Role of Cell Extrinsic and Intrinsic Factors in Regulating Mesenchymal Stem Cell Properties

A proposal submitted for the partial fulfillment of requirements for the award of

Doctor of Philosophy

Under the supervision of

Dr. Bithiah Grace Jaganathan

Submitted by

Chinnapaka Somaiah
Roll No: 11610624

Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati-781 039, Assam, India

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

GUWAHATI-781039

DECLARATION

I do hereby declare that the research findings of this thesis entitled **‘‘Role of cell extrinsic and intrinsic factors in regulating mesenchymal stem cell properties”** is the results of research work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the supervision of **Dr. Bithiah Grace Jaganathan.**

I acknowledge Dr. Atul Kumar for help in gene expression studies and cell death analysis.

Date: **Chinnapaka Somaiah**

(Candidate)

Roll No: 11610624

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

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CERTIFICATE

It is certified that the work described in this thesis entitled **''Role of cell extrinsic and intrinsic factors in regulating mesenchymal stem cell properties"** by Mr. Chinnapaka Somaiah (Roll No: 11610624) for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India, and this work has not been submitted elsewhere for the award of any other degree.

Date:

Dr. Bithiah Grace Jaganathan

(Thesis Supervisor)

ALCAM: activated lymphocyte cell adhesion molecule

ALL: acute lymphoblastic leukemia

AML: acute myeloid leukemia

APML: acute promyelocytic leukemia

ARS: alizarin Red S

Bcl-2: B-cell lymphoma-2

BM: bone Marrow

CASR: Ca2+-sensing receptor

CD: cluster of differentiation

Cdc42: cell division control protein 42

cDNA: complementary DNA

CLL: chronic lymphocytic leukemia

CML: chronic myeloid leukemia

COL: collagen

CON: control

CPC: cetyl pyridinium chloride

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

CYT: cytarabine

DAPI: 4',6-diamidino-2-phenylindole

DAU: daunorubicine

DMEM-HG: dulbeccos modified Eagles medium-high glucose

DMEM-LG: dulbeccos modified Eagles medium-low glucose

DMSO: dimethyl sulphoxide

DNA: deoxyribonucleic acid

dNTP: deoxy nitro triphospahates

dT: deoxy thymidines

ECM: extra cellular matrix

eGFP: enhanced green fluorescent protein

ESCs: embryonic stem cells

EtBr: ethidium Bromide

F-actin: Filamentous-actin

FAK: focal adhesion kinases

FBN: fibronectin

FBS: fetal bovine serum

FITC: fluoroscene iso thiocyanate

Flt3: FMS-like tyrosine kinase 3

GAP: GTPase activating protein

GAPDH: glyceroldehyde-3-phosphate de hydrogenase

GAPs: GTPase-activating proteins

GDI: guanine nucleotide dissociation inhibitors

GDP: guanine nucleoside di phosphate

GFP: green fluorescent protein

GM-CSF: granulocyte macrophage-colony stimulating factor

GTP: guanine nucleoside tri Phosphate

GTPases: guanosine triphosphatases

HEK 293FT: human epithelial kidney 293FT

HIF1α: Hypoxia inhibitory factor 1-alpha

Hl60: human promyelocytic leukemia cells

HLA: human leucocyte antigen

HSCs: hematopoietic stem cells

HSPCs: haematopoietic stem and progenitor cells

IBMX: 3-iso butyl methyl-1-xanthane

IL-1β: interleukin 1-beta

IL-2: interleukin- 2

IL-6: interleukin-6

IL-8: interleukin-8

IRES: intra ribosomal entry site

ITGA3: integrin α4β3

ITGA4: integrin α4β1

LTR: long terminal repeats

MEMNEAA: minimum essential medium non-essential amino Acids

MFI: mean fluorescence intensity

MHC II: major histocompatibility complex II

MSCs: mesenchymal stem cells

MTT: 3-(4, 5-Di methylthiazol-2-yl)-2, 5-di phenyltetrazolium bromide]

NOD: non-obese diabetic

OCN: osteocalcin

OPN: osteopontin

ORO: Oil Red O

OTIR: objective type-total internal reflection

PBS: phosphate buffered saline

PC: primary leukemic cells

PE: phycoerythrin

PEI: Poly Ethylene Imine

PI: propidium iodide

Pim-2: proviral integrations of moloney virus-2

PLL: poly-l-lysine

PML-RARα: Promyelocytic locus gene-retinoic acid receptor alpha

PPARγ: peroxysome proliferating activator receptor gamma

Rac1: ras-related C3 botulinum toxin substrate 1

RBC: red blood cell

RHO: Ras homolog gene family

RN19: Rho dominant negative N19

RNA: ribonucleic acid

ROS: reactive oxygen species

RPMI-1640: rose well park memorial institute1640

RT: reverse transcriptase

RV14: RHOA constitutively active V14

SCF: stem cell factor

SCID: severe combined immunodisease

SCs: stromal cells

SDF1α: stromal cell derived factor alpha

SFFV: Spleen Focus Forming Virus

SFRP1: secreted frizzled-related protein 1

SIEW: Sin vector with IRES regulating eGFP reporter Woodchuck Hepatitis Virus

SOC: super optimal broth with catabolite repression

SPP1: secreted phosphoprotein 1

TE: tissue engineering

TGFβ1: transforming growth factor beta 1

THP1: Tamm-Horsfall Protein 1

TIRF: total internal reflection microscope

TMRE: Tetramethylrhodamine, ethyl ester

TNFα: tumor necrosis factor alpha

TPO: thrombopoietin

TRITC: tetramethyl rhodamine isothiocyanate

UNTR: untreated

VCAM: vascular cell adhesion molecule

VIN: vincristine

WBC: white blood cells

WPRE: Woodchuck Hepatitis Virus

Synopsis:

Bone marrow niche is composed of cellular and non-cellular compartments. Cellular environment includes hematopoietic stem cells (HSCs), non-hematopoietic stem cells and their derivatives. HSCs maintain the blood cell development. HSCs fate is determined by endosteal niche made of osteoblasts and stromal cells, and sinusoidal niche made of endothelial cells. HSCs proliferation and differentiation is regulated by interaction between the cells directly or indirectly. Stromal cells, their derivatives and secretory factors like growth factors, cytokines regulate the stem cells present in the bone marrow. It is a highly regulated process, imbalance in regulation leads to pathological conditions. In addition, it is composed of network like structure known as extra cellular matrix (ECM) which is made of collagen, fibronectin and proteoglycans, also known to regulate the stem cell properties. Mesenchymal stem cells (MSCs) from the non-hematopoietic stem cell compartment known to regulate the HSCs maintenance, proliferation and differentiation. The MSCs have self-renewal capacity to produce same type of stem cells and undergo differentiation to give rise to specialized mature cells like adipocytes, osteocytes, and chondrocytes. MSCs have role in wound healing, immune suppression, immune modulation, and angiogenesis. MSCs were first isolated from the bone marrow and also isolated from the various other sources like cord blood, peripheral blood, adipose tissue, placenta, cartilage etc. MSCs can be identified by expression of cell surface markers CD73, CD90 and CD105 and they are negative for hematopoietic markers CD34, CD45 and CD14. MSCs can be used to treat diseases such as neurodegenerative diseases, heart diseases, leukemia, muscular dystrophy, bone diseases and diabetes.

MSCs were used for cell therapy to treat several osteogenic disorders and have potential to be used for treatment of spinal cord injury, cardiac diseases. Hematopoietic engraftment has been facilitated in patients when MSCs was co-injected with the bone marrow cells (Jaganathan BG et, al. 2010; Chen S et, al. 2006; Hare JM et, al. 2009; Li J et, al. 2013; Park JH et, al. 2012). The clinical use of MSCs and its therapeutic outcomes were limited due to its low survival rate during *in vivo* injection. Rapid proliferation is also essential if these cells have to be used for autologous transplantation soon after the injury to achieve high clinical benefit. *In vitro* cell expansion is performed prior to *in vivo* administration where a large number of cells are required ($1x10^6$ cells/Kg body

weight) which needs a rapid expansion system (Jaganathan BG et, al. 2010). To facilitate tissue repair, during tissue engineering, the cells are directly injected into the injury site or seeded on to scaffold and implanted. (Kim HJ et, al. 2012). There are several reports which show that MSCs underwent rapid cell death soon after injection intravenously and the cells could not be identified when tracked after few months. In the present study, analysis of the cell characteristics of MSCs such as proliferation, survival, differentiation and migration was performed to understand the suitable extracellular matrix that could be utilized for MSCs expansion as well as coating on scaffold for tissue engineering.

In the current study, it was found that when MSCs were cultured on collagen (COL) fibers, they promoted high cell proliferation compared to other matrices such as fibronectin (FBN), poly-l-lysine (PLL) or normal tissue culture treated surface. In addition, cells cultured on COL showed higher resistance to apoptosis induced by oxidative and nutrient stress which might occur *in vivo* during ischemia (Rodrigo R et, al. 2013). COL also promoted faster cell adhesion to the culture surface, within 2hr and ROS production on COL surface was significantly low. These results suggest that COL could be utilized as a suitable coating on cell culture plates to promote higher proliferation or on scaffold for greater survival *in vivo*.

MSCs cultured on COL also showed higher osteogenic differentiation compared to other matrices. Interestingly, cells that were cultured on other matrices, when induced to differentiate on COL surface showed higher osteogenic differentiation. Importantly, culturing on COL surface was sufficient to promote higher osteogenesis even when differentiated on non-COL matrices. High osteogenic differentiation on COL also correlated with increased RHOA GTPase activity. Although chondrogenic differentiation is an important property of MSCs, it was not tested in the current study as chondrogenic differentiation *in vitro* was usually performed as a pellet culture where the cells do not have high contact with the culture surface during differentiation. Conversely, MSCs differentiated into adipocytes on control tissue culture treated (CON) surface showed significantly higher percentage of adipocytes, which correlated with the high expression of linage marker gene *PPAR gamma* whereas no difference in *Osteocalcin* levels were observed on different ECM. Since COL promotes higher osteogenic potential, we identified the role of ECM switching on MSCs differentiation. MSCs were expanded on different ECM and differentiated on different ECM. Our analysis showed that whenever cells were cultured or differentiated on COL, they showed high osteogenic potential.

