pre-treatment of MSC with CYD during normal growth media and then inducing the cells to differentiate in the absence of CYD resulted in increased adipogenic differentiation and decreased osteogenic differentiation. These results indicate that actin plays an important role in MSC differentiation and integrity of actin cytoskeleton was an important contributing factor for controlling adipogenic and osteogenic differentiation. Further analysis of the molecular pathways affected by actin modifications during differentiation showed that actin modifications signal through p38MAPK pathway to regulate osteogenic and adipogenic differentiation of MSC.

To briefly conclude, the results show that actin modification was an important initial event that controls MSC differentiation.

Malignancies such as leukemia and lymphoma are disorders of hematopoietic system. The hematopoietic cells either due to mutations or exposure to genotoxic agents become abnormal, divide uncontrollably and are not able to carry out their normal cellular functions. These abnormal cells were also found to alter the properties of different stromal populations in the bone marrow. The altered marrow microenvironment cells might help the leukemic cells in proliferation and evasion of chemotherapy resulting in disease relapse. Understanding the changes in properties of cells of the microenvironment during hematologic malignancies might help in understanding the disease progression and to design therapeutic strategies for complete eradication of leukemic cells.

In the second part of the study, MSC were isolated from bone marrow of patients with different hematologic diseases and their phenotypic properties were analysed. MSC were isolated from bone marrow of patients diagnosed for acute myeloid leukemia

(AML), chronic myeloid leukemia (CML), multiple myeloma (MM), aplastic anemia (AA) and acute lymphoblastic leukemia (ALL). MSC from all the samples displayed spindle-shaped morphology and did not possess any chromosomal abnormality. The isolated MSC readily differentiated into osteocytes and adipocytes but osteogenic differentiation potential was variable in MSC from different patients. A 40%-50% reduction in osteogenic differentiation capacity was seen in MSC isolated from samples diagnosed for AML, ALL and AA. Age related reduction in osteogenic potential was observed where MSC isolated from aged donors had lower osteogenic differentiation compared to young donors.

Expression of cell surface markers CD13, CD73, CD90 and CD105 were analysed in MSC isolated from different patients by flow cytometry. A significant decrease in CD90 cell surface expression was seen in MSC from patients diagnosed for AML, MM, ALL and AA. Changes in CD90 expression was also determined during osteogenic and adipogenic differentiation of MSC. There was a significant increase in CD90 expression within 24 hours after addition of induction media. However, the levels returned to normal thereafter.

To test whether decrease in CD90 expression in MSC was disease induced, MSC from normal bone marrow were co-cultured with human monocytic leukemia cell line THP-1 and human promyelocytic leukemia cell line HL-60 *in vitro*. Although no changes in CD90 expression was seen initially, a significant down-regulation was seen after 4 weeks of co-culture with leukemic cells.

Thus, these results show that MSC differentiation could be controlled by actin modification. In addition, MSC isolated from patients with hematologic disorders have an altered phenotype and osteogenic differentiation capacity.

Table of Contents

1.1.Mesenchymal stem cells

Mesenchymal stem cells are one of the most studied adult stem cells. They have multilineage differentiation potential and can be easily isolated from various sources in the body, expanded and manipulated in *ex vivo* cultures (Hass, Kasper et al. 2011). Mesenchymal stem cells are non-immunogenic and have immunosuppressive properties. These can be used for allogenic transplantation without immune suppression (Abumaree, Al Jumah et al. 2012; De Miguel, Fuentes-Julian et al. 2012). MSC also secrete many biologically active molecules which have been reported to serve important functions both *in vivo* and *in vitro* (Schinkothe, Bloch et al. 2008). MSC have been reported to be important components of the hematopoietic stem cell niche in the bone marrow and helps to maintain hematopoietic stem cells in the bone marrow (Frenette, Pinho et al. 2013). Use of MSC for therapeutic applications don't raise any ethical concerns as these can be isolated from various sources from the patient's own body and can be expanded to large numbers *in vitro*. Because of the ease of isolation and large scale *in vitro* expansion capabilities along-with paracrine effects, MSC have attracted great interest as potential candidates for tissue engineering and therapeutic applications (Brooke, Cook et al. 2007). However, many fundamental aspects like *in vivo* cellular identity and localization and exact functions *in vivo* are yet to be properly understood (Nombela-Arrieta, Ritz et al. 2011; da Silva Meirelles, Caplan et al. 2008). The properties of mesenchymal stem cells have to be properly understood for efficient use of these cells for tissue engineering and therapeutic applications.

1.1.1. Isolation and source of mesenchymal stem cells

Mesenchymal stem cells were first reported by Friedenstein et al., (1966) from the bone marrow. They had reported fibroblast like adherent population of cells with osteogenic differentiation potential from a mixed population of cells isolated from rat bone marrow (BM). A heterogeneous population of cells was observed after few days of seeding the bone marrow cells and after around 4-5 days, fibroblast cells with potential to differentiate into bone or cartilage were observed. These cells were termed colony forming unit-fibroblast CFU-F (Friedenstein, Piatetzky et al. 1966; Friedenstein, Gorskaja et al. 1976). Following reports of Friedenstein et al., MSCs have been isolated from a variety of other sources like cartilage, synovial fluid, dental pulp, muscles,

tendons etc. MSC were also isolated from a variety of fetal tissues, umbilical cord blood etc. (Gronthos, Mankani et al. 2000; Hu, Liao et al. 2003; in't Anker, Noort et al. 2003; Romanov, Svintsitskaya et al. 2003). Sarah et al. isolated MSC from adult BM, cord blood and peripheral blood and found that the frequency of MSC was highest in the BM. The frequency of MSC in adult bone marrow was found to be approximately one in 3.4 x 10^4 BM mononuclear cells (Wexler, Donaldson et al. 2003).

The most widely used approach for the isolation of mesenchymal stem cells is their ability to adhere to plastic surfaces of tissue culture flask. Initially heterogeneous population of cells are observed after seeding the bone marrow mononuclear cells, but after about 2-3 weeks of culture, homogenous population of cells are observed which express cell surface markers like CD13, CD29, CD44, CD73, CD71, CD90, CD105, CD146, CD166, STRO-1 and are negative for CD11b, CD34, CD45 and had potential to differentiate into osteocytes, adipocytes, chondrocytes etc. (Simmons and Torok-Storb 1991; Majumdar, Thiede et al. 1998; Pittenger, Mackay et al. 1999). As per directions laid down by International Society for Cellular Therapy (ISCT), positivity for CD73, CD90, CD105 and negativity for CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR along-with multilineage differentiation potential is a must to meet the criteria to be termed as MSC (Dominici, Le Blanc et al. 2006). MSC can be expanded *in vitro* in DMEM containing 10% fetal bovine serum without any loss of proliferative or differentiation potential (Ayatollahi, Salmani et al. 2012).

Table1.1. Different sources of mesenchymal stem cells reported in humans

Site of isolation	Reference
Adipose tissue	(Zuk, Zhu et al. 2001; Zuk, Zhu et al. 2002)
Pancreas	(Hu, Liao et al. 2003)
Bone marrow	(Pittenger, Mackay et al. 1999)
Liver	(Campagnoli, Roberts et al. 2001)
Blood	(Campagnoli, Roberts et al. 2001)
Amniotic fluid	(In 't Anker, Scherjon et al. 2004)
Umbilical cord blood	(Zhang, Hirai et al. 2011)
Dental tissues	(Akiyama, Chen et al. ; Gronthos, Mankani et al. 2000)
Synovial fluid	(Harvanova, Tothova et al. 2011)

Table.1.2. Surface antigens expressed on cultured human mesenchymal stem cells

Surface antigens	Expression levels	Reference
CD13, CD29, CD44, CD49e, CD73, CD90, CD146, CD166, SSEA-4, STRO-1	50-100% expression	(Noort, Oerlemans et al. ; Rozemuller, Prins et al. 2012)
CD271	<5% expression	(Rozemuller, Prins et al. 2010)
CD31, CD34, CD45, CD117, Sca-1	No expression	(Noort, Oerlemans et al. ; Rozemuller, Prins et al. 2012)

1.1.2. Differentiation and proliferation potential of mesenchymal stem cells

MSC have multilineage differentiation potential and can be readily cultured and expanded *in vitro* without loss of proliferation or multilineage differentiation potential. MSC have potential to differentiate into cells of various mesodermal lineages like osteocytes, adipocytes, chondrocytes (Pittenger, Mackay et al. 1999; Chamberlain, Fox et al. 2007) and into cells of other lineages like vascular smooth muscles (Galmiche, Koteliansky et al. 1993), endothelial cells (Oswald, Boxberger et al. 2004), neurons (Woodbury, Schwarz et al. 2000), hepatocytes (Pournasr, Mohamadnejad et al. 2011) etc. MSC can be proliferated in culture until 10-38 population doublings, but the differentiation and proliferation potential of MSC has been reported to decline with progressive passaging in culture (Bara, Richards et al. 2014; Digirolamo, Stokes et al. 1999). MSCs in culture are genetically stable and were reportedly being used for various clinical and therapeutic applications without any ethical limitations (Caplan 1991; Short, Brouard et al. 2003; Barry and Murphy 2004). Although the safety of *in vitro* culture expanded MSC was questioned in the beginning, their safety and efficacy was highlighted in many clinical trials thereafter.

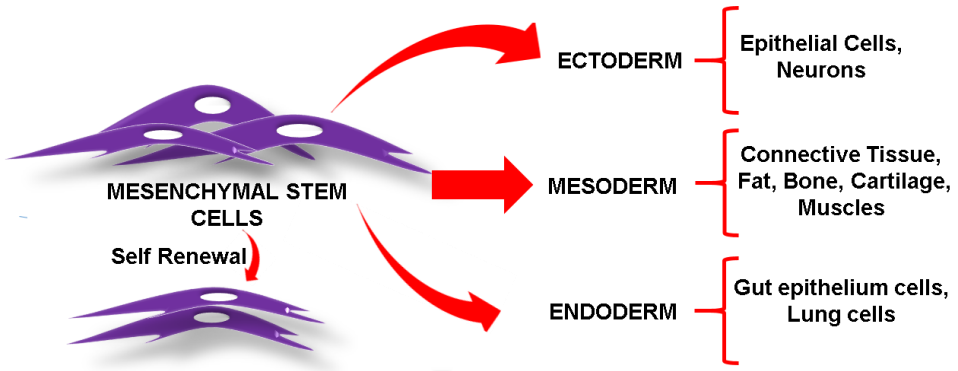


Figure.1.1. Multilineage differentiation potential of mesenchymal stem cells. Apart from self-renewal, MSC has potential to differentiate into various ectodermal, mesodermal and endodermal lineages (Adapted from: Amy M. DiMarino et al., Frontiers in Immunology, 2013, Sep 4;4:201).

1.1.3. Therapeutic and clinical applications of mesenchymal stem cells

Mesenchymal stem cells can be used for a wide variety of therapeutic applications. Use of MSC have been reported in various clinical and pre-clinical therapeutic applications like facilitating HSC engraftment in the BM, as immune modulators and in regenerative medicine as enhancers of endogenous repair mechanisms (Frenette, Pinho et al. 2013; Krampera, Pizzolo et al. 2006; Giordano, Galderisi et al. 2007). Transplanted MSC helped in tissue repair and regeneration and also formed an important component of the microenvironment secreting factors which helped in repair or rejuvenate diseased cells and tissues (Caplan and Dennis 2006; Hwang, Zhang et al. 2009). The first documented use of MSC for regenerative therapy in humans was for skeletal tissue repair. MSC were successfully used for the treatment of Osteogenesis imperfecta (OI) in children. Accelerated increase in patients bone mineral density and growth velocity during a 6-month follow up period was seen in patients infused with MSC (Horwitz, Gordon et al. 2002). Apart from this, MSC were reported to have wide range of potential applications for repair of cartilage defects, skin tissue engineering, cardiac tissue engineering etc. (Dehghanifard, Shahjahani et al. 2013; Marion and Mao 2006).

In Crohn's disease of gastrointestinal tract, infusion of autologous adipose tissue derived MSC led to successful closure of fistulas in MSC infused patients (Dalal, Gandy et al. 2012). Autologous MSC infusion in patients with ischemic stroke led to significant

clinical improvement. Several ongoing trials with autologous bone marrow derived MSC for the treatment of patients with chronic ischemia, acute myocardial infarction and patients suffering from myocardial contractility problems had yielded positive results and trials are further being carried forward for large scale practical applications (Jin, Zhao et al. 2011; Piepoli, Vallisa et al. 2010; Pittenger, Mackay et al. 1999; Bang, Lee et al. 2005; Katritsis, Sotiropoulou et al. 2005; Kunter, Rong et al. 2007).

MSC form an important component of the hematopoietic stem cell niche in the bone marrow and were reported to support hematopoietic stem cells. MSC secrete cytokines important for hematopoiesis and facilitate hematopoietic stem cell engraftment during transplantation. Rapid hematopoietic recovery was observed in breast cancer patients infused with MSC along-with autologous peripheral blood progenitor cells after chemotherapy. No long term toxicity was observed and the patients displayed a disease free survival (Koc, Gerson et al. 2000). MSC were also reported to support the growth of hematopoietic stem cells during *ex vivo* culture and were found to secrete many regulatory molecules and cytokines important for hematopoietic maintenance (Li and Wu 2011).

Table.1.3. HSC regulatory factors secreted by MSC

Secreted factors	Functions
CXCL12 (SDF-1)	Adhesion, expansion, migration and homing of hematopoietic stem cells Regulating production of inflammatory cytokines and chemokines
Flt3 ligand	Regulation of hematopoietic growth, maintain HSC proliferation and self-renewal
GM-CSF	HSC engraftment
SCF	HSC proliferation, self-renewal, hematopoietic growth, HSC engraftment
VCAM, E-selectin, collagen-I, fibronectin	HSC homing and adhesion

MSC were also reported to have immunosuppressive and immunomodulatory properties that makes them promising candidates for the treatment of autoimmune and inflammatory disorders (Gebler, Zabel et al. 2012; Le Blanc and Mougiakakos 2012). The immunomodulatory effects of mesenchymal stem cells were reported to be either

due to direct modulation of T-cell response or cell-cell interactions or via secreted soluble factors (Di Nicola, Carlo-Stella et al. 2002; Krampera, Glennie et al. 2003).

Co-transplantation of MSC helped in the treatment and prevention of graft versus host disease (GvHD). Infusion of MSC during allogenic transplantation greatly enhanced allogenic engraftment. Infusion of MSC showed significant improvement in cases of terminal graft versus host disease (GvHD) also. Even in cases of steroid resistant severe GvHD, transplantation of MSC during allogenic transplantation led to significantly lower transplantation related mortality in MSC infused patients compared to non-MSC recipient patients (Baron and Storb 2012; Le Blanc, Rasmusson et al. 2004; Le Blanc, Frassoni et al. 2008). Use of MSC has also shown promise for therapy of various neurological complications like Hurler's Syndrome, Metachromatic Leukodystrophy (MLD), Amyotrophic Lateral Sclerosis (ALS) etc. Patients diagnosed for Hurler's syndrome and Metachromatic Leukodystrophy (MLD) who received MSC infusion showed significant improvement in nerve conduction velocities and increased bone mineral density (Koc, Day et al. 2002; Mazzini, Fagioli et al. 2003).

Mesenchymal stem cells have migratory potential and can be used for targeted drug delivery to tumor cells and cancer suppression by the release of immunomodulatory and anti-inflammatory molecules. Apart from migration to sites of tissue injury to facilitate tissue repair, MSC were reported to migrate to tumor sites which allow the use of these cells as vehicles for drug delivery (Kidd, Spaeth et al. 2009). MSC showed tropism to gliomas and MSC were found to be attracted towards gliomas through cytokine networks. Cytokines like VEGF, IL-8, TGF-β1 secreted in the tumor sites attracted MSC to the tumor sites. MSC efficiently homed into the tumor sites regardless of the site of injection (Birnbaum, Roider et al. 2007; Kidd, Spaeth et al. 2009). This property of MSC can be efficiently exploited for targeted drug delivery. MSC can also be either engineered to produce inhibitory chemokines and cytokines like IL-2 or IFN-β, which have the potential to stimulate T-cells and produce anti-tumor effects (Ho, Toh et al. 2013). MSC were also efficiently engineered to deliver drug loaded nanoparticles (Gjorgieva, Zaidman et al. 2012; Hu, Fu et al. 2010; Yeo, Lai et al. 2013) and prodrug converting enzymes to tumors sites for effective therapy (Sun, Nong et al. 2011). Elegantly modified nanocarriers for drug delivery which have enhanced uptake and release capability have been developed for efficient drug delivery. These modified nano-

particles were easily taken up by the cells and can be made to efficiently deliver their therapeutic compounds at specific sites for effective therapy (Gao, Zhang et al. 2013; Hu, Fu et al. 2010).

MSC engineered to produce cytokines were found to be effective in producing anti-proliferative effects in many tumors (Shah 2012). Cytokines like IFN-β or IL-12 or IL-18 produced by engineered MSC were effective in exerting inhibitory effects over melanoma cell lines (Studeny, Marini et al. 2002), gliomas (Kosztowski, Zaidi et al. 2009), renal carcinomas (Gao, Ding et al. 2010) etc. However, the use of MSC for therapeutic applications has various pros and cons which have to be addressed and overcome before these cells can be efficiently used for their potential highlighted therapeutic applications. Apart from tissue engineering applications for skin, bone and cartilage repair, mesenchymal stem cell therapy have been reported to be of great hope for the treatment of type-1 diabetes, liver diseases, Parkinson's disease, cardiac myopathies and also various old age associated degenerative disorders (Nishimura and Takahashi 2013; Qi, Feng et al. 2012; Sanganalmath and Bolli 2013; Muraca, Gerunda et al. 2002).

1.2. MSC for therapeutic applications: the challenges

Stem cells are an important tool for regenerative medicine and therapy which eliminates the need for organ replacement. Regenerative medicine aims at promoting organ repair and regeneration in vivo by using multipotent stem cells. Stem cells take part in the repair process by either differentiating into the native tissue types or by paracrine effects where the stem cell secreted factors may initiate the repair and regeneration process. Stem cell therapy has enormous potential for the treatment of certain diseases which were previously considered incurable.

Autologous stem cells are the cell of choice for stem cell therapy and regenerative medicine as they eliminate the need to suppress the recipient's immune system for minimizing allogenic rejection. This is where mesenchymal stem cells come into light as the most preferred candidate stem cell for various therapeutic applications as they have multilineage differentiation potential and are non-immunogenic and don't elicit any immune response.

Although MSC has lot of therapeutic potential, and has been designated by US-FDA as safe for clinical transplantation in human subjects, certain clinical studies have shown long term potential risk associated with MSC. The process of MSC initiating tissue repair in regenerative medicine and therapy involves either one of the following approaches:

1. Replacement of tissue by multipotent differentiation (Quevedo, Hatzistergos et al. 2009).
2. Immunomodulatory, anti-inflammatory and anti-apoptotic affects (Aggarwal and Pittenger 2005; Meirelles Lda, Fontes et al. 2009).
3. By secretory functions and releasing molecules which help in tissue repair and regeneration (Baraniak and McDevitt 2010; Du, Wei et al. 2013).

But the exact mechanisms taking place *in vivo* in the repair and regeneration process are yet to be clearly understood. All these factors need to be properly addressed before MSC can be declared safe for clinical applications. A brief account of the problems associated with use of mesenchymal stem cells for clinical applications is presented below.

1.2.1. Differentiation of transplanted MSC *in vivo*

The process of *in vivo* differentiation of mesenchymal stem cells and the manner in which they carry out therapeutic responses are not yet clearly understood. Contrary to the reported evidences which showed that the injected stem cells themselves differentiate into the concerned tissue types, some reports showed that MSC themselves don't differentiate, but they modulate the local responses in the tissue microenvironment and promoted wound healing and regeneration by either modulating inflammatory responses (Kode, Mukherjee et al. 2009) or released various growth factors and promoted angiogenesis facilitating tissue repair (Tang, Zhao et al. 2004). For long term safety and efficacy of transplantation, the cellular and extracellular cues which might be important for maintaining cellular behavior has to be properly understood or else clinical failures in various transplantation experiments are bound to happen (Fong, Gauthaman et al. 2010; Goldring, Duffy et al. 2011; Amariglio, Hirshberg et al. 2009).

Signals from the microenvironment or *in vivo* conditions were shown to direct the fate of transplanted mesenchymal stem cells. Multiple factors in the *in vivo* niche have been postulated to regulate the cell fate of stem and progenitor cells (Discher, Mooney et

al. 2009). When injected into appropriate niches, depending upon the cues from the microenvironment, MSC differentiate into the concerned tissue types. Importance of microenvironmental cues in directing differentiation were shown by De Bari et al., when synovial membrane derived MSC were not able to form cartilage even after pre-induction to chondrogenesis before transplantation. While the same cells during *in vitro* culture conditions showed expression of chondrogenic markers indicative of cartilage differentiation (De Bari, Dell'Accio et al. 2004). Even formation of bone tissue in heart by mesenchymal stem cells after infusion for myocardial infarction was reported (Breitbach, Bostani et al. 2007). Hence, the *in vivo* differentiation of MSC is a matter of concern. To address these potential differentiation associated problems, novel strategies for efficient differentiation of transplanted cells like biomaterial mediated mimicking of microenvironmental cues have been developed lately which might go a long way for efficient regenerative therapies (Toh, Spector et al. 2011).

1.2.2. Migration of transplanted MSC

Cell based therapy using stem cell involves the infusion of stem cells either by systemic infusion or by local delivery. It has been postulated that the injected cells migrate to the sites of injury and under the influence of the intrinsic signals or either by cues from the microenvironment differentiated into the cells of the desired phenotype and helped in the repair process. But the migration and *in vivo* distribution of the injected stem cells is not yet properly understood. Efficient tracking of MSC after transplantation of MSC for cardiac repair to quantify the actual number of MSC that have actually engrafted and helped in the repair process was not possible (Pittenger and Martin 2004; Bagi and Kaley 2009). After infusion, MSC were found trapped in the lungs at pre-capillary level which created problems like irregular blood flow and possible fatal injury. Although strategies like treatment with vasodilator drugs during infusion have yielded positive results but these problems have to be addressed properly to ensure safety of systemic injection procedures of MSC for therapy (Gao, Dennis et al. 2001; Barbash, Chouraqui et al. 2003; Schrepfer, Deuse et al. 2007; Toma, Wagner et al. 2009).

1.2.3. Transformation during *ex vivo* expansion

The safety and efficacy of *in vitro* culture expanded MSC was questioned by some authors (Prockop and Olson 2007). Clinical settings using mesenchymal stem cell

based therapy requires large number of mesenchymal stem cells for transplantation which necessitates large scale *in vitro* expansion of these cells for ready availability for clinical use. But reports have shown that MSC undergo spontaneous mutation and transformation in long term culture which is a matter of serious concern (Bentivegna, Miloso et al. 2013; Bonab, Alimoghaddam et al. 2006; Rosland, Svendsen et al. 2009). MSC were found to undergo spontaneous mutation in long term culture which was further associated with replicative senescence and cancerous or non-cancerous transformations (Estrada, Torres et al. 2013; Roemeling-van Rhijn 2013, de Klein et al. 2013). Replicative senescence of MSC during *in vitro* culture has been reported by authors and if these cells are used for clinical applications, these aged and transformed cells can undergo maldifferentiation. MSC giving rise to tumors in mice following transplantation in certain cases was reported (Ljujic, Milovanovic et al. 2013; Lepperdinger, Brunauer et al. 2008). Mouse mesenchymal stem cells were reported to undergo chromosomal aberrations during *in vitro* culture and these cells gave rise to sarcomas and tumors following transplantation (Ljujic, Milovanovic et al.; Tolar, Nauta et al. 2007). There are a multitude of sources in the human body from which mesenchymal stem cells could be isolated, expanded and used for clinical applications. MSC from different sources have been shown to be normal and similar in their properties. Even MSC isolated from benign tumors and expanded in culture were found to be normal without any *in vivo* tumorigenicity (Wang, Huso et al. 2005). But conflicting reports describing normal MSC undergoing transformation during *in vitro* culture and forming tumors after transplantation have raised concerns regarding the safety of MSC for use in clinical applications (Wang, Huso et al. 2005). Transformed MSC had increased proliferation rate with altered morphology and phenotype and were highly tumorigenic (Rosland, Svendsen et al. 2009). The process of transformation was found to be a highly coordinated event involving distinct molecular signatures (Rubio, Garcia et al. 2008). But majority of the reports show that MSC in culture are normal and questions are being raised as to whether MSC undergo spontaneous transformation during *in vitro* culture (Binato, de Souza Fernandez et al. 2013; Dominina, Fridliandskaia et al. 2013; Fernandez Vallone, Romaniuk et al. 2013; Haack-Sorensen, Hansen et al. 2013; Bernardo, Zaffaroni et al. 2007).

1.2.4. Modulation of tumor microenvironment *in vivo*

Transformation of MSC *in vivo* and modulation of the microenvironment is a matter of serious concern during MSC transplantation. During chemotherapy, inflammatory cytokines and chemokines released in the tumor sites were reported to attract MSC to the tumor sites (Klopp, Spaeth et al. 2007) which have been reported to alter the microenvironment properties. Mesenchymal stem cells in the tumor microenvironment in acute lymphoblastic leukemia (ALL) were reported to secrete asparagines which modulated chemotherapeutic response of asparaginase therapy for ALL (Iwamoto, Mihara et al. 2007). MSC were also reported to promote metastasis and proliferation of breast cancer cells by production of CCL5 (Karnoub, Dash et al. 2007). Apart from these, MSC themselves were reported to give rise to tumors upon transplantation. Although the reported findings of MSC giving rise to tumors after transplantation were from mouse MSC. The reported incidence of human MSC undergoing transformation was later on attributed to cross contamination of cell lines (de la Fuente, Bernad et al. 2010; Rubio, Garcia-Castro et al. 2005) and MSC use for therapy was termed safe, but a thorough study of MSC safety for clinical applications must be carried out before they are termed same.

Hence, despite extensive research, the properties of mesenchymal stem cells are not yet clearly understood. The basic mechanisms underlying differentiation and proliferation of mesenchymal stem cells, regulatory processes *in vivo* which govern the biology of mesenchymal stem cells etc. need to be clearly understood for safe and efficient use of these cells for clinical and therapeutic applications.

The differentiation potential of mesenchymal stem cells is the major exploited domain for tissue engineering applications. The current area of focus for controlling MSC differentiation is the niche mediated signals and particularly the ECM mediated signals. Cell shape, cytoskeleton, integrins etc. play an important role in the integration of ECM signals that control differentiation, lineage commitment and various other cellular properties. A brief account of these factors is presented as follows.

1.3. Controlling differentiation of mesenchymal stem cells

The efficient use of stem cells in regenerative medicine and cell replacement therapies including the transfer of these stem cell therapies from laboratory to the clinic requires the following fundamental biological and bioengineering aspects to be addressed: **(a)** control of stem cell self-renewal **(b)** directed differentiation to specific lineages **(c)** proper *in vivo* delivery and integration into the host tissue.

As stated earlier, understanding the process of differentiation and directed lineage commitment will open new avenues and help eradicate much of the problems associated with stem cell therapy. Stem cells transplanted *in vivo* are influenced by various mechanical stimuli and paracrine signals from the microenvironment and these play important regulatory roles in fate determination of the transplanted cells. A brief overview of the micro-environmental factors which might play an important role in the process of lineage commitment of MSC *in vivo* is presented in the succeeding section.

1.3.1. Interactions with the microenvironment, mechanical signals and differentiation

Signals from the microenvironment are an important determining factor in lineage commitment. Signals from the microenvironment were reported to be important regulators of differentiation (Engler, Sen et al. 2006), migration (Gardel, Sabass et al. 2008), morphogenesis (Wozniak, Desai et al. 2003) and proliferation (Provenzano, Inman et al. 2009) in different cell types. During tissue development the cells sense the micro-environmental signals and convert them to biochemical processes which affect cellular processes. The shortage of organ for transplant and the need for artificial implants have fuelled research in areas of biomaterials and bio-implants which could be incorporated with stem cells to augment wound healing and therapeutic response and lead to efficient regeneration of damaged tissues and organs. Apart from the interactions with the *in vivo* microenvironmental factors, interactions of the transplanted cells with the artificial biomaterial are an important aspect of cell biology as these also provide cues for directing cellular fate.

Cell sensing of the microenvironment factors is done with receptors like integrins, laminin receptors, syndecans etc. Signals from integrins interaction with the

ECM are translated to cytoskeletal modifications which are further translated to biochemical signals affecting cellular processes (Ross, Coon et al. 2013). Downstream signaling molecules affected by binding of cells to ECM and cytoskeletal modifications include SMADS, Rho GTPases, ERKs etc. have been reported to affect cellular transcriptional machinery or gene expression (Miyamoto, Teramoto et al. 1995). The cytoskeletal organization and particularly the actin filaments regulated the differential behavior of stem cells in response to the change in environmental cues and play an important role in cell differentiation (Treiser, Yang et al. 2010). Thus, understanding the cytoskeletal elements during differentiation might provide new insights into the role of cytoskeleton during the process of lineage commitment and differentiation.

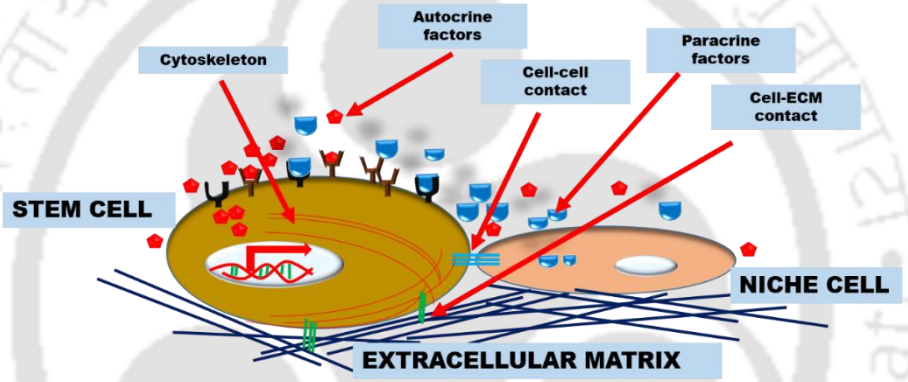


Figure.1.2. Factors affecting stem cell properties *in vivo*. Diagrammatic representation of the various factors in the microenvironment of a stem cell which might modulate the properties of stem cells. Expression of genes, lineage commitment etc. is determined to a large extent by the interactions with the external environment. (Adapted from: Dennis E. Discher et al., Science, 324, 1673. 2009).

1.4. Cytoskeleton of a cell: sensing the mechanical signals

Cytoskeleton in a cell plays an important role in maintaining cellular morphology, signaling, intracellular transport, cell division, growth etc. The cytoskeleton is composed of three different types of polymeric components: **microtubules, intermediate filaments and actin filaments**. The cytoskeletal elements differ in their mechanical properties and polymerization, the type of monomers that form the polymeric units and also in their functions in the cell.

Microtubules are the stiffest component of the cytoskeleton and are composed of heterodimeric subunits composed of α and β tubulin. They are involved in cell division,

organization of intracellular structure, intracellular transport and ciliary and flagellar motion.

Microfilaments provide general mechanical stability to the cell. Subunits of intermediate filaments are composed of two symmetric dimers. Various classes of intermediate filaments are reported and the prominent ones are vimentin and keratin.

Actin is the most abundant intracellular protein found in cells and has been reported to perform essential biological functions in cells like maintaining structural support, cellular morphology, cell movement etc. (Fletcher and Mullins 2010; Pollard and Cooper 2009). Actin forms a major component of the cell cytoskeleton and exists as monomeric G-actin and polymeric F-actin. The binding and release of monomeric G-actin subunits to form polymeric F-actin filaments is a polymerization mechanism wherein the filaments grow at one end and shorten at the other (Wegner 1976).

Cytoskeleton form connections of the cells to the external environment. Sensing of the mechanical properties of the extracellular matrix (ECM) is done by the cytoskeleton and the tension generated by the contracting cytoskeleton in response to signals from the ECM has been reported to affects cell behavior (Discher, Janmey et al. 2005). The mechanical forces from the ECM were also reported to alter focal adhesions and adherens junctions in cells, which formed an important component of signal transmission network from the ECM to the cell (Janmey and McCulloch 2007).

The ECM properties and mechanical signals were also found to play important roles during the process of differentiation and lineage commitment of MSC and MSC derived progenitors. ECM has been reported to play an important role in osteogenic differentiation. Modulating cell attachment and ECM composition was reported to affect lineage commitment of pre-committed osteo-progenitors. Attachment to ECM and spreading of pre-osteoblastic progenitors enhanced osteogenic differentiation and increase in osteogenic differentiation genes was observed (Carvalho, Schaffer et al. 1998; Thomas, Collier et al. 2002). Matrix mediated induction of osteogenic differentiation by engineering the matrix for providing osteo-inductive signals is of great relevance in regenerative medicine (Zouani, Rami et al. 2013). But this area is still in infancy and can be utilized to full potential only if the downstream effectors are identified properly. Cytoskeleton forms an important bridge from the outside of the cell

to inside in transducing matrix or microenvironment cues and hence the properties of cytoskeleton have to be properly understood to modulate and regulate the cellular differentiation processes.