This indicates that switching of cells from one ECM to other help to achieve better differentiation potential for therapeutic applications.

The actin cytoskeleton plays an important role in regulating cellular properties. To determine the actin modification and cell shape, MSCs were cultured on different matrices and stained for F-actin. MSCs cultured on PLL showed polygonal arrangement of F-actin, whereas MSCs cultured on COL, FBN or CON surfaces acquired parallel F-actin arrangement. Actin also mediates the attachment of cells with the cell culture surface through integrin and other proteins. To identify the cell contact points, MSCs were cultured on ECM proteins, stained for F-actin and examined with total internal reflection fluoroscence (TIRF) microscope. MSCs cultured on FBN, COL or PLL showed high number of contact poins, whereas cells cultured on CON surface showed very few contact points. Another important feature that is required during tissue remodeling and repair is active migration of cells. COL surface promoted higher cell migration, cell attachment and significantly high cell-surface contact points were observed in MSCs cultured on COL. Culturing MSCs on COL resulted in significantly high CD49e cell surface expression and CD49e was shown to mediate migration of hematopoietic stem cells (Sahin AO et, al. 2012) and an earlier study from our group also showed that CD49e cell surface expression increased during osteogenesis (Sonowal H et, al. 2013). This increase in CD49e expression on COL might be associated with its increased osteogenic differentiation potential. However, no significant difference in CD29 and CD49D expression level were observed in all the matrices. Furthermore, MSCs cultured on collagen showed mitochondrial distribution pattern similar to that observed in stem cells, where it was predominantly peri-nuclear mitochondrial distribution suggesting that COL might promote or maintain more stem cell-like state in MSCs.

In our earlier study, high RHOA levels were observed on COL grown MSCs. RHOA is essential for proliferation, differentiation, migration and other cellular functions. Inorder to understand the RHOA signaling pathway in MSCs, we expressed constitutively active (RHOAV14) or dominant negative inactive (RHOAN19) forms of RHOA in MSCs. In our study, we transduced the MSCs with lentiviral vector for stable expression and after 48 hours of transduction, morphology change was observed. Control MSCs showed spindle shape with normal fibroblastic morphology but MSCs expressing RHOAV14 lost their normal spindle shape and showed stellate like morphology. To demonstrate the role

of RHOA in MSCs proliferation, MTT cell viability assay was performed. The results of MTT assay showed that there was a significant reduction in proliferation in MSCs expressing RHOAV14 compared to control. Furthermore, we elucidated the role of RHOA in actin modification. Parallel F-actin arrangement was seen in control MSCs, whereas crisscross arrangement and increased stress fiber formation was observed in MSCs transduced with RHOAV14. The MSCs transduced with RHOAV14 showed significant less migration potential than others. For evaluation of differentiation potential, RHOA transduced MSCs were subjected to osteo and adipogenic differentiation for 21 days and stained with alizarin red (ARS) for osteocytes and Oil red O (ORO) for adipocytes. There was an increase in osteogenesis and decrease in adipogenic potential of MSCs transduced with RHOAV14. However, no difference was seen in MSCs transduced with dominant negative RHOAN19.

Leukemic stem cells modify or manipulate the environment by secreting factors required for cancer cells. To study the changes in MSCs due to interaction with leukemic cells, MSCs were co-cultured with primary leukemic cells for 1 week and expression of surface markers CD13, CD29, CD49E, CD73, CD90 and CD95 was determined by flowcytometry. Decrease in CD73, CD90 and CD95 expression was observed in MSCs during co culture with primary leukemic cells. Furthermore, in order to understand the fate of MSCs during leukemic state, MSCs were co cultured with leukemic cells for 1 week and induced to differentiate into adipocytes and osteocytes with respective induction media for 28 days. The MSCs co cultured with primary leukemic cells showed reduced adipogenic potential compared to control, whereas no change was observed in osteogenic differentiation. MSCs were co cultured with THP1 and HL60 leukemic cell lines and screened for the expression of MSCs surface markers. No significant difference was seen in MSCs cell surface marker expression during THP1 or HL60 co-culture.

Understanding the changes in MSCs after chemotherapeutic treatment is essential to determine their potential for cell therapy; we treated MSCs with cytrarabine (CYT), daunorubicine (DAU) and vincristine (VIN) and evaluated their properties. The cells treated with VIN induced a marked change in cell shape, converting spindle shaped cells to round shape, whereas no change in morphology was observed in CYT and DAU treated MSCs after 48hr. We observed following changes in cell surface marker expression profiles a) significant decrease in CD49E expression in CYT treated MSCs, b) MSCs treated with DAU showed reduced levels of CD13, CD29, CD49E, CD90 and

CD95 expression, c) MSCs treated with VIN down regulated the expression levels of CD13, CD49E and CD90. Significant reduction in MSCs proliferation was observed after 48hr of drug treatment. To evaluate the effect of chemo drugs on differentiation potential, we stained post-differentiated MSCs with Oil Red O (ORO) and Alizarin Red S (ARS) after adipocytes and osteocytes differentiation respectively. We observed significantly less adipogenic and osteogenic potential in all the drug treated MSCs.

MSCs treated with chemotherapeutic drugs were allowed to recover and their cell surface antigen expression was determined. DAU treated MSCs showed reduction in CD13 and CD29 expression after 7 days of recovery. However, VIN treated MSCs completely recovered from chemo drugs and showed normal surface marker expression. MSCs recovered from VIN and CYT retained their normal adipo and osteo differentiation potential.

To check if recovered MSCs could potentially support the growth of leukemic cells, MSCs were co cultured with AML cells HL60 and treated with CYT, DAU and VIN. Increased apoptosis was observed in HL60 after treatment with all three drugs, whereas treatment with drugs in the presence of MSCs reduced their apoptotic percentage except during VIN treatment.

We also evaluated the effect of antimitotic drugs, colchicine and nocodazole on MSCs properties, using phenotypic markers CD13, CD29, CD49E, CD90 and CD95. In colchicine treatment we found decreased expression of CD49E and CD90, whereas, CD13 and CD90 expression was reduced by nocodazole treatment. To understand the effect of drug treatment on cell differentiation, MSCs were treated for 24hr and 1 week with colchicine and nocodazole in differentiation media. We observed, reduced adipogenic and osteogenic differentiation in both colchicine and one week nocodazole treated MSCs. However, no significant difference was seen after 24hr nocodazole treatment. After removing the nocodazole, MSCs recovered and regained normal differentiation potential. However colchicine treated MSCs did not achieve their normal differentiation potential after the drug removal.

In conclusion, the present study demonstrates that extracellular matrices control the characteristics of MSCs by altering their cell proliferation, migration and osteogenic differentiation. Our study shows that MSCs could be cultured or pre-differentiated on COL surface to obtain high osteogenic differentiation and culturing them on uncoated

tissue culture plastic primed them to adipogenesis. Increased proliferation can be achieved by coating the cell growth surface with COL and when used in tissue engineering, COL coated scaffolds will promote high cell migration, proliferation, survival and osteogenic differentiation. MSCs treated with chemotherapeutic drugs recovered their cell surface expression and differentiation potential after removal of the drugs and thus might be suitable for cell therapy.

Chapter1

Scientific Literature

1. Scientific Literature

1.1. Introduction

Though prevalent use of stem-cell-based therapies is still a long way off, it could benefit large numbers of patients with cancer (Jayesh Sagar et, al. 2007) neurodegenerative disorders (Olle Lindvall et, al. 2010), and heart diseases sometime in the future (Hotkar AJ et, al. 2012).

Cells which produce blood cells, known as hematopoietic stem cells (HSCs) (Bryan A. Anthony et, al. 2013), are located in a dynamic microenvironment termed as bone marrow (BM) niche (Morrison SJ et, al. 2014). It is composed of various types of cells, including stromal cells and their derivatives (Jian Huang et, al. 2012). Mesenchymal stem cells (MSCs) have the potential to produce different mesenchymal lineages like adipocytes, osteocytes, chondrocytes, cardiocytes and myeocytes (Majumdar et, al. 2000; Pittenger et, al. 1999). Other than these, MSCs can proliferate and migrate to injured regions (Kristine C. Rustad et, al. 2012). A number of extrinsic and intrinsic factors control the fate of MSCs (Tzyy Yue Wong et, al. 2015). One of the intrinsic factor called Ras homolog gene family guanosine triphosphatases (RHO GTPases), which is expressed endogenously, is known to regulate stem cell fate (Farshid Guilak et, al. 2009). Extra cellular matrix (ECM) present or secreted by cells present in the BM is also known to regulate the stem cell behavior (Farshid Guilak et, al. 2009). In our study we used ECM proteins collagen (COL), fibronectin (FBN) and poly-l-lysine (PLL) and chemotherapeutic drugs cytarabine (CYT), daunorubicine (DAU) and vincristine (VIN) as an extrinsic factors. RHOA GTPases and leukemic cells were studied as an intrinsic factors for studying the MSCs properties. MSCs applications are still limited due to lack of information about their response under different environments. It is essential to improve the efficiency of MSCs for the wide range of applications and also to understand the role of MSCs during chemotherapy, and the change in their properties in that particular environment.

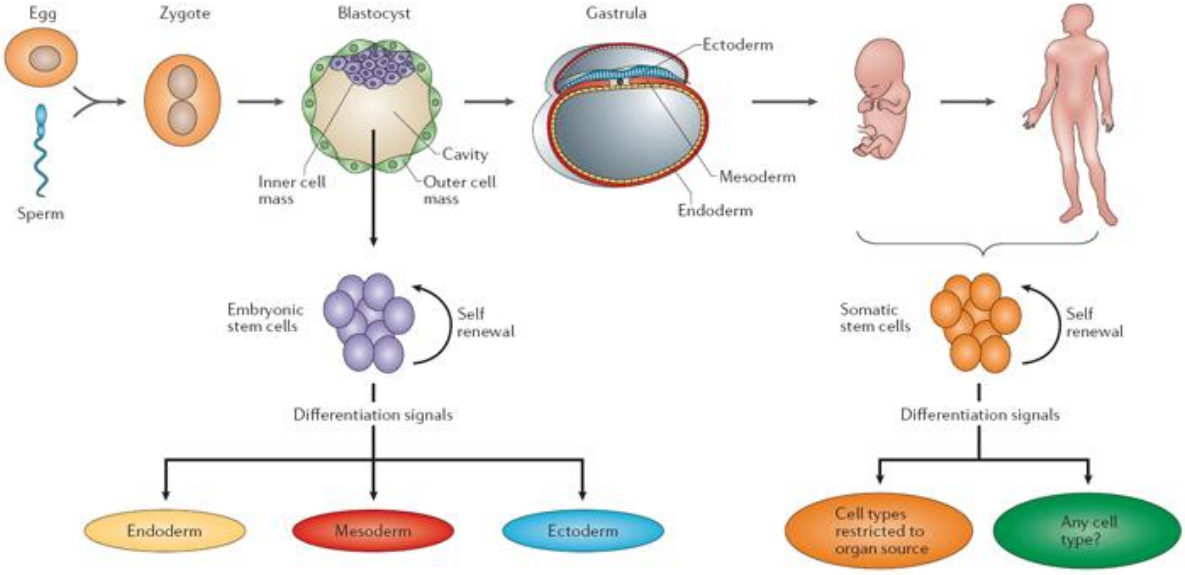
Here, we used genetically modified RHOA GTPases to elucidate the role of RHOA in MSCs. ECM proteins are used to create an artificial microenvironment to find out their effects on MSCs fate. By studying the chemotherapy affected MSCs properties and their role in the protection of leukemic cells from chemo drugs. It is essential to understand the effect of leukemic cells on MSCs for therapeutic purposes.

1.2. Stem cells

Modern therapeutics has given a lot of hope in stem cell research to replace destroyed or damaged cells with healthy or genetically modified cells through transplantation or cell therapy (Jayesh Sagar et, al. 2007; Olle Lindvall et, al. 2010). Proliferation and differentiation capacity makes stem cells unique in nature and are used in wide range of therapeutic applications (Ingmar Glauche et, al. 2009). In right conditions and with right signals, these stem cells can be transformed into different types of cells with distinct characteristics (Shigeo Takashima et, al. 2013). Their progeny are affected by combination of intrinsic and extrinsic gene expression to form molecular and structural pattern of specific tissue to perform specific function (Farshid Guilak et, al. 2009).

1.3. Embryonic stem cells (ESCs)

Mammalian stem cells are classified into embryonic stem cells (ESCs), which were isolated from inner cell mass of developing embryo (Berenika Plusa et,al. 2014) and stem cells isolated from adult tissue sources (Stefania Montagnani et, al. 2016). ESCs are totipotent and can be used to treat all diseases (Maureen L. Condic. 2014). Very few studies are present for ESCs because of ethical issues (Bernard Lo et, al. 2009). Also, ESCs are very limited in clinical applications because of allogenic immune reactions (Shengwen Calvin Li et, al. 2009). The main role of stem cells and progenitor cells in adult organisms is to function as repair the dmaged tissues and replace with normal healthy cells to regenerate tissues like skin or blood (Benoit Biteau et, al. 2011).

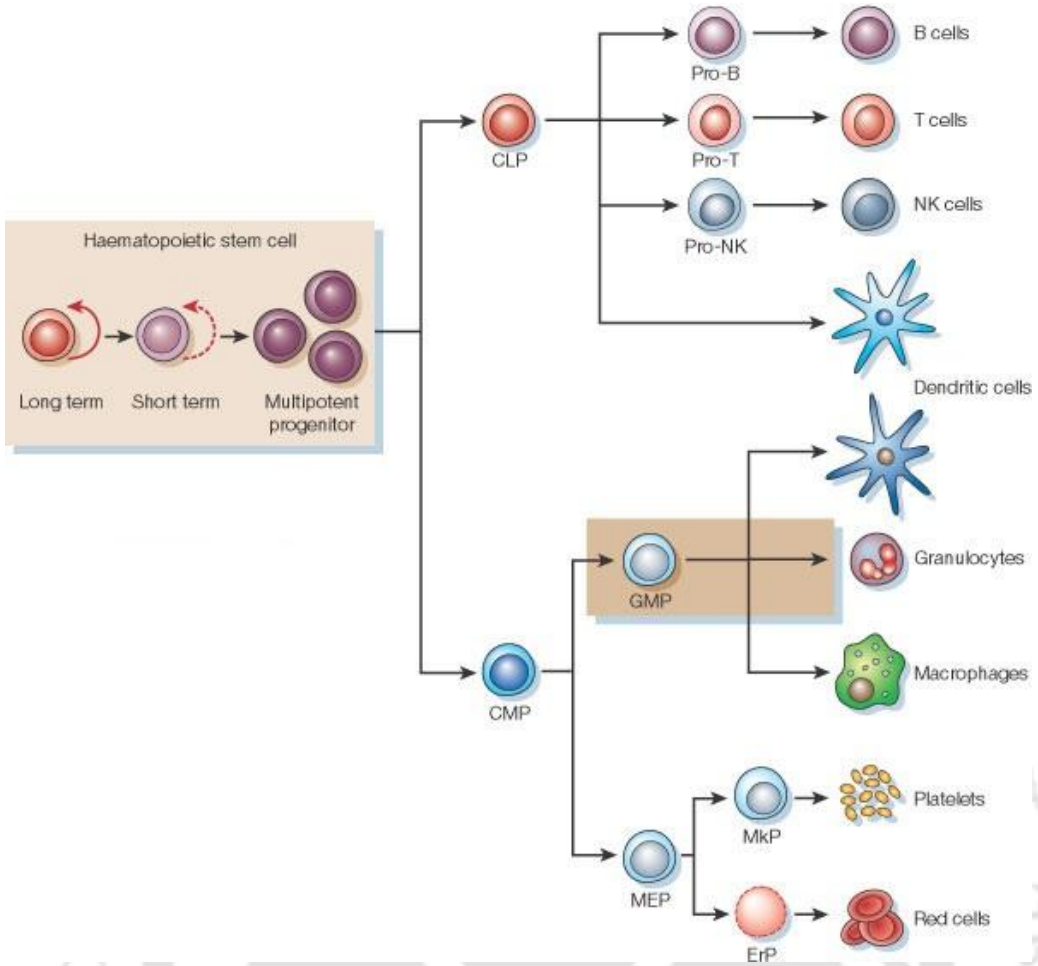


(O cannor et, al. 2006)

Fig.1.1. Stem cell self-renewal and differentiation capacity

1.4. Hematopoietic Stem Cells (HSCs)

HSCs are one of the adult stem cell populations present in the BM microenvironment and involve in blood cell production. Erythrocytic red blood corpuscles (RBC) transport molecular oxygen to the cells or tissues (R.Lipowsky . 1995). Platelets which is derived from megakaryocytes help in blood clotting (Laki. 1972). Granulocytes such as neutrophils, basophils and eosinophils and macrophages which is collectively known as myeloid cellscan fight infections of bacteria, fungi, and other parasites such as nematodes (ubiquitous small worms) (Katherine Y. King et, al. 2011; Arielle Glatman Zaretsky et, al. 2014). While B-lymphocytes generally produce antibodies (Miroslav Holub. 1958). Further, many virus-infected cells and cancer cells are directly killed by T-lymphocytes. Additionally, T-lymphocytes also isolate cells recognized as foreign to the body (Patrick J. Brennan et, al. 2013).



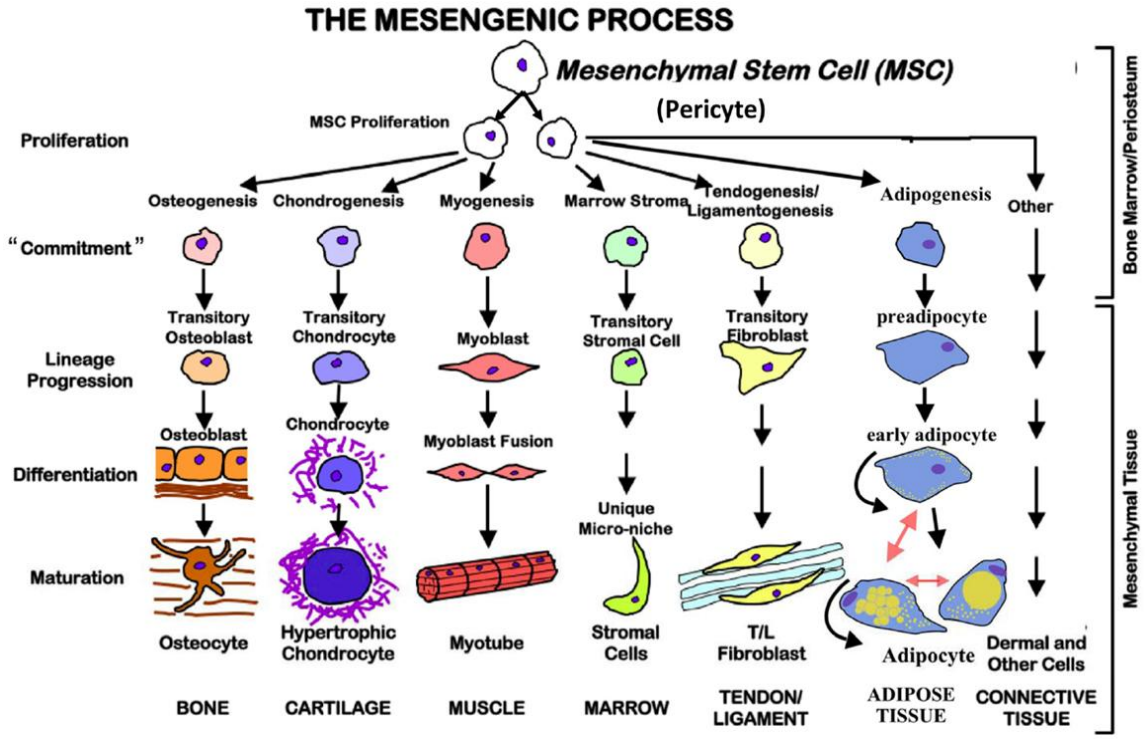
(T Reya et, al. 2001)