1.4.1. Cell shape, actin cytoskeleton and differentiation of mesenchymal stem cells

The importance of cell shape in the process of differentiation of mesenchymal stem cells were first reported by Spiegelman and Ginty, who showed that changes in cell shape altered the differentiation of pre-committed mesenchymal progenitors. Cell shape and cytoskeleton are related and have been shown to affect differentiation and lineage commitment. Cytoskeletal integrity has been reported to be an important necessary factor for differentiation. The cytoskeletal elements transduce signals from the external environment to the cells (Toma, Ashkar et al. 1997; Pavalko, Chen et al. 1998).

Cell shape and cytoskeleton have also been reported to be important for adipogenesis. When adipogenic cell line 3T3-F442A was allowed to spread and attach on surfaces coated with fibronectin, inhibition of adipogenic differentiation was observed. Decreased expression of adipogenic genes were observed and the phenomenon could be reversed and adipogenesis reverted back by keeping the cells in rounded structures or by modifications of the actin cytoskeleton (Spiegelman and Ginty 1983). Yourek et al., have reported that changes in cell morphology during osteogenic and chondrogenic differentiation of mesenchymal stem cells might be associated with the actin cytoskeleton changes within the cell (Yourek, Hussain et al. 2007). Mesenchymal stem cells grown on rounded micropatterned structures underwent adipogenic differentiation, while cell spreading, which allowed cells to flatten and adhere on substrate favored osteogenesis.

Change in cytoskeleton properties affects the mechanical properties of mesenchymal stem cells. Polymeric F-actin filaments play an important role in the regulating the elastic properties of the cells. Actin disassembly has been reported to change the elastic modulus of mesenchymal stem cells (Titushkin and Cho 2009). The viscoelastic behavior of mesenchymal stem cells were reported to be dependent on the integrity of F-actin filaments in the cells (Tan, Pan et al. 2008).

Disruption of actin cytoskeleton by Cytochalasin-D, an inhibitor of actin polymerization was reported to affect expression of differentiation genes. Disruption of actin cytoskeleton by Cytochalasin-D led to decreased actin tension and the cells attained a rounded morphology. Changes in expression of PPAR gamma, Runx2 and downstream affector RhoA was observed with change in cytoskeletal dynamics induced by Cytochalasin-D which indicated that cell shape and actin cytoskeleton were interlinked and important regulators of differentiation (Arnsdorf, Tummala et al. 2009). Changes in contractile properties of actin cytoskeleton were reported to direct fate of mesenchymal stem cells. A more contractile cytoskeleton with larger focal adhesions and stress fibers was reported to promote osteogenesis of mesenchymal stem cells (Meyers, Zayzafoon et al. 2005).

Actin cytoskeleton also affected adipogenic differentiation of mesenchymal stem cells. Stable actin cytoskeleton was reported to inhibit adipogenesis of mesenchymal stem cells and cells with less organized and less stiff actin cytoskeleton were reported to favor adipogenic differentiation (McBeath, Pirone et al. 2004).

Blocking cytoskeletal dynamics was even reported to affect differentiation of committed precursors. Blocking F-actin dynamics inhibited generation of mature osteoblasts from precursor osteoblasts and modification of F-actin network and microtubules in the cell was found to be an important necessary factor after BMP-2 induced osteoblast differentiation in pre-osteoblasts (Zouani, Rami et al. 2013).

Actin cytoskeleton was found to play an important role during chondrogenic differentiation of mesenchymal stem cells. Disruption of actin cytoskeleton by Cytochalasin-D affected chondrogenesis of mesenchymal stem cells and it was reported to affect chondrogenesis by induction of PKCα expression in MSC, an important regulator of chondrogenesis (Lim, Kang et al. 2000).

Actin cytoskeleton also forms an important component of integrin signaling network. Actin cytoskeleton through integrin network mediate adhesive interactions with the ECM and change in ECM properties are sensed by the integrins which were reported to alter actin cytoskeleton dynamics and regulated cell shape, growth and differentiation (Maniotis, Chen et al. 1997; Geiger, Bershadsky et al. 2001). Adhesive interactions with

the ECM or other cells were also reported to be necessary for survival of cells during *ex vivo* culture (Discher, Mooney et al. 2009).

1.4.2. Cytoskeleton related signaling pathways during differentiation

Rho GTPases are important regulators of cellular mechanotransduction and respond to extracellular signals from the microenvironment. Extracellular signals regulated Rho GTPases, which in turn were reported to direct the stability and assembly of actin cytoskeleton (Nobes and Hall 1995). Rho GTPases in turn affected Rho kinases (ROCK) which further affected actin binding proteins like cofilin (Lappalainen and Drubin 1997). Phosphorylation of cofilin by kinases like LIM in turn affected actin stabilization and total cellular actin content of the cells (Bamburg, McGough et al. 1999). Change in cell shape affected RhoA activity which affected the lineage commitment of mesenchymal stem cells. Expression of constitutive active RhoA in MSC promoted osteogenic differentiation of MSC and dominant negative RhoA promoted adipogenic differentiation of MSC. Addition of actin inhibitor Cytochalasin-D affected osteogenic differentiation in MSC expressing constitute active RhoA, which indicated that actin cytoskeleton formed an important component of the RhoA mediated control of lineage commitment of MSC (McBeath, Pirone et al. 2004).

Assembly of focal adhesion kinases also changes during osteogenic differentiation of MSC. MSC were reported to have more focal adhesion kinases (FAK) compared to undifferentiated MSC (Titushkin and Cho 2009). ROCK and FAK were found to regulate osteogenic phenotype of MSC and FAK affected osteogenic differentiation by signaling through ERK1/2 (Shih, Tseng et al. 2011), which further activated transcription factors Runx2/Cbfa-1 etc. and controlled osteogenic differentiation (Salasznyk, Klees et al. 2007). While the disruption of FAKs in mesenchymal stem cells were reported to promote adipogenic differentiation of MSC (Luo, Shitaye et al. 2008).

Hence, from the above discussion, it is clear that actin cytoskeleton plays an important role in the regulation of differentiation of mesenchymal stem cells. But the events are not yet clearly understood and it might be possible that MSC differentiation could be regulated by controlling the properties of actin cytoskeleton.

1.5. Hematopoiesis and the hematopoietic stem cell niche

Hematopoiesis is a highly regulated and sequential process. During embryonic development, hematopoiesis first occur in the extra-embryonic tissues and then move to the aorta-gonad-mesonephros areas, further shifting to the fetal liver and spleen and finally to the bone marrow which is the major site of hematopoiesis in the adult body (Dzierzak and Speck 2008). Inside the bone marrow, the hematopoietic stem cells (HSC) are housed in a homeostatic microenvironment, wherein the HSCs, various other stromal cells and extracellular matrix components form a microenvironment that is supportive of the stem cells and maintains a balance between self-renewal and differentiation of HSC. Such a spatial arrangement of referred to as the **"*stem cell niche*"** (Martinez-Agosto, Mikkola et al. 2007). Thus, the hematopoietic stem cells giving rise to billions of blood cells in our body are housed in homeostatic microenvironment termed as ***"hematopoietic stem cell niche".*** The term stem cell niche was first coined by **Schofield** in 1978 for the hematopoietic system (Schofield 1978) and since then the hematopoietic stem cell niche and its role in hematopoietic maintenance has been extensively studied (Renstrom, Kroger et al. 2010; Grossman 1986; Arai, Hirao et al. 2005). Deregulation or manipulation of the stem cell niche was even postulated to contribute to tumorigenesis and various disorders (Li and Neaves 2006). The bone marrow microenvironment has been reported to maintain the HSC in normal conditions and protect them from external damaging agents (Morrison and Spradling 2008; Wagner, Horn et al. 2008).

1.5.1. Structure and cellular composition of the hematopoietic stem cell niche

The bone marrow is a highly complex structure of cellular and extracellular matrix (ECM) network and is devoid of lymphatic system. Structurally the bone marrow has been reported to consist of two different types of cells: the hematopoietic stem cells and the stromal cells (Xie, Yin et al. 2009). Areas near the endosteum and the sinusoids were initially denoted as potential niche areas in the bone marrow (Lo Celso, Fleming et al. 2009). Representative HSC populations with phenotype of Sca-1^{+}Kit^{+}CD41^{-}CD48^{-} CD150^{+} were found to be distinctly located in areas of sinusoidal endothelium within the bone marrow and spleen which indicated that these spaces within the bone marrow were potential niche areas (Kiel, Yilmaz et al. 2005).

Two definitive HSC niches have been reported in the bone marrow, the endosteal niche and the vascular niche (Zhang, Niu et al. 2003). The endosteal niche lies in close proximity to the bone cells or osteocytes and helped in maintaining HSC quiescence whereas the vascular niche consisted of highly vascularized areas and signaled for HSC proliferation and differentiation (Winkler, Barbier et al. 2012; Zhang, Niu et al. 2003; Kopp, Avecilla et al. 2005). The HSC niche comprises of a multitude of factors like adhesion molecules, soluble cytokines and interplay of signaling pathways which regulated HSC by providing unique extrinsic cues and were distinctive based on their distinct anatomical locations (Rossi, Lin et al. 2012).

The stromal population in the BM which composes the hematopoietic stem cell niche consists of adipocytes, osteocytes, reticular cells, macrophages, vascular endothelial cell, smooth muscle cells and mesenchymal stem cells. These cells have been reported to collectively produce the ECM components, cytokines, growth factors, cell surface receptors etc. which create a specialized microenvironment important for various stages of hematopoietic development and proliferation (Anthony and Link 2014; Tokoyoda, Egawa et al. 2004).

The signals from the niche not only maintain a balance between self-renewal and differentiation, but also maintain a constant supply of differentiated progenitors during normal and emergency conditions. During conditions of stress or situations when stem cell reinforcements are needed for tissue repair and regeneration, the stem cell niche or microenvironment is transformed from a resting place to a command center. The niche instructs the stem cells to proliferate, differentiate or migrate to circulation as per the requirements of the body (Kiefer 2011).

1.5.2. Hematologic malignancies and the hematopoietic stem cell niche

As described earlier, the cellular and non-cellular components of the niche play an important role in the maintenance of the hematopoietic stem cells in the bone marrow. Apart from its role in normal hematopoiesis, the hematopoietic stem cell niche also plays an important role during hematologic disorders.

Blood cell disorders or hematologic disorders arise from defects in the hematopoietic stem cells and affect the production and function of the blood cells in the

body. Hematologic malignancies can be divided into two major categories: ***(a)*** *Lymphoid malignancies* ***(b)*** *Myeloid malignancies,* which are subdivided further based on the type of mutation in the cell type and also the proliferative stage in which the hematopoietic cells are (Vardiman, Thiele et al. 2009).

In malignancies like leukemia, the hematopoietic stem cells accumulate mutations and in due course of time, give rise to abnormal progenitors which don't differentiate properly and are not able to carry out their normal functions. Leukemia has been postulated to originate from a primitive stem cell population termed leukemic stem cell (LSC) (Bonnet and Dick 1997; Johnsen, Kjeldsen et al. 2009). The LSC share similar characteristics to HSC in their self-renewal and differentiation properties but were reported to differ in their ability to metastasize, produce abnormal non-functional progenitors and at times become resistant to chemotherapeutic drugs (McCulloch 1983; Bonnet and Dick 1997). The LSCs keeps on proliferating and accumulating in the marrow and ultimately affect hematopoiesis and also migrate to peripheral circulation affecting vital functions of the body. Although advancement has been made in the treatment strategies for various hematological malignancies but the survival rate of patients has been reported to be low. A very low survival rate has been observed during a long term follow up period in majority of the patients. Inspite of intensified and modified treatment strategies for leukemia, if the treatment is discontinued, the disease re-occurs or relapses after certain years. Relapse of leukemia has been attributed to the leukemic stem cells which evade chemotherapy and survive in the BM and give rise to the disease later on. The homeostatic BM microenvironment has been reported to provide a survival advantage to the leukemic stem cells (Lane, Scadden et al. 2009). Majority of the leukemic cells are highly proliferative and are affected by chemotherapy but the leukemic stem cells housed in the bone marrow were found to become quiescent and developed resistance to chemotherapeutic drugs (Nwajei and Konopleva 2013). Therapeutic targeting of the leukemia-microenvironment interactions has been postulated to be the strategy of the future for complete eradication of the leukemic stem cells and to achieve a complete remission (Uy 2012, Rettig et al.; Brendel and Neubauer 2000).

1.5.3. An altered hematopoietic stem cell niche during malignancies protects the leukemic cells

Altered BM microenvironment is reported in many hematologic malignancies like myelodysplastic syndromes, myelofibrosis, acute myeloid leukemia, multiple myeloma etc. During hematologic malignancies, the leukemic cells in the bone marrow alter the cytokine profile in the marrow and also effect the cell-cell interaction among the various cells in the marrow microenvironment. This altered cell-cell interaction in the marrow microenvironment has been postulated to provide surviving signals to the leukemic cells. Multistage transformations have been reported during primary myelofibrosis including alterations in the stromal compartment of the niche. The leukemic cells have been reported to condition the stromal compartment in the niche and the stromal cells in turn reciprocated by creating a pathologic microenvironment that participates in the maintenance of the malignant hematopoietic stem cells (Lataillade, Pierre-Louis et al. 2008).

The microenvironment has been postulated to affect the properties of tumor cells like functional heterogeneity of tumors, chemoresistance, metastatic potential, quiescence etc. Properties of tumor cells have been reported to be determined to a large extent by the ECM composition, cytokine and chemokine profile and cell-cell adhesion in the microenvironment (Alexander and Friedl 2012; Ungefroren, Sebens et al. 2011). Stromal cells in the microenvironment were reported to secrete factors that induced tumor proliferation and presumably created a sanctuary for tumor cells by helping them evade chemotherapy. Stromal cells in the microenvironment have been reported to protect leukemic cells by VCAM mediated adhesion (Mudry, Fortney et al. 2000). Adhesion of leukemic cells to stroma has been reported to induce production of Stat-3 (Bewry, Nair et al. 2008) Galectin-3 (Yamamoto-Sugitani, Kuroda et al. 2011) etc., which helped in survival of leukemic cells and promoted drug resistance. CXCR4 mediated adhesion of CML cells to ECM and stromal cell was also found to be an important mediator of drug resistance (Vianello, Villanova et al. 2010; Weisberg, Azab et al. 2012). Integrin mediated adhesion of myeloma cells to the ECM (Damiano, Cress et al. 1999) and release of molecules like IL-6 by the stromal cells promoted drug resistance and induced osteolysis during multiple myeloma (Michigami, Shimizu et al. 2000).

Apart from this, the abnormal stromal compartment during hematologic disorders has been reported to affect hematopoietic engraftment after ablative chemotherapy for cancer. Leukemic cells in the BM have been reported to cause disruption in the normal bone marrow microenvironment structure which led to formation of malignant niches that compete with the normal hematopoietic niches for the engraftment of transplanted normal hematopoietic stem cells during transplantation. The normal hematopoietic stem cells engrafted into these abnormal niches were reported to be defective in their hematopoietic repopulation capacities (Colmone, Amorim et al. 2008). Thus, alteration in bone marrow microenvironment during hematologic malignancies plays an important role during disease progression and also affects hematopoietic recovery after therapy.

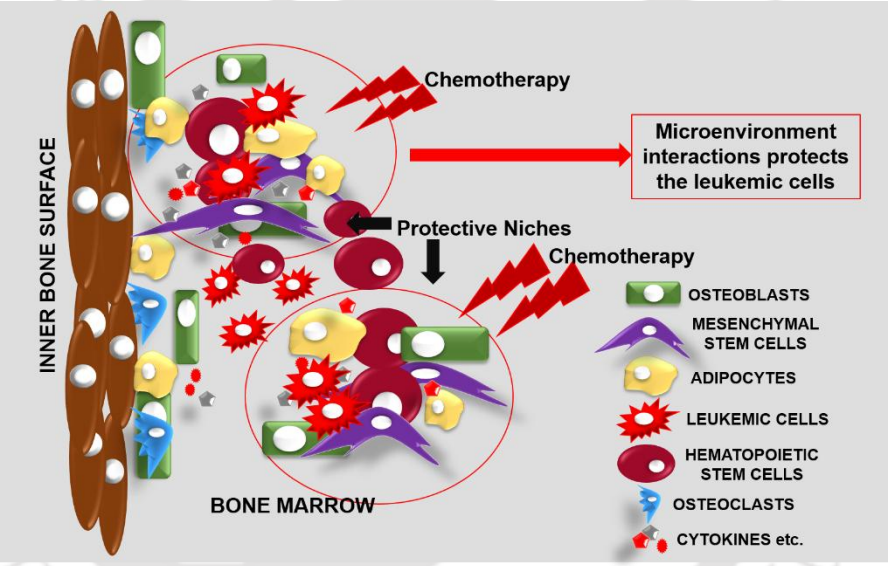


Figure.1.3. The bone marrow microenvironment during hematologic malignancies. Diagrammatic representation of the interaction of leukemic cells with stromal cells in the bone marrow. These interactions contribute to leukemic cell survival and proliferation and also contribute to protection from chemotherapeutic drugs (Adapted from: Kiel and Morrison, Nat Rev Immunol. 2008 Apr;8(4):290-301).

1.6.Mesenchymal stem cells: an important component of the bone marrow microenvironment

MSC in the BM were first described as stromal progenitor cells and were initially hypothesized to serve one primary role in the bone marrow: replenishment of marrow stromal populations. But until recently, MSCs in the BM have been proved to serve alternate functions. MSCs in the BM have been found to be important niche element

regulating hematopoietic stem cells (HSC). MSCs secrete soluble mediators which have hematopoietic support capability and also regulate HSC biology by cell-cell interactions (Frenette, Pinho et al. 2013; Ugarte and Forsberg 2013).

The importance of MSC in HSC maintenance came to light when autologous MSC supported HSC engraftment and recovery of hematopoiesis during HSC transplantation after BM ablative chemotherapy for cancer (Koc, Gerson et al. 2000).The role of MSC in the maintenance of hematopoietic stem cell (HSC) has been studied by many authors. MSC and HSC have been linked and MSCs were reported to be supportive of HSC right from ontogeny. MSC have been co-localized with hematopoietic sites right from the time of development (Mendes, Robin et al. 2005). Before the onset of hematopoiesis in fetal liver and BM, fetal hematopoiesis occurs in the aorta-gonad-mesonephros and placenta and MSC were predominantly found in these sites and have been reported to form embryonic niches which provide signals for hematopoietic maintenance (Dazzi, Ramasamy et al. 2006; Dzierzak and Speck 2008; Bowman and Zon 2009). MSC were reported to be found in BM as early as after 9 weeks of gestation, long before hematopoiesis was established in the BM (Charbord, Tavian et al. 1996).

Although the *in vivo* identity of MSC in the bone marrow microenvironment is not clearly defined but strong evidences suggest that these are the major components of the hematopoietic stem cell niche. Nestin$^+$ MSCs were found to be important component of the HSC niche. Purified HSC after transplantation were observed to preferentially migrate to regions containing Nestin$^+$ MSC in irradiated mouse. Depletion of Nestin$^+$ MSC in the bone marrow led to decreased HSC numbers, which showed that these cells were important for hematopoietic maintenance (Mendez-Ferrer, Michurina et al. 2010).

MSC in the BM produce a wide variety of cytokines and growth factors which are important for various hematopoietic functions like maintenance of HSC quiescence, HSC growth and differentiation and regulation and maintenance of differentiated progenitors (Li and Wu 2011). Factors like SDF-1, IL-6, GM-CSF, SCF, adhesion molecules like VCAM, E-Selectin and ECM components like collagen, fibronectin etc. secreted by MSC form a very important component of the hematopoietic microenvironment *in vivo* (Burness and Sipkins 2010; Lam and Adams 2010; Mishima, Nagai et al. 2010; Campagnoli, Roberts et al. 2001; Burns, Summers et al. 2006; Sugiyama, Kohara et al.

2006; Zhang and Li 2008). MSC have been reported to support the growth of hematopoietic stem cell in *ex vivo* cultures. Co-culture of hematopoietic progenitor cells with MSC *ex vivo* have been reported to induce HSC proliferation and maintained a primitive hematopoietic stem cell phenotype for longer durations (Rodriguez-Pardo and Vernot 2012). HSC expanded *ex vivo* with MSC cocultures have been reported to have greater engraftment potential compared to hematopoietic progenitors cocultured with osteoblasts (Walenda, Bork et al. 2010). These results showed that MSC maintains HSC in an undifferentiated state *in vitro* and this function of HSC maintenance is also postulated to be carried out by the mesenchymal stem cells *in vivo*. Apart from the MSC themselves playing a pivotal role in HSC maintenance in the BM, MSC derived progenitors like adipocytes and osteocytes also form an important component of the hematopoietic microenvironment in the bone marrow and have been reported to perform HSC regulatory functions.

Osteoblast lineage cells have been postulated to control the hematopoietic stem cell niche size in the bone marrow and immature HSC progenitors were found to be localized in areas close to the endosteum (Lord, Testa et al. 1975). Selective deletion of osteoblasts in the BM led to decreased BM cellularity and subsequent loss of both lymphoid and erythroid progenitors from the BM was observed (Visnjic, Kalajzic et al. 2004). Osteoblasts have been reported to secrete factors which help in the maintenance of HSC *in vitro*. Increased number of hematopoietic long term culture initiating cells (LTC-IC) and hematopoietic progenitors were reported in *ex vivo* cultures of HSC with osteoblasts (Taichman, Reilly et al. 1996). Factors important for HSC migration, proliferation and differentiation like CXCL12 and osteopontin (de Barros, Takiya et al.), G-CSF (Taichman and Emerson 1994), GM-CSF, M-CSF, IL-1, IL-6, IL-7 (Taichman 2005), Notch, Wnt, thrombopoietin (Mercier, Ragu et al. 2011; Stier, Cheng et al. 2002; Qian, Buza-Vidas et al. 2007; Yoshihara, Arai et al. 2007; Fleming, Janzen et al. 2008), VCAM-1, ICAM-1, CD44, CD164, osteopontin, Annexin II (Raaijmakers, Mukherjee et al. 2010; Verfaillie 1998; Nilsson, Johnston et al. 2005; Jung, Wang et al. 2007), BMPs (Bhatia, Bonnet et al. 1999) etc. were reported to be expressed by osteoblasts in the bone marrow. Osteoblasts have been found to play an important role in the maintenance of B-cells (Nagasawa 2006). Selective deletion of osteoblasts led to significant decrease in the number or pre-pro-B-cells and pro B-cells *in vivo* (Zhu, Garrett et al. 2007) . Not only

osteoblasts but mature osteocytes were also found to play an important role in the maintenance of HSC in the BM (Fulzele, Krause et al. 2013). Mature osteocytes were reported to create a calcium gradient in the BM which helped in migration of HSC to the bone marrow (Adams, Chabner et al. 2006).

Apart from the osteoblasts, MSC derived adipocytes have been reported to play an important role in HSC maintenance. Initially adipocytes were postulated to play a passive role in the BM by acting as "space fillers" in the bone marrow cavity (Gimble, Robinson et al. 1996). But recent reports have shown that adipocytes were associated with hematopoiesis and adipocyte numbers in the bone marrow were inversely correlated with hematopoiesis. Age related decrease in hematopoietic stem cell function was correlated with increased adipocyte numbers in the bone marrow (Justesen, Stenderup et al. 2001). Associated with increased fat content, changes in cytokine levels like IL-6, IGF-1, SDF-1 were also observed in fatty marrows of elderly which were of importance for hematopoietic stem cell maintenance in the marrow (Tuljapurkar, McGuire et al. 2011; Rosen, Ackert-Bicknell et al. 2009).

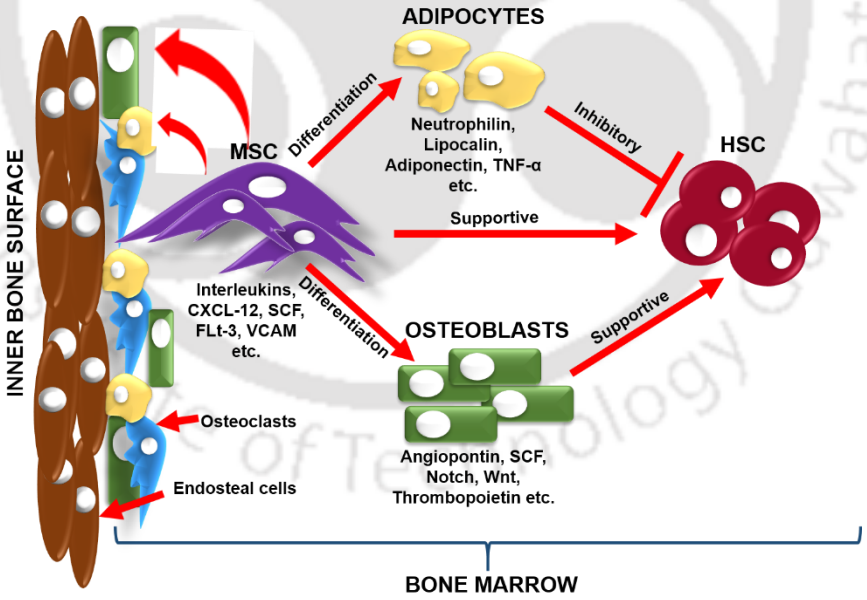


Figure.1.4. Functions of mesenchymal stem cells in the bone marrow. MSC apart from replenishing the stromal components of the bone marrow perform important hematopoietic regulatory functions in the bone marrow. MSC derived adipocytes and osteocytes also perform HSC regulatory functions in the bone marrow. (Adapted from: F.E. Mercier et al., Nature Reviews Immunology 12.1 (2011): 49-60).

A perfect balance between osteocytes and adipocytes in the marrow microenvironment was reported to be necessary for proper maintenance of the hematopoietic stem cell niche in the bone marrow (Sugimura and Li 2010). During ageing, the balance between adipocytes and osteocytes in the marrow was reported to be altered and adipogenesis was favored in the BM which affected hematopoietic function (Naveiras, Nardi et al. 2009). Adipocyte secreted molecules like Neutrophilin-1, Lipocalin-2, TNF-α etc. were reported to affect myeloid differentiation of HSC (Bilwani and Knight 2011; Zhang, Harada et al. 1995; Nilsson, Johnston et al. 2005; Miharada, Hiroyama et al. 2008). But the suppression of hematopoietic stem cell proliferation by adipocytes might have positive effects. In response to stress or injury, the BM adipocytes were found to be increased in number promoting hematopoietic stem cell quiescence which probably protected the HSCs in the marrow (Lo Celso, Fleming et al. 2009; Xie, Yin et al. 2009).

1.7. Properties of mesenchymal stem cells from bone marrow of patients with hematologic disorders

Abnormal hematopoietic progenitors in the bone marrow during hematologic disorders alter the cellular and cytokine properties of the bone marrow microenvironment. MSC which form the most important component of the bone marrow microenvironment have been reported to be affected by the altered cellular and cytokine profile of the BM. MSC during normal conditions are supportive of the hematopoietic stem cells in the bone marrow. Interaction with leukemic cells in the bone marrow during hematologic malignancies leads to malignant transformation of the bone marrow microenvironment. This is a multistep process and ultimately leads to creation of an altered bone marrow microenvironment that might support the leukemic cells (Raaijmakers, Mukherjee et al. 2010; Bernasconi 2008).

Exposure to leukemic factors in the bone marrow during disease has been reported to affect the properties of mesenchymal stem cells. Enhanced proliferation and modulation of immunogenic properties was reported in MSC after exposure to necrotic tumor tissues (Lotfi, Eisenbacher et al. 2011). Even conditioned media from tumor cells was reported to alter the properties of MSC. MSC cultured with conditioned media from tumor cells acquired characteristics of carcinoma associated fibroblast (CAF) and produced high

levels of chemokines like SDF-1, which promoted tumor growth *in vivo* and *in vitro* (Mishra, Humeniuk et al. 2008).

Culture of cord blood derived normal hematopoietic progenitors with stromal cultures obtained from bone marrow of patients with MDS led to acquisition of myelodysplastic phenotype which provided evidence that the stromal compartment has an important role in the development of MDS (Duhrsen and Hossfeld 1996; Borojevic, Roela et al. 2004). The properties of stromal cells during hematologic malignancies might be altered which may have important implications in the development of the disease. Functional defects like altered differentiation and proliferation potential including gene expression and cytokine profile and also cytogenetic abnormalities have been reported in MSC isolated from BM of patients with hematologic malignancies. A brief account of which is presented in the succeeding sections.

1.7.1. Proliferation and hematopoietic support

Reduction in proliferation and hematopoietic support capabilities was reported in MSC from patient with hematologic disorders. In MDS, AML and CML derived BMMSC, decrease in CFU-F along-with decreased hematopoietic support was reported (Lisovsky and Savchenko 1995; Flores-Figueroa, Montesinos et al. 2008). MSC from oncohematological disorders like AML have been shown to be abnormal in their hematopoietic support, differentiation and proliferation potential (Isaikina, Kustanovich et al. 2006; Zhao, Liang et al. 2007). In a separate study of MSC from MDS patients, reduction in proliferation together-with reduced hematopoietic support capabilities was reported (Geyh, Oz et al. 2013; Varga, Kiss et al. 2007), which was attributed to contribute to loss of hematopoietic support during hematologic disorders.

Nagao et al. had reported decrease in CFU-F in patients diagnosed with AML and CML. But following treatment, significant increase in CFU-F was observed in AML patients. After busulfan treatment, increase in CFU-F was observed and this increased further during remission, although the values were less than normal samples. But in BM samples from patients undergoing relapse, CFU-F levels reduced again and were of similar levels as during diagnosis (Nagao, Yamauchi et al. 1983). Decreased CFU-F in MSC from patients diagnosed for ALL and AML was correlated with poor prognosis and normal CFU-F was correlated with favorable prognosis (Hirata, Katsuno et al. 1986).

Some authors had reported no difference in CFU-F in MSC from aplastic anemia, hematopoietic dysplasia and CML but found stage specific decrease in CFU-F in MSC from AML patients (Kaneko, Motomura et al. 1982). No difference in hematopoietic support capacity was reported in BMMSC from CML patients. Normal or enhanced hematopoietic support capability for $CD34^{+}CD38^{-}$ primitive and $CD34^{+}CD38^{+}$ mature populations compared to normal were reported in BMMSC from CML patients (Sparrow, O'Flaherty et al. 1997).

1.7.2. Genetic properties

Chromosomal abnormalities and defects in gene and protein expression had been reported in MSC isolated from MM bone marrow and MM (Reagan and Ghobrial 2011; Arnulf, Lecourt et al. 2007). Chromosomal aberrations like t(1;7), t(4;7), t(7;9), t(7;10), t(7;19), t(15;17) were reported in MSC from MDS and AML (Blau, Hofmann et al. 2007). Chromosomal alterations like translocations, inversions and partial deletions detected in mesenchymal stem cells from BM of patients with MDS and AML were different from the genetic alterations found in the hematopoietic cells (Blau, Baldus et al. 2011). The cytogenetic abnormalities in MSC from patients with hematologic malignancies might be related to the disease prognosis. No cytogenetic aberrations were detected in MSC from patients having favorable cytogenetics in hematopoietic cells during hematologic disorders. Although conflicting reports of BMMSC from patients with B-ALL harboring the MLL-AF4 fusion gene, which is among the common chromosomal translocations like TEL-AML1, AML1-ETO that were observed in leukemia had been reported (Menendez, Catalina et al. 2009). However in reports by some authors, no cytogenetic abnormalities were detected in MSC isolated from bone marrow of patients with ALL and CLL (Campioni, Bardi et al. 2012; Jootar, Pornprasertsud et al. 2006).

The observed differences in results regarding the chromosomal translocations in MSC from hematologic malignancies might be due to the experimental differences as many authors have reported difficulty in separating mesenchymal stem cells from pathologic lymphocytes during *in vitro* culture. The presence of these abnormal lymphocyte populations in culture and chances of those contributing to the observed

genetic defects in mesenchymal stem cells cannot be denied (Campioni, Bardi et al. 2012; Ramakrishnan, Awaya et al. 2006).

1.7.3. Phenotypic properties

Immunophenotype heterogeneity has been reported in MSC from patients with hematologic malignancies. Decreased expression of CD73, CD90 and CD105 was reported in MSC isolated from patients with hematologic disorders (Campioni, Moretti et al. 2006). Atypical phenotype of $CD45^{+}CD166^{-}$ was reported in MSC from patients with multiple myeloma but during later passages phenotype of $CD45^{-}CD166^{+}$ was observed (Yeh, Chang et al. 2005). No difference in expression of markers CD13, CD44, CD49e, CD90 were reported in MSC from multiple myeloma patients (Arnulf, Lecourt et al. 2007). Similarly, no difference in expression of CD29, CD44, CD90 and MHC class II was reported in MSC isolated from normal and ALL patients (Vicente Lopez, Vazquez Garcia et al. 2014). An increase in expression of HLA-DR was reported in MSC from patients with hematologic malignancies.

1.7.4. Differentiation potential

Defects in differentiation potential have been reported in MSC from hematologic disorders. An increase in adipogenic differentiation potential was reported in MSC isolated from ALL patients (Vicente Lopez, Vazquez Garcia et al. 2014). Defects in neuronal and adipogenic differentiation potential were reported in MSC from MDS patients while no difference in osteogenic differentiation potential was observed MDS donors (Varga, Kiss et al. 2007). Separate reports showed reduction in osteogenic differentiation potential in MSC isolated from MDS patients (Geyh, Oz et al. 2013). But some authors have shown that MSC from hematologic malignancies are normal without and defects in differentiation potential. MSC from B-CLL patients were reported to be normal without any defects in adipogenic and osteogenic differentiation potential (Pontikoglou, Kastrinaki et al. 2013).