Fig.1.2. Hematopoisis (Blood cell development)

1.5. Mesenchymal Stem Cells (MSCs)

During embryonic development 3 different types of germ layers are formed: ectoderm, mesoderm and endoderm. MSCs were first discovered by A.J.Friedenstine and are characterized by their ability to adhere to plastic surface and generate fibroblastic like morphology and can be repeatedly passaged upon proliferation (HorwitzEM et, al. 2005; Friedenstein AJ et, al. 1976). They are not only involved in the bone formation but also support hematopoietic microenviroment of BM stroma (Friedenstein AJ et, al. 1976). The small percentage of cells present in the BM populating cells (the number is around 0.01 to 0.001% of mono nuclear cells) are isolated by density gradient (Ficoll/Percoll)

which give rise to plastic adherent like colonies (Pittenger MF et, al. 1999). MSCs reside in the BM as a non-hematopoietic stem cell population. MSCs can be expanded without losing proliferation capacity for several passages (Rahaman MN et, al. 2005). They show self-renewal and can produce progenitor cells and differentiate into particular lineages depending on the stimuli (Pittenger et, al. 1999; Majumdar et, al. 2000). They have broad range of differentiation potential and can differentiate into neurons (Kopen et, al. 1999) cardiac muscle cells (Mkino S et, al. 1999), hepatocytes (Oswald J et, al. 2000), endocrine cells (Hee-Sook Jun et, al. 2009), epithelial cells (Ortiz LA et,al.2003) and hepatocytic cells (Lee KD et, al. 2004). Therapeutic potential of MSCs can be used in clinical fields like neurology, cardiology, hematology and orthopaedics (Wang S et, al. 2012). MSCs are able to adhere to plastic surface and show fibroblastic like morphology. According to stem cells classification committee international society for cellular therapy (ISCT), 95% of MSCs express CD73, CD90, and are negative for CD34, CD45, CD14 or CD11b, CD79a or CD19 and human leucocyte antigen (HLA) class II (Dominici et, al. 2006). MSCs generally do not express major histocompatibility complex (MHC) II but their expression can be up regulated during cytokine exposure or during MSCs differentiation (Le blank K et, al. 2005). MSCs secrete various cytokines and growth factors which are important to regulate various physiological functions. During tissue damage and inflammation these protective factors formed a matrix and helps in healing. Apart from the BM, MSCs can also be found in the non-hematopoietic BM (Suva D et, al. 2004), umbilical cord blood (Erices A et, al. 2000), umbilical vein (Covas DT et, al. 2003), peripheral blood (Fernandez M et, al. 1997), adipose tissue (Zuk PA et, al. 2000), post-partum placenta (Zhang Y et, al. 2004). The animal studies data suggested that MSCs elicit allogeneic immune responses and can be rejected by the recipient (Eliopoulos et, al. 2005). This allogenic response can be overcome by MSCs immunomodulatory effect (Marcella Franquesa et, al. 2012). The number of MSCs decreases with age. Highest number of MSCs is found in neonates and decrease by one half by the age of 80. Highest number of circulating MSCs is found in first trimester and decline during second trimester to 0.0001%, and further decline to 0.00003% in nucleated cord blood (Campognoli et, al. 2001).



(Calpan AI et, al. 2006)

Fig.1.3. Mesengenesis. A multi-potent stem cell found in adult bone marrow and other.

HSCs are found in a complex cellular microenvironment composed of osteoclasts, endothelial cells, stromal cells (SCs), mesenchymal progenitor cells and adipocytes along with components of the ECM. All these cellular elements combined with ECM form the hematopoietic niche. Its main functions are controlling stem cell pool size and fate of HSCs. The stromal cells and their different lineages and their secretory products and structures are established to affect HSCs fate and function.

Osteoblastic cells and bone endosteal surface were identified as the first component of HSCs niche in BM microenvironment (Calvi et, al. 2003). Recent studies are trying to find out if a specific subpopulation of osteoblats interact with HSCs. The ECM component osteopontin (OPN) or secreted phosphoprotein 1 [SPP1] secreted by activated lymphocyte cell adhesion molecule (ALCAM) and osteoblasts were known to involve in HSCs function in BM microenvironment (Nakamura et, al. 2010). Also reports have suggested that nestin positive MSCs interact with HSCs and affect the cellular functions (Mendez-Ferrer et, al. 2010). The localization study shows that tahey

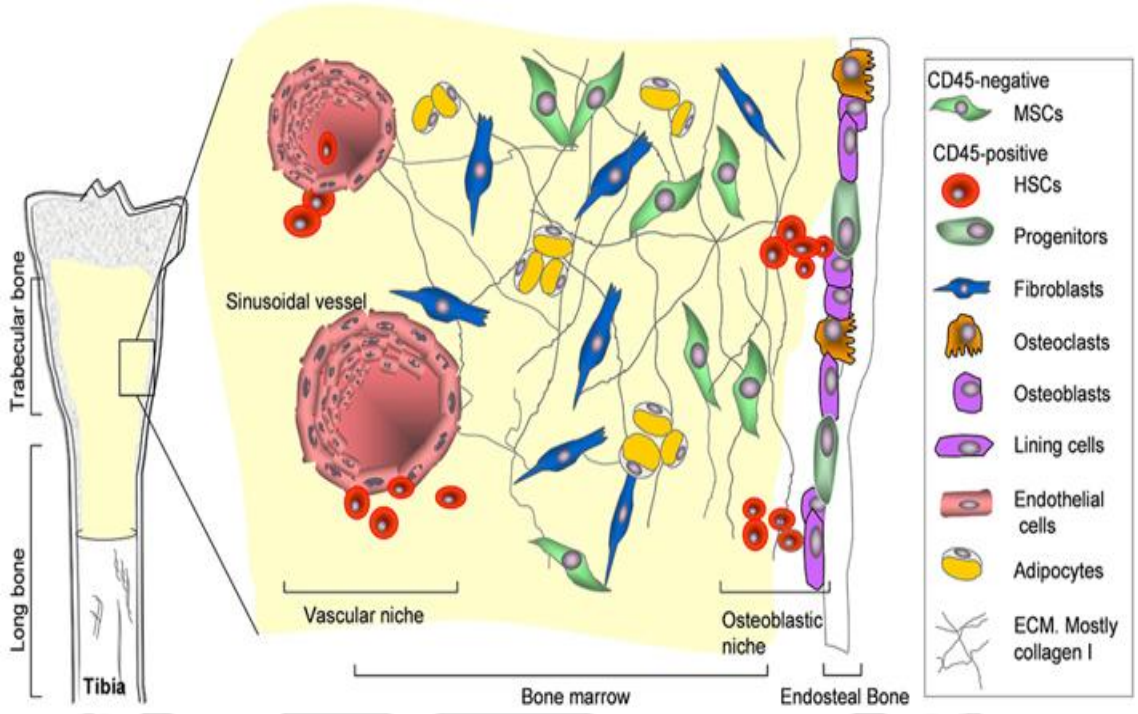
are mainly found in areas of bone marrow containing HSCs niches (Morikawa et, al. 2009). Most of the reports concluded that osteoblasts derivatives from stromal cells regulates the maintenance and support of HSCs functions.

Earlier reports have shown that HSCs engraftment can be inhibited by perivascular (Sugiyama et, al. 2006) and adipocytes (Naveiras et, al. 2009). This influences HSCs mobilization (Katayama et, al. 2006). Furthermore, a number of cells of hematopoietic origin have a role in HSCs niche.

From the finding that even inside the marrow different HSPCs localizations exist, and a variety of cell types involved in HSCs regulation, and above all studies that suggest heterogeneity of even highly purified HSPCs populations are indicative of complex microenvironment through which HSCs navigate (Dykstra et, al. 2007). Osteoblastic niche responsible for maintenance of HSCs in dormancy condition, whereas HSCs in quiescent state maintained by perivascular niche. During right signal, HSCs can be activated from quiescent state and become differentiated progeny or back to HSCs dormancy condition. This modulation is depends on the requirement of an organism (Malhotra et, al. 2009).

1.6. Bone marrow (BM) microenvironment

The location of stem cells present in the BM known to be BM microenvironment. It provides all the factors required for maintenance and support of HSCs. Earlier studies reported that molecular regulators present in the BM microenvironmentwere responsible for HSCs maintenance (Tong Yin et, al. 2006). It includes stromal cells and their derivatives such as osteoblastic cells and secreted factors from multiple cell types (Mansour A et, al. 2012).



(Grassel et, al. 2007)

Fig.1.4. Bone marrow niche shows interaction of stromal cells with HSCs

1.6.1. The Extracellular Matrix (ECM)

Any type of cell requires ECM to carry out their physiological functions (H. William Schnaper et, al. 1993). Initially it was thought like an inert material, but later on its imperative role was elucidated (Teti A. 1992). Main function of ECM is to converts extrinsic signals into intrinsic signals by engaging with integrins and cell surface receptors (Yue Gao et, al. 2014). They do it by connecting ECM environment to the intercellular cytoskeleton (Hynes RO et, al. 2002). The biological relevance of ECM to cell adhesion and signaling has been extensively documented (Hynes RO et, al. 2002). Stem cell niche provide extracellular cues which allow stem cell survival and maintain a balance between proliferation and differentiation (Ting Xie et, al. 2000; Sean J. Morriso et, al. 2008). In addition to this, ECM provides mechanical support to maintain stem cells homeostasis. ECM interacts with cells to form ECM-cell interaction and regulate signaling pathways (Disher DE et, al. 2009). ECM is a structural entity which regulates cell properties. Several reports have demonstrated that ECM affects cell adherence,

growth and migration (Hutchings et, al. 2003), proliferation and differentiation (Moiseeva. 2001). ECM is composed of 3 major classes of proteins which are structural proteins (collagen and elastin), structural proteins (fibrillin, fibronectin and laminin) and proteoglycans (Xiao et, al. 2007). ECM is known to regulate MSCs properties. ECM stiffness modulates the MSCs differentiation into SMC and chondrogenic lineages in response to transforming growth factor beta (TGF-β) (Jennifer S Park et, al. 2012). Stiffness of ECM also guides the differentiation of MSCs into osteoblasts, skeletal and neuronal lineages (Engler AJ et, al. 2006). Micro patterned islands regulate the stem cells fate. Especially, cell spreading favours osteogenesis and rounding promotes adipogenesis (McBeath et, al. 2004). Further investigation showed that, geometry of ECM also regulates differentiation capacity (Kilian et, al. 2012). Elasticity of ECM decides the lineage commitment; stiff matrix favours osteogenesis, whereas soft substrates favour adipogenesis (Engler AJ et, al.2006). Substrate modulus direct the neuronal differentiation, softer gel promotes neuronal, whereas stiffer gels favors glial cells differentiation in the presence of serum.

With the development in the field of cell based tissue engineering (TE), researchers have been trying to identify novel scaffolds or modify the existing in order to achieve the best performance for faster and effective tissue engineering. To improve the ECM-cells interaction in TE another approach can be taken which involves coating the biomaterial surface with desired proteins that will increase the cell adhesion or enhance specific differentiation.

In this study, we have selected collagen (COL), fibronectin (FN) from extracellular components and poly-l-lysine (PLL) as a cell adhesion factor, to evaluate their effect on the *in vitro* behavior of MSCs. This study aimed at understanding the role of specific ECM proteins on physiological properties like proliferation, migration, differentiation and cell survival.

COL is the most abundant protein in the human body (30%) provides strength to tissues or organs by acting as a structural element (Lisa D. Muiznieks et, al. 2012). COL acts as a structural frame work for connective tissues such as bone, dermis or tendons, also it has a role in elasticity of the tendons (M.D. Shoulders et, al. 2009). COL fibers involve in tissue repair and regeneration applications. Moreover, COL contributes the wide range of

biological functions include cell adhesion, cell migration, differentiation and tumor suppression (K.E. Kadler et, al. 2007).

FBN is an ECM component secreted by most of the cells present in the human body. FBN exist as a homodimer and present in two different forms: soluble FBN secreted by haptocytes of liver cells and insoluble FBN form secretd by most of the cells such as fibroblasts (Pankov R et, al. 2002). Earlier reports have demonstrated that FBN contribute the biological functions such as cell adhesion, growth, differentiation and migration (Pankov R et, al. 2002). Also, it has an important role in development of embryo and wound healing capacity (Pankov R et, al. 2002). FBN regulates the cellular functions by interacting with cells and other ECM components present in the human body (George EL et, al. 1993).

1.6.2. Adhesion molecules

Adhesion molecules present on the cell surface, majorly involves in cell-cell or cell-ECM interactions. Integrin α4β1 (ITGA4) mediates HSCs retention within BM microenvironment (Priestley et, al. 2006), whereas integrins α1β1 and α5β1 (ITGA3 and ITGA5) mediate adhesion of HSCs to ECM proteins such as SPP1 (Nilsson et, al. 2005). Also, all the three integrins regulate the function of stromal derived factor 1 (SDF1), which is expressed on stromal cells (Peled et, al. 2000). Also reports have demonstrated that integrin expression of HSCs is associated with WNT signaling pathway. Loss of HSCs progeny is associated with expression of constitutive active mutant of β-catenin and higher expression of integrins α2, β1 and β7 on HSPCs surface (Kirstetter et, al. 2006).

1.6.3. Chemical gradients

The concentration of chemicals also plays an important role in the maintenance of HSCs function in the BM. Cells present in the BM secrete various types of chemicals through their metabolism. Osteoclasts releases Ca^{2+} during bone resorption. This leads to the formation of a concentration gradient that spreads out from endosteal surface (Levesque et, al. 2010). HSCs express G-protein coupled Ca^{2+}-sensing receptor (CASR), which is responsible for HSCs function and localization in peri-endosteal regions (Adams et, al. 2006).

However, the role and relevance of oxygen tension in the in the HSCs niche in not clear. Both of these endosteal and non-endosteal LT-HSPCs are localized near vasculature (Lo Celso et, al. 2009). Therefore, to detect the presence of O_2 gradients in BM microenvironment and their role in cellular functions, *in vivo* real time measurements of BM O_2 concentration are necessary. Earlier reports have demonstrated that oxygen levels affect the expression of WNT transcription factor, which is associated with the maintenance of HSCs (Levesque et, al. 2007).

1.6.4. MSCs – HSCs interaction

With increasing knowledge about the marrow microenvironment and the stromal cells in the marrow, researchers have been trying to find out the ECM components that play an important role in the modulation of signaling pathways and the maintenance of the HSCs and the leukemic stem cells in disease condition. Cytokine induced proliferation of acute myeloid leukemia (AML) cells by different marrow components is also studied by many authors. Human AML blast cells are induced to produce interleukin 8 (IL8), a pro-angiogenic cytokine which increases vascularization and increase the survival of AML. Literatures reports that osteoblastic cells regulate the HSCs niche (Calvi et, al. 2003) but the role of cytokines in the survival and maintenance of the HSCs pool was not its sole responsibility. Induction of promyelocytic locus gene-retinoic acid receptor alpha (PML-RARα), a translocation product in Acute promyelocytic leukemia (APML) cells results in up-regulation of functional leptin receptor isoform in bone marrow derived adipocytes which results in production of anti-apoptotic signals and increased proliferation of leukemic cells (Yoko Tabe et, al. 2013). Adipocytes provide a protective niche during chemotherapy and promotes survival of leukemic cells by increased expression of two pro-survival signals B-cell lymphoma-2(Bcl-2) and proviral integrations of moloney virus-2 (Pim-2) (James W. Behan et, al. 2009). Although many authors have shown the possible mechanisms of interactions, but the exact way in which they interact is not yet clear.

1.6.4.1. Cytokines secreted by MSCs on HSCs

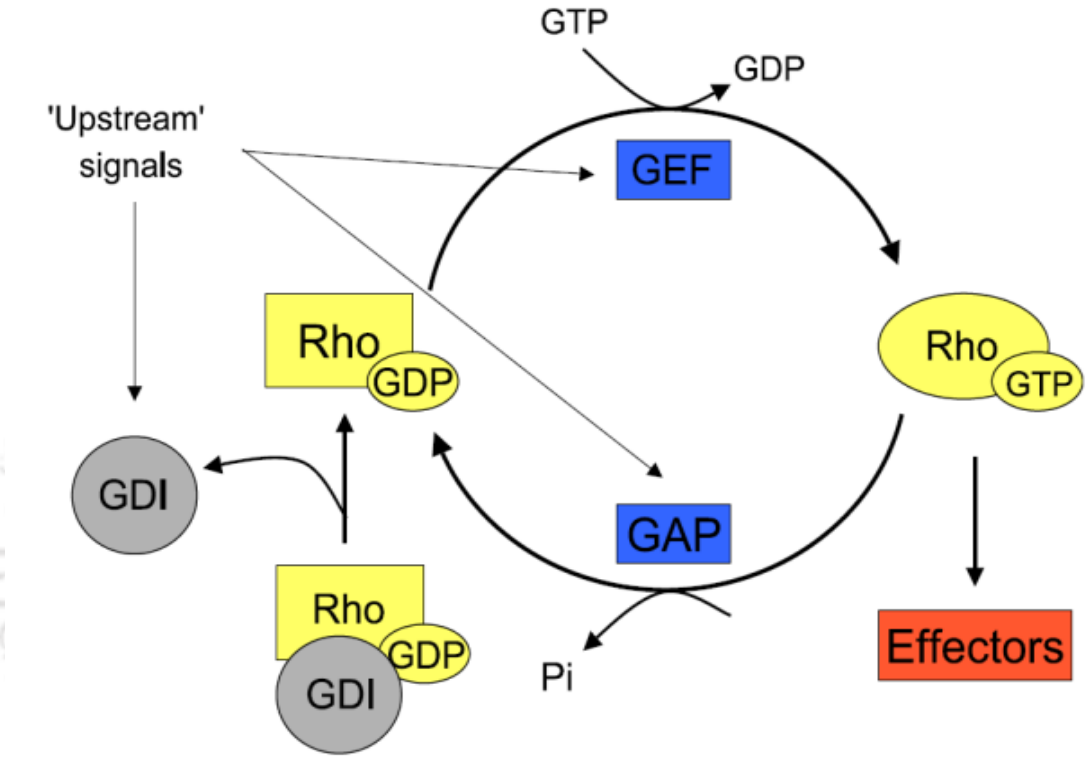
CXCL2	Regulates the adhesion, expansion, migration and homing of HSCs (Seiji Fukuda et, al. 2007)
SDF-1α	Reduces the production of inflammatory cytokines and chemokines (Orit Kollet et, al. 2003)
FLt3	Maintains HSCs proliferation and self-renewal, regulate hematopoietic growth (S. Haihua Chu et, al. 2013)
IL-6,TPO	Influences HSCs proliferation and differentiation (Jun Seita et, al. 2011)
GM-CSF	Regulates HSCs engraftment (Bendall et, al. 2014)
SCF	Maintains HSCs proliferation and self-renewal, regulates hematopoietic growth, regulates HSCs engraftment (Linda J. Bendall et, al. 2014)
VCAM-1, E-selectin, collagen, fibronectin	Regulates HSCs homing and adhesion (Knittel T et, al. 1999)

1.7. Ras homologous guanosine triphosphatases (RHO GTPases)

A wide variety of cellular activities involving actin dynamics are regulated by the RHO subfamily of small GTPases (Etienne-Manneville et, al. 2002). Studies have shown that compared with undifferentiated cells, stem cells are softer and more sensitive to local stress stimulation, and the extraordinary sensitivity of embryonic stem cells to stress is closely regulated by the RHO family of GTPases (Chowdhury et, al. 2010). RHO GTPases are involved in integrin-mediated mechanotransduction (Burridge et, al. 2004; Wennerberg et, al. 2004) and regulate stress fiber formation in adult stem cells under mechanical stimulation (Discher et al., 2009). Ras-related C3 botulinum toxin substrate 1 (Rac1) plays an important regulatory role in cytoskeletal assembly during lamellipodia formation in cells under mechanical stimulation (Hu et, al. 2002).