1.7.5. Cytokine and gene expression properties

MSC from hematological malignancies show alterations in cytokine and adhesion molecule expression including altered immunosuppressive capacity. Significant increase in IL-1β was seen in MSC from MDS, MM and AML patients (Wallace, Oken et al.

2001; Flores-Figueroa, Montesinos et al. 2008). Excessive IL-6 production was reported in MSC from bone marrow of MM patients which contributed to proliferation of MM plasma cells in the BM (Reagan and Ghobrial 2011; Arnulf, Lecourt et al. 2007). Reduced immunosuppressive potential along-with high IL-6 production was reported in MSC from MM patients and these effects persisted in culture for around 3-weeks without the presence of multiple myeloma cells (Arnulf, Lecourt et al. 2007). Adhesion of MM cells to stromal cells was reported to induce the expression of IL-6 in stromal cells (Chauhan, Uchiyama et al. 1996) which inhibited drug induced apoptosis in MM plasma cells and helped in their proliferation (Lichtenstein, Tu et al. 1995).

Apart from these, higher levels of expression of IL-32 from chronic myelomonocytic leukemia (Marcondes, Mhyre et al. 2008), BMP-4 from acute lymphoblastic leukemia (Vicente Lopez, Vazquez Garcia et al. 2014) was reported in BMMSC isolated from the bone marrow. Higher levels of cytokines CXCL1, IL-8, IL1β. CXCL3, CXCL2, CXCL3 etc. which are reported to be involved in acute inflammatory responses was reported in BMMSC co-cultured with leukemic cells (Civini, Jin et al. 2013).

Thus, the development of hematologic malignancies affects the mesenchymal stem cells which form an important niche element in the bone marrow. The properties of mesenchymal stem cells might be altered with abnormal secretory profile, expression of adhesion molecules and also harbor chromosomal aberrations which might contribute to pathophysiology of the disease. A clear understanding of the properties of mesenchymal stem cells from the bone marrow will lead to utilization of these cells for helping hematopoietic support and recovery during disease and might also lead to the development of new therapeutic target for hematologic disorders.

2. Aims and objectives of the present investigation:

From the review of literature presented in the previous section, it is clear that despite immense potential for various tissue engineering and therapeutic applications, mesenchymal stem cells are still not being fully utilized to their true potential. After the initial clinical therapeutic applications of mesenchymal stem cells, various potential problems were highlighted which have to be addressed properly before these cells can be termed safe for clinical applications. The differentiation potential of MSC is one of the major areas of exploitation for MSC therapeutics. But the process of lineage commitment and directed differentiation of MSC has not been possible to date. Apart from this, MSC in the bone marrow also form an important component of the hematopoietic microenvironment *in vivo*. MSC and their derivatives like adipocytes and osteocytes have been postulated to play important regulatory role during normal hematopoiesis and also during hematologic disorders. MSC are also reported to interact with HSC through various cell surface receptors. These interactions have been postulated to be important for HSC maintenance in the bone marrow. Alterations in the balance of adipocytes and osteocytes in the bone marrow have been reported to affect hematopoiesis. Properties of MSC and their progenitors have been reported to affect the properties of leukemic cells and therapeutic outcome during hematologic malignancies. Hence, understanding the phenotype and differentiation properties of MSC from the bone marrow during normal and pathological conditions may shed light into the roles of MSC in the bone marrow.

Thus, to understand the properties of mesenchymal stem cells, the properties of actin cytoskeleton during the process of adipogenic and osteogenic differentiation was analyzed. In addition, the differentiation properties and phenotype of MSC isolated from bone marrow of patients with hematologic disorders was analyzed. This might provide new insights into the functions and roles of MSC during hematologic malignancies. The results obtained from the research findings are presented and discussed in this current thesis.

3.1. MATERIALS

3.1.1. General laboratory chemicals

All the general lab chemicals were obtained from Merck, India, unless specifically mentioned**.**

3.1.2. Composition of reagents and buffers

(i). Phosphate buffered saline (1X)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2mM KH_2PO_4

The components are dissolved in de-ionized water with stirring, the pH was adjusted to 7.2 -7.4 with 1N HCl and sterilized by autoclaving at 121°C and 15psi for 20 mins.

(ii). RBC lysis buffer

Ammonium chloride (NH_4Cl): 0.15M

Potassium carbonate ($KHCO_3$):10mM

EDTA: 0.1mM

Dissolved in distilled water and sterile filtered with 0.22µM and stored at 4°C.

(iii). Citrate acetone formaldehyde fixative

Citrate: 25ml

Acetone: 65ml

37% Formaldehyde: 8ml

Mixed properly and stored at 4°C. The fixative was brought to room temperature before use.

(iv). Carnoy's fixative

3:1 ratio of Methanol: acetic acid.

(v). Hypotonic KCl solution

0.075M potassium chloride dissolved in milliQ water.

(vii). 0.1% Triton X-100

Prepared a solution of 0.1% (v/v) in PBS.

(viii). Formalin solution 10%

Diluted from 37% paraformaldehyde stock (Merck) in distilled water to a final concentration of 10% (v/v).

(ix). Paraformaldehyde 4%

4% paraformaldehyde solution was prepared by dissolving powdered paraformaldehyde powder in PBS over a heating plate or water bath set at 55°C. When the solution became clear and fully dissolved, the pH of the solution was adjusted to the range 7.2-7.4 and aliquots were stored at -20°C until use.

(x). Alkaline phosphatase substrate solution

Reagents were part of Leukocyte alkaline phosphatase kit (Sigma-86C-1KT). 250µl of sodium nitrate solution was mixed with 250µl FBB alkaline solution and added to 11.25 ml of deionized water. Finally 250µl of Napthol AS-Bl-Alkaline solution was added and the substrate was ready for use. The reagents were mixed in an amber glass bottle and kept away from light. The solution was prepared fresh before use each time.

3.1.3. Cell culture media

(i). DMEM low glucose (Sigma D2902)

DMEM low glucose (Sigma), supplemented with 10% FBS, 50U/ml Penicillin, 50µg/ml Streptomycin. Filter sterilized with 0.22µM filter and stored at 4°C.

(ii). RPMI-1640 Medium (SigmaR8755)

RPMI 1640 (Sigma), supplemented with 10% FBS, 50U/ml Penicillin, 50µg/ml Streptomycin. Filter sterilized with 0.22µM filter and stored at 4°C.

(iii). Adipogenic differentiation media

DMEM high glucose (4.5g glucose/l) supplemented with:

Dexamethasone: 1 µM (Sigma. D-2915)

Indomethacin: 0.2mM (Sigma. I-7378)

IBMX: 0.5mM (Sigma. I-5879)

Insulin: 0.01M (Sigma I-5500)

10% Fetal bovine serum (PAA laboratories Cat A15-101)

50U/ml Penicillin

50µg/ml Streptomycin

Filter sterilized with 0.22µM filter and stored at 4°C.

(iv). Osteogenic differentiation media

DMEM high glucose (4.5g glucose/l) supplemented with:

B-glycerophosphate: 10mM (Sigma. G9891)

Dexamethasone: 0.1µM (Sigma D2915)

Ascorbic acid-2-phosphate: 0.05mM (Sigma. A8960)

10% Fetal bovine serum (PAA laboratories Cat A15-101)

50U/ml Penicillin

50µg/ml streptomycin

Filter sterilized with 0.22µM filter and stored at 4°C.

(v). Cell freezing solution

90% Fetal bovine serum (v/v)

10% DMSO (v/v), sterile filtered with 0.22µM and stored at 4 °C.

(vi). Trypsin solution

0.25% trypsin solution (TCL-047) from Himedia Laboratories, India.

(vii). Tissue culture plastic

All the tissue culture flasks, plates and dishes were obtained from BD biosciences.

3.1.4. Dyes and stains

(i). Phalloidin TRITC (P1951 Sigma)

Solution: 1mg/ml (w/v) in DMSO (Storage -20°C). Working solution: diluted from stock 1:1000; 1:3000 in 2% FBS in PBS.

(ii). Propidium iodide (P4170, Sigma)

Dissolved in PBS (pH 7.2-7.4) and stored at 4°C at stock concentration of 5mg/ml (w/v).

(iii). Oil-red O stock solution (Sigma O-0625)

Stock solution: 1% stock solution in isopropanol (v/v).

Working solution:

Three parts of 1% Oil-red O stock solution+2 parts of dH_2O. Mixed and allowed to stand for 1 hour. The solution is filtered with 14µM filter paper.

(iv). Antibodies for flow cytometry

All the antibodies for flow cytometry were obtained from BD biosciences (BD Pharmingen™) USA. These monoclonal antibodies were either conjugated with Fluorescein-isothiocyanate (FITC) or Phycoerythrin (PE).

The details are as follows:

Antibody	Fluorochrome
CD13	PE
CD29	PE
CD34	PE
CD45	FITC
CD49a	PE
CD49b	FITC
CD49e	PE
CD49d	PE
CD73	PE
CD90	FITC

CD105	PE
CD146	PE
SSEA-4	PE
CD271 (NGF Receptor)	PE
p38MAPK	PE
pERK1/2	PE

3.2. METHODS

3.2.1. Isolation of mesenchymal stem cells

Mesenchymal stem cells were isolated from bone marrow aspirates of patients referred to Gauhati Medical College and Hospital (GMCH), Guwahati, Assam, India following institutional ethical guidelines and after informed consent from the individuals. Bone marrow aspirates were taken by doctors of GMCH from the iliac crest. The aspirates were collected in heparin tubes and transferred to laboratory under ambient conditions.

Red blood cell (RBC) lysis of the bone marrow aspirate was carried out by using RBC lysis buffer containing ammonium chloride under sterile conditions. Briefly, the bone marrow aspirate was mixed with ice-cold lysis buffer in a ratio of 1:5 (marrow aspirate: buffer) and was incubated for 7mins on ice. The reaction was stopped by adding fetal bovine serum (FBS) at a final concentration of 10%. The lysate was then centrifuged for 7mins at 280g, supernatant was discarded and the pellet containing mononuclear cells was re-suspended in DMEM low glucose medium containing 10% FBS and penicillin/streptomycin. The cells were then seeded onto fibronectin coated (10ng/cm^2) tissue culture flasks at a density of 1×10^5 cells/cm^2 in DMEM low glucose media.

The flasks were kept in CO_2 incubator maintained at 37ºC and 5% CO_2. 50% of old media from the flasks was discarded every 2-3 days and the cultures were supplemented with same amount of fresh media.

After around 2-3 weeks, adherent, spindle shaped cells were observed which were sub-cultured and expanded and used for experiments. After adherent cells were

observed in the culture flasks, complete media change was done for the flasks every 2-3 days. The cells were trypsinized when 60-70% confluent.

3.2.2. Differentiation of mesenchymal stem cells

Differentiation of mesenchymal stem cells was carried out according to previously established protocols. Briefly, MSC were seeded at densities of 5000 cells/cm^2 for osteogenic and 25,000-30,000 cells/cm^2 for adipogenic differentiation. After 24 hours of seeding, normal media was replaced by induction media containing inducers for adipogenesis and osteogenesis (composition described in materials section). The cells were induced to differentiate for 14 days. Media change was done every 2-3 days.

Osteogenic differentiation was analyzed at the end of 14 days by staining the osteo-differentiated cells for alkaline phosphatase activity by using Leukocyte Alkaline Phosphatase Kit (Sigma Aldrich, 86C-1KT). Staining was done following manufacturer's protocol. Briefly the cells were fixed with citrate acetone formaldehyde fixative and incubated with substrate for alkaline phosphatase for 15 mins in dark. The cells were then washed with de-ionized water and nuclei were counterstained with neutral red. The stained cells were counted under microscope and photographed.

Adipogenic differentiation at the end of 14 days was analyzed by staining the oil droplets in the adipo-differentiated cells with Oil-red O (Sigma Aldrich). Oil-red O is a lysochromediazo dye used for staining neutral triglycerides and lipids. Briefly, for staining the differentiated cells, initially the cells were washed with PBS and fixed with 10% formalin. The fixed cells were then rinsed with 60% isopropanol gently and then stained with Oil-red O for 20 mins. The stained cells were then washed with de-ionized water and were counted under the microscope and photographed.

For each time point during adipogenic and osteogenic differentiation, the number of cells that differentiated into adipocytes and osteocytes were calculated by counting the number of Oil-Red O or Alkaline Phosphatase positive cells respectively. The total number of cells in each particular well was calculated by staining the nuclei. Percentage of adipocytes and osteocytes in each well was determined and the final data plotted.

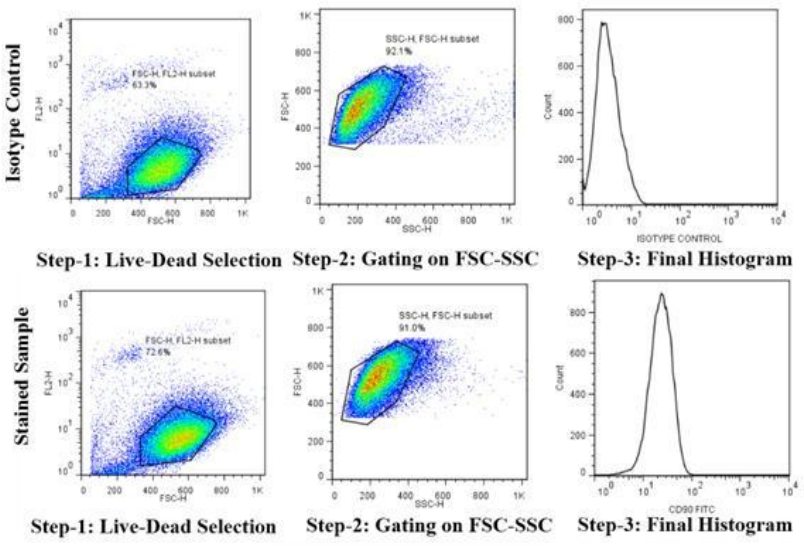
3.2.3. Phenotyping of mesenchymal stem cells

Phenotype of isolated mesenchymal stem cells was analyzed by flow cytometry. The cells were stained with fluorescence conjugated monoclonal antibodies against CD13, CD29, CD34, CD45, CD49a, CD49b, CD49e, CD73, CD90, CD146, SSEA-4 and NGFR and analyzed with BD FACS Calibur Flow cytometer. Briefly, the cells were trypsinized and re-suspended in 2% FBS in PBS and were incubated with fluorescence conjugated monoclonal antibodies for 30 mins in dark on ice. The cells were then washed with cold 2% FBS in PBS and re-suspended in 2% FBS containing propidium iodide for live dead discrimination. The cells were then analyzed in a BD FACS Calibur flow cytometer.

Analyzing cells by flow cytometry:

Cell surface marker analysis:

Data acquisition was done with BD Cell Quest™ Pro software provided with BD FACS caliber flow cytometer equipped with a 488nm blue laser. Initially live cells were selecting by gating and then expression of the fluorescence conjugated monoclonal antibody was analyzed in live cells in either FL-1 (Green emission 530/30 nm band pass filter) or FL-2 (Red/orange Emission 585/42nm band pass filter) or FL-3 (650 nm low pass filter) channels depending upon the type of fluorochrome used. The gating strategy is depicted as follows. The final histograms obtained in step 3 for isotype control and stained sample are overlayed to obtain the histogram shown for each MSC marker.



DNA content analysis:

Initially debris was gated out in FSC/SSC plot and then doublet discrimination was done using FL2-A/FL2-W plot. Single cells were then analyzed for fluorescence for DNA content in a FL2-A histogram.

3.2.4. Karyotyping of mesenchymal stem cells

Metaphase chromosome spreads of MSC were prepared and stained with Giemsa. Briefly, MSC at 50%-60% confluence were treated with 10µg/ml Colchicine overnight in culture. The cells were then trypsinized and pelleted by centrifugation (1600rpm x 10 mins). The supernatant was removed and around 500µl of the liquid was left in the tube. The pellet was re-suspended by gently flicking the tube with fingers. 10ml of 0.075M potassium chloride was added to the tube and incubated for 10 mins in a 37ºC water bath. 3-5 drops of pre-chilled Carnoy's fixative was added and the cells were pelleted by centrifugation (1600rpm x 10mins). The supernatant was removed and around 500µl of KCl was left behind. 10ml of freshly prepared Carnoy's fixative was added and the cells were pelleted by centrifugation. This step was repeated twice. Finally the cells were re-suspended in 500µl of fixative. Around 20µl of the cell solution was dropped from a distance of around 45 cm on preheated clean glass slides at 55ºC. The slides were then allowed to dry and stained with Giemsa. Excess stain was removed by washing with tap water and the slides were mounted and observed under microscope.

3.2.5. Scanning electron microscopy

Mesenchymal stem cells were seeded onto fibronectin coated glass slides. These were grown in normal medium or differentiated into osteocytes or adipocytes by addition of differentiation medium. After 14 days of differentiation, the differentiated cells or undifferentiated cells were fixed and analyzed by scanning electron microscope (SEM). Briefly, the media was removed and the cells were washed 2-3 times with 0.2µM filtered phosphate buffered saline (PBS). The cells were then fixed with 2.5% gluteraldehyde for 2-3 hours in dark. After fixation, the cells were washed gently 2-3 times with PBS and dehydrated with grades of alcohol starting with 30%, 50%, 70%, 90% (2 x 10 minutes) and 100% (2 x 10 minutes) for 10 mins each. The dehydrated cells were then dried in a desiccator overnight. The cells were then gold coated with a sputter coater and analyzed by SEM (Leo 1430vp, Germany).

3.2.6. Actin staining with phalloidin TRITC

Actin cytoskeleton of mesenchymal stem cells were stained with TRITC conjugated phalloidin (P1951- Sigma Aldrich, Bangalore). Phalloidin binds to polymeric F-actin and TRITC conjugated phalloidin has excitation at 540-545nm and emission at 570-573 nm. MSC grown on fibronectin coated coverslides were fixed with paraformaldehyde and stained with phalloidin TRITC. Briefly, after growing the cells in normal media or differentiating them to either adipocytes or osteocytes, the media was removed and the cells were washed 2-3 times with PBS. The cells were then fixed with 4% paraformaldehyde for 30 mins in dark. The fixative was removed and the cells were washed 2-3 times with PBS. Permeabilization of the cells was done by 0.1% Triton X-100 and then blocking was done with 5% FBS for 1 hour in dark at room temperature. Actin staining was done overnight at 4ºC by incubating with TRITC conjugated phalloidin. After staining, the cells were washed 4-5 times with PBS in dark and observed under fluorescence microscope (NIKON TS100F equipped with CCD camera).

For flow cytometric analysis of F-actin fluorescence in MSC stained with Phalloidin-TRITC, above mentioned protocol was followed. Mesenchymal stem cells were trypsinized, fixed immediately with 4% paraformaldehyde and the above mentioned protocol was followed. After staining, the cells were analyzed with BD FACS Calibur Flow cytometer. Cells not stained with Phalloidin-TRITC but processed through all the steps in the fixation and staining protocol were used to obtain background fluorescence during flow cytometric analysis.

3.2.7. Staining with phosphoprotein-specific antibodies for flow cytometry

Cells were fixed with 4% formaldehyde and permeabilised with 100% ice cold methanol. Cells were then immediately stained with fluorescent conjugated antibodies that specifically bind to the phosphorylated form of the proteins for 1 hour at 37ºC in dark. The cells were then analyzed by flow cytometry.

3.2.8. Propidium iodide staining of MSC for cell cycle analysis

MSC were fixed and stained with propidium iodide (Sigma Aldrich) and DNA cell cycle analysis was done by flow cytometry. Briefly, the cells were trypsinized, washed with PBS and fixed with 70% ice cold ethanol. Ethanol was added by gentle

vortexing and the cells were incubated at 4ºC for 30 mins. (* can be stored indefinitely at this stage). The cells were then washed with PBS and treated with RNAse A (50µg RNAse A). The cells were then washed with PBS and stained with propidium iodide (Sigma) (50µg/ml) and analyzed by flow cytometry.

All the centrifugation steps were carried out at 4ºC with zero de-acceleration to minimize loss of cells during centrifugation.

3.2.9. Inhibition of actin polymerization

Inhibition of actin polymerization was done by addition of Cytochalasin-D (Sigma). Cytochalasin-D was added at different concentrations (10-1000ng/ml) directly to the media during culture. Media was supplemented with the drug every 24 hours as the drug became ineffective in inhibiting actin after 24 hours. Media change was done every 2-3 days and Cytochalasin-D was added accordingly.

3.2.10. Inhibition and recovery of actin polymerization during differentiation

Inhibition of actin polymerization during differentiation was done by addition of Cytochalasin-D to the induction media as mentioned before for normal media.

Recovery of actin polymerization was done by removal of Cytochalasin-D from the media. The media was completely removed from the cells and the cell layer was washed gently 2 times with sterile phosphate buffered saline. Normal induction media was added after that and the cells were allowed to differentiate for the required time period. At the end of the induction period, the cells were fixed and stained with Phalloidin-TRITC as mentioned before.

The washing steps were done carefully so that the cell layer doesn't come off or the viability of the cells is affected during the washing steps.

3.2.11. RNA isolation

RNA was isolated using TRIzol Reagent (Applied Biosystems, USA). All the plastic-wares like centrifuge tubes, microtips etc. were DNAase, RNAase free. RNA was isolated following manufacturer's protocol. Briefly, the cells were trypsinized and pelleted by centrifugation. Cells were then lysed with TRIzol. Chloroform was added to the lysate and mixed properly. After centrifugation, the aqueous phase was collected in a

new tube. 100% isopropanol was added to the tubes. Finally RNA was pellet by centrifugations and the pellet was washed with 75% ethanol. The pellet was finally dissolved in 100µl of RNAse/DNAse free water and quantified with a spectrophotometer.

3.2.12. Reverse transcription

RNA was reverse transcribed into cDNA by using high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Protocol provided by the manufacturer was followed. Briefly, 2X reverse transcription master mix was prepared by mixing the following kit components for a 15 µl reaction. All the reagents were kept at 4ºC and enzymes were kept at -20ºC. The components of 2X master mix were as follows:

1. 10X RT buffer-1.5µl
2. 25X dNTP mix-0.6µl
3. OligodT primers-0.9µl
4. Reverse transcriptase-0.5µl
5. RNase inhibitor-0.5µl
6. Nuclease free water-1µl

5µl of the 2X master mix was pipette in PCR tubes and finally 10µl of RNA was added. Reverse transcription was carried out in a Veriti® Thermal Cycler (Applied Biosystems, USA).

The conditions for reverse transcription were as follows*:*

Holding: 25°C for 10 mins**.**
Extension: 37°C for 120 mins**.**
Holding: 85°C for 5 mins**.**
Holding: 4 degree ∞
cDNA was stored in -20°C until use.

3.2.13. Real time PCR

Real time PCR was performed using Power SYBR® Green PCR master mix (Applied Biosystems, USA) in an Applied Biosystems® 7500 Real time PCR system. A 10µl reaction mixture was set up and the following composition of reagents was used.

1. SYBR® green PCR master mix (2X)-5µl.
2. cDNA -4µl.
3. Primer mix (Forward and Reverse) (500nM)-0.5µl.
4. Nuclease free water-0.5µl.

PCR cycling conditions are as follows.

Holding stage:

50°C for 2 mins**.**

95°C for 10 mins**.**

Cycling stage (40 cycles):

95°C for 0.15 mins**.**

60°C for 0.30 mins**.**

Melt curve stage:

72°C for 10 mins**.**

95°C for 0.15 mins**.**

60°C for 1 mins**.**

95°C for 1 min**.**

60 °C for 0.15 mins**.**

Final holding stage:

4°C ∞

3.2.14. Primers for real time PCR

All the primers for real time PCR were obtained from Sigma Aldrich. The primer sequences were as follows:

Gene	Sequence (5'-3')	Tm (°C)
GAPDH	Forward: GGGAAGGTGAAGGTCGGAGT Reverse: GGGTCATTGATGGCAACAATA	60
ADIPONECTIN	Forward: CCATCTCCTCCTCACTTCCA Reverse: GTGCCATCTCTGCCATCAC	61
PPAR gamma	Forward: GACCACTCCCACTCCTTTGA Reverse: CGACATTCAATTGCCATGAG	61
OSTEOCALCIN	Forward: GTGCAGAGTCCAGCAAAGGT Reverse: TCAGCCAACTCGTCACAGTC	60
CD90	Forward: GTTCGTGAAGAGGGAAGCCA Reverse: TGTGGCTGAGAATGCTGGAG	60

3.2.15. Analysis of Real time PCR data

Real time PCR data was analyzed by using $\mathbf{2^{-\Delta\Delta C_T}}$ method. C_T values in triplicates were collected for the target gene and the housekeeping gene. The mean C_T values were taken and then data was analyzed as follows:

Initially $\mathbf{\Delta C_T}$ values were calculated for the experiment and control sample.

ΔC_T Experiment= (C_T target gene-C_T housekeeping gene) Experiment.

ΔC_TControl= ($C_T$target gene-$C_T$housekeeping gene) Control.

Then, $\mathbf{\Delta\Delta C_T}$ value was calculated as follows:

$\Delta\Delta C_T$=ΔC_T Experiment-ΔC_T Control.

Finally, the fold change in gene expression compared to control was determined by $\mathbf{2^{-\Delta\Delta C_T}}$.

3.2.16. Coating of tissue culture flask and glass surfaces

Tissue culture flasks and glass surfaces were coated with fibronectin. Before coating glass surfaces, these were cleaned by overnight treatment with chromic acid, rinsed vigorously with de-ionized water, cleaned with isopropanol and sterilized by autoclaving. Apart from fibronectin, collagen and poly-L-lysine was also used for coating tissue culture flasks. The coating procedures were as follows:

(i). Fibronectin

Fibronectin was diluted in sterile PBS and added to tissue culture or glass surfaces at a concentration of 10ng/cm^2 of growth area. These were incubated for 1 hour at 37ºC. After 1 hour, fibronectin was removed and the coated surface was washed with PBS twice. Cells were then seeded onto these surfaces.

(ii). Collagen

Collagen was diluted in PBS and coating of tissue culture plates was done by incubating for 3 hour at 37ºC (Concentration: 5µg/cm^2). Collagen was removed and the coated surfaces were dried for 1hour in laminar flow and sterilized with UV radiation.

These plates are used directly for seeding cells or stored in sterile conditions at 4ºC until use.

(iii)Poly-L-Lysine

Poly-l-lysine was diluted with PBS and tissue culture plates were coated with collagen at a final concentration of 2µg/cm^2 by incubating for 1 hour at 37ºC. After incubation was over, the plates are washed with PBS and used directly for seeding or stored in sterile conditions at 4ºC.

3.2.17. Drug treatment of MSC

MSC were treated with cytarabine at concentrations of 5-100µM. Cytarabine was obtained as a solution and diluted to required concentration in sterile water. The drug was added directly to DMEM media for the required time period.

For recovery after drug treatment, media containing drug was completely removed and the cell layer was washed with PBS twice. Normal media was then added to the cells and the cells were allowed to grow for the required time periods. Media was changed after every 2-3 days and the cells were trypsinized when confluent.

3.2.18. MTT assay

Effect of drug treatment on mesenchymal stem cells was analyzed by MTT assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The dye can be quantified colorimetrically by dissolving in DMSO and measuring the absorbance at 570 nm. EZcount™ MTT cell assay kit from Himedia Laboratories (Product Code: CCK003), India was used and analysis of cell viability was done as per manufacturer's instruction. Briefly, after the incubation period with the drug was over, MTT reagent was added to the cell culture media at a final concentration of 10%. The plate was wrapped with aluminium foil and transferred to incubator at 37ºC for 4 hours. After 4 hours incubation, the cells were observed under microscope and when purple precipitate was seen, 100µl of solubilization solution was added to the cells. The solution was then mixed gently and the absorbance was measured at 570nm (reference wavelength 650nm). The percentage viability was then calculated for the cells. All the conditions were in quadruplets and average absorbance value was taken.

3.2.19. Co-culture of mesenchymal stem cells with leukemic cells

Human promyelocytic leukemia cells HL-60 and human acute monocytic leukemia cell THP-1 were maintained in RPMI-1640 media or DMEM low glucose containing 10% FBS and penicillin/streptomycin.

For co-culture of MSC with leukemic cells, MSC and leukemic cells were seeded onto fibronectin coated tissue culture flasks in a ratio of 1:3 (Leukemic cells: MSC) in DMEM with 10% FBS. MSC adhere to the cell culture flask but the leukemic cells remain in suspension. Some leukemic cells were found loosely attached to MSC layers. The cells were supplemented with fresh media after every 2-3 days and sub-cultured when confluent. Co-cultures were carried out for 28 days and cells were used for immunophenotyping or cell cycle analysis after that. MSC and leukemic cells were counted regularly during subculture and uniform ratio of 1:3 was maintained while reseeding the cells during co-culture.

3.2.20. Maintenance of cells in culture

(i). Mesenchymal stem cells:

Mesenchymal stem cells were isolated from bone marrow of donors. When the cells became confluent during culture, media was removed and the cell layer was washed with PBS twice. The cell layer was then trypsinized by addition of 0.25% Trypsin. After 3-4 mins of trypsinization, the reaction was stopped by addition of complete DMEM. The cells were pelleted by centrifugation, counted and when required reseeded at density of 3000 cells/cm^2 for continuation of culture or frozen for later use.

Cells were frozen in freezing mix containing 10% DMSO in serum. The pellet was re-suspended gently in 1ml of freezing mix on ice and transferred to cryovials. The cryovials were then kept in -80°C deep freezer in a Cryo 1 Degree cooler to achieve a uniform rate of cooling. After keeping them overnight in -80°C, the cryovials were transferred to a liquid nitrogen container and stored in the vapor phase.

(ii). Leukemic cells

Human promyelocytic leukemia cells HL-60 and human acute monocytic leukemia cell THP-1 were obtained from repository at National Centre for Cell Sciences

(NCCS, Pune, India). The leukemic cells were grown in RPMI-1640 or DMEM supplemented with 10% FBS containing penicillin/streptomycin. The cells were initially seeded at 1×10^5 cells/ml and media change was done every 2-3 days. The cell suspension settled down to the bottom of the flask when kept in the incubator. Media was changed by gentle aspiration from the top without disturbing the cells or by centrifugation and discarding the old media. The cells were split 1:5 and frozen stocks were maintained as mentioned before.

3.2.21. Data analysis

Results were expressed as Mean±SD. Experiments where median values are depicted are specifically mentioned. Statistical analysis was done with Student's t–test using Microsoft excel.

Flow cytometry data was analyzed with either Flow Jo (Treestar) or with BD Cell Quest™ Pro software provided with BD FACS Calibur. Percentage positive cells were calculated by defining gates based on unstained or isotype control. The geometric mean of fluorescence intensity was used for determining mean fluorescence intensity (MFI) values.

4.1.MSC differentiation into adipocytes and osteocytes

4.1.1. Cell morphology and size

Bone marrow derived mesenchymal stem cells have the ability to differentiate into the major cellular components of the bone marrow niche namely, adipocytes and osteocytes. In order to understand the events that activate and control MSC differentiation, MSC were differentiated into osteocytes and adipocytes by addition of specific induction media. The cells underwent significant changes in the cell morphology during differentiation into adipocytes and osteocytes. Under normal *ex-vivo* culture conditions, MSC were spindle shaped with high spindle ratio. On differentiation into adipocytes, the cells increased in both cell size and volume. The cells attained a globular shape enclosing the fat droplets which could be detected by Oil Red O staining (Figure.4.1.1).

However on osteogenic differentiation, the cells lost their spindle shape and became angular with increased cell extensions and mineralization. In order to get a better understanding of the morphological changes, scanning electron microscopy was performed on MSC differentiated into adipocytes and osteocytes. As shown in the figure.4.1.2, adipocytes were globular with fat droplets and osteocytes had a polygonal shape.

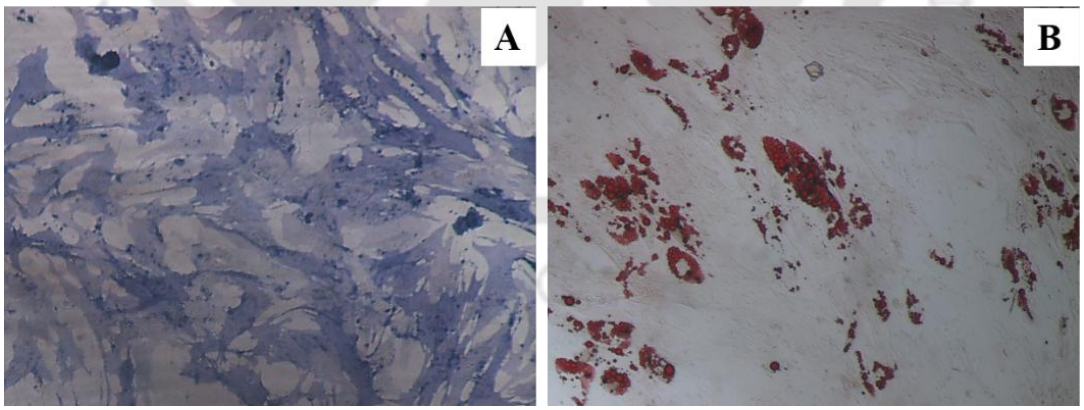


Figure.4.1.1. MSC differentiation. MSC were differentiated into osteocytes or adipocytes in their specific induction media for 14 days and stained for **(A)** alkaline phosphatase activity to detect osteogenic differentiation and **(B)** Oil Red O to determine adipogenic differentiation. Representative photomicrographs are shown. Magnification 100x.