Their best-characterized function is in the regulation of actin dynamics. TC studies (carried out originally in fibroblasts, but later in many other cell types) using constitutively active and dominant negative, interfering forms, have shown that RHO regulates the assembly of contractile, actin:myosin filaments, while Rac and cell division

control protein 42 (Cdc42) regulate the polymerization of actin to form peripheral lamellipodial and filopodial protrusions, respectively. Also, they can influence a wide range of other biochemical activities apart from their effect on actin, RHO, Rac and Cdc42. Most importantly, Cdc42 is only required for the establishment of cell polarity, whereas microtubule cytoskeleton and gene transcription were affected by all three of them (Etienne-Manneville et, al. 2002).



(SM. Raftopoulou et, al. 2004)

Fig.1.5. The RHO GTPase cycle.

RHO, Rac and Cdc42 are the three important members of RHO family . Generally, when a cell polarizes in response to a chemotactic or chemokinetic signal cell migration starts accordingly. RHOA signaling regulates the different modes of cell migration includes amoeboid or rounded. However, elongated mode of cell movement is driven by Rac. A polarized interaction at the leading edge between COL fibres with integrin β1 and matrix metalloproteinases co-clusters at these sites, migratory behavior was characterized (Horwitz R et, al. 2003).

MSCs differentiate into osteocytes, adipocytes in the bone marrow and act as a source of connective tissues, ECM (Majumdar et, al. 2000; Pittenger et, al.1999). Cells resulting from MSCs also play a central role in the HSCs niche and control the localization, self-renewal and differentiation of HSCs (Seiji Fukuda et, al. 2007; Orit Kollet et, al. 2003). In the bone marrow niche, the SCs provide recognition sites for the HSCs such as cell surface ligands for the adhesion molecules on the HSCs surface. HSCs express a range of integrins and selectins which bind to the ECM substances or the SCs (Knittel T et, al.1999).

In addition to this we will be trying to find out the in-vitro maintenance of HSCs co-culture with RHOA transduced MSCs. Many reports have already shown that MSCs regulating the HSCs functions in vivo and in vitro but still the exact mechanism is not clear.

1.8. Leukemia

More and more occupation of BM by abnormal cells results in the very less space for normal cells and the person who suffers with abnormality becomes ill (H M Amin et, al. 2005). Furthermore, on the basis of disease progression in different lineages of blood cell development, leukemia can be classified into four major classes includes acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), AML and chronic myeloid leukemia (CML) [(A Tefferi et, al. 2008)]. It is more common in adults as compared to children and more often seen in men as compared to females (E Manara et, al. 2016). 40% of chemotherapy treated patients undergo complete remission for 5 years. AML diagnosis is carried out by staining of BM smears and looking for presence of multiple aur rods. These rods consist of clumps of azurophilic granules which are present only in AML samples (Olsen RJ et, al. 2008).

With increasing knowledge about the BM and the SCs in the marrow, researchers have been trying to find out the exact cellular components that play an important role in the modulation of signaling pathways and the maintenance of the HSCs and the leukemic stem cells in disease condition (Rizo A1 et, al. 2006). The role of MSCs and their progenitors, including osteocytes and adipocytes, have been studied by many researchers. Co-culture of MSCs with CML cells inhibits the proliferation of CML cells by the production of interferon alpha (IFN-α) (Zhang et, al. 2009). Cytokine induced

proliferation of AML cells by different marrow components has also been studied by many authors.

The function of osteoblast in the growth and support of the HSCs pool in the BM has been studied by many authors (Taichman, R.S. et, al. 1994). The bone reabsorbing cells "osteoclasts" are also involved in the maintenance of dormant leukemic cell by maintaining the cells in G_0 quiescent stage by releasing soluble factors like TGF-β (Yokota et, al. 2010). Some literatures suggest that MSCs derived adipocytes plays a role in promotion of survival of leukemic cells.

Leukemic stem cells are present in the BM microenvironment during diseased condition. The growth and maintenance of leukemia cells is not only because of tumor cells, but also involves various other types of cells present in the BM tumor microenvironment, including endothelial cells, SCs and their derivatives like osteocytes, adipocytes and so on (Hong-Sheng Zhou et, al. 2016). The mechanism of regulating tumor cells by MSCs can be direct or indirect (Catharina Melzer et, al. 2016). Interaction between tumor cells and MSCs is a reciprocal relationship (Catharina Melzer et, al. 2016). In the indirect mechanism, MSCs and tumor cells are mediated by secretion of various types of growth factors, cytokines and chemokines (Catharina Melzer et, al. 2016). These molecules act as substrates for specific receptors and activate intracellular signaling pathways and secrete metabolites and shows effect on other cells through paracrine mechanism or through transfer of RNA and proteins via micro particles like microvescicles and microsomes. Direct interaction between tumor cells and MSCs are mediated by Notch signaling pathway, gap junctions, nanotube formation, membrane patches and membrane fusion to generate hybrid cells (Lazennec Gwendal et, al. 2016).

Leukemic cells are protected from chemo toxic agents by them and they are mainly responsible for relapse and re-occurrence of the disease after treatment (A John Barrett et, al. 2012). Although many authors have shown the possible mechanisms of interactions, but the exact way in which they interact is not yet clear.

1.9. Chemotherapeutic drugs for therapy

Chemotherapy is the treatment by which cancer is cured with the help of anti-cancer drugs (Pierre A. Stryckmans et, al. 1973). The main purpose of chemotherapy is to improve the standard of life for the patient. Chemotherapy has capability to annihilate

cancer cells anywhere in the body even the cells that spread in different parts of human body (Pierre A. Stryckmans et, al. 1973). Even for patients undergoing surgery, chemotherapy can be given before performing surgery to reduce the size of the tumor (neoadjuvant therapy). It is essential to understand that which type of drug and in which ratio is best to cure cancer. Chemo drugs can be given differently on the basis of type of drugs and cancer. Whereas, the main objective is to target tumor cells and kill them. Depending upon drugs, it can be introduced in the body by different routes, as the accessibility of these drugs is different inside the human body. Chemo therapy can be given in different ways: orally, it can be in the form of pill, capsule or liquid. Intra muscular chemotherapy can be injected to the muscle. In addition, other methods are also available to pump the drugs through other routes viz. intra-arterial (arteries), intra-lesional [(tumor in the skin), intra-peritoneal (abdominal cavity), intrathecal (spinal cord) (Kenneth R. Hande. 1996)].

The chemotherapies have been used for cancer treatment for several years (Pierre A. Stryckmans et, al. 1973). The goal of the anti-cancer drugs is to eradicate the cancer cells completely. Major sources of anti-cancer drugs are natural compounds, about 30%. Majority of these drugs either targets growth, proliferation or division of the tumor cells (Arnold L.Demain et, al. 2010). Depending upon the action of drug on tumor cell cycle it can be classified in two categories, first one cell cycle specific (G1/S stages of the cell cycle) and the other one cell cycle nonspecific (G2 stage of the cell cycle) [(Geoffrey I et, al. 1999)]. One type of drug can be used for several cancers with varying effects and efficiency. Some tumors are very sensitive, but some are more resistant to same type of drug. Several drugs have been approved for the clinical use; they are derived from different types of sources. Anti-cancer drugs were classified into different types based on their mechanism of action. It includes, alkylating agents/ deoxy ribonucleic acids (DNA) damaging agents, antimetabolites, plant alkaloids and terpenoids, phodophyllotoxin, taxanes, topoisomerase inhibitors, antitumor antibiotics, hormones and monoclonal antibodies. In our study we are trying to find out the effect of 3 different types of chemo drugs: cytarabine (CYT), daunorubicine (DAU) and vincristine (VIN), which are widely used for treating the acute myeloid leukemia. It is very difficult to understand the resistance of tumor cells to chemotherapy due to which the outcome of therapy turns out to be very poor sometimes.

1.9.1. Cytarabine (Cytarabine Arabinoside) (CYT)

CYT is a synthetic pyrimidine nucleoside. It belongs to the antimetabolite category of chemotherapeutic drugs. After entering the cell, antimetabolites render the cell unable to divide. Activated CYT triphosphate will be incorporate into DNA and inhibits the DNA polymerase by competing with deoxy-cytosine triphosphate resulting in inhibition of DNA synthesis (Pui C-H et, al. 2007). CYT incorporation inhibits synthesis of DNA and ribonucleic acids (RNA) and leads to cytotoxic effect (Pui C-H et, al. 2007). Mechanism of action of CYT is cell cycle specific and it blocks S-phase.

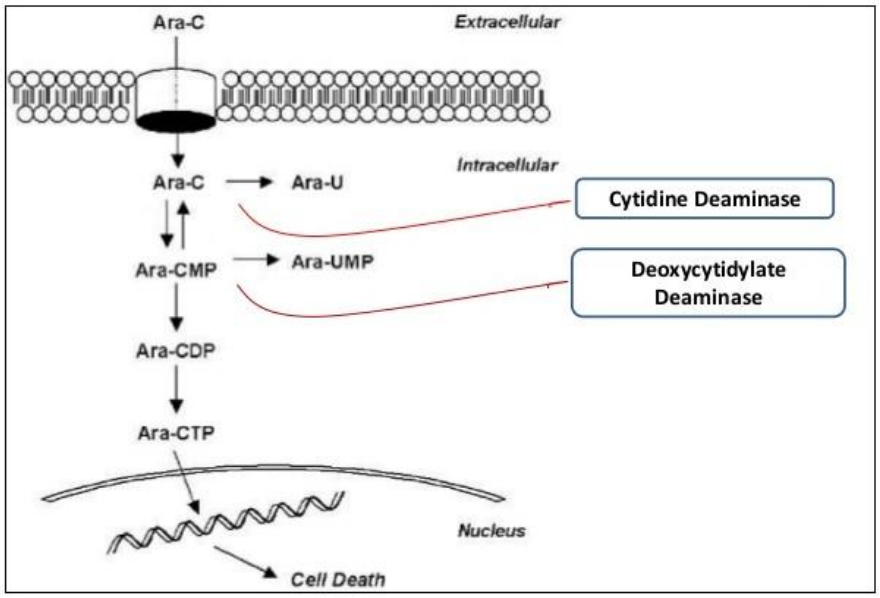


Fig.1.8. CYT mechanism of Action

(Pui C-H et, al. 2007)

Patients, who undergo chemotherapy with CYT show severe side effects, such as pulmonary toxicity in patients who undergo chemotherapy for leukemia (F Forghieri et, al. 2011). Other effects includes cerebellar toxicity at a high dosage (Brown C, 2010), polyneuropathy (Openshaw H et, al. 1993), Corneal toxicity (Hopen G et, al. 1991; J Lochhead et, al. 2003), ocular toxicity (Kumar L et, al. 1987) etc. Even at lower dosage CYT shows corneal and conjunctival toxicity in patients with leukemia (Barletta et, al. 1992). Lung injury (Kopterides P et, al. 2005) acryl erythemia (Ozmen S et, al. 2013), Myelosupression (Drenberg CD et, al. 2016) and intestinal toxicity (Camera A et, al.