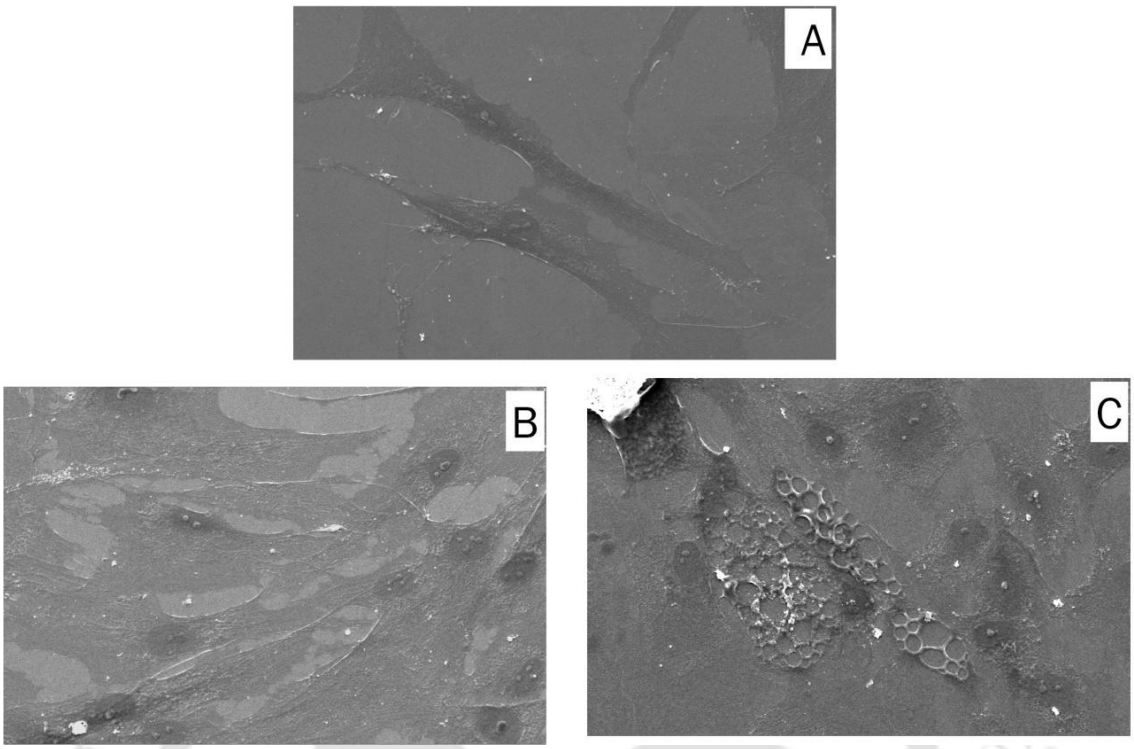


Figure.4.1.2. SEM images of adipocytes and osteocytes. Scanning electron microscopy was performed on **(A)** control cells or cells differentiated into **(B)** osteocytes, **(C)** adipocytes in their respective induction media for 14 days. Representative images are shown. Magnification 500x.

The time required to obtain complete differentiation of MSC into either osteocytes or adipocytes was determined to be 14-21 days. For this, MSC were differentiated into either adipocytes or osteocytes for different time periods (24hr, 3days, 7 days and 14 days) and the level of differentiation was monitored by biochemical staining of the differentiated cells (Figure.4.1.1). In order to detect osteogenic differentiation, the cells were stained for alkaline phosphatase activity (ALP) and the positive cells were enumerated microscopically (Figure 4.1.1).

Adipogenic differentiation was determined by Oil Red O staining and cells were counted microscopically. The percentage of MSC differentiating into either adipocytes or osteocytes increased with increasing time (Figure.4.1.3).

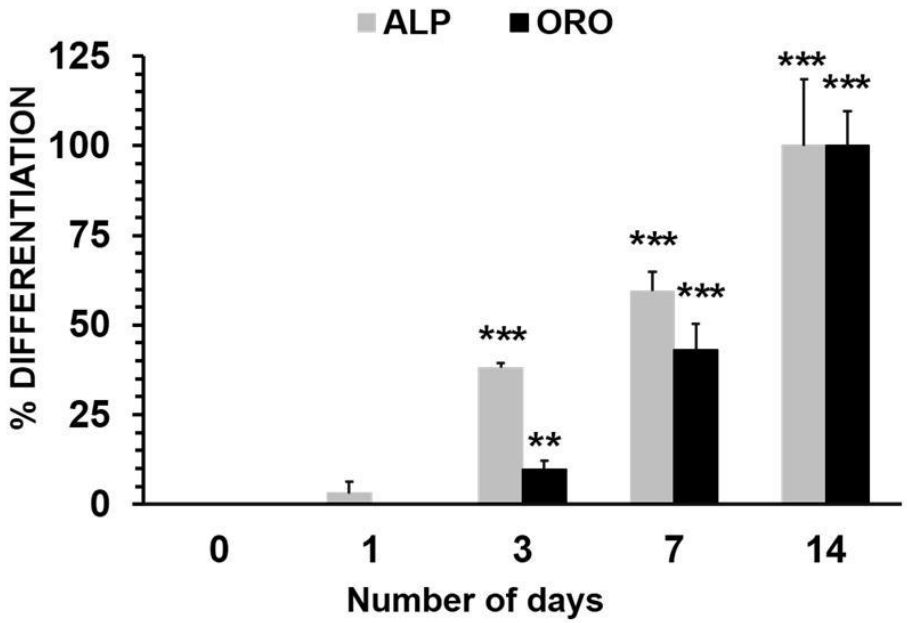


Figure.4.1.3. MSC differentiation into adipocytes or osteocytes. MSC were differentiated for different time periods (Day 1, 3, 7 or 14) into either adipocytes or osteocytes in their respective induction media. Adipogenic and osteogenic differentiation was analysed by staining for Oil Red O (ORO) and alkaline phosphatase (ALP) respectively. Differentiation at day 14 was taken as 100%. Values are means±SD, n=6-7 **$p<0.005, ***$p<0.0005.

To understand the changes in cell size during MSC differentiation, flow cytometric analysis was performed on differentiated cells. MSC were differentiated into adipocytes or osteocytes for different time periods and their forward and side scattering properties were analysed. During adipogenic differentiation, there was a gradual increase in the cell size as detected by increased forward scatter starting from day one until day 14. However during osteogenic differentiation, the cells did not undergo changes in their cell size as evidenced by minimal or no change in the forward scatter until 14 days of induction even though increase in alkaline phosphatase expressing cells were seen during this time period (Figure 4.1.4).

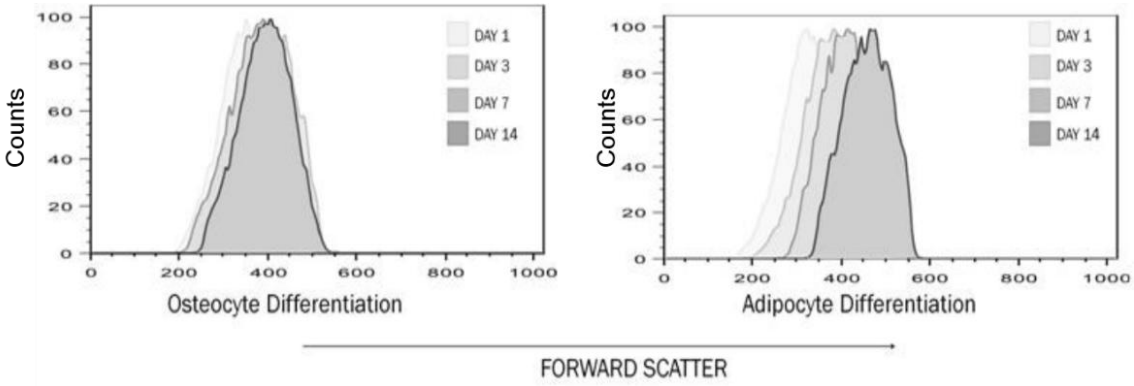


Figure.4.1.4. Cell size analysis by flow cytometry. MSC were differentiated into either adipocytes or osteocytes for different time periods; day 1, day 3, day 7 and day 14 and their cells size was determined by forward scatter measurement in a flow cytometer. Y-axis represents the event number and X-axis represents forward scatter (FSC). Representative histograms from three independent experiments are shown. The analysis for all the samples was performed on the same day to determine the changes in cell-size.

4.1.2. Actin modifications during MSC differentiation

In order to gain more insights into the initial cellular events that occur during MSC differentiation, the actin cytoskeleton arrangement was studied. Actin is an important component of cellular network that was shown to control the cell shape, cell migration and other cellular events. In undifferentiated MSC, the actin filaments were arranged parallel to each other running from one end of the cell to the other. Within 24 hours of addition of either adipogenic or osteogenic induction media there was a significant modification in the actin cytoskeleton arrangement. During adipogenesis, the regular actin arrangement was lost but converted into a criss-cross network like structure (Figure.4.1.5). On osteogenic induction, there was an increase in actin polymerization with increased stress fibers forming peri-nuclear actin bundles (Figure.4.1.5). The actin rearrangement continued until 14 days accompanied by increased oil droplets formation in adipocytes and alkaline phosphatase activity in osteocytes.

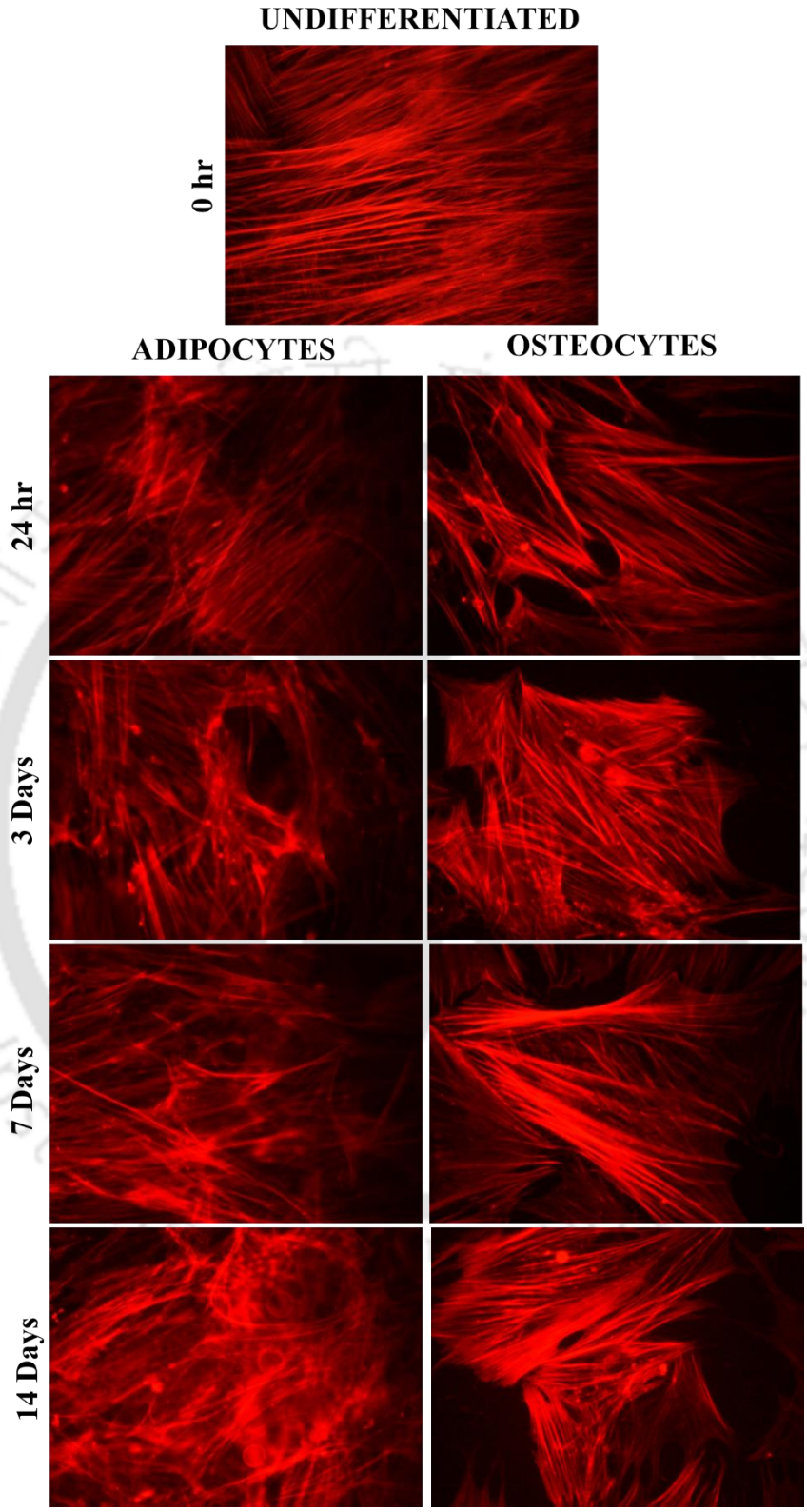
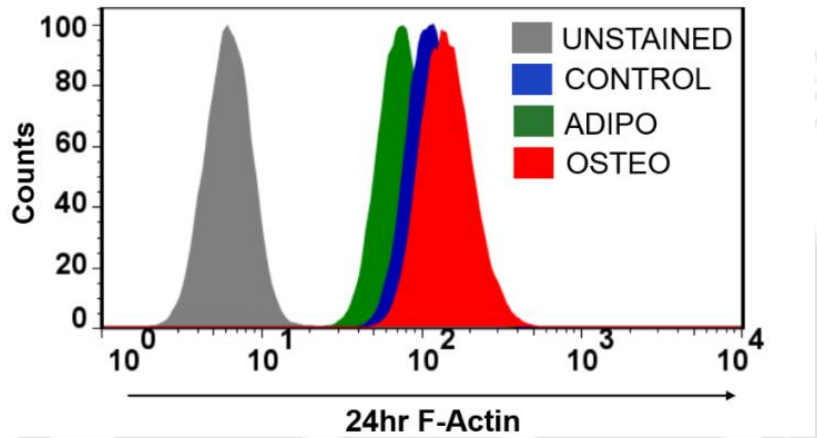


Figure.4.1.5. Actin cytoskeleton during MSC differentiation. MSC were differentiated into osteocytes or adipocytes for indicated time periods and stained with phalloidin-TRITC to visualize actin modification. Representative photomicrographs are shown. Magnification 200x.

To determine if the changes observed in actin arrangement and polymerization resulted in changes in F-actin content of adipocytes or osteocytes, the quantity of F-actin in each cell type was analysed. Filamentous actin (F-actin) content was determined by staining the cells with fluorescent conjugated phalloidin (Phalloidin-TRITC). The increase or decrease in the fluorescent levels which would reflect the F-actin content of the cells was checked by flow cytometry. Surprisingly, within 24 hours of adipogenic induction, there was a decrease in F-actin content whereas F-actin levels increased during osteogenesis (Figure.4.1.6).

A



B

Sample	Mean Fluorescence Intensity (MFI)		
	Unstained Control	TRITC Stained	Ratio
Control	6.17	114	18.476
ADIPO differentiated	6.65	76.2	11.458
OSTEO differentiated	7.61	142	18.659

Figure.4.1.6. F-actin levels in adipocytes and osteocytes. (A) MSC were left uninduced (CONTROL) or induced with osteogenic (OSTEO) or adipogenic (ADIPO) induction media for 24 hours and their F-actin content was determined by flow cytometry. F-actin was stained with phalloidin-TRITC. Y-axis represents the number of events and X-axis represents the F-actin fluorescence intensity. Representative histograms from three independent experiments are shown. Unstained cells were used to determine the background fluorescence levels during flow cytometry. **(B)** Table showing the mean fluorescence intensity of TRITC stained and unstained cells of a representative data set. The experiments were carried out in duplicates and the representative histogram from an independent experiment is shown.

However, there was a significant change in the cell size during MSC differentiation into adipocytes or osteocytes starting from an early time point of 24 hours. In order to make sure that the changes in actin polymerization during MSC differentiation was not due to the cell size change, a restrictive gate which selects a homogenous subpopulation of cells with similar size was made in the forward/side scatter flow cytometry plot during F-actin analysis. This again showed decreased F-actin during adipogenesis and increase in F-actin during osteogenesis (Figure 4.1.7).

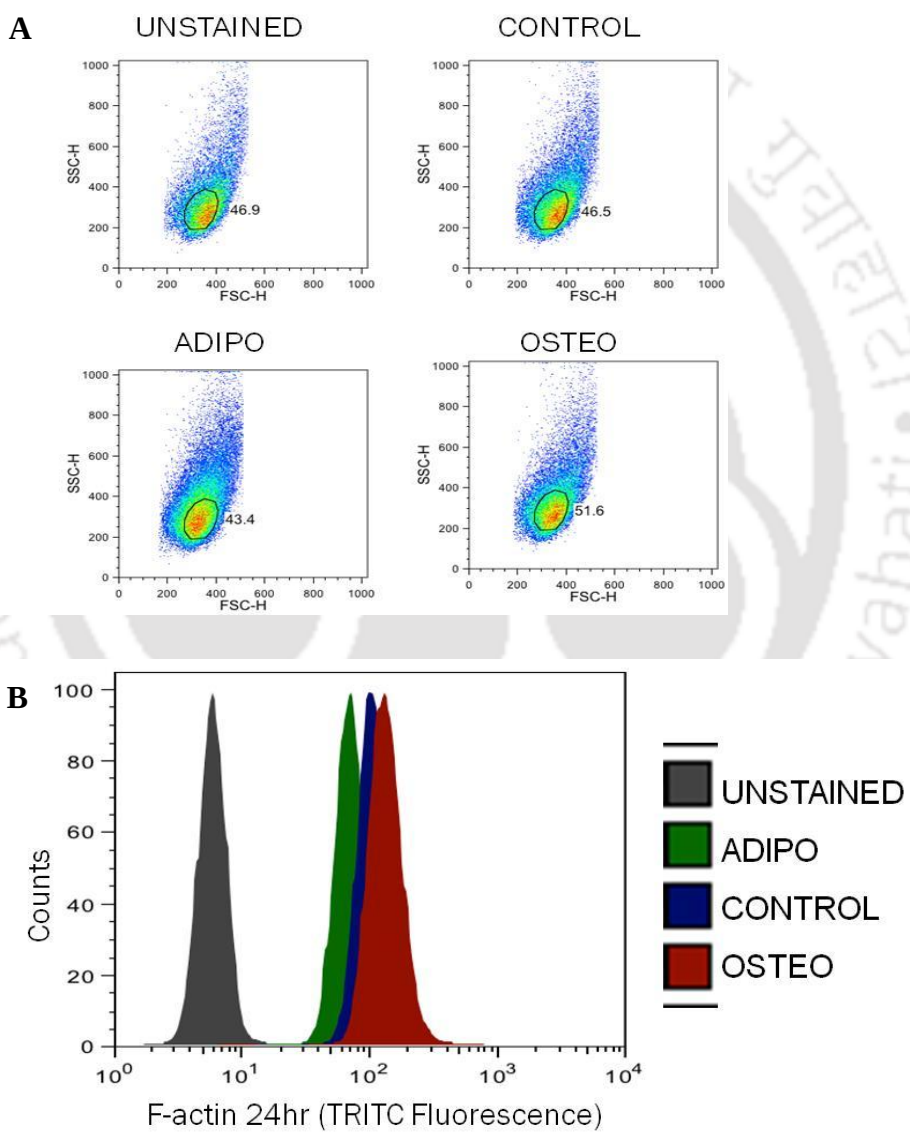


Figure 4.1.7: (A). Flow cytometry dot plot representing forward and side scatter profiles of control MSC (CONTROL) and MSC differentiated into adipocytes (ADIPO) or osteocytes (OSTEO) for 24 hours. **(B).** TRITC fluorescence representing F-actin content in control MSC and MSC differentiated into adipocytes (ADIPO) or osteocytes (OSTEO).

These results show that the changes in actin during MSC differentiation were not only positional but also structural where the cells changed their F-actin content during differentiation.

To study the changes in actin cytoskeleton during MSC differentiation further, actin polymerization was inhibited by addition of Cytochalasin D (CYD). CYD has been reported to bind to the ends of actin fibres thereby inhibiting the addition of actin monomers to the growing actin polymer. Firstly, different concentrations of CYD (1-1000ng/ml) were added to control MSC and their actin cytoskeleton changes was determined by phalloidin TRITC staining (Figure.4.1.8).

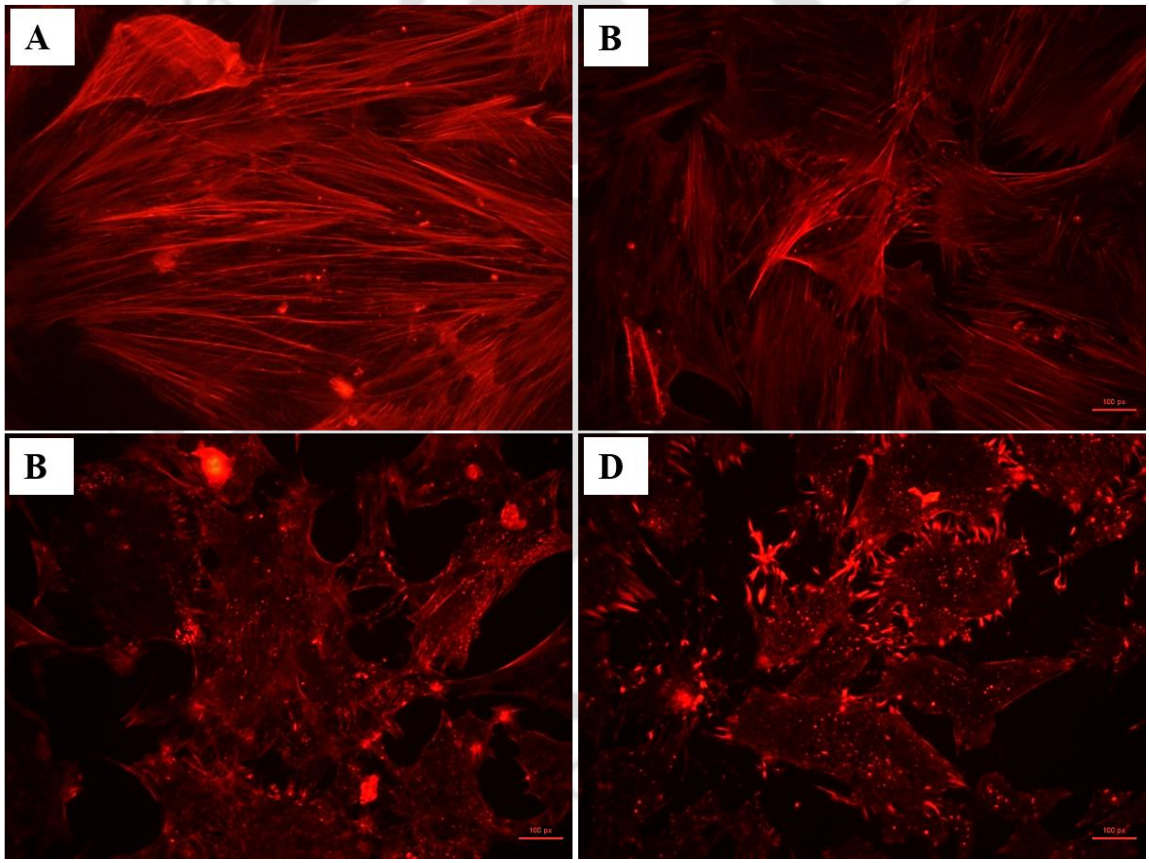
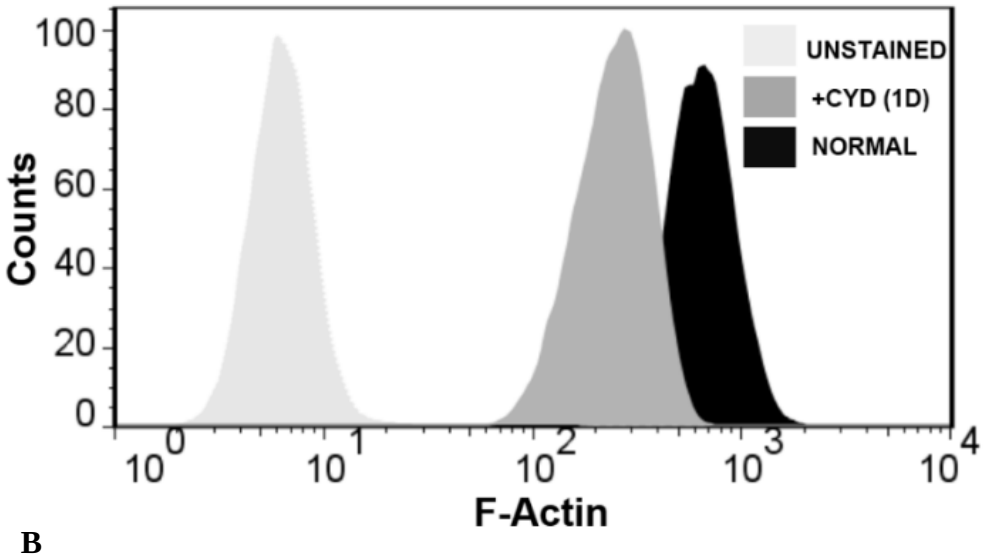


Figure.4.1.8. Actin cytoskeleton in MSC after CYD treatment. MSC were treated with different concentration of CYD **(A)** control **(B)** 10ng/ml **(C)** 100ng/ml **(D)** 1000ng/ml for 24 hours and stained with phalloidin-TRITC to visualize F-actin. Representative images are shown. Magnification 200x.

The optimal concentration of CYD was found to be 1000ng/ml which when treated for 24 hours in the complete culture media, it significantly reduced the F-actin formation without affecting the cell viability of MSC (Figure.4.1.8.D, Figure. 4.1.9).

A



B

Sample	Mean Fluorescence Intensity (MFI)		
	Unstained Control	TRITC Stained	Ratio
NORMAL	12.1	588	48.595
Cytochalasin Treated (+CYD)	8.56	229	26.752

Figure.4.1.9. Flow cytometric analysis of MSC treated with CYD. Flow cytometric analysis was performed to detect F-actin fluorescence in MSC stained with Phalloidin-TRITC after treatment with CYD (1000ng/ml) for 24 hours. Y-axis presents the number of events and X-axis represents the F-actin fluorescence. **(A)** Representative histograms from three independent experiments are shown. **(B)** Values of Mean fluorescence intensity of representative histograms.

CYD treatment altered the cell size and since flow cytometry measures number of fluorescent molecules per cell, a restrictive gate selecting a small population of similar cell size was made in the forward/side scatter flow cytometry plot. This small population was analysed for their change in F-actin fluorescence which again showed a decrease in F-actin content upon CYD treatment (Figure 4.1.10).

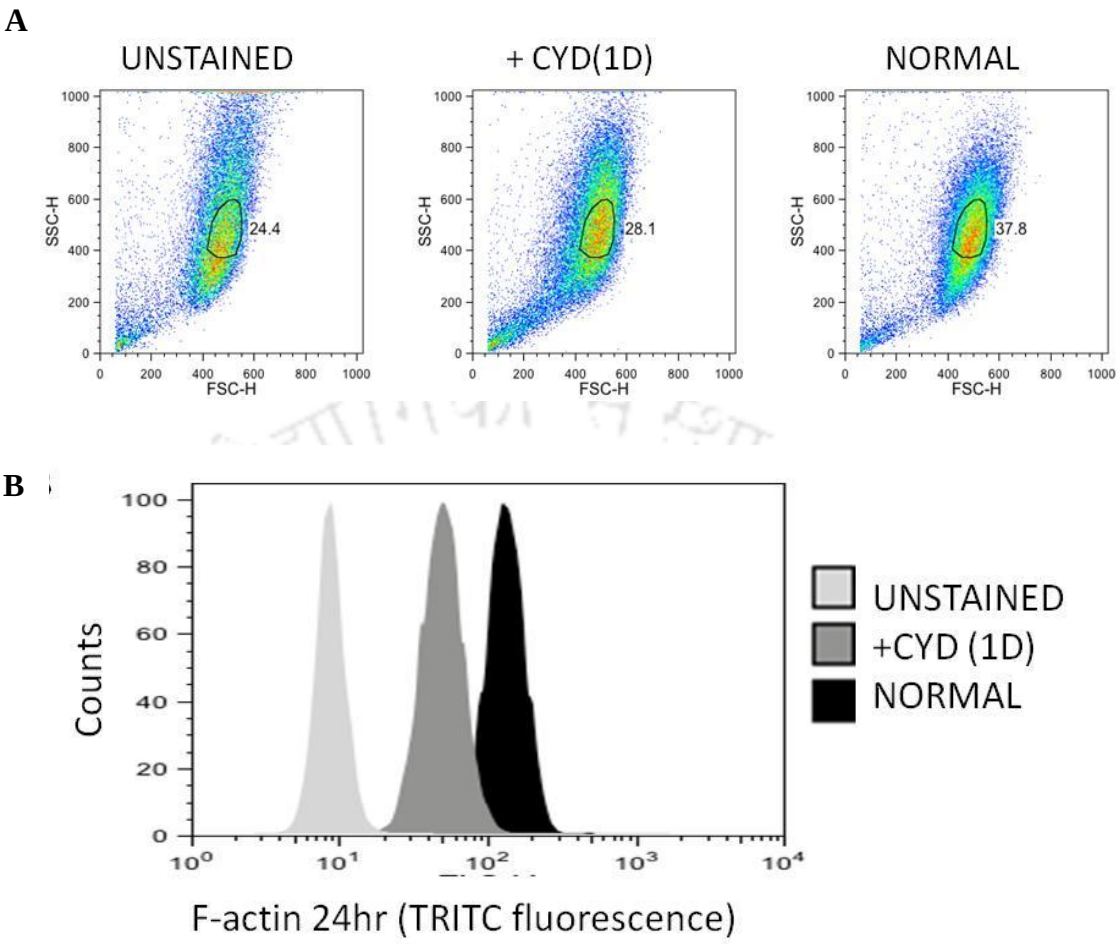


Figure 4.1.10 Flow cytometric analysis of MSC treated with CYD. Flow cytometric analysis was performed to detect F-actin fluorescence in MSC stained with Phalloidin-TRITC after treatment with CYD (1000ng/ml) for 24 hours. **(A)** FSC-SSC profile **(B)** Histogram representing F-actin fluorescence. Representative histograms from three independent experiments are shown.

Upon removal of CYD from the media, the cells re-polymerised their actin filaments within 24 hours (Figure.4.1.11.C). Flow cytometric analysis confirmed the microscopic results where there was a decrease in Phalloidin-TRITC fluorescence upon CYD treatment indicating reduced F-actin content (Figure 4.1.9, Figure.4.1.10).

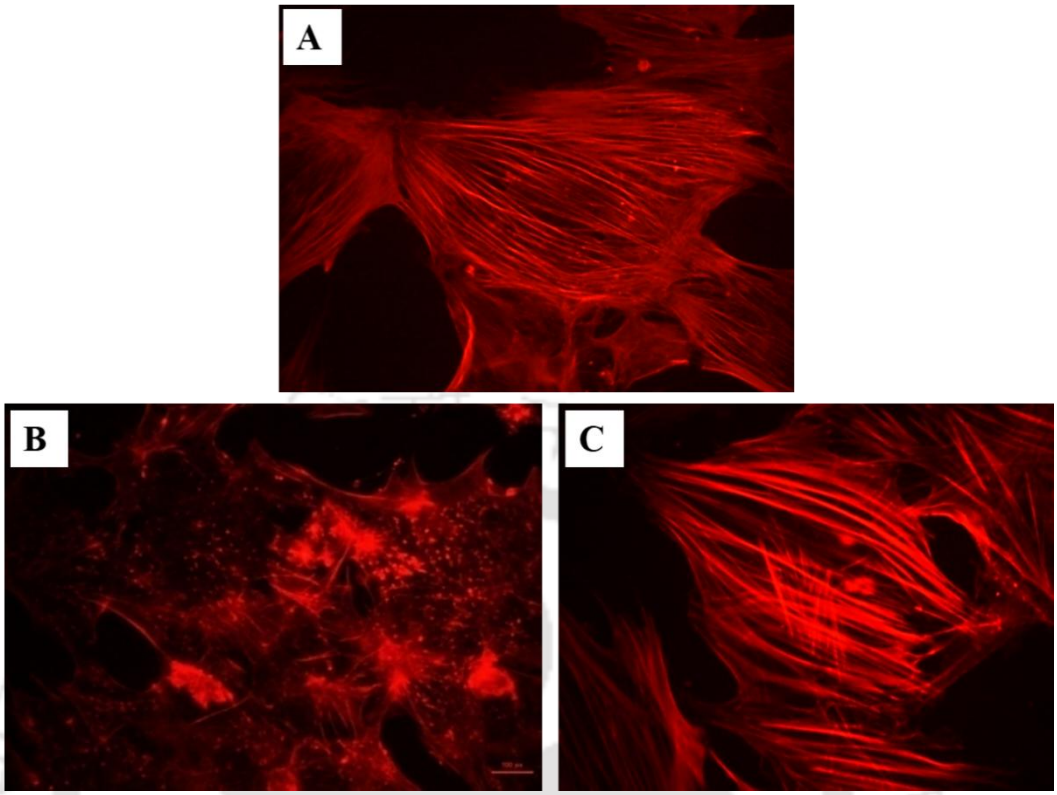


Figure.4.1.11. Reversal of actin polymerization after treatment with CYD. MSC were left **(A)** untreated or **(B)** treated with CYD (1000ng/ml) for 24 hours and **(C)** treated with CYD (1000ng/ml) and grown in normal media without CYD for 24 hours. F-actin was visualized by staining with phalloidin-TRITC. Representative images are shown. Magnification 200x.

4.1.3. Actin polymerization inhibition decreases osteogenesis

To understand further the importance of actin cytoskeleton in determining MSC differentiation, the cells were treated with CYD for different time periods during differentiation. MSC were differentiated into osteocytes by addition of specific induction media without or with CYD. Minimal actin polymerization was observed after CYD addition at various time points in the presence or absence of osteogenic induction media (Figure.4.1.12). ALP staining was performed to detect osteogenic differentiation in cells differentiated into osteocytes in the presence or absence of CYD. At all time points tested there was a significant decrease in the percentage of cells that differentiated into osteocytes when CYD was added to the induction media (Figure.4.1.13.A). A 13 fold decrease in osteogenic differentiation after 7 days and 10 fold decrease after 14 days in the presence of CYD compared to cells differentiated in the absence of CYD was observed. (Figure.4.1.13.A).

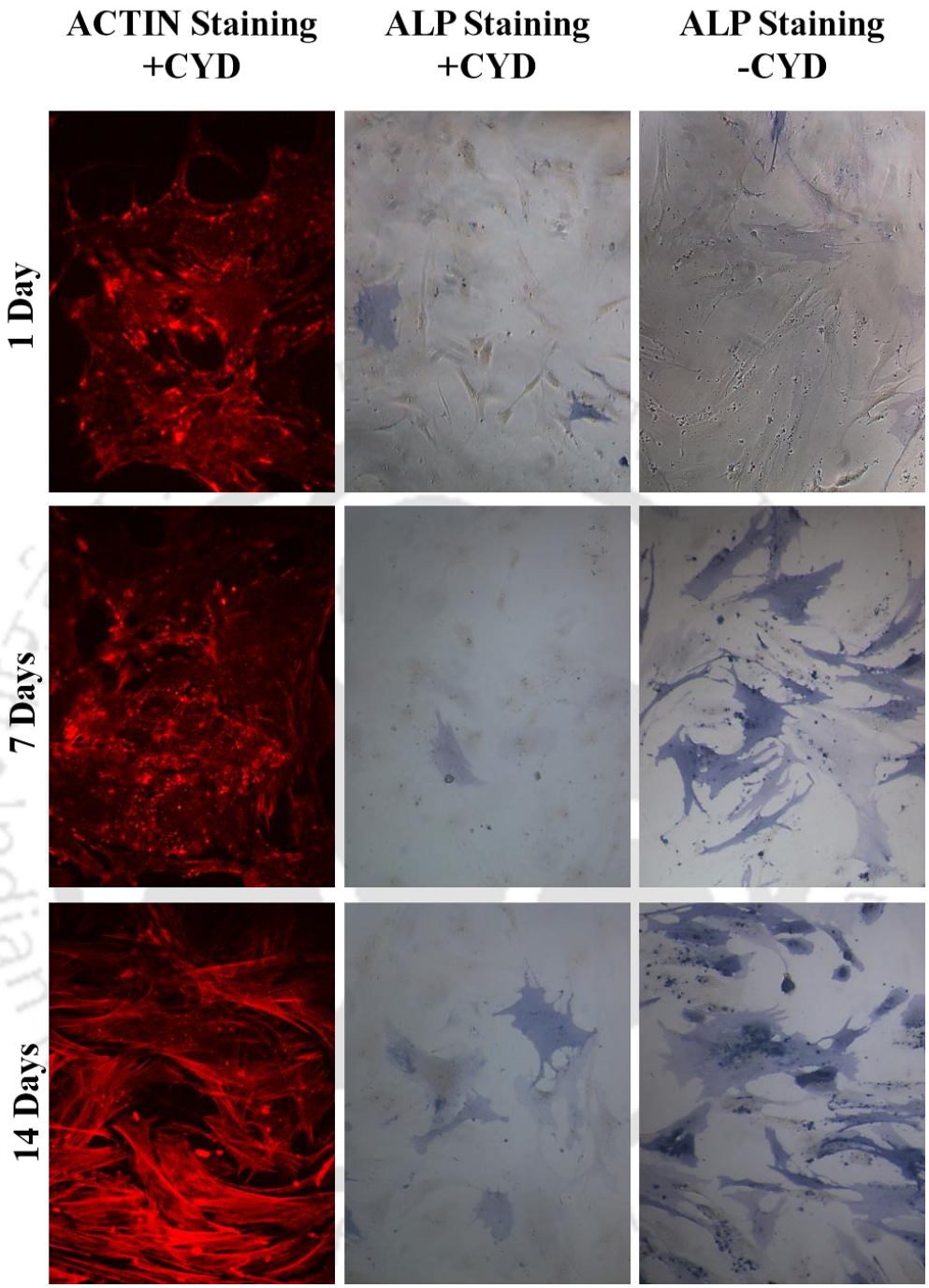


Figure.4.1.12. Osteogenic differentiation after CYD treatment. MSC were treated with osteogenic induction media for indicated time points in the presence (+CYD) or in the absence (-CYD) of CYD. Osteogenic differentiation was determined by staining for alkaline phosphatase activity (ALP staining) and F-actin (ACTIN) was visualized by staining with phalloidin-TRITC. The concentration of CYD used was 1000ng/ml. Magnification 200x. Representative images are shown.

In addition to ALP staining, *OSTEOCALCIN* expression levels was determined by real-time PCR in MSC differentiated into osteocytes in the presence or absence of

CYD for different time periods. There was an eight-fold reduction in *OSTEOCALCIN* expression after 7 days when MSC were differentiated in the presence of CYD and a 30-fold reduction after 14 days (Figure.4.1.13.B). This result clearly demonstrates actin polymerization was an important event during MSC differentiation into osteocytes and inhibition of actin polymerization with CYD resulted in inhibition of osteogenic differentiation.

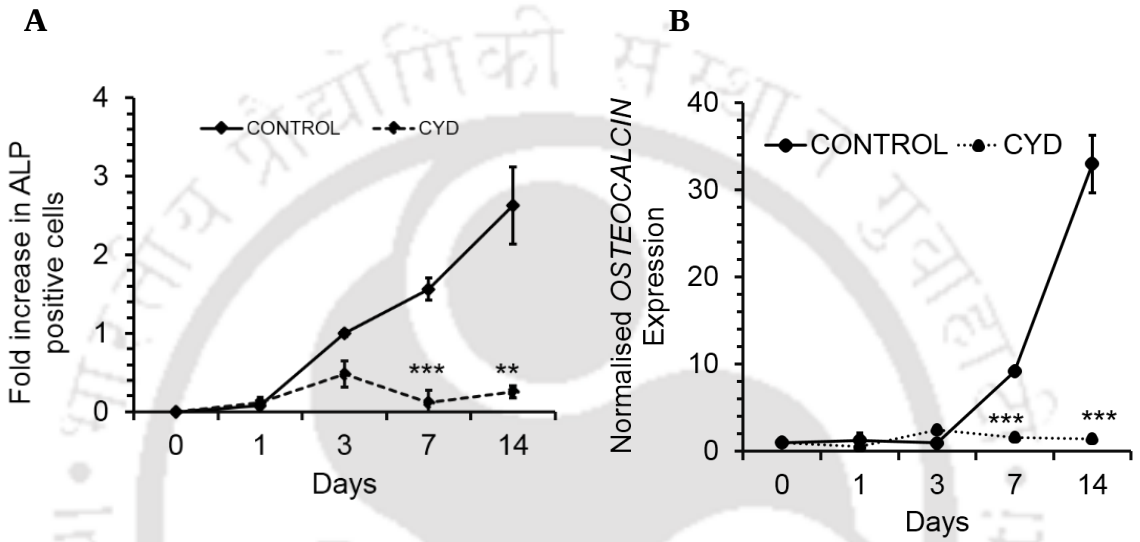


Figure.4.1.13. Effect of CYD treatment on osteogenesis. MSC were differentiated into osteocytes in the presence (CYD) or absence (CONTROL) of CYD for the indicated time periods. **(A)** Osteogenic differentiation was determined by staining for alkaline phosphatase (ALP) activity in the differentiated cells. **(B)** *OSTEOCALCIN* mRNA levels in CYD treated (CYD) and control (CONTROL) differentiated cells were analysed by real-time PCR. Values are mean±SD, n=3 **p<0.005 and ***p<0.0005.

To study the role of actin polymerization in osteogenic differentiation further, MSC were treated with CYD for a short period in the initial stages of differentiation and then allowed to differentiate further. In the first experiment, differentiation was analyzed after 3 days of induction where CYD was added only for the initial 24 hours. The actin polymerization which was inhibited during the initial 24 hours changed into a pattern seen during normal osteogenic differentiation after the removal of CYD (Figure.4.1.14.D). However, there was a 50% reduction in osteogenic differentiation in cells treated with CYD just for 24 hours compared to untreated cells as evidenced by decrease in ALP positive cells (Figure.4.1.14.A).

Secondly, when MSC were treated for 3 days with CYD during 7 days induction period, there was a 3-fold reduction in osteogenic differentiation potential compared to cells differentiated for 7 days without CYD (Figure.4.1.14.B). Consistent with the decreased ALP positive cells during CYD treatment, there was a significant reduction in *OSTEOCALCIN* levels during the above treatment conditions in the presence of CYD (Figure.4.1.14.C).

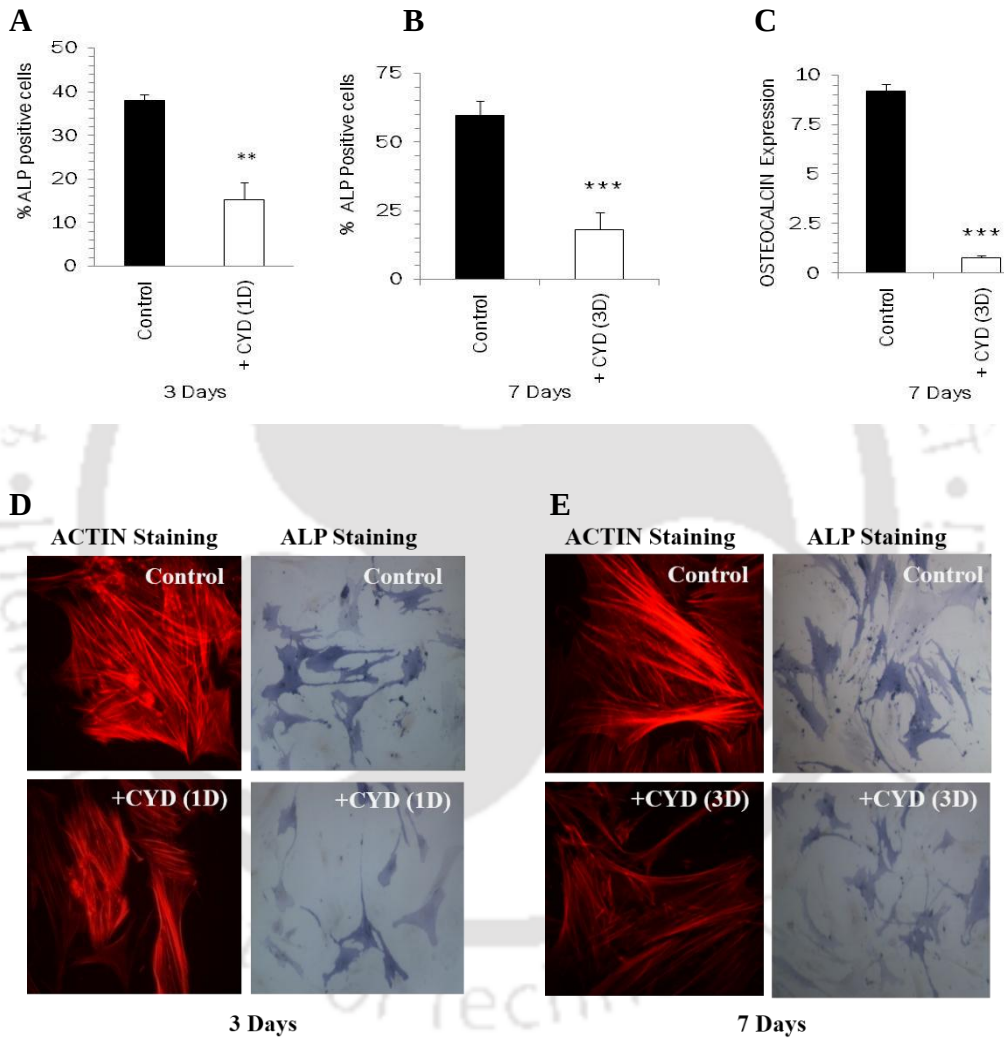


Figure.4.1.14. Effect of CYD treatment on osteogenesis. Percentage of Alkaline phosphatase (ALP) positive cells during **(A)** 3 days induction, **(B)** 7 days induction and **(C)** *OSTEOCALCIN* mRNA expression in MSC induced to undergo osteogenesis without (Control) or with CYD (+CYD) treatment. **(D, E)** osteogenic differentiation was determined by staining for alkaline phosphatase activity (ALP staining) and F-actin was visualized with phalloidin-TRITC staining. CYD treatment was given for initial one day (+CYD (1D)) during 3 days of osteogenic induction or for initial 3 days (+CYD (3D)) during 7 days osteogenic induction. Values are mean ± SD, n = 3-4.** $p < 0.005$, *** $p < 0.0005$.

4.1.4. Inhibition of actin polymerization enhances adipogenesis

A decrease in actin polymerization was observed during adipogenic differentiation and to determine the effect of actin modification in controlling adipogenic differentiation, actin polymerization inhibition was carried out by CYD treatment. MSC were induced to differentiate into adipocytes by addition of adipogenic specific induction media in the presence or absence of CYD. Adipogenic differentiation was determined after one day, three days, seven days and 14 days after adipogenic induction with or without CYD treatment by Oil Red O staining (Figure.4.1.15). F-actin staining with Phalloidin TRITC confirmed that within 24 hours of CYD treatment there was a reduction in actin polymerization and actin remained unpolymerised as long as CYD was present in the media (Figure.4.1.15). Oil Red O staining showed that there was a significant increase in adipogenic differentiation in CYD treated cells compared to untreated adipo-induced cells (Figure.4.1.16.A). For instance, when CYD was added to the induction media for 7 or 14 days, there was approximately a 3-fold increase in adipogenic differentiation compared to the respective untreated but adipo-induced controls (Figure.4.1.16.A).

There was an increased expression of adipogenic lineage specific genes *ADIPONECTIN* and *PPAR gamma* when the cells differentiated into adipocytes in the normal induction media. However, when CYD was added to the differentiating cells, there was an increased expression of *ADIPONECTIN* and *PPAR gamma* corresponding to the increased Oil Red O staining (Figure.4.1.16.B).

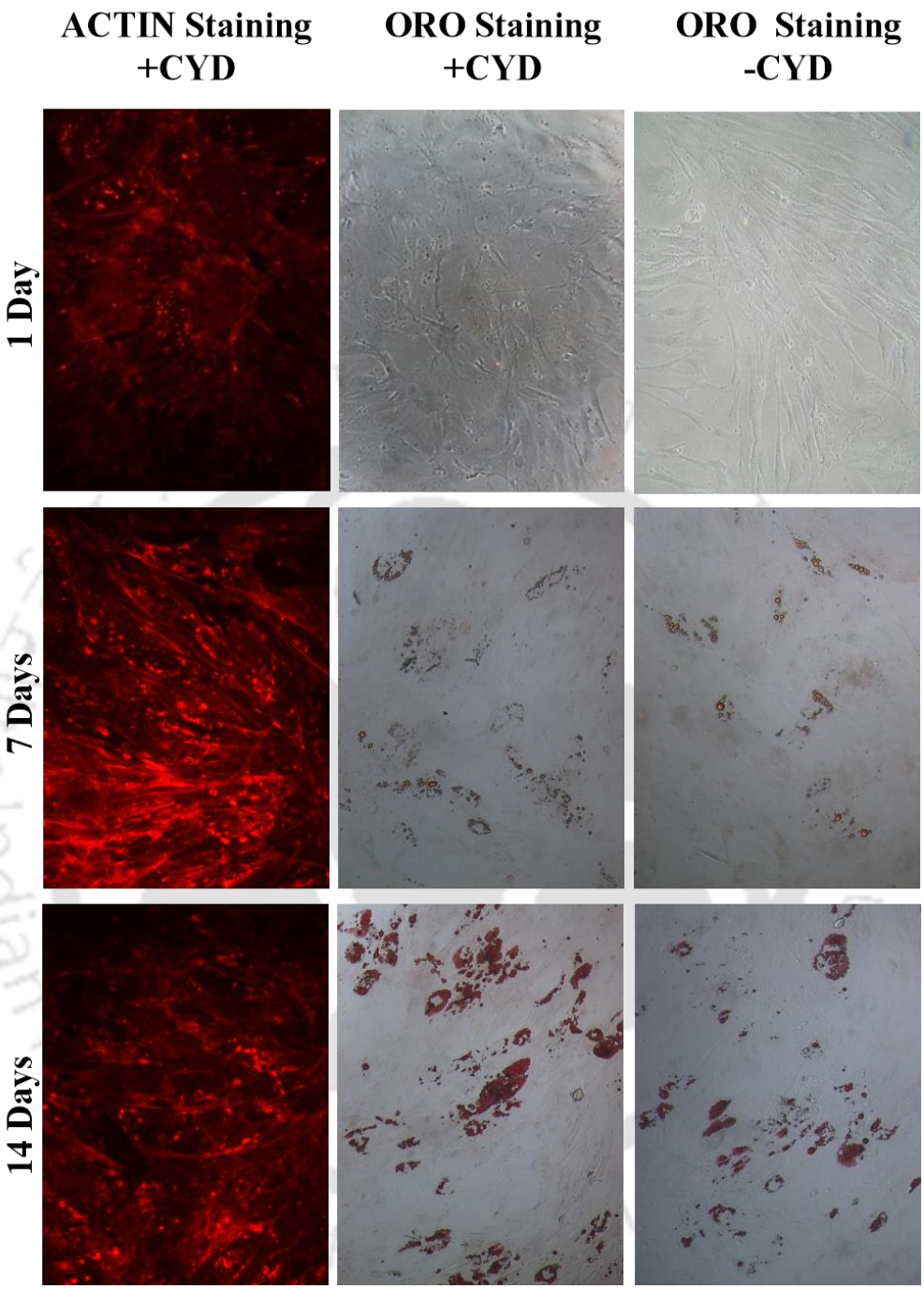


Figure.4.1.15. Adipogenic differentiation after CYD treatment. MSC were treated with adipogenic induction media for indicated time points in the presence (+CYD) or in the absence (-CYD) of CYD. Adipogenic differentiation was determined by staining with Oil Red O (ORO staining) and F-actin (ACTIN) was visualized by staining with phalloidin-TRITC. The concentration of CYD used was 1000ng/ml. Magnification 200x. Representative images are shown.

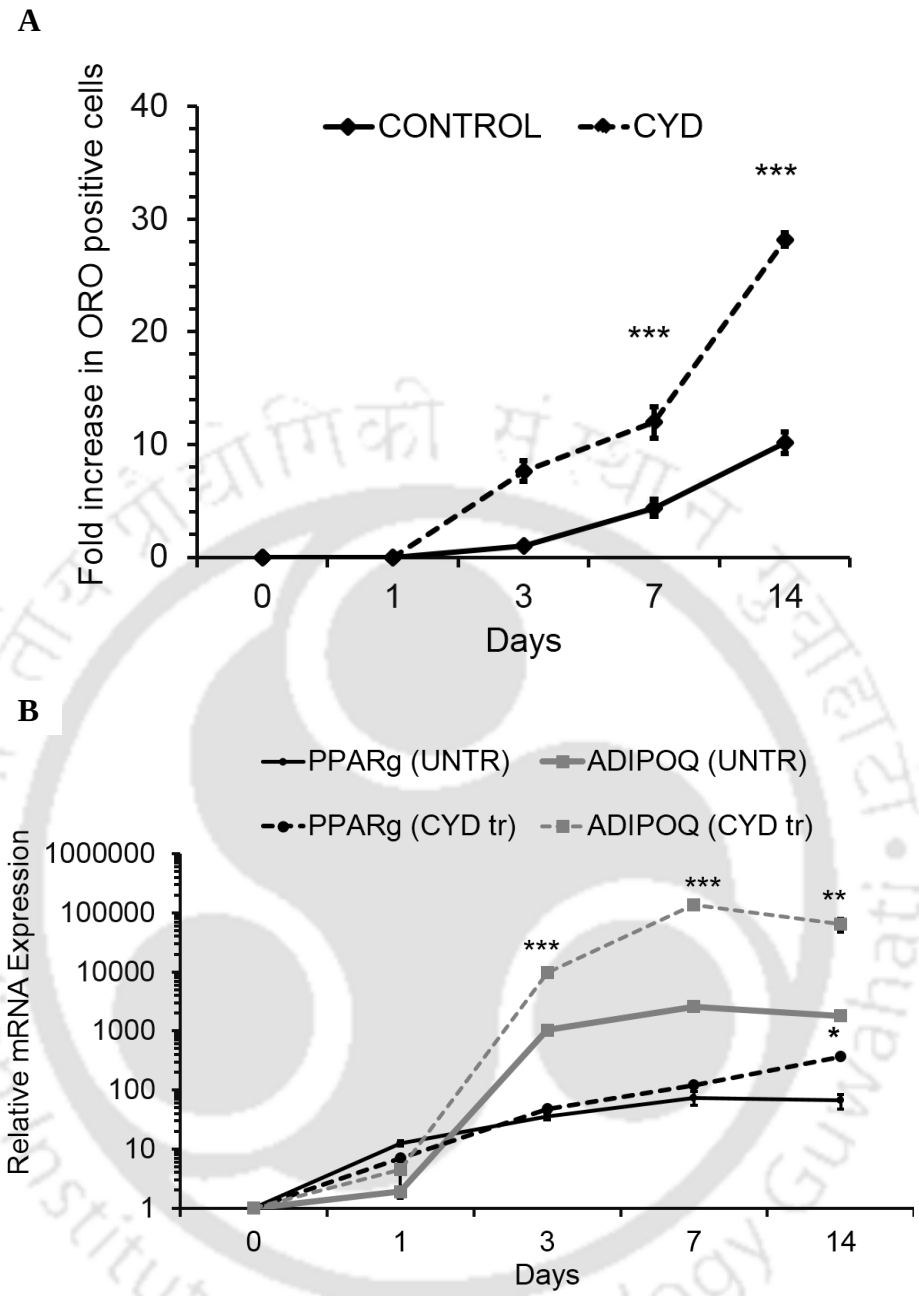


Figure.4.1.16. Effect of CYD on adipogenesis. (A) Fold change in Oil-red O (ORO) positive cells in MSC induced to undergo adipogenesis without (CONTROL) or with CYD (CYD) for the indicated time periods **(B)** Adiponectin (*ADIPOQ*) and PPAR gamma (*PPARg*) mRNA levels in untreated (CONTROL) or CYD treated (+CYD) MSC. Values are mean±SD n=3-4. *p<0.05, **p<0.005 and ***p<0.0005.

For further analysis, when MSC were treated with CYD for initial 3 days during 7 days adipogenic induction there was a 33% increase in adipogenic differentiation compared to cells induced into adipogenic differentiation without CYD (Figure.4.1.17.A). There was a corresponding increase in the expression of *ADIPOQ* in

CYD treated cells in the above conditions compared to untreated adipo-induced cells (Figure.4.1.17.B). Thus, inhibition of actin polymerization enhanced adipogenesis.

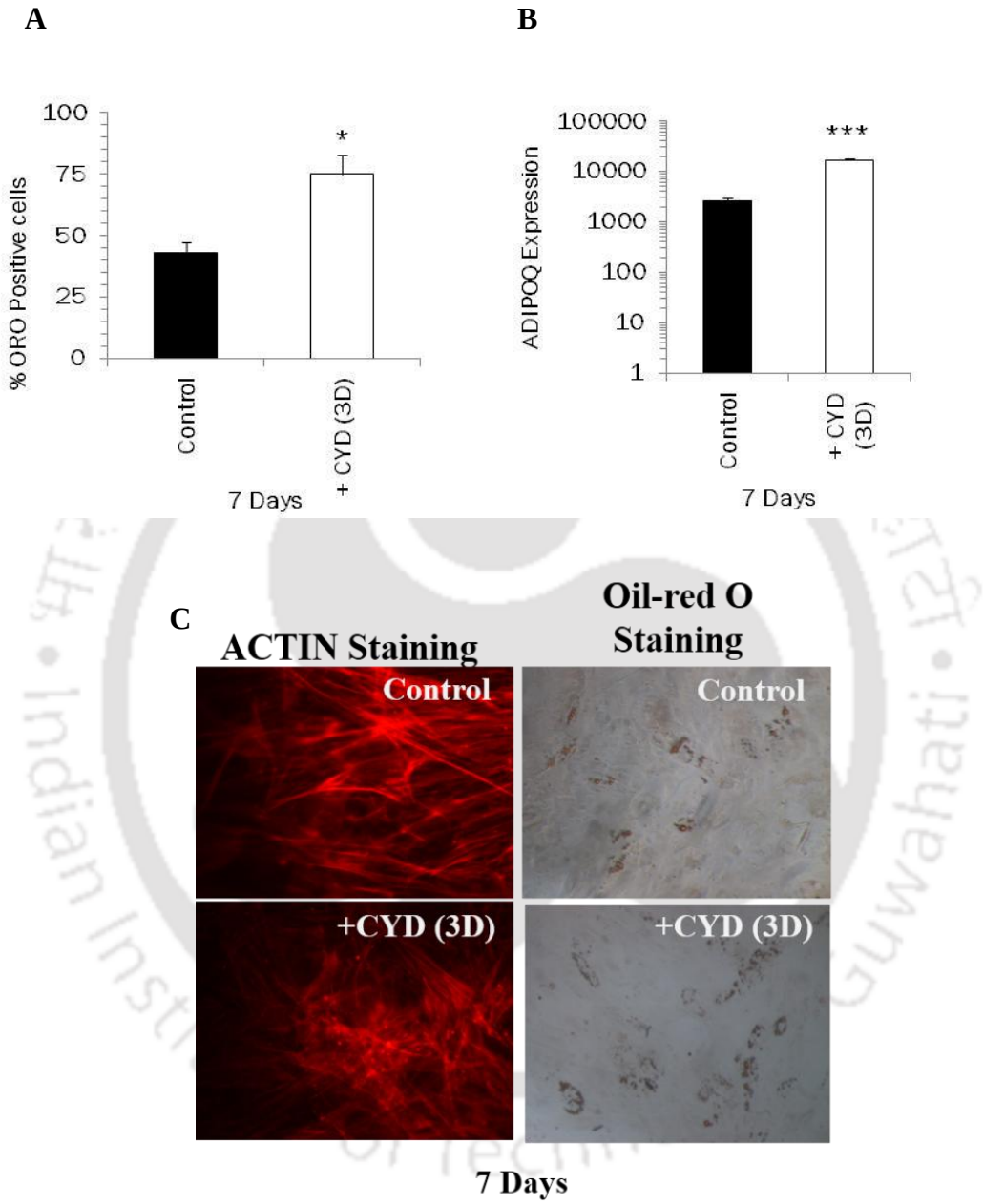


Figure.4.1.17. Effect of CYD treatment on adipogenic differentiation. (A) Percentage of Oil Red O (ORO) positive cells and **(B)** ADIPONECTIN (*ADIPOQ*) mRNA expression in MSC induced to undergo adipogenesis without (Control) or with CYD (+CYD) treatment for 7 days. CYD treatment was given for initial 3 days (+CYD (3D)) during 7 days of adipogenic induction. Values are mean±SD, n = 3-4. *p<0.05, ***p<0.0005. **(C)** Actin and Oil Red O staining in MSC during 3 days of CYD treatment (+CYD (3D)) during 7 days of induction. Control represents adipogenic differentiated cells without CYD. Representative images are shown.

The results so far confirmed that actin cytoskeleton modification was an early event during MSC differentiation into adipocytes or osteocytes and inhibition of actin polymerization resulted in differential effects on adipogenesis and osteogenesis. However, the question remains whether modifying the actin cytoskeleton without the induction factor could prime the cells into a particular lineage. For this, MSC were pretreated with CYD in the normal growth media for one day or three days and the cells were differentiated into either adipocytes or osteocytes in their respective induction media. Differentiation into adipocytes or osteocytes was determined by staining for Oil Red O or alkaline phosphatase respectively. An increase in adipogenic differentiation (Figure.4.1.18.B) but a significant decrease in osteogenic differentiation (Figure.4.1.18.A) was observed.

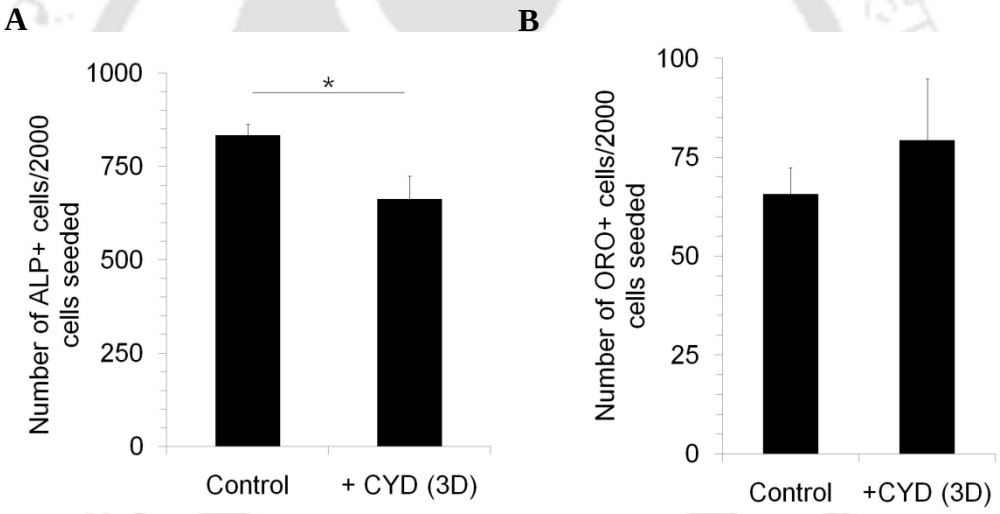


Figure.4.1.18. Effect of CYD pretreatment on MSC differentiation. MSC were pretreated with CYD in normal growth media for 3 days and thereafter induced into **(A)** Osteogenic and **(B)** Adipogenic lineage for 14 days in normal induction media without CYD. ALP stands for alkaline phosphatase staining and ORO represents Oil Red O staining. Values are mean±SD, n=3-5 *p<0.05.

Next, MSC were treated for 7 days in normal growth media with CYD in the absence of induction media. The expression levels of *OSTEOCALCIN* and *PPARg* was analysed. Surprisingly, there was a significant increase in *PPARg* expression (Figure.4.1.19.B) and a significant decrease in *OSTEOCALCIN* expression (Figure.4.1.19.A) in uninduced MSC treated only with CYD compared to untreated control cells suggesting that actin modification with CYD might affect gene expression.

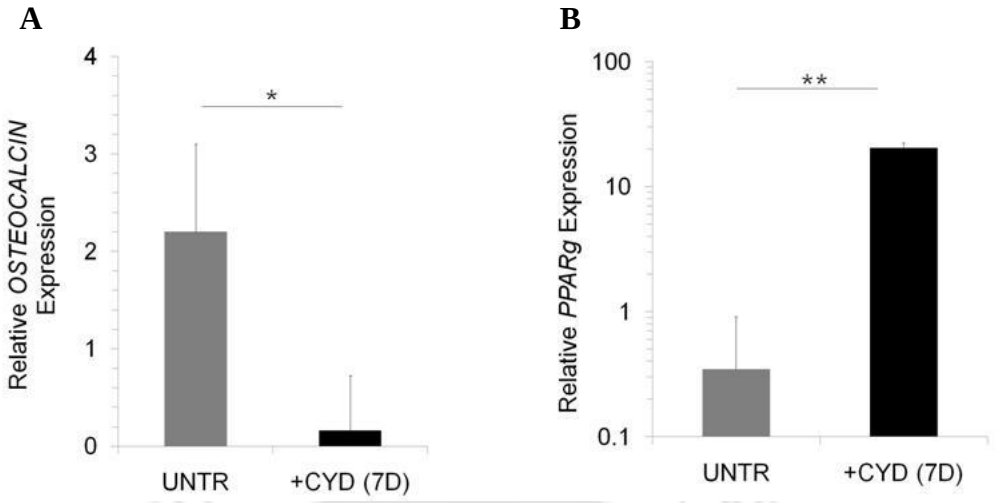


Figure.4.1.19. Gene expression changes after pretreatment of MSC with CYD. Real-time PCR analysis of **(A)** *OSTEOCALCIN* and **(B)** *PPARg* mRNA expression levels in MSC without (UNTR) or with CYD (+CYD (7D)) pretreatment in normal growth media for 7 days. Values are mean ± SD, n = 3. *p <0.05, **p< 0.005.