2003) has also been observed as side-effects in patients. Fatal cardiac toxicity was seen in patients with leukemia (Trigg ME et, al. 1987). Hepatic damage and renal dysfunction was seen in myelodysplasia syndrome patients who were taking low doses of CYT (Tanaka M et, al.1999).

1.9.2. Daunorubicine (DAU)

According to anti-cancer drugs classification DAU is an antibiotic belongs to the anthracycline family. It is isolated from the pigment-producing *Streptomyces peuctius* early in the 1960 (Camerino B et, al. 1960). It has antimitotic effect, and capable of inhibiting the topoisomerase II enzyme activity. DAU bind to sugar phospahate moiety of DNA and block the unwinding of the DNA which results in the inhibition of DNA synthesis and DNA dependent RNA synthesis (G J Quigley et, al. 1980). It also inhibits polymerase activity, regulates gene expression and generates free radicals, hydrogen peroxides, inducing toxicity for the cells. Action of DAU is cell cycle nonspecific, although it shows maximal effect at S phase of the cell cycle (Pang B et, al. 2015).

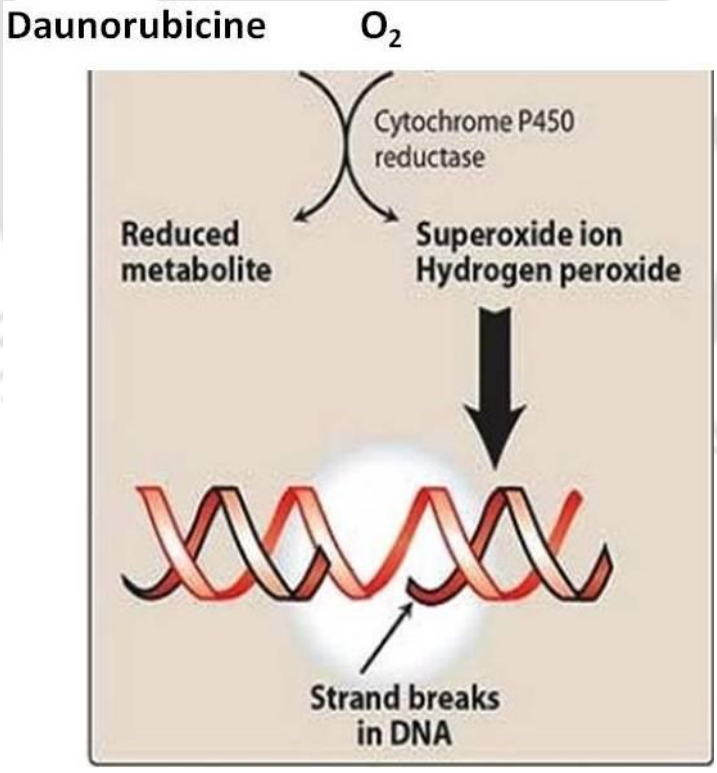


Fig.1.7. DNR Mechanism of action

(Pang B et, al. 2015)

DAU is known to induce side effects, such as cardiac problems either early or late in patients. This is due to generation of free radicals and cardiac tissue is more sensitive to highly reactive oxygen species (Seiter K. 2005). Hyper pigmentation (Kelly TM et, al. 1984) and nail pigmentation has been observed with daunorubicin therapy (deMarinis M et, al. 1978). DAU induces cardiomyopathy (Singal PK et, al. 1998). Renal failure (Burke JF Jr et, al. 1977) and nephrotic syndrome was seen in patients treated with DAU (Thomson M et, al. 1989).

1.9.3. Vincristine (VIN)

VIN is an antimitotic drug, classified into alkaloids. It is derived from natural sources like plant leaves. Vinca alkaloid derived from madagascar periwinkle *Catharanthus roseus* (Ravina et, al. 2011). Action of VIN is cell cycle specific, it block the cells in S and M phases. It is used to treat several tumors. VI N does not alter the DNA structure or function but it inhibits the microtubule function during mitosis, destabilize the microtubule formation by binding to tubulin proteins and inhibit polymerization. Spindle fibers formed during DNA replication are made of microtubules (which are formed by smaller units of tubulin proteins). Without formation of spindle fibers cells can't divide and lead to cell death. VIN induces the tumor protein p53 and p21 and changes the protein kinase activities. The activated protein kinase inactivates the Bcl 2 function by phosphorylation. Inactivation of Bcl2 function leads to increased levels of p53 and p21, which leads to apoptosis (Long G. Wang et, al. 1999).

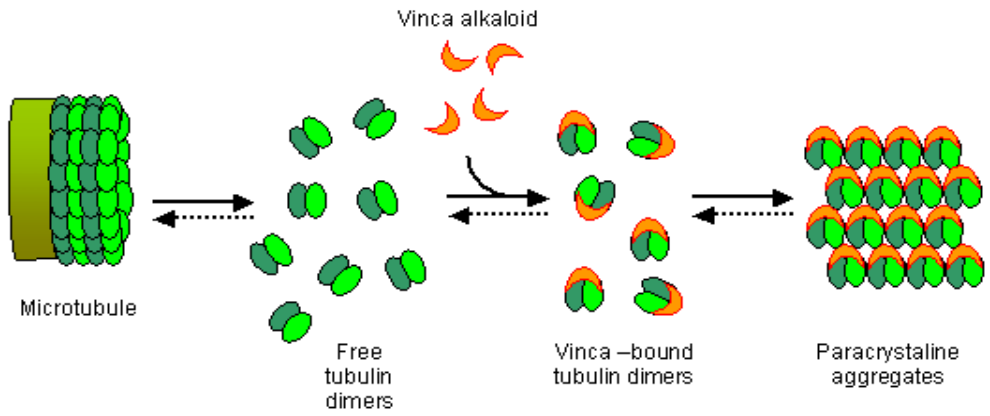


Fig.1.8. VIN mechanism of action

(Long G. Wang et, al. 1999)

Patients undergoing VIN therapy shows serious side effects. It affects nervous system and damages neuronal cells. Neuronal toxicity was seen in leukemic patients (LeghSS et, al. 1986). Rats treated with VIN shows hepatic toxicity (NagiS et, al. 1984). Patients have been observed to suffer from visual hallucinations during vincristine therapy (Ghosh K et, al. 1994). In addition to this, VIN induces erythero toxicity (Neville AJ et, al. 1982), myocardial infarction (Mandel EM et, al. 1975) and gastrointestinal diseases (Rosenberg RF et, al. 1990). Furthermore, VIN associated diseases include hepatic (Nagi S El Saghir et, al. 2006), cortical blindness (Byrd RL et, al. 1981), optic atrophy (Shurin SB et, al. 1982), optic neuropathy (Sanderson PA et, al. 1976).

1.9.4. Antimitotic drugs (Colchicine and Nocodazole)

We also studied the effect of antimitotic drugs, colchicine and nocodazole on MSCs. Both drugs are known to depolymerize the microtubules in fibroblasts (Stargell LA et, al. 1992), P388D1macrophages, human polymorpho nuclear leucocytes (Michael Kuhn. 1998; H.U.Keller et, al. 1989), oviduct (EB-Ulrich et, al. 1989) and inhibit microtubule synthesis.

1.10. MSCs in disease

Because of their multipotent and proliferation MSCs used to treat diseases. MSCs have the capacity to specialized into bone forming cells and are used to treat orthopedic background. MSCs have shown therapeutic effects after transplantation into patients suffering from ostogenesis imperfecta, osteoporosis and hypophasphatasia. Patients with bone disorders who underwent BM transplantation show normal development in bone formation (Horwitz EM et, al. 2001). In other studies MSCs were transplanted *in utero* and shows normal bone development in fetus suffering from ostogenesis imperfecta (Le Blanc K et, al. 2005). To examine the potential of cartilage differentiation, MSCs were used to treat cartilage repair in which MSCs were loaded with COL gels and transplanted the knee joins of the patients with cartilage defects. Reports suggested that transplantation of MSCs showed normal cartilage function but the exact mechanism is not yet known (Wakitani S et, al. 2004). Chemotherapy and radiation therapy causes BM damages and requires BM transplantation. To avoid or reduce the toxicity during or after HSCs transplantation because of graft versus host disease, MSCs were co injected with HSCs (Baron F et, al. 2010). Heart diseases and heart attack leads to increase the

mortality rates. MSCs are a desirable source to treat the cardiac diseases. In clinical studies MSCs have been shown to repair the cardiac tissue after transplantation (Zhang S et, al. 2006). MSCs have been used to treat liver cirrhosis in clinical trials. Report on MSCs transplantation showed that they can repair the liver function (Kharaziha P et, al. 2009). Cancer microenvironment secretes various molecules for attracting MSCs. This is the advantage; MSCs can be used as a vehicle for treating the cancer. Also by using genetically modified MSCs for secretion of various antitumor proteins could be used to treat cancer. Studies have reported that allo or autologous MSCs treat auto immune disorder like sever immune combined immune deficiency. MSCs are also used to treat the multiple sclerosis disease (Gao P et, al. 2010).

1.11. MSCs in therapy

Various therapeutic properties exist for MSCs. Firstly MSCs are involved in cell repopulation through proliferation and specialization. Diseases cause physical or chemical damage to healthy cells or tissues, which can be treated by introducing patient's own stem cells into defected tissue (Alsberg E et, al. 2006). Studies demonstrated that MSCs reconstitute the stem cell population in many tissues like BM, adipose tissue or cartilage tissues (Prockop DJ. 1998) Also investigation continues till today (Mareddy S et, al. 2007). Also many reports stated that MSCs investigation for direct repair of various types of tissues like kidney, skin and heart.

Also studies have demonstrated the role of MSCs in delivering a therapeutic transgene for treating the disease. Stem cell gene therapy treated disease like parkinson disease (Reiser J et, al. 2005) and severe combined immunodisease (SCID) (Gaspar BH et, al. 2005). Mutated or dysfunctional gene or allele which is responsible for causing the disease can be replaced with functional gene into patient stem cells and transplantation into defected cell types to expand and produce the products for restoration of the function (Reiser J et, al. 2005). MSCs transplanted in defective tissues showed normal development in cardiac (Kraitchman DL et, al. 2005), bone (Lee K wt, al. 2001), neuronal tissues (Torrente Y et, al. 2008). Also it has been shown that MSCs transplanted in non-obese diabetic (NOD) /SCID can maintain their normal expansion and differentiation potential (Lee K wt, al. 2001).

Migration of transplanted MSCs towards site of inflammation is an important therapeutic target for treating the disease and ability to make transduced MSCs is a desirable property for therapy. Migration mechanism of MSCs requires further research; it has been shown that CXCL 12 released during inflammation induces genes involved in MSCs migration and cellular movement (Stich S et, al. 2009). Also several studies demonstrated that secreted factors including hepatocyte growth factors or SDF-1 have a role in attraction of MSCs towards damaged or inflammatory tissues or regions (Rosova I et, al. 2008). Because of their ability to migrate, MSCs have been used as a therapeutic agent for treating tumor like glioblastoma multiformae (Sasportas LS et, al. 2009).

MSCs also involved in tissue repair mechanisms to maintenance or support of other cells in the microenvironment. MSCs are a stromal cell component and known to support the hematopoiesis in BM. Also it has been shown that allogenic transplantation of MSCs increases the engraftment of HSCs (Almeida-Porada G et, al. 1999). MSCs directly interact with cells and give physical support and also produce various types of cytokines, chemokines and growth factors for maintenance of expansion and differentiation (Ball LM et, al. 2008). Also several reports suggest-that MSCs from other tissues also involved in the repair and restoration of the function. (Le Blanc K et, al. 2005). MSCs have been shown to home to hypoxic and ischemic regions like myocardial infarction and regenerate the tissues. Functional tissue losses and leads to death of an individual during myocardial infarction. Transplantation of MSCs for cardiac repair has been used because of their ability to regenerate cardiac tissue. Also reports have shown that transplanted MSCs migrate towards necrotic tissues and retain their proliferation and differentiation capability (Li W et, al. 2007).

The most important feature of MSCs in therapy is their immunomodulatory effect. It has been shown that allogenic MSCs do not show any immune response. MSCs have been shown to modulate the proliferation and differentiation of B-lymphocytes (Nauta AJ et, al. 2007). MSCs have important functions like immunosuppressive and anti-inflammatory effect for therapy. Lack or lower expression of MHC class II molecules in MSCs makes them to suppress the identification between donor and recipient system and suppress immune reactions. It has also been reported that direct and indirect interactions between cells and secretary factors including TGF-β suppress the immune reactions (Marigo I et, al. 2011). Production of interleukins and activation of T regulatory cells

makes them to suppress the immune reactions. In addition, production of cytokines like IFN-ɣ also makes to immunosuppressive activity (Di Ianni M et, al. 2008).

In earlier studies MSCs were directly transplanted into injured regions but in recent tissue engineering applications MSCs are cultured on synthetic or bioengineered scaffold are utilized for therapy (Tuan RS et, al. 2003). Scaffold cultured MSCs secrete growth factors, chemokines and cytokines which involves in tissue repair mechanisms.

Chapter2

Aims and Objectives

2. Aims and Objectives

From the literature and studies reported it is clear that MSCs have a great potential to be used for therapy of several degenerative diseases. To obtain the conceivable therapeutic effect, a large amount of cells are required to be injected since these cells undergo frequent apoptosis in the *in vivo* system. So, understanding the conditions for *in vitro* expansion is important for proper utilization of these cells. Moreover, the usability of MSCs for regenerative therapy from patients who underwent chemotherapy also need to be understood. Thus the following objectives were formulated for the study:

1. Role of cell extrinsic factors such as extracellular matrix components on MSCs characteristics.

2. Effect of chemotherapeutic drugs on the cellular characteristics of MSCs.

Chapter3

Materials and Methods

Materials

3.1.Mammalian cell culture Media

3.1.1. Growth Media

(i). DMEM-LG (Dulbeccos Modified Eagles Medium-Low Glucose)

DMEM-LG, supplemented with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) and 100x Pencillin/Strptomycin sterilized with 0.22 µM filter paper and stored at 4^0C.

(ii). DMEM-HG (Dulbeccos Modified Eagles Medium-High Glucose)

DMEM-HG, supplemented with 3.7 g/L sodium bicarbonate, 10% FBS and 100x Pencillin/Strptomycin sterilized with 0.22 µM filter paper and stored at 4^0C.

3.1.2. Differentiation Media

(i). Adipogenic differentiation media

DMEM-HG supplemented with 10% FBS, 1 µM Dexamethasone, 0.2 mM IBMX, 0.01 mM Insulin and 100x Pencillin /Streptomycin filtered with 0.22 µM filter and stored at 4^0C.

(ii). Osteo differentiation media

DMEM-HG supplemented with 10% FBS, 10 mM β-Glycerol-3-Phosphate, 0.1 µM Dexamethasone, 0.05 mM Ascorbic acid-2-Phospahte and 100x Penicillin/Streptomycin sterilized by 0.22 µM filter and stored at 4^0C.

3.2. Solutions and Buffers

(i). Phosphate Buffered Saline (PBS)

The mixture of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 was dissolved in sterile distilled water and adjusted pH to 7.2-7.4 by adding 1N HCl. Sterilised by autoclave at 121^0C and stored at room temperature.

(ii). Red cell lysis buffer

0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA were dissolved in distilled water and adjusted pH to 7.4 by using 1N HCl or 1N NaOH. Filtered with sterile autoclave 0.22 µM filter and stored at 4^0C.

(iii). 0.1% Triton-x-100

0.1% Triton-x-100 solution prepared with PBS (v/v)

(iv). 10% Formalin solution

37% Paraformaldehyde stock solution was diluted in distilled water to make 10% (v/v) solution.

(v). 4% Paraformaldehyde solution

Paraformaldehyde (4%) solution was prepared by dissolving paraformaldehyde power in PBS (w/v) and warm at 55^0C by setting up water bath. When pH of the solution was adjusted to the range of 7.2 to 7.4, the solution became clear and dissolves completely, make aliquot and stored at -20^0C until use.

(vi). Trypsin solution

2.5% Trypsin solution was diluted in PBS and stored at -20^0C until use.

3.3. Stains and Dyes

(i). Phalloidin-tetramethyl rhodamine isothiocyanate (TRITC)

1mg/ml solution was made in dimethyl sulphoxide (DMSO) (w/v) and aliquots stored at -20^0C until use. Working solution (1:3000) was made from stock solution by diluting with 2% FBS and protected from light.

(ii). Propidium Iodide (PI) solution

1 mg/ml stock solution of PI was dissolved in PBS and stored at 4^0C and protected from light.

(iii). 4',6-diamidino-2-phenylindole (DAPI) solution

DAPI powder was reconstituted in double distilled water to make 1mg/ml stock solution and stored at -20^{0}C until use. For working solution (1:2000), DAPI was dissolved in PBS.

(iv). Tetramethylrhodamine, ethyl ester (TMRE)

TMRE was reconstituted in Methanol and stored in -20^{0}C until use.

(v). Oil Red O (ORO)

1% stock solution of ORO prepared with isopropanol (w/v), ORO was dissolved in isopropanol by warming and stored at room temperature. Working solution of ORO prepared by mixing 3 parts of 1% ORO stock and 2 parts of distilled water. Prepared mixture was filtered with Whatman filter paper and used immediately.

(vi). Alizarin Red S (ARS)

0.19g ARS was dissolved in10ml of distilled water and adjusted pH to 4.2 and stored at room temperature.

(vii). Cetylpyridinium chloride (CPC)

10% CPC was dissolved in 10 mM NaH_2PO_4 (w/v). The pH was adjusted to 7.4 and stored at room temperature.

(viii). Antibodies

CD13-PE, CD29-PE, CD44-FITC, CD49a-PE, CD49b-FITC, CD49d-PE, CD49e-PE, CD73-PE, CD90-FITC/PE, and CD95-PE antibodies were purchased from BD Biosciences.

3.4. Cells

(i). Bone marrow mesenchymal stem cells

BM samples were aspirated from iliac crest of patients from Haematology department of Guwahati Medical College Hospital (GMCH) after proper consent from the patients and as per GMCH ethical guidelines. RBC lysis buffer were added to BM samples with the ration of 1:5 (1 volume bone marrow sample+5 volume lysis buffer). Incubated the samples on ice for 7 minutes and added FBS to stop the reaction. Centrifugation was done for the undisturbed layers for 5 minutes with the speed of 270g at 4ºC. Collected the supernatant without disturbing the cell pellet, resuspnded the mononuclear cells in DMEM-LG. The mono nuclear cells were plated in a media containing 10% FBS with seeding density of $1x10^5$ cells/cm^2. The flasks were maintained at 37^oC and 5% of CO_2 in the incubator. The non-adherent cells were removed from plate after 48hr. Media change was done for every 2-3 days in a week. The colonies containing spindle shaped adherent cells appeared as MSCs within 2-3 weeks in culture. The MSCs used in our experiments were from 2-5 passages. For late passage experiments, we used MSCs derived from 10-12 passages.

(iii). Human Epithelial Kidney 293FT (293FT) Cells

293FT cells were obtained from Invitrogen and maintained with the DMEM-HG medium containing L-Glutamine (100x), MEM Non-Essential Amino Acids (100x), Sodium Pyruvate (100x) and Penicillin-Streptomycin (100x). The flasks were maintained at 37^oC and 5% of CO_2 incubator.

(iv). Leukemic cell lines