4.1.5. Integrin expression during MSC differentiation into adipocytes and osteocytes

Integrins connect the actin cytoskeleton to the cell exterior through which they interact with the extracellular matrix substances. Since there were significant changes in actin cytoskeleton during MSC differentiation into adipocytes or osteocytes, the level of changes in integrin expression in these differentiated cells was analysed. The expression of integrins such as CD29, CD49a, CD49b and CD49e were analysed by flow cytometry in the cells differentiated into either adipocytes or osteocytes. This was further compared with MSC differentiated into adipocytes or osteocytes in the presence of CYD where CYD was added to the cells during the entire differentiation period. No difference was observed in CD49a expression where the expression levels were low in undifferentiated and differentiated MSC. There was a reduction in CD29 cell surface expression when the cells differentiated into adipocytes but CYD treatment did not have any effect on CD29 expression during both adipogenesis and osteogenesis (Figure.4.1.20.A).

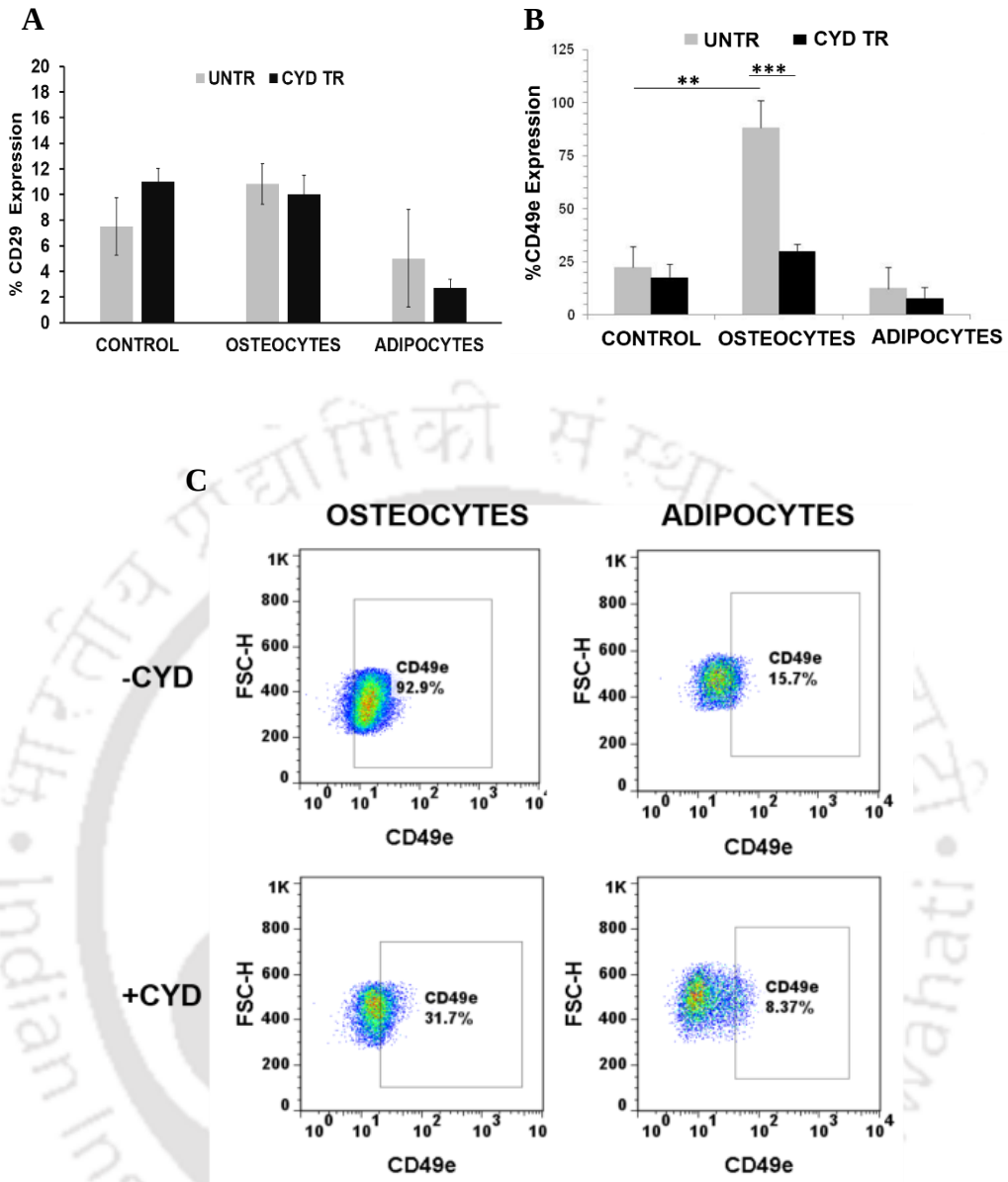


Figure.4.1.20. Integrin expression during adipogenic and osteogenic differentiation of MSC. MSC were differentiated into osteocytes or adipocytes in the absence (UNTR) or presence of CYD (CYD TR) for 14 days and expression of integrin **(A)** CD29 **(B)** CD49e were analyzed by flow cytometry. Values are mean±SD, n=3-4. **p<0.005, ***p<0.0005. **(C)** Dot plots showing percentage of CD49e expression in MSC after 14 days of adipogenic or osteogenic differentiation without (-CYD) or with CYD (+CYD). Gates selecting CD49e expression was made based on background fluorescence levels of isotype controls for each condition. Representative flow cytometric plots are shown.

There was a decrease in CD49b expression when MSC were differentiated into adipocytes or osteocytes, however, CYD treatment did not have any effect on these cells. Interestingly, there was a significant increase in CD49e expression when MSC were differentiated into osteocytes but no changes were observed during adipogenic

differentiation. In addition, when CYD was added to the cells during differentiation, there was 50% reduction in CD49e expression in osteocytes. In contrast, CYD treatment did not have any effect on CD49e expression in control or adipo-differentiated cells (Figure.4.1.20.B).

4.1.6. Actin modification signals through p38MAPK in osteocytes

The study was further extended to understand the molecular pathways affected by actin cytoskeleton changes in MSC. For this, the activation levels of p38MAPK and ERK1/2 MAPK in MSC was analysed during adipogenic and osteogenic differentiation in the presence or absence of CYD.

Significant increase in the phosphorylated levels of both p38MAPK (Figure.4.1.21.A) and ERK1/2 MAPK (Figure.4.1.22) was seen when MSC were differentiated into osteocytes. On CYD treatment, there was a reduction in p38MAPK (Figure.4.1.21.A) phosphorylated levels but not ERK1/2 MAPK (Figure.4.1.22) phosphorylated levels in osteo-differentiated cells.

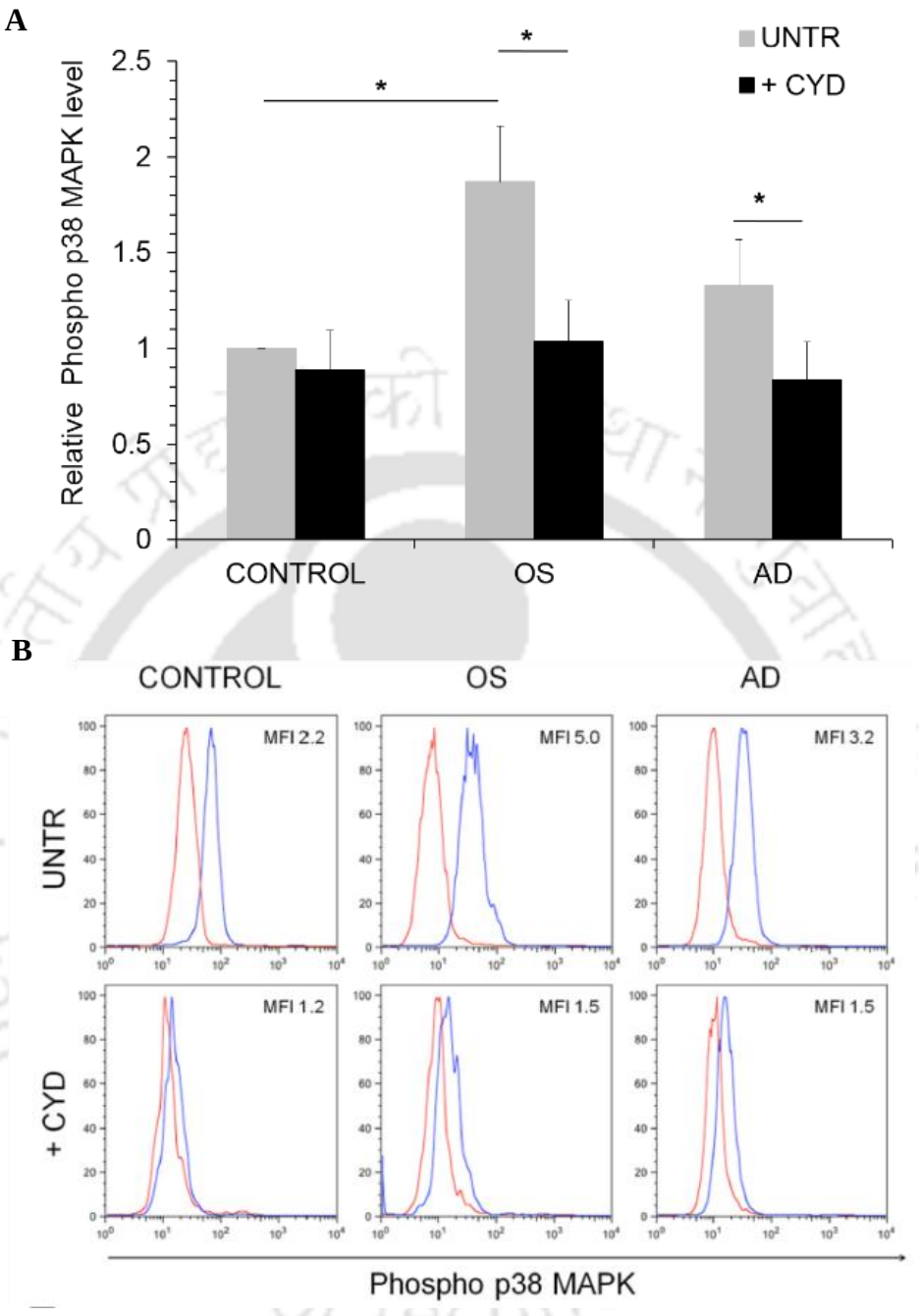


Figure.4.1.21. p38MAPK expression changes during MSC differentiation. MSC were differentiated into osteocytes (OS) or adipocytes (AD) in the absence (UNTR) or presence of CYD (+CYD) for 3 days and the phosphorylated levels of **(A)** p38 MAPK was determined by flow cytometry. The values represented in Y-axis are MFI sample/MFI isotype and these ratios are normalized against untreated CONTROL. **(B)** Representative flow cytometric histograms of MSC differentiated into osteocytes (OS) or adipocytes (AD) which were left untreated (UNTR) or treated with CYD (+CYD) and stained for phosphorylated form of p38MAPK. Red line is the isotype control and blue line is the sample. CONTROL represents undifferentiated MSC. Values are mean±SD, n=3, *p<0.05.

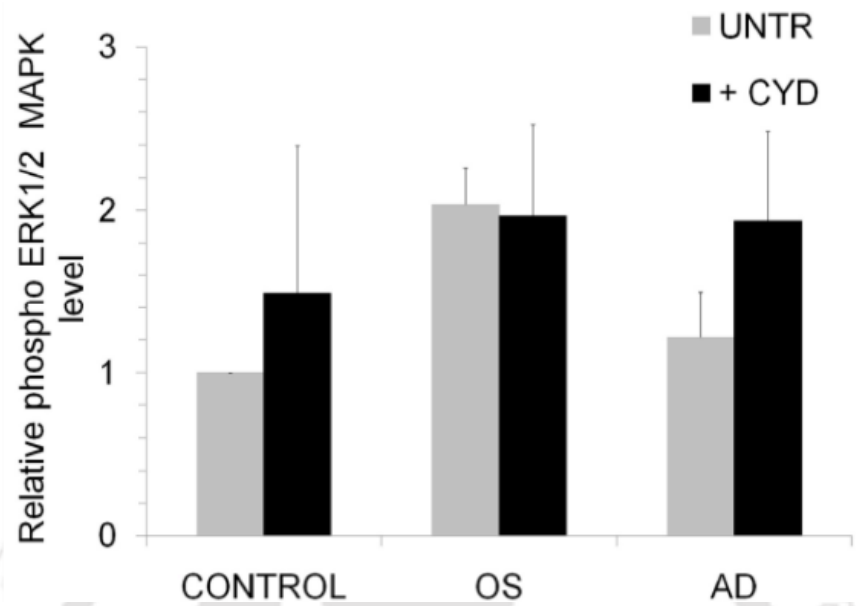


Figure.4.1.22. ERK1/2 expression changes during MSC differentiation. MSC were differentiated into osteocytes (OS) or adipocytes (AD) in the absence (UNTR) or presence of CYD (+CYD) for 3 days and the phosphorylated levels of ERK1/2 was determined by flow cytometry. Y-axis shows the relative phosphorylated levels of ERK1/2 levels compared to undifferentiated untreated control cells (CONTROL). Values are mean±SD, n=3.

Taken together, these results strongly suggest that cytoskeletal changes were very important for MSC differentiation into adipocytes and osteocytes and it was a very early cellular event which precedes the gene expression changes. Actin modification seemed to regulate osteogenic differentiation through p38MAPK pathway.

4.2.Isolation and characterization of mesenchymal stem cells from patients with bone marrow disorders

4.2.1. Morphology

MSC were isolated from several patients reported to possess bone marrow disorders. The cells under study were collected from patients diagnosed for hematologic disorders such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL) and Non-Hodgkin's lymphoma (NHL). The bone marrow cells obtained were at different stages of the disease; during diagnosis, treatment or after remission. Here, in the current study, no attempt was made to classify the above disorders into respective sub-types, but the pre- and post-treatment samples were studied separately and compared.

The samples obtained for our study represents cancer type and incidence in North East India. An attempt was made to classify different hematologic malignancies based on the age at which diagnosis was made. The following plot represents the age-group in which a particular cancer type is prevalent.

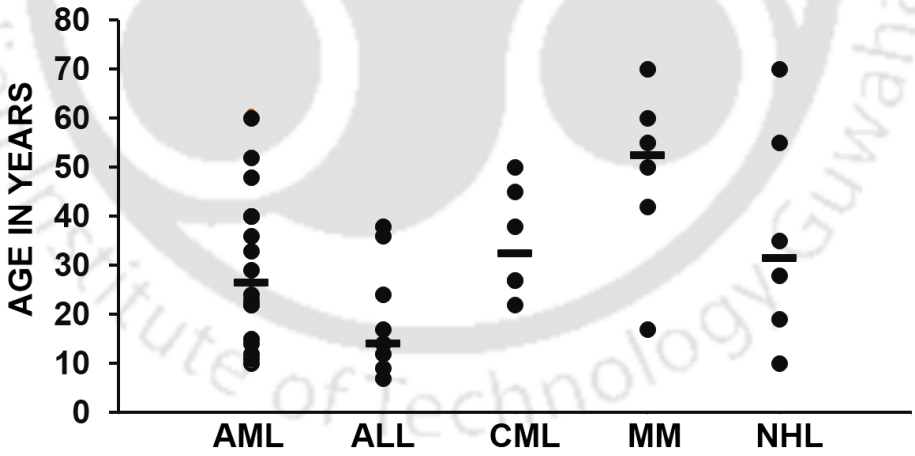


Figure.4.2.1. Age distribution of patients diagnosed for hematologic disorders. The bone marrow samples obtained were divided into broad cancer types and age distribution was studied. Plot showing age distribution in patients diagnosed for acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), multiple myeloma (MM) and non-hodgkin's lymphoma (NHL). Y-axis represents the age in years. Each dot represents a patient. Bars represent median value.

MSC were isolated from more than 80% of the bone marrow samples, however the isolation percentage was lower in cases diagnosed for CML. Initial isolation cultures of all samples had spindle shaped cells, however in later passages MSC isolated from ALL patients changed into flat cells with less proliferative capacity.

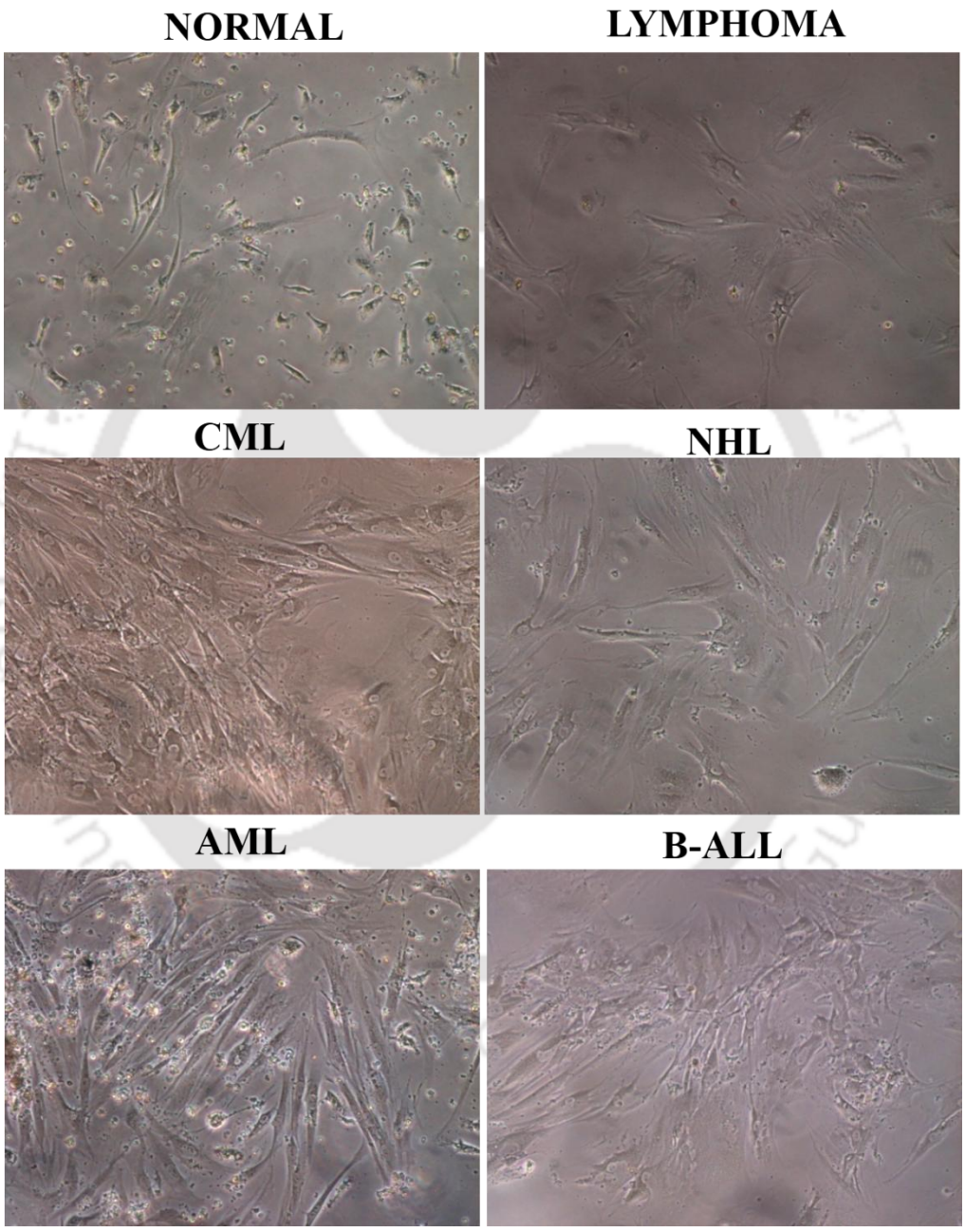


Figure.4.2.2. Morphology of MSC from different patients. Photomicrographs showing morphology of MSC after isolation from bone marrow of patients diagnosed for lymphoma, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), non-hodgkin's lymphoma (NHL) and acute lymphoblastic leukemia (B-ALL). Magnification 100x. Representative images are shown.

MSC isolated from different patients were karyotyped to check for chromosomal abnormality. MSC isolated from all the patients had normal karyotype with 46 chromosomes although translocations were not determined for these cells (Figure.4.2.3).

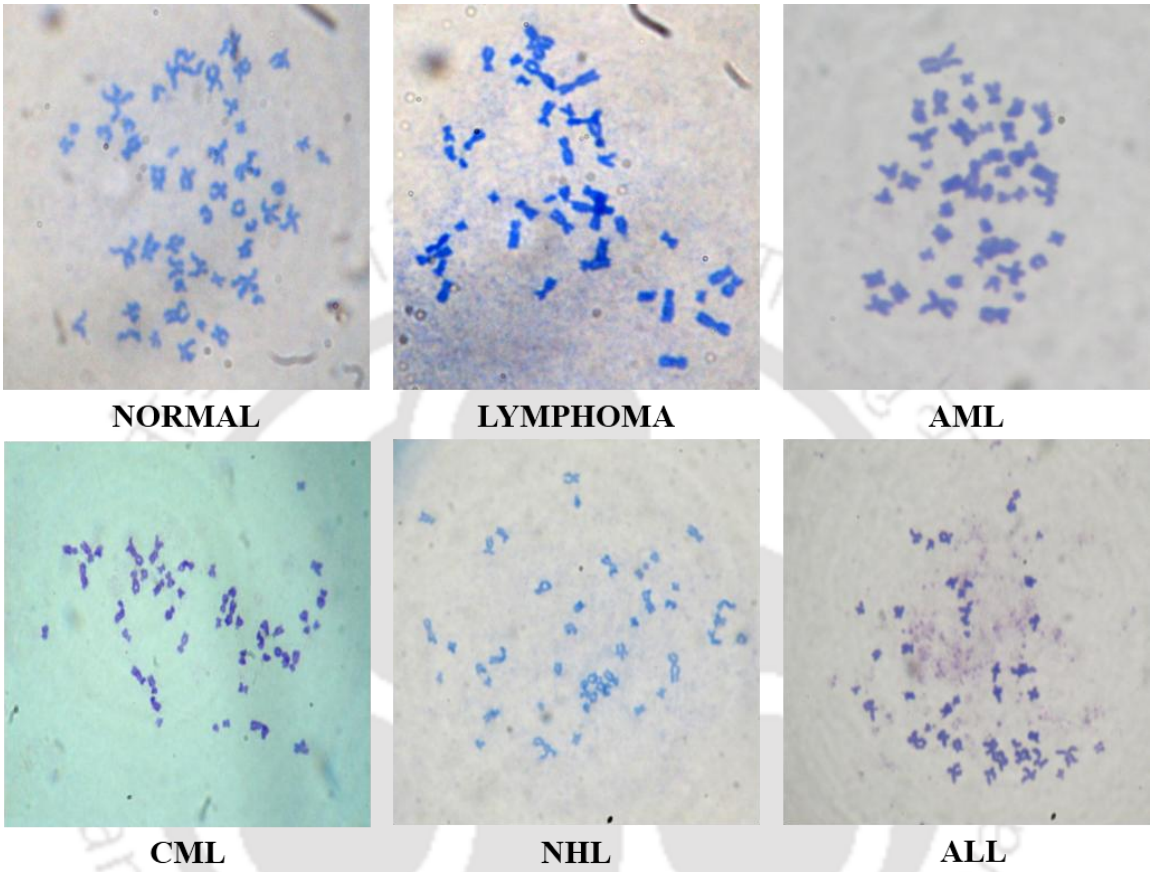


Figure.4.2.3. Karyotype of MSC. Photomicrographs showing metaphase chromosomes prepared from colchicine (10µg/ml) treated MSC isolated from patients diagnosed for acute myeloid leukemia (AML), chronic myeloid leukemia (CML), non-hodgkin's lymphoma (NHL) and acute lymphoblastic leukemia (ALL). Chromosomes were stained with Giemsa and visualized under phase contrast microscope. Magnification 1000x. Representative images are shown.

4.2.2. Differentiation of MSC

One of the main characteristic features of stem cells is their ability to differentiate into multiple cell types. MSC from bone marrow were found to be capable of adipogenic, osteogenic and chondrogenic differentiation. In this study, adipogenic and osteogenic differentiation potential of MSC were studied in detail. MSC from different

patients were induced with specific induction media and allowed to differentiate into either adipocytes or osteocytes for 14 days. The osteogenic differentiation was detected by alkaline phosphatase staining and adipogenic differentiation by Oil Red O staining.

No difference in adipogenic differentiation was observed in MSC obtained from patients of different hematologic disorders. However, there was a significant reduction in osteogenic differentiation potential in MSC from patients diagnosed for AML, CML, MM and AA (Figure.4.2.4).

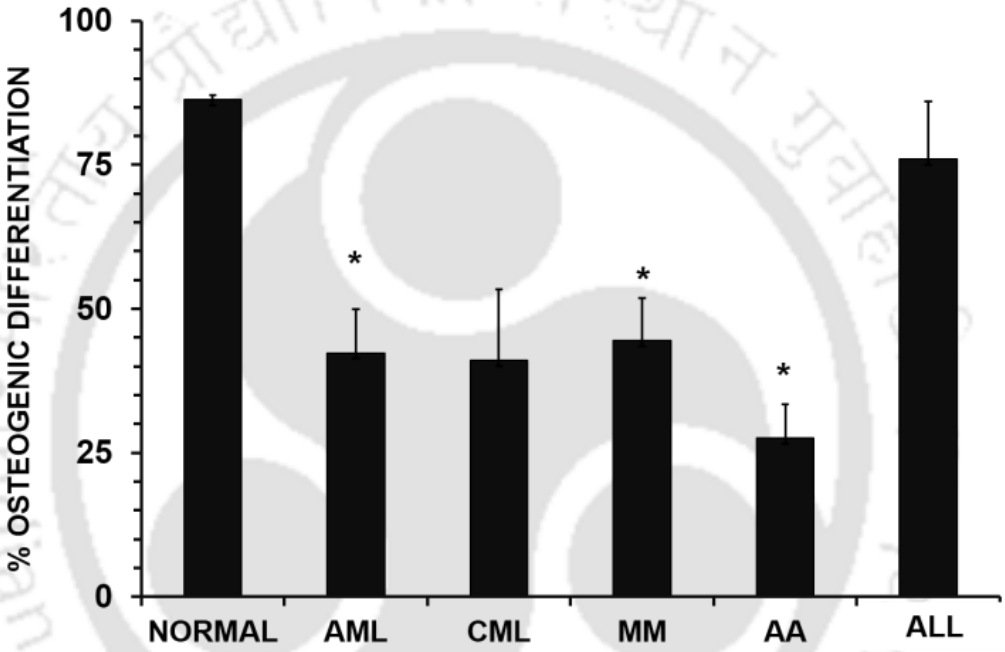


Figure.4.2.4. Osteogenic differentiation in MSC from patients with hematologic disorders. MSC from bone marrow of patients with hematologic disorders were differentiated into osteocytes by addition of osteo-induction media for 14 days and differentiation was determined by alkaline phosphatase (ALP) staining. Values are mean±SD, n=6-10. *p<0.05.

In addition to analyzing differentiation potential on different groups of patients based on their cancer type, the age related differentiation in osteogenic potential was also studied. Again, patients were grouped into different age groups of young children and adults: young (0-25 years), adults (26-50 years) and older individuals (51-80 years). As reported earlier by others, the osteogenic differentiation potential was higher in younger individuals and it decreased significantly in adults and older individuals (Figure.4.2.5).

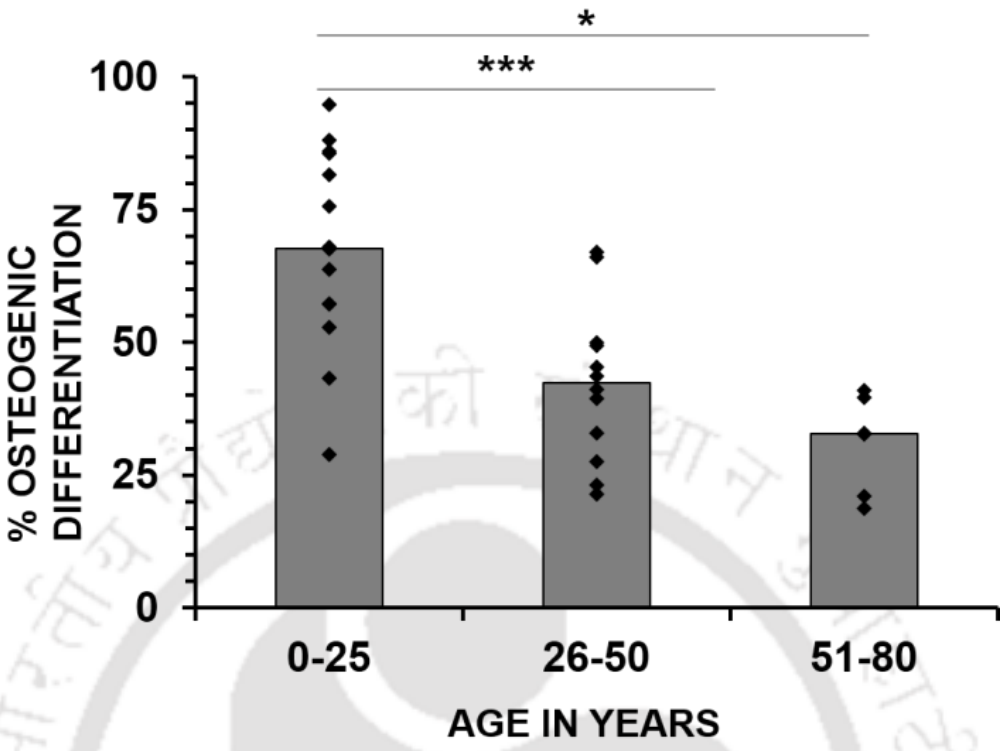


Figure.4.2.5. Osteogenic differentiation in MSC from different age groups. MSC obtained from bone marrow of patients from different age groups ((0-25), (26-50), (51-80) years) were differentiated into osteocytes. Osteogenic differentiation was determined by alkaline phosphatase staining. Each dot represents a patient and histogram represents the median value. *p<0.05, *p<0.0005.

4.2.3. Phenotype of MSC

Although no specific cell surface marker has been reported for MSC isolation and identification, MSC are defined by the presence and absence of a set of cell surface markers. All the cells isolated were tested for cell surface expression of CD13, CD34, CD44, CD45, CD49a, CD49b, CD49e, CD49d, CD73, CD90, CD105, CD146, HLA Class I, NGFR and SSEA4 by staining with fluorescent conjugated antibodies against each specific marker. MSC were found to be positive for mesenchymal lineage specific markers CD13, CD44, CD49a, CD49b, CD49e, CD73, CD90, CD105, HLA Class I and SSEA4. They were negative for hematopoietic lineage markers CD34, CD45 and they were negative also for CD49d, CD146 and NGFR (Figure.4.2.6).

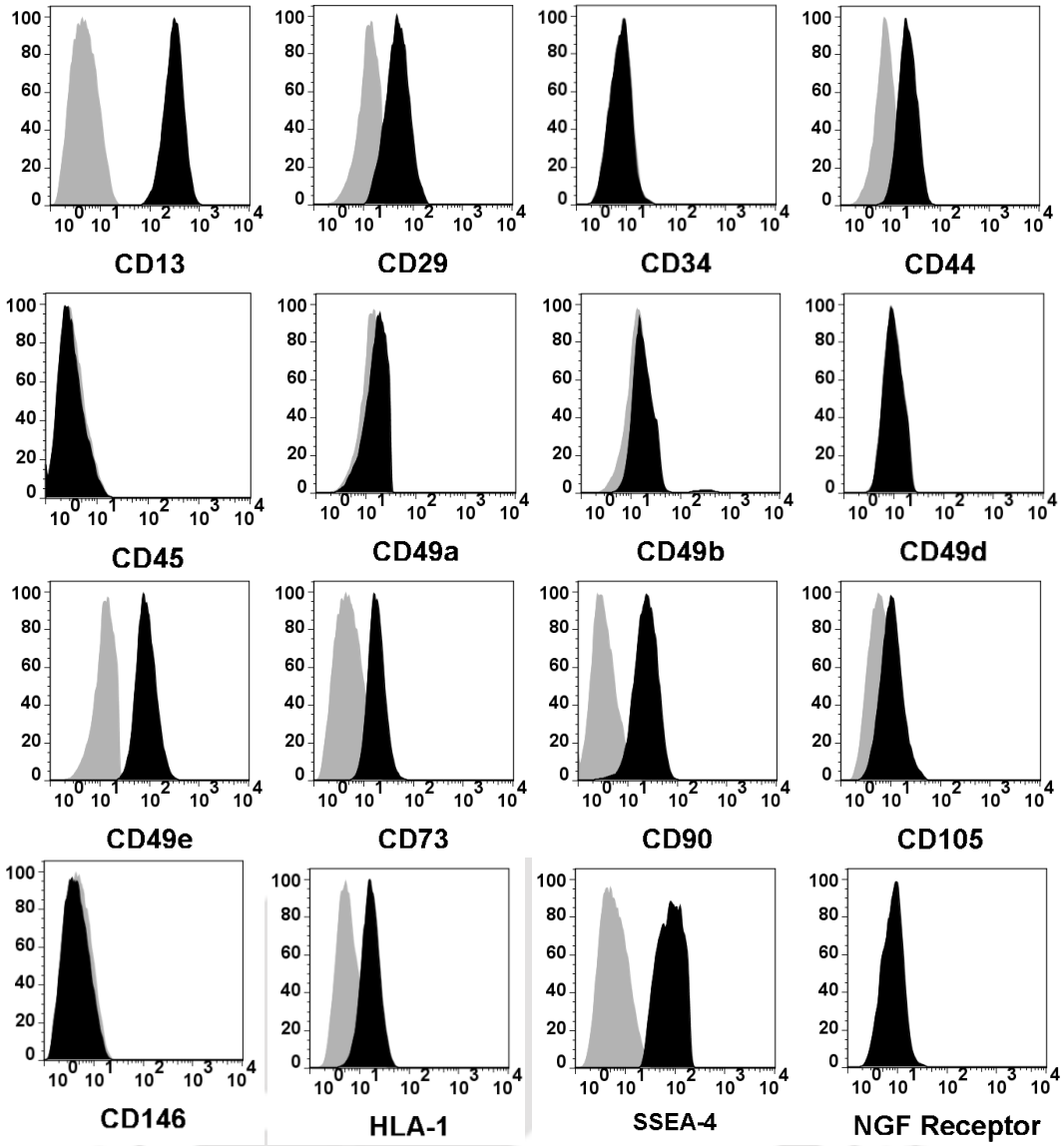


Figure.4.2.6. Cell surface marker expression in MSC. Bone marrow MSC were labelled with fluorescent conjugated monoclonal antibodies against CD13, CD29, CD34, CD45, CD49a, CD49b,CD49d, CD49e, CD73, CD90, CD105, CD146, HLA-1, SSEA-4, NGF Receptor and the expression was analyzed by flow cytometry. Y-axis in the histogram represents the number of events and X-axis represents the fluorescence intensity. Grey filled histogram represents isotype control and black filled are the marker stained sample. Representative results are shown.

Although all the isolated cells showed similar phenotype, variability among different groups of patient was observed. No significant difference in the expression levels of CD13 (Figure.4.2.7.A) and CD105 (Figure.4.2.7.C) was observed between different patients groups.

However, CD73 was found to be differentially expressed among different patient groups studied who were diagnosed for acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), multiple myeloma (MM) and aplastic anemia (AA) (Figure.4.2.7.B). In addition, a significant difference in the cell surface expression of CD90 was found among different patient groups. There was a significant downregulation in the cell surface expression of CD90 in patients diagnosed for AML, ALL, AA and MM but not in CML patients (Figure.4.2.7.D).

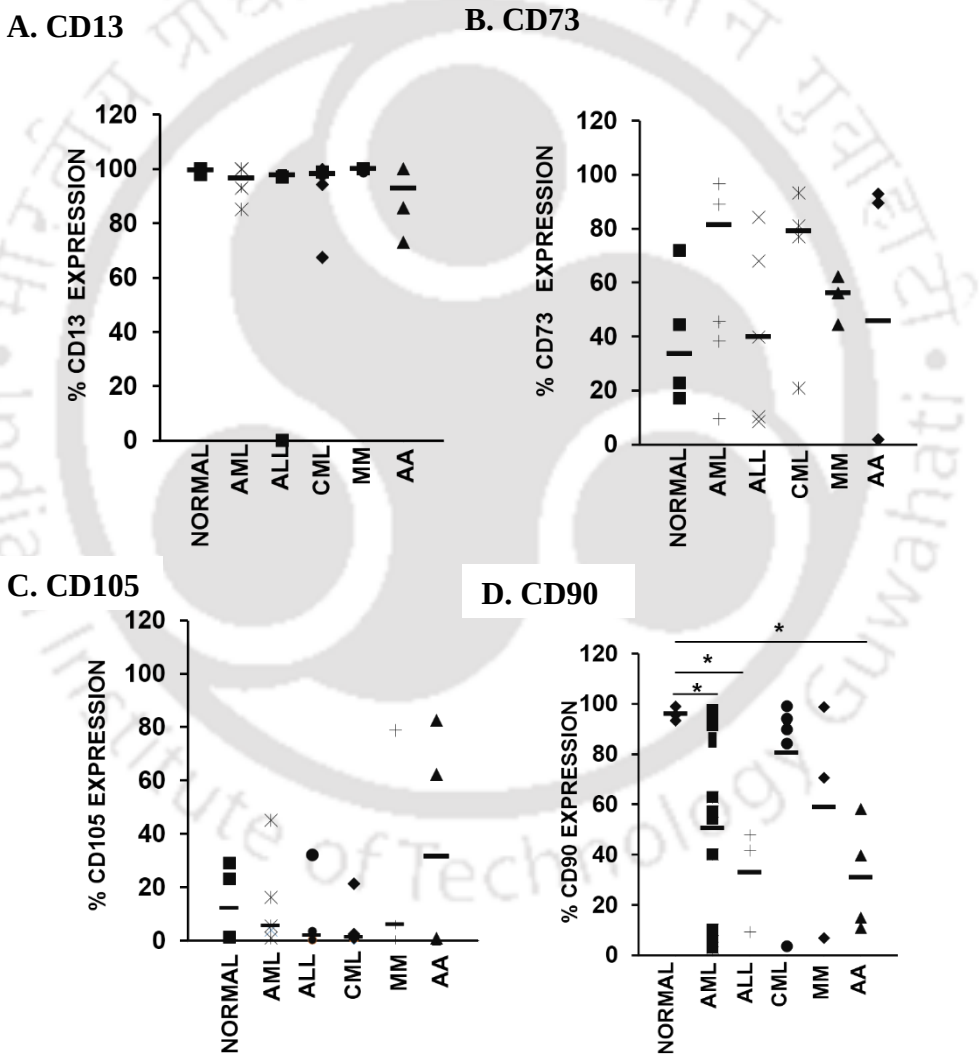


Figure.4.2.7. Cell surface marker expression in MSC from different patients. Cell surface expression of **(A)** CD13 **(B)** CD73 **(C)** CD105 and **(D)** CD90 was analysed in MSC from unaffected donors (NORMAL) or patients with AML, ALL, CML, MM and AA patients by flow cytometry. Each dot represents a single donor. Bars represent median value. *****$p<0.05$.

To check whether the CD90 expression levels in MSC vary according to the disease status of the patient, CD90 cell surface expression levels were detected during diagnosis and post-chemotherapeutic treatment (Figure.4.2.8). In patients diagnosed for AML and MM no significant difference in cell surface CD90 levels were seen between samples obtained at diagnosis and after treatment. Nevertheless, there was a significant increase in CD90 expression levels in acute lymphoblastic leukemia (ALL) patients after chemotherapeutic treatment (30% during diagnosis and ~80% post-chemotherapeutic treatment) (Figure.4.2.8.B)

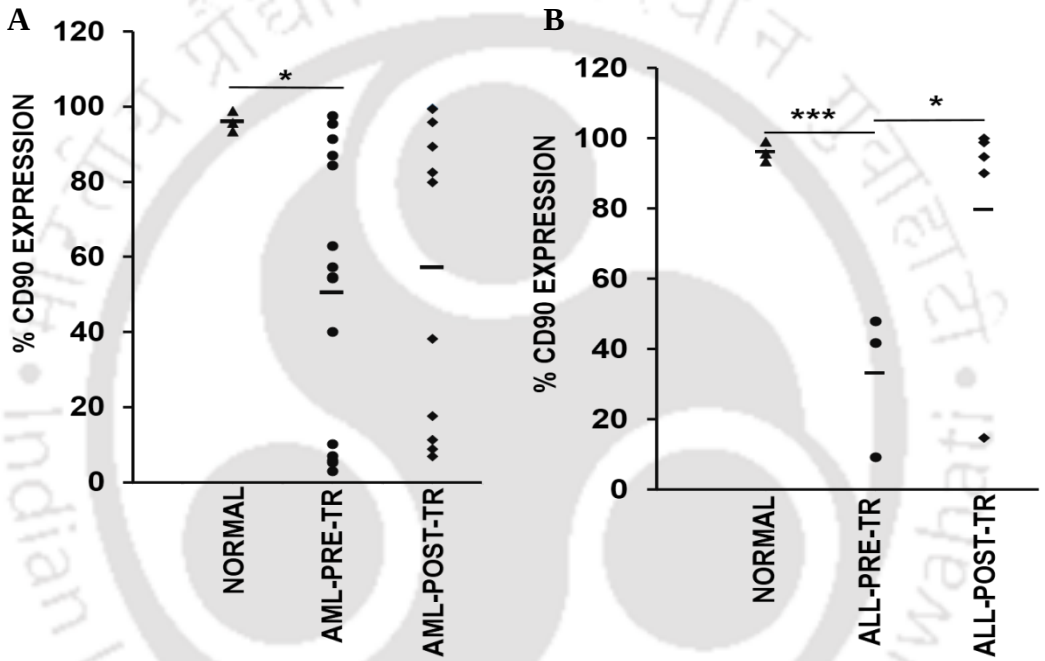


Figure.4.2.8. CD90 expression pre- and post-treatment. CD90 expression in MSC isolated from patients during diagnosis (PRE-TR) and after treatment (POST-TR) for **(A)** Acute Myeloid Leukemia (AML) **(B)** Acute Lymphoblastic Leukemia (ALL). Each dot represents a single donor. Bars represent median value. ***p<0.05, ****p<0.0005.

Since there was a significant reduction in CD90 expression in MSC obtained from different cancer types, the effect of age in CD90 expression was also analyzed. The patients were divided into younger (5-20 years) and older (51-80 years) age groups. CD90 cell surface expression was determined flow cytometrically. There was a significant reduction in CD90 expression in MSC obtained from patients belonging to an older age group of 51-80 years. Whereas the average percentage expression of CD90 was

~90% in patients belonging to 5-20 years age group, there was a ~30% reduction in CD90 expression percentage in patients of 51-80 years age (Figure.4.2.9).

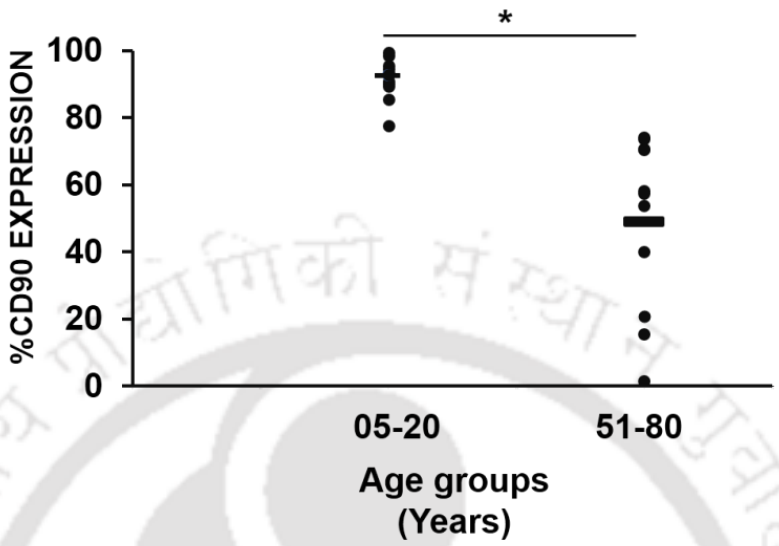


Figure.4.2.9. Cell surface expression of CD90 in young and aged donors. Flow cytometric analysis of CD90 expression in MSC isolated from bone marrow of young (5-20) and elderly (51-80) donors. Each dot represents a single donor. Bar represents the median value. *p<0.05.

There was an indication from the earlier experiments that cells that expressed low levels of cell surface CD90 also exhibited low osteogenic differentiation. To determine the correlation between CD90 expression and differentiation into osteocytes or adipocytes, MSC were differentiated into adipocytes or osteocytes. The cell surface expression level of CD90 was determined at different time points during differentiation. During adipogenic differentiation, there was a significant increase in the cell surface CD90 expression 24 hours after induction but the expression levels decreased to pre-induction levels at day 3 and remained unchanged until 14 days when the oil droplets were visible (Figure.4.2.10.B). Similarly, during osteogenic differentiation, there was a significant increase in CD90 expression 24 hours after osteogenic induction. In addition, high levels of CD90 expression was observed at 3 days and 7 days during osteogenic differentiation and reached pre-induction levels at day 14 indicating a possible role for CD90 cell surface expression in initial stages of osteogenic differentiation (Figure.4.2.10.A).

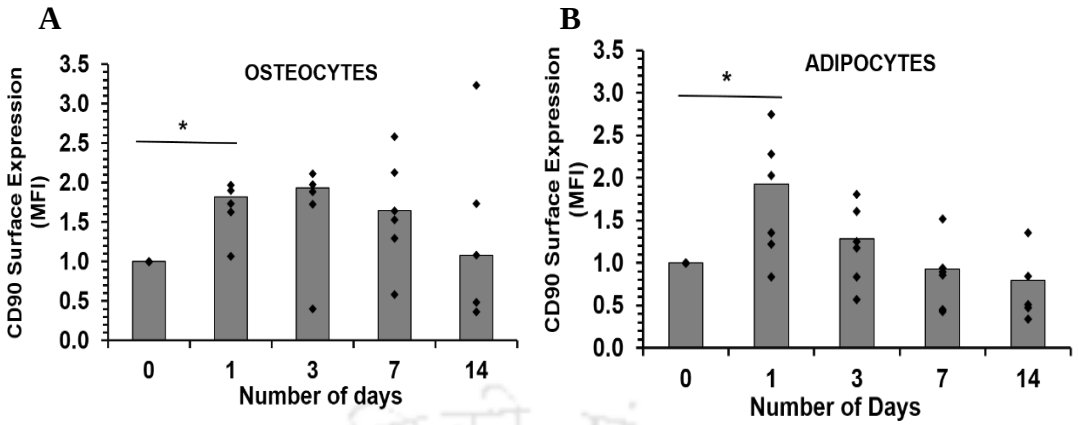


Figure.4.2.10. CD90 surface expression in osteocytes and adipocytes. MSC isolated from normal donors were differentiated into **(A)** osteocytes or **(B)** adipocytes and CD90 surface expression was analysed after 0, 1, 3, 7, and 14 days of differentiation by flow cytometry. Each dot represents a single sample and the histogram represents the median value. *p<0.05.

In addition, the transcript level of CD90 during osteogenic differentiation showed a similar trend to the observed cell surface expression levels. There was an increase during first 24 hours and a high CD90 mRNA level was observed until 7 days and it decreased to pre-induction levels at day 14 (Figure.4.2.11).

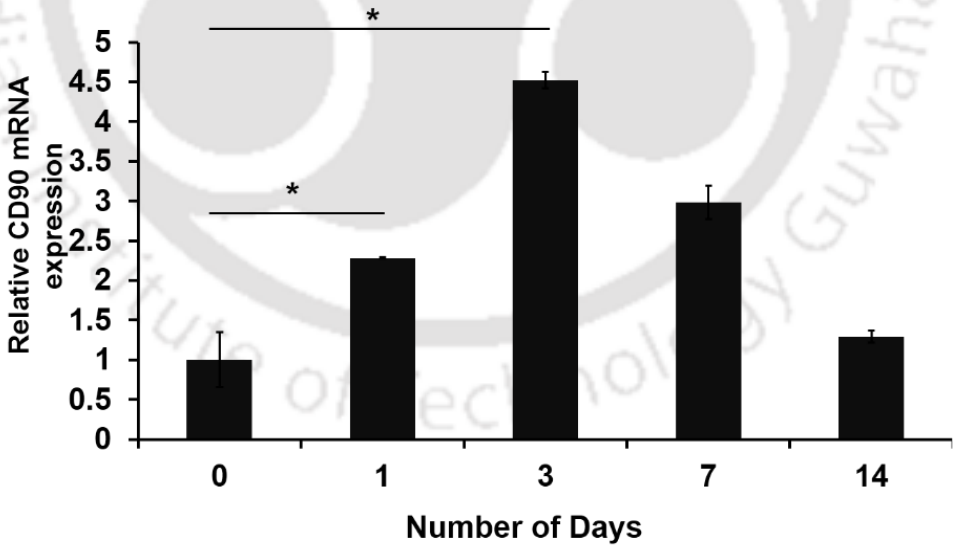


Figure.4.2.11. CD90 mRNA expression during osteogenic differentiation. Real time PCR analysis of CD90 mRNA expression in MSC after 0,1,3,7 and 14 days of osteogenic induction. Values are mean±SD, n=3. *p<0.05.

To study further the changes in cell surface expression, especially CD90 expression observed in MSC from different patients, different *in vitro* culture conditions

were tested. MSC were cultured for several passages and cell surface expression levels of CD90 was analysed. Increase in passage number did not significantly alter the expression levels of CD90 (Figure.4.2.12.A). Other *in vitro* conditions such as cell density, extra cellular matrix components were also examined.

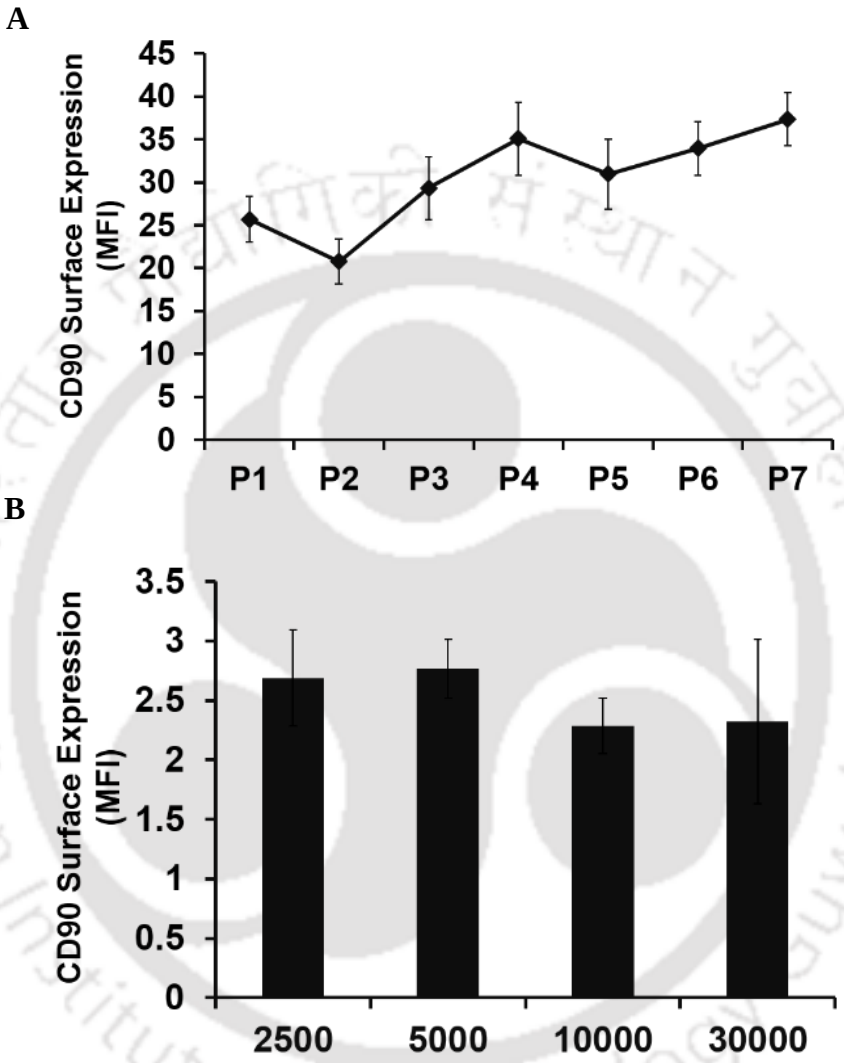


Figure.4.2.12. Effect of *in vitro* conditions on CD90 expression. (A) CD90 expression during different passages in culture. (B) CD90 expression in MSC seeded and grown for 48 hours at cell densities 2500 cells/cm^2, 5000 cells/cm^2, 10,000 cells/cm^2 and 30,000 cells/cm^2. Values are mean±SD, n=4-7.

When MSC were grown in different cell density (0.5 to 30 x 10^3 cells/cm^2), no significant difference in CD90 cell surface expression levels was observed (Figure.4.2.12.B). Similarly, CD90 expression levels were determined in MSC grown on uncoated glass or various extra cellular matrix substances such as fibronectin, poly-l-

lysine or collagen IV. All the tested conditions did not alter the CD90 cell surface expression levels (Figure.4.2.13).

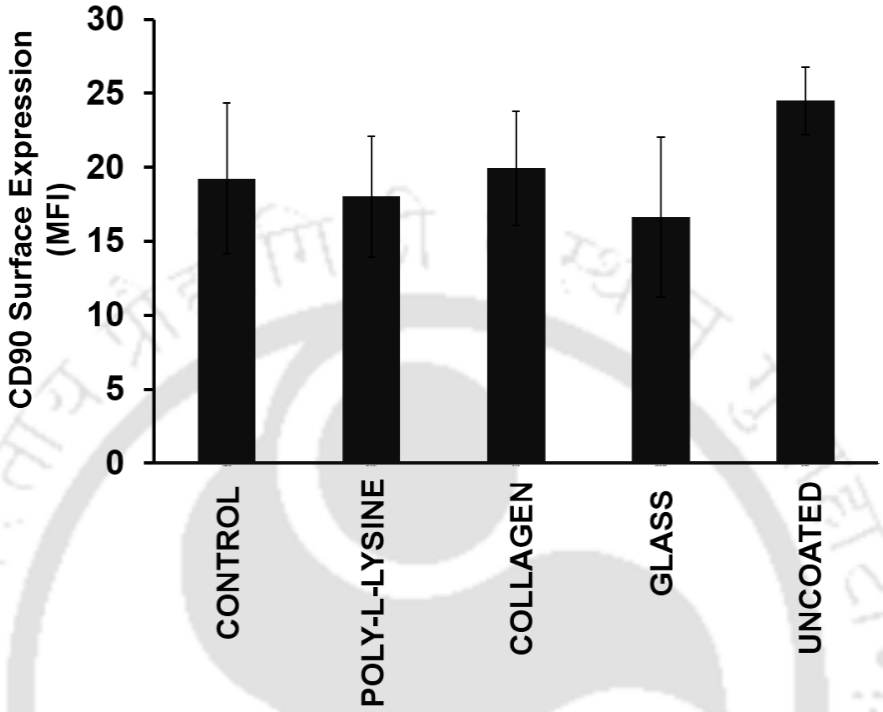


Figure.4.2.13. CD90 expression of MSC grown on different ECM. CD90 expression in MSC grown on different ECM surfaces such as fibronectin coated (CONTROL), poly-l-lysine, collagen or on uncoated tissue culture flask or glass surface. Values are mean±SD, n=3.

In addition, the effect of various serum components in the MSC culture media on CD90 expression was also analysed. MSC cultured in normal growth media was compared with those cultured with growth factors (basic fibroblast growth factor (bFGF), epidermal growth factor (EGF)) and also MSC cultured under serum starvation (0.1%) or high serum (40%) conditions. When MSC were cultured in media containing bFGF for 72 hours, there was a significant decrease in CD90 surface expression, whereas other serum or media composition did not significantly alter CD90 surface expression (Figure.4.2.14).

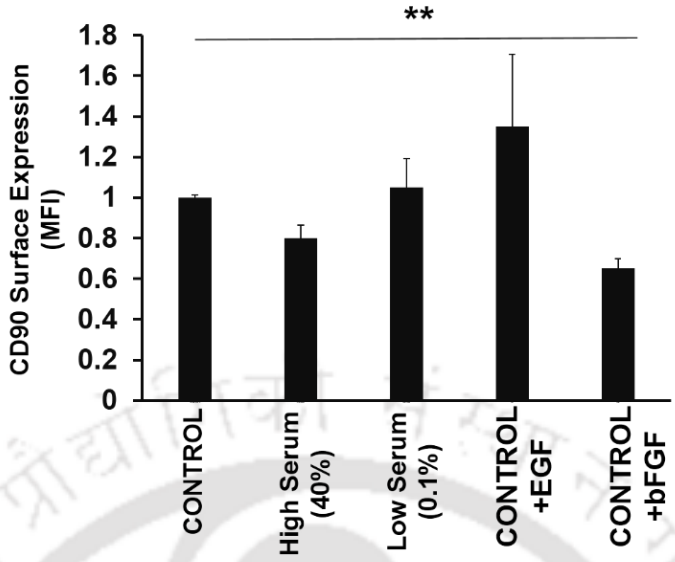


Figure.4.2.14. Effect of media components on MSC CD90 expression. MSC were cultured for 72 hours in DMEM containing 10% serum (CONTROL), 40% serum (High serum), 0.1% serum (Low serum), or media containing EGF or bFGF and the expression of CD90 was analyzed by flow cytometry. Values are mean±SD, n=3, **p<0.005.

For further analysis, MSC were cultured in the presence of cytarabine (Ara-C), a DNA synthesis inhibitor, clinically administered drug to treat AML and ALL. Various drug concentrations did not affect the viability of MSC until 48 hours. However, there was a significant decrease in CD90 surface expression in MSC treated with Ara-C for 48 hours (Figure.4.2.15.A). Nevertheless, the CD90 expression levels reverted to normal levels within 5 days after the removal of Ara-C from the culture media (Figure.4.2.15.B).

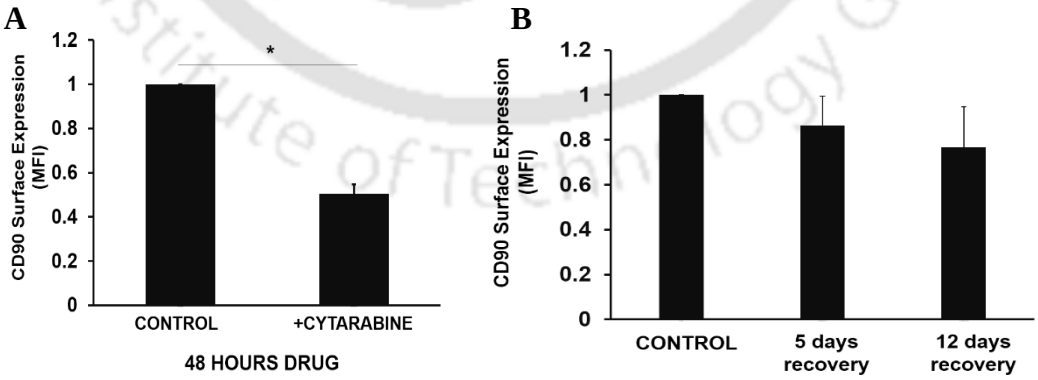


Figure.4.2.15. CD90 expression in MSC after drug treatment. **(A)** MSC were treated with chemotherapeutic drug cytarabine for 48 hours and the expression of CD90 was analyzed by flow cytometry. **(B)** MSC were allowed to recover for 5 days and 12 days in media without cytarabine after initial treatment with cytarabine and CD90 expression was analysed. Values are mean±SD, n=3.

The above results suggest that CD90 surface expression was down regulated in MSC obtained from patients with some hematologic disorders but not affected by *ex vivo* culture conditions or the chemotherapeutic drug used. In order to understand the reasons for CD90 down regulation further, CD90 expression was studied in MSC co-cultured with leukemic cell lines. For this, two different leukemic cell lines, THP-1 and HL60 were utilized. Firstly, unaffected MSC were co-cultured with THP-1 or HL60 for 4 weeks and their effect on MSC, especially the changes in CD90 cell surface expression was analyzed.

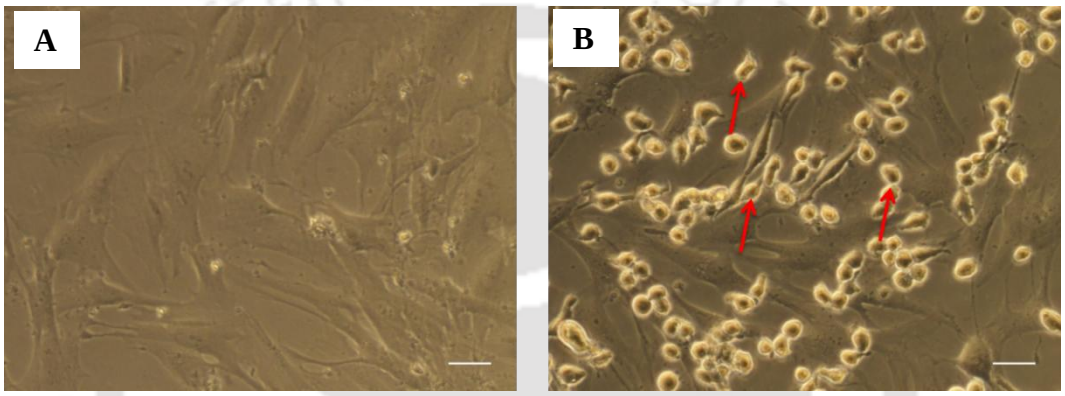


Figure.4.2.16. Co-culture of MSC with leukemic cells. Phase contrast photomicrographs showing MSC during **(A)** normal culture conditions and (B) MSC co-cultured with leukemic cells THP-1. Red arrows in **(B)** shows THP-1 cells during co-culture. Representative images are shown. Magnification 100x.

There was a significant reduction in the CD90 cell surface expression in MSC when co-cultured for 4 weeks with either THP-1 or HL60 indicating that in the presence of leukemic cells, MSC down regulated their CD90 cell surface expression (Figure.4.2.17). Analogous to the results obtained with co-culture, MSC down regulated their cell surface CD90 expression in the cell free leukemic conditioned media when added to unaffected MSC for 4 weeks. Thus, factors from leukemic cells might be responsible for altering the stromal compartment of the bone marrow during disease.

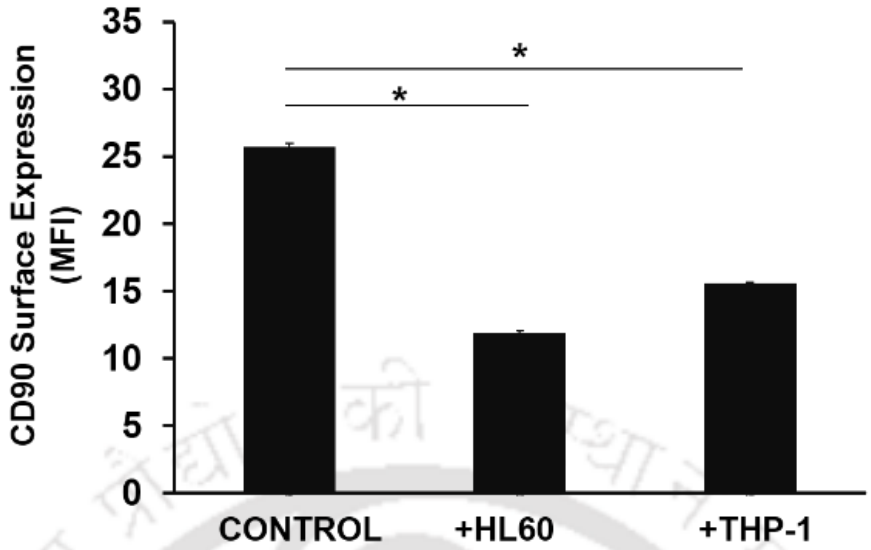


Figure.4.2.17. CD90 expression in MSC after co-culture with leukemic cells. MSC were co-cultured with leukemic cells (HL60 and THP-1) for 4 weeks and the expression of CD90 was analysed by flow cytometry. Values are mean±SD, n=3.

Thus, the changes in the differentiation and CD90 cell surface expression in MSC obtained from bone marrow of patients with hematologic disorders are not due to *ex vivo* culture conditions but might be disease induced. Further studies are required to validate the above results.

5. Discussion:

5.1. Role of cytoskeleton in MSC differentiation

MSC have multilineage differentiation potential and are extensively used for various tissue engineering and therapeutic applications (Mohal, Tailor et al. 2012). However, the prediction of *in vivo* lineage commitment of MSC still remains a challenge (Wang and Chen 2013). The properties of MSC during differentiation have to be properly understood for effective *in vivo* and *in vitro* manipulation and guided differentiation of MSC. Only then these cells can be effectively used for the various tissue engineering and therapeutic applications. In addition to the cell intrinsic transcription factors (Harada and Rodan 2003), interaction of stem cells with the cellular and non-cellular components *in vivo* have been reported to affect the properties of stem cells in the body. Integration of a complex array of signals from the microenvironment mediated by cell-cell contact, cell-matrix adhesions, biochemical signals like growth factors etc. have been reported to guide the differentiation and proliferation of stem cells *in vivo* (Fuchs, Tumbar et al. 2004; Moore and Lemischka 2006).

In the current study, the initial events controlling the osteogenic and adipogenic differentiation of MSC were analyzed. MSC were differentiated into either adipocytes or osteocytes by the addition of respective induction media. MSC underwent significant changes in morphology during adipogenic and osteogenic differentiation. Undifferentiated MSC were spindle shaped and after 14 days of differentiation, MSC differentiated into adipocytes became globular and oil droplets were formed inside the cell. While during osteogenic differentiation, MSC attained an angular shape with increased cell extensions. The differences were clearly observed by analyzing differentiated cells by scanning electron microscope (SEM). During flow cytometric analysis, increase in forward scatter (FSC) was observed in MSC during different days of adipogenic differentiation whereas during osteogenic differentiation, little or no change in forward scatter was observed. The change in cell area and morphology has been reported in MSC during adipogenic differentiation. An increase in 75% in total cell area compared to undifferentiated MSC was observed in MSC differentiated into adipocytes. While during osteogenic differentiation, the area of cells was reported to increase initially but decreased during later stages of differentiation (Yu, Tay et al. 2010).

Change in morphology also affects the mechanical properties of cells. The changes in mechanical properties are contributed by the underlying cytoskeleton of the cell consisting of microtubules, intermediate filaments and actin filaments. Apart from the use of chemical modulators for directing lineage commitment of mesenchymal stem cells, modulating mechanical forces or mechanical processes have been found to be effective in regulating differentiation of mesenchymal stem cells (Li, Batra et al. 2004; Sumanasinghe, Bernacki et al. 2006; Sen, Xie et al. 2008; Arnsdorf, Tummala et al. 2009). However, the mechanisms through which these mechanical signals got converted to biochemical cascade and affected the gene and protein expression and ultimately modulated cellular functions are yet to be properly understood.

Actin is the major cytoskeletal element contributing to maintenance of cell shape which senses the changes in mechanical properties of a cell and affect gene expression. Actin fibers are dynamic in their organization and were reported to undergo modifications during cell shape change and differentiation. The organization of actin cytoskeleton and focal adhesions of MSC grown on surfaces like polystyrene and collagen I, which allowed spreading and attachment of cells were reported to be similar to osteoblasts. Processes like spreading of cells with more attachment to the surrounding extracellular matrix (ECM) were found to mimic adhesion and morphological properties of osteoblasts which indicated a possible relation between cell shape, actin cytoskeleton and differentiation of mesenchymal stem cells (Docheva, Padula et al. 2008). Disruption of actin cytoskeleton with Cytochalasin-D was reported to affect the mechanical properties of MSC. Actin disruption led to decreased elastic modulus in MSC with decreased stiffness and increased viscosity (Tan, Pan et al. 2008; Titushkin and Cho 2009). Thus, actin cytoskeleton, cell shape and mechanical properties of MSC are related and actin cytoskeleton might play an important role in determination of morphology, shape and mechanical properties of MSC.

In addition to the morphological changes during adipogenic and osteogenic differentiation of mesenchymal stem cells, differential actin cytoskeleton modifications were also observed in differentiating MSC. Thick actin bundles with increased stress fibers were observed in MSC induced into osteocytes. In a fully differentiated cell, actin filaments were seen as peri-nuclear filaments framing the angular cell body with increased stress fibers. While on the other hand during adipogenic differentiation, the

actin filaments became discontinuous and formed a criss-cross network and the actin filaments formed a disrupted network around the oil droplets in a differentiated cell.

These observations were similar to previous reported findings wherein different pattern of actin cytoskeleton organization was reported in MSC differentiated into osteoblasts. Osteoblasts derived from mesenchymal stem cells were reported to contain robust actin filaments with high amount of criss-cross actin filaments and bulky stress fibers (Docheva, Padula et al. 2008). This differential rearrangement of actin filaments might be due to the different mechanical strength required for adipocytes and osteocytes (Naito, Dohi et al. 2011). Bone tissues withstand more mechanical strength and hence increased actin polymerization and formation of actin bundles might render osteocytes suitable for physiological functions of bone (Titushkin and Cho 2011). Whereas, fat cells don't have to endure such mechanical strength as osteocytes and hence they might favor a disrupted actin network giving the adipocytes its peculiar morphological and functional attributes.

In fact, signals from different ECM components, which induce modifications in actin cytoskeleton has been reported to be an important contributing factor for osteogenic differentiation of MSC (Treiser, Yang et al. 2010; McBeath, Pirone et al. 2004; Yourek, Hussain et al. 2007). ECM derived signals have been reported to play an important role in lineage commitment of MSC and the fate of MSC could be altered by engineering matrices to direct differentiation and lineage commitment (Frith, Mills et al. 2012). These authors had highlighted the importance of different ECM composition, cytoskeletal tension and downstream signaling like changes in effector Rho proteins during the process of differentiation. Although studies had depicted that actin cytoskeleton is an important factor during differentiation. However, it was not well understood whether the changes in actin cytoskeleton organization during differentiation of MSC were a pre-requisite for differentiation or just an associated effect of differentiation. In the present work, MSC were cultured on uniform fibronectin coated surface and the changes in actin cytoskeleton during differentiation was analyzed to find out the role of actin cytoskeleton during osteogenic and adipogenic differentiation.

Analyzing actin cytoskeleton remodeling and associated gene expression changes during adipogenic and osteogenic differentiation of MSC, actin cytoskeleton

modifications was found to be a very early event. Actin cytoskeleton modifications occurred as early as 12 hours after differentiation induction, much before the differentiation genes were expressed in the differentiating cell (undocumented observation). Early differentiation genes like *OSTEOCALCIN* for osteocytes and *ADIPONECTIN* for adipocytes were observed only after 48 hours of differentiation induction.

The active role of actin cytoskeleton during the process of differentiation was determined by inhibition of actin polymerization during the process of differentiation by addition of Cytochalasin-D (CYD) to the induction media. CYD inhibits actin polymerization by preventing formation of F-actin polymers (Schliwa 1982; Cooper 1987). The effect of CYD on actin polymerization was reversible and actin polymerization could be reverted back on removal of CYD from the media.

Interestingly it was observed that inhibition of actin polymerization during the process of differentiation led to decrease in osteogenic differentiation and increase in adipogenic differentiation potential of MSC. A significant decrease in osteogenic differentiation was observed when the cells were treated with CYD during the induction period compared to control differentiated cells without CYD in the induction media. Even 24hr treatment of CYD during osteogenic differentiation in a 3 day induction period led to 50% decrease in osteogenic differentiation. Little or no actin filaments were observed in MSC treated with CYD during osteogenic differentiation. There are reports of decreased osteogenic differentiation in MSC after treatment with CYD (Rodriguez, Gonzalez et al. 2004) but the role of cytoskeletal organization during osteogenic differentiation was not studied in detail by the authors. Differences in pattern of actin polymerization recovery and relating differentiation potential of MSC after removal of CYD during osteogenic differentiation clearly indicated that actin cytoskeleton modifications play an important role during osteogenic differentiation of MSC. During osteogenic differentiation, even though the cytoskeleton became normally polymerized with formation of actin bundles and stress fibers after CYD was removed from the media and the differentiation was continued in induction media without CYD, osteogenic differentiation was not restored. Thus, a clear indication that actin remodeling and osteogenic differentiation of MSC were interlinked and integrity of actin cytoskeleton was a necessary factor for osteogenic differentiation of MSC was observed. It might be

possible that early actin modifications during osteogenic differentiation may control the expression of osteo-specific genes during differentiation. While no reports on the role of actin modifications in controlling the osteogenic differentiation of MSC are available, integrity of actin cytoskeleton has been reported to be necessary for differentiation of mammary epithelial cells (Zoubiane, Valentijn et al. 2004). This suggests that presence of filamentous actin was important for osteogenesis.

In contrast, when the cells were treated with CYD during adipogenic differentiation, increase in adipogenic differentiation was observed. Significant increase in Oil Red O positive cells was observed in MSC treated with CYD compared to normal induction and no polymerized F-actin filaments were observed. The differential affects after CYD treatment during adipogenesis and osteogenesis of MSC was an effect of modifications of the pattern of actin polymerization in differentiating MSC by CYD. During osteogenic differentiation, more actin polymerization occurred and addition of CYD prevented actin polymerization resulting in decreased osteogenic differentiation. Whereas during adipogenic differentiation, actin filaments became disrupted and little or no polymerized F-actin filaments was observed in differentiating cells. Addition of CYD further prevented the formation of F-actin polymers and increase in adipogenic differentiation was observed. In both the processes, a common factor i.e., actin cytoskeleton modifications was influenced and the differences in differentiation were observed, which indicated that actin cytoskeleton modification is an important factor for adipogenic and osteogenic differentiation of MSC. Previous studies have shown that actin filaments were associated with lipid droplets and beta actin was involved in regulation of intracellular lipid metabolism (Fong, Wu et al. 2001). Apart from this, monomeric G-actin was reported to facilitate cholesterol transport in steroid responding cells (Hall 1995; Hall and Almahbobi 1997). Similar roles of actin during adipogenic differentiation of MSC might facilitate increased adipogenesis.

The importance of actin cytoskeleton during the process of lineage commitment of MSC was further confirmed when inhibition of actin by done by addition of CYD prior to differentiation induction. A 7.5-fold increase in PPAR gamma expression and significant decrease in *OSTEOCALCIN* expression was observed just by actin inhibition without differentiation induction. When the cells pre-treated with CYD for different periods were induced to undergo adipogenesis and osteogenesis, increased adipogenic

differentiation and decreased osteogenic differentiation was observed in MSC. Actin inhibition primed the cells for adipogenesis and reduced osteogenesis. Thus, it was clear from the results that actin cytoskeleton modification plays an important role in the osteogenic and adipogenic differentiation of MSC but the roles were differential.

Alterations in integrin expression may also influence differentiation of MSC by affecting actin cytoskeleton. Variation in integrin expression was reported in differentiating MSC. Integrin α5 was reported to be up-regulated during days 7 of osteogenesis and increased integrin α6 expression was reported during the entire duration of adipogenic differentiation of mesenchymal stem cells (Hamidouche, Fromigue et al. 2009; Kundu, Khatiwala et al. 2009). Integrin expression was reported to effect downstream signaling pathways affecting osteogenesis through actin and Rho GTPase. Rho GTPases have been reported to play important roles during differentiation and migration of mesenchymal stem cells (Provenzano and Keely 2011; Jaganathan, Ruester et al. 2007; Khatiwala, Kim et al. 2009). Apart from these, cytoskeletal changes during osteogenic differentiation were reported to be integrin dependent (Shih, Tseng et al. 2011; Salasznyk, Klees et al. 2007). Integrins mediated adhesion to ECM during differentiation have been reported to be important regulators of cytoskeletal modifications during differentiation. No cytoskeleton modifications were observed in MSC which were prevented from attaching to the substratum during osteogenic differentiation. The cytoskeleton modifications were observed only after the cells attached to the substratum (Treiser, Yang et al. 2010). Thus, integrins also formed an important component of the actin cytoskeleton signaling machinery affecting differentiation of MSC.

The pattern of integrin expression was analyzed during adipogenic and osteogenic differentiation of MSC. Expression of integrins CD29 (integrinβ1), CD49a (integrin α1), CD49b (integrin α2) and CD49e (integrin α5) were analyzed in MSC after differentiation into osteocytes and adipocytes with or without CYD in the induction media. CD49e was found to be upregulated during osteogenic differentiation of MSC as reported by others (Hamidouche, Fromigue et al. 2009). Addition of CYD during osteogenic differentiation led to decrease in CD49e expression. Thus, both actin polymerization and CD49e were affected by CYD during osteogenic differentiation of MSC and CD49e was reported to be an important factor during osteogenic

differentiation. Thus, CD49e, actin cytoskeleton and osteogenic differentiation might be related. They might either act independently or may be interconnected forming important components for differentiation regulation.

Various signaling pathways were reported to be affected during the process of differentiation of MSC. Components of MAPK pathway have been reported to be important regulators of osteoblast specific transcription factors (Xiao, Jiang et al. 2000). RhoA regulated cytoskeleton modifications and cell shape mediated lineage commitment (McBeath, Pirone et al. 2004). In addition to Rho/ROCK and Focal adhesion kinases, ERK1/2 have also been found to integrate signals from the microenvironment affecting osteogenic differentiation of MSC (Bai, He et al. 2013). p38MAPK might be an important regulator of osteogenic differentiation and it has been reported that induction of osteogenic differentiation by bone morphogenic protein-9 (BMP-9) in rat dental follicle stem cells activated MAPK pathway (Li, Yang et al. 2012). Actin binding was also reported to affect p38MAPK activity (Yang, Jiang et al. 2003).

Increase in phosphorylation of p38 MAPK and ERK1/2 was observed during osteogenic and adipogenic differentiation of MSC. It was observed that inhibition of actin cytoskeleton by CYD affected phosphorylation of p38MAPK during osteogenic and adipogenic differentiation. This showed that actin cytoskeleton modifications during differentiation might modify or regulate the phosphorylation status of p38MAPK based on a correlation although not directly demonstrated. However, addition of p38MAPK inhibitor (SB208530) during differentiation had no effect on osteogenic differentiation of MSC. No difference in phosphorylated levels of ERK1/2 was observed after CYD treatment during adipogenic and osteogenic differentiation of MSC. From these results, it could be concluded that actin modifications might signal through p38MAPK pathway and regulate osteogenic and adipogenic differentiation of MSC.

5.2. Properties of mesenchymal stem cells isolated from bone marrow of patients with hematologic disorders

Apart from their application in tissue engineering and regenerative medicine, mesenchymal stem cells also form an important component of the hematopoietic stem cell niche in the bone marrow. In continuation with the findings described in the previous section, wherein the role of actin cytoskeleton modifications during adipogenic and osteogenic differentiation of MSC was elucidated, further studies were carried out on MSC and the phenotypic and differentiation properties of MSC isolated from bone marrow of patients with hematologic diseases was analyzed.

Although MSC could be isolated from most of the donors, lower isolation percentage was observed in patients diagnosed with chronic myeloid leukemia (CML). Previous findings have reported that marrow aspirate volume affected the CFU-F of MSC from leukemia patients. Low frequency of CFU-F was reported but the proliferation rate was not reported to be affected (Li, Wong et al. 2011). Apart from this, during hematologic disorders, the chemotherapeutic drugs administered for therapy were reported to have deteriorating effect on MSC populations in the bone marrow. Mixed morphology and a rounded membrane structure with loss of proliferative potential was reported in MSC isolated from patients receiving high dose chemotherapy (Kemp, Morse et al. 2010). However, in the present work, successful isolation of MSC from patients undergoing therapy for acute lymphoblastic leukemia (ALL), multiple myeloma (MM) and acute myeloid leukemia (AML) was done. The isolated cells were spindle shaped with fibroblast morphology and proliferated normally in culture. However, some samples from acute lymphoblastic leukemia patients (ALL) were later found to become flattened with decreased proliferation rate during culture.

Conflicting reports regarding the properties of MSC isolated from patients with different hematologic disorders exist in literature. MSC with abnormal cytogenetic and differentiation properties or even perfectly normal MSC were reported in bone marrow of patients with different hematologic disorders (Flores-Figueroa, Montesinos et al. 2008). Chromosomal abnormalities were detected in MSC isolated from multiple myeloma patients (Arnulf, Lecourt et al. 2007). MLL-AF4 fusion gene was detected in MSC from MLL-AF4 positive acute leukemia which indicated the presence of a

common factor inducing transformation in MSC and hematopoietic stem cells (Menendez, Catalina et al. 2009). Apart from that, certain chromosomal translocations, inversions, and partial deletions were reported in MSC from bone marrow of patients with Myelodysplastic Syndromes (MDS) and Acute myeloid leukemia (AML) which were completely different from the chromosomal alterations found in the abnormal hematopoietic cells of that particular patient which indicated that the transformations occurring in MSC were independent of the hematopoietic lineage cells (Ramakrishnan, Awaya et al. 2006). However, in some cases, MSC from CML patients positive for BCR-ABL were found to be normal without any mutations or translocations (Jootar, Pornprasertsud et al. 2006) and the cytogenetic abnormalities detected in MSC derived from acute and chronic lymphoproliferative disorders were reported to be due to contaminating hematopoietic cells in culture. The cytogenetic abnormalities detected were attributed to the abnormal hematopoietic cells which grew in cultures along-with mesenchymal stem cells (Campioni, Bardi et al. 2012).

In the current study, since most of the isolates had normal morphology and growth rate, the cytogenetic abnormalities were not looked at. However, uniform karyotype with normal 46 chromosomes in MSC was observed in patients with AML, ALL, CML, MM and NHL.

MSC derived adipocytes and osteocytes are important components of the hematopoietic microenvironment in the bone marrow and osteocytes have been shown to play an important role in hematopoietic maintenance in the bone marrow (Chitteti, Cheng et al. 2010; Liu, Wu et al. 2011; Taichman and Emerson 1998). In this study, a significant reduction in osteogenic differentiation potential was observed in MSC isolated from patients diagnosed for acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma (MM) and aplastic anemia (AA). This reduction in osteogenic differentiation potential in MSC from patients diagnosed with hematologic diseases indicates that MSC were altered in their properties during hematologic diseases. The results were in accordance with the published data from other groups where MSC from MDS and AML patients were reported to have reduced osteogenic differentiation potential (Geyh, Oz et al. 2013; Isaikina, Kustanovich et al. 2006; Zhao, Liang et al. 2007). Reduced osteogenic and adipogenic differentiation potential were also reported in MSC from children with aplastic anemia (Chao, Peng et al. 2010). However, some

studies had reported no difference in differentiation potential of MSC isolated from patients with hematological diseases like MDS and multiple myeloma (Arnulf, Lecourt et al. 2007; Varga, Kiss et al. 2007). This might be due to the actual disease status of the individual or the differences in the isolation and differentiation procedure employed.

Analyzing osteogenic differentiation in MSC from different age groups, a significant reduction in osteogenic differentiation was observed in MSC isolated from elderly donors. MSC isolated from young individuals had higher osteogenic differentiation compared to elderly individuals. Some studies have shown that MSC from aged donors displayed a shift towards adipogenesis (Kim, Kim et al. 2012). During ageing, reduced hematopoietic activity was reported (Henry, Marusyk et al. 2011), which was also associated with increased fat content in the bone marrow (Tuljapurkar, McGuire et al. 2011). Osteocytes form an important component in the hematopoietic stem cell niche and maintain hematopoietic stem cell function. Thus, decrease in osteogenic differentiation observed in MSC from patients with hematologic disorders might be related to impaired hematopoiesis during hematologic disorders. Osteocytes have also been reported to support hematopoietic stem cells engraftment during transplantation (Calvi, Adams et al. 2003) and an impaired osteogenic differentiation of MSC from hematologic disorders might also affect hematopoietic recovery after transplantation. Apart from hematopoietic support, osteocytes in the bone marrow are also important for maintenance of bone mass and defects in osteogenesis has been related to osteoporosis or other age related disorders (Kim, Kim et al. 2012).

Together with morphology and differentiation potential, immunophenotype characteristics also forms an important parameter for defining the characters of mesenchymal stem cells. Positivity for CD73, CD90 and CD105 is among the basic panel of surface markers for identification of MSC (Dominici, Le Blanc et al. 2006). Changes in properties of MSC might affect the expression of these markers, which may be an indicator of heterogeneity or defects in functional properties of MSC.

A significant reduction in CD90 expression was observed in MSC isolated from patients diagnosed for acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and multiple myeloma (MM). All these samples were obtained from patients at the time of diagnosis and none of them were administered any kind of therapy. The function of

CD90 in mesenchymal stem cells has not been reported till date. Without any description regarding the functional role, reduction in CD90 expression was already reported in MSC from aplastic anemia patients (Shevela EY, et al., 2013). Apart from this, low levels of CD90 expression was reported in MSC from MDS patients with no difference in expression of other markers like CD29 and CD105 (Flores-Figueroa, Arana-Trejo et al. 2005). While some other studies have reported a reduction in CD90 and CD105 expression in MSC from AML, ALL, NHL, MM and MDS patients (Campioni, Moretti et al. 2006; Campioni, Rizzo et al. 2009). Some studies have even reported normal immunophenotype in MSC isolated from patients diagnosed with autoimmune neutropenia (AIN), idiopathic thrombocytopenic purpura (ITP) and B-cell acute lymphoblastic leukemia (B-ALL). All these samples had higher than 90% BM infiltration and no difference in expression of markers CD14, CD29, CD44, CD90, CD105 and CD146 was reported (Dimitriou, Linardakis et al. 2008).

In the present work, apart from CD90, other markers like CD13, CD73 and CD105 were also analyzed but no difference in the surface expression pattern of CD13, CD105 was observed. The expression of CD73 was found to be variable without any correlation with disease of the donor. Thus, from the present work and reported literature available, data regarding the expression of surface markers in MSC isolated from bone marrow of patients diagnosed with hematologic disorders seems to be inconclusive and various conflicting reports were observed.

CD90 or THY-1 is a 25-30 kDa GPI anchored protein. The gene encoding Thy-1 is located on long arm of 11 on position 11q22.3 (Seki, Spurr et al. 1985). It was the first T cell antigen to be identified and was first reported in murine thymocytes, T Lymphocytes and on neuronal cells. The expression of CD90 have been reported to be developmentally and post transcriptionally regulated (Xue, Calvert et al. 1990; Xue and Morris 1992).

CD90 is expressed by different cells like fibroblasts, mesenchymal stem cells, hematopoietic stem cells, endothelial cells, neurons, T-cells, ovarian follicular cells, cancer cells etc. CD90 has been postulated to serve many immunogenic and non-immunogenic functions. It has been postulated to serve important roles in various cellular functions like cell adhesion, migration, differentiation, apoptotic signaling etc.

(Haeryfar and Hoskin 2004; Rege and Hagood 2006; Rege and Hagood 2006). The highest expression of CD90 has been reported in neurons, where it has been postulated to serve important neurological and cognitive roles (Mayeux-Portas, File et al. 2000; Chen, Wang et al. 2005). During early development, low expression of CD90 was observed in neurons but as maturation occurs, levels of CD90 increased (Barlow and Huntley 2000). CD90 expression was also reported in cancer cells and was linked to cancer stem cell (CSC) like properties of glioblastoma. CD90 was reported to be a potential marker for high grade gliomas and related to metastatic potential of glioblastomas (He, Liu et al. 2012). Even in gastric cancer and hepatocellular carcinoma, $CD90^+$ tumor cells showed higher tumorigenic capacity and tumorigenic potential (Jiang, Zhang et al. 2012; Sukowati, Anfuso et al. 2013)

Although CD90 expression was linked to tumorigenicity and cancer stem cell like properties of cancer cells, but several reports highlighted the possible role of CD90 as a tumor suppressor gene. There were reports of CD90 expression in human ovarian cancers and nasopharyngeal carcinomas which was associated with tumor suppression and lack of tumorigenicity. CD90 overexpression resulted in significant tumor suppression in ovarian cancer cell line SKOV-3 and CD90 expression in non-tumorigenic clone resulted in restoration of tumorigenicity (Abeysinghe, Pollock et al. 2004). CD90 has been reported to be associated with tumor suppression in nasopharyngeal carcinoma also and CD90 overexpression was associated with loss of invasiveness of nasopharyngeal carcinoma cell lines (Lung, Cheung et al. 2010). Downregulation of CD90 was observed in diseases like nasopharyngeal carcinoma and immunopulmonary fibrosis and the decrease in CD90 was attributed to promoter hypermethylation (Lung, Bangarusamy et al. 2005; Sanders, Pardo et al. 2008).

A broad range of functions have been attributed to CD90, but the molecules affected by CD90 interaction and the downstream signaling pathways are still unknown, which is mainly due to the lack of a definite ligand for CD90 (Barker and Hagood 2009). An important interacting partner of CD90 molecule is integrin $\alpha_v\beta_3$ on astrocytes. Interaction of CD90 to $\alpha_v\beta_3$ have been reported to affect focal adhesions and cytoskeleton via Rho GTPase (Leyton, Schneider et al. 2001; Avalos, Arthur et al. 2004). CD90 through its GPI anchor have been reported to regulate FAK and SFK phosphorylation and plays an important role in lung fibrosis (Barker, Grenett et al. 2004; Cohen, Breuer

et al. 2009). CD90 has been reported to signal heterotypically (in *trans*) by binding to potential CD90 ligand like β2 and β3 integrins on target cells (Hermosilla, Munoz et al. 2008) or homotypic signaling (in *cis*) by interacting with T-Cell Receptor, CD90 crosslinking or by mechanisms unknown (Shenoy-Scaria, Kwong et al. 1992; Barker and Hagood 2009). CD90 expression is of relevance in regulation of phenotype of many different cell types and affects cellular events through mechanisms not clearly known (Barker and Hagood 2009; Bradley, Ramirez et al. 2009).

Thus, various potential roles of CD90 and the mechanisms by which they signal downstream and affect cellular processes have been postulated, but the mechanisms are yet not clear and many questions are yet to be answered. Many contradictory roles of CD90 have been postulated in tumor cells and the role of CD90 needs to be further investigated (Rege and Hagood 2006; Rege and Hagood 2006).

To check whether CD90 expression in MSC varies according to disease status of the patient, CD90 surface expression was analyzed in MSC isolated from patients during diagnosis and post chemotherapeutic treatment. In patients diagnosed for AML and MM no significant difference in cell surface CD90 levels was observed between samples obtained at diagnosis and after treatment. Surprisingly, there was a significant increase in CD90 expression levels in acute lymphoblastic leukemia (ALL) patients after chemotherapeutic treatment. This is the first reported incidence of MSC CD90 expression during diagnosis and post chemotherapeutic treatment and the expression of CD90 was found to be normal in acute lymphoblastic leukemia (ALL) patients post-therapy. These results suggest that atleast in case of ALL, CD90 could be used as a biomarker to understand bone marrow recovery after successful therapy, although a thorough and detailed investigation is required before it can be used effectively in the clinical setting.

In MSC isolated from patients diagnosed with hematologic disorders reduction in CD90 expression along-with decreased osteogenic differentiation was observed. The decrease in CD90 expression and osteogenic differentiation were correlated which indicated a possible relation between CD90 expression and osteogenic differentiation. Anticipating a possible link between CD90 expression and differentiation of MSC, the expression of CD90 was analyzed during different days of osteogenic and adipogenic

differentiation of MSC from normal bone marrow. During adipogenic differentiation, significant increase in CD90 cell surface expression was observed after 24 hours of induction but the expression levels decreased to pre-induction levels at day 3 and remained unchanged until day 14 of adipogenic differentiation. Similar pattern of CD90 expression with increase in expression after 24 hours was observed during osteogenic differentiation but unlike adipocytes, high levels of CD90 expression was observed until 3 days and 7 days of osteogenic differentiation but returned to pre-induction levels at day 14. Concomitant with the increased CD90 cell surface expression, CD90 mRNA levels also showed a similar trend during osteogenic differentiation.

The results indicated that the expression of CD90 may be related to osteogenesis of MSC. Available reports from literature also depict a possible relation between osteogenesis and CD90 expression. Murine stromal cell lines which were initially negative for CD90, expressed CD90 during osteogenic differentiation. CD90 expression was higher during the initial proliferative phase but as the cells mature, the percentage of cells positive for CD90 decreased (Chen, Qian et al. 1999). Higher osteogenic differentiation was reported in CD90 positive MSC compared to CD90 negative MSC isolated from lipoaspirates. When transplanted *in vivo*, CD90 positive MSC had greater potential to repair calvarial defects and displayed higher *de novo* bone regeneration compared to CD90 negative MSC (Chung, Liu et al. 2013). Hence, CD90 expression and osteogenic differentiation might be related. A separate report wherein MSC were differentiated into osteocytes by applying mechanical stimulation, decrease in CD90 expression was observed during differentiation as the cells mature and attained a differentiated phenotype (Wiesmann, Buhring et al. 2006).

Apart from these reports, *in vivo* analysis of localization of osteoprogenitors during endochondral ossification in rats have showed that CD90 positive cells possessed high osteogenic activity and CD90 could be a potential marker for osteo-progenitors (Nakamura, Yukita et al. 2010). Primary rat and human osteoblast had high expression of CD90 but the expression of CD90 decreased in mature osteoprogenitors (Chen, Qian et al. 1999). Campioni et al., had reported reduced CD90 expression in MSC from hematologic malignancies but no difference in adipogenic, osteogenic and chondrogenic differentiation potential was observed (Campioni, Rizzo et al. 2009).

To further elucidate the expression of CD90 in MSC, various *ex vivo* factors like passage number, seeding density, different ECM composition and various serum concentrations were tested and it was observed that CD90 expression in MSC was not affected by these factors. However, when MSC were cultured in media containing bFGF for 72 hours, a significant decrease in CD90 surface expression was observed. The presence of cytokines in the growth media was reported to exert a negative effect on CD90 expression in MSC. An irreversible decrease in CD90 expression was reported in MSC cultured in DMEM supplemented with cytokines but MSC grown in DMEM without cytokines were normal and didn't show any such variation in marker expression (Campioni, Lanza et al. 2008).

Chemotherapeutic drugs used for the treatment of hematologic malignancies might also alter the properties of MSC. A reversible decrease in CD90 expression was observed in MSC after treatment with cytarabine for 48 hours. The decrease in CD90 expression was found to become normal in MSC after 5 days of culture in media without drug. This was a preliminary investigation to study the effects of cytarabine on MSC. Differential effects of chemotherapeutic drugs have been reported in mesenchymal stem cells. Some drugs were reported to be toxic while others were not (Li, Law et al. 2004). Lenalidomide have been reported to alter the properties of MSC and had impaired $CD34^+$ support capacity but supported CFU-E in cocultures (Ferrer, Wobus et al. 2013). Although the results depict no effect of cytarabine on CD90 expression in MSC but during *in vivo* chemotherapeutic strategies, various combinations of drugs are used and the effects of the drugs *in vivo* cannot be imitated *in vitro* and differential affects might be observed on MSC. Other properties of MSC might also be affected by the drugs which were not analyzed in this study.

The results obtained clearly showed that variation in CD90 expression in MSC was not affected by the *in vitro* conditions during culture and gave an indication that leukemic cell associated factors in the bone marrow during hematologic diseases might be responsible for the variation in CD90 expression. To further confirm this, MSC obtained from normal samples were either co-cultured with leukemic cell lines HL-60 and THP-1 or grown in conditioned media obtained from leukemic cells and the expression of CD90 was analyzed. Cancer cell secreted factors were reported to induce morphological and genetic changes in MSC (Al-Toub, Almusa et al. 2013). Most of the

reports available on the coculture of mesenchymal stem cells with leukemic cells concentrated on the anti-apoptotic and survival effects of MSC on leukemic cells (Nwabo Kamdje, Bassi et al. 2012; Wei, Chen et al. 2009). However, leukemic cells were also reported to induce changes in MSC like activation of ERK and Akt signaling pathways (Ding, Nowakowski et al. 2009) or induction in IL-17, IL-8 expression (Civini, Jin et al. 2013), which might induce proliferation , differentiation or alteration of various other properties of MSC.

Decrease in CD90 expression in MSC was observed after coculture with leukemic cells for 28 days. Downregulation of CD90 was observed even in MSC cultured with conditioned media from leukemic cells. Thus, leukemic cell secreted factors were sufficient to induce downregulation of CD90 expression in MSC. Thus, the results obtained during *in vitro* exposure to leukemic cells were in tune with the results obtained from the patient samples analyzed where a reduction in CD90 was observed in MSC from hematologic disorders. Hence, it could be concluded that microenvironmental alterations in bone marrow during hematologic malignancies might be responsible for the reduction in CD90 expression and osteogenic differentiation observed. But further experiments have to be performed to confirm the hypothesis and to establish a role of CD90 expression in MSC from bone marrow of patients with hematologic disorders.

6. Conclusions:

The differentiation of stem cells is a complex process that is dependent on a multitude of cell intrinsic and external factors. Mesenchymal stem cells have a wide variety of applications in the field of tissue engineering and clinical therapeutics. Understanding the processes that control the differentiation and other cellular properties of mesenchymal stem cells will help in efficient use of these cells for various applications. Taken together, the findings of the current work describe some important aspects of mesenchymal stem cells during differentiation and also during the development of hematologic disorders in the bone marrow, which might affect the properties of mesenchymal stem cells. To briefly conclude, it was found that actin cytoskeletal modification was a very important event during adipogenic and osteogenic differentiation of mesenchymal stem cells. Actin cytoskeleton modification precedes gene expression and might regulate osteogenic and adipogenic differentiation of mesenchymal stem cells by signaling through p38MAPK pathway. These findings might have important implications for regulation of MSC differentiation and might be of relevance in controlling MSC differentiation and contribute to efficient use of MSC in regenerative medicine and therapy. Different types of scaffolds and tissue implants are being developed nowadays, wherein the cells are seeded and transplanted for facilitating accelerated tissue repair. Designing scaffolds and implants in such a way they maintain cell shape and morphology will help in efficient differentiation of MSC to the desired lineage. It might even be possible to direct the differentiation of MSC to a particular lineage by such approaches. Actin cytoskeleton is the key player in regulation of cell shape and morphology. From the results discussed in this thesis, it is clear that actin plays an important role during the process of adipogenic and osteogenic differentiation of MSC. So controlling the actin cytoskeleton by altering the cell surface contacts or drug induced modification of actin cytoskeleton can be utilized for directed differentiation and lineage commitment of MSC. Apart from this, *in-vivo* adipogenesis or osteogenesis of transplanted MSC can also be controlled by addition of drugs modifying actin cytoskeleton to facilitate the regeneration processes.

Apart from this, MSC isolated from patients with hematologic disorders were found to have an altered phenotype and differentiation capacity. A reduction in CD90 surface expression and osteogenic differentiation potential was observed in MSC from

hematologic disorders. Leukemic cell associated factors were found to affect the differentiation and CD90 surface expression. These findings might provide insights into an altered bone marrow microenvironment, which has been postulated to affect leukemic progression and hematopoietic recovery during hematologic malignancies. The results also indicate a possible correlation between CD90 expression and osteogenic differentiation of mesenchymal stem cells from patients with hematologic disorders. But further experiments are required to prove this hypothesis. Silencing of CD90 expression during osteogenic differentiation will provide insights into the role of CD90 during osteogenic differentiation of MSC. Apart from this, analyzing the osteogenic differentiation potential of CD90 negative and CD90 positive fractions of MSC and studying the signaling pathways affected during differentiation will provide further insights into the signaling pathways affected by CD90 during differentiation in MSC.

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Journal:

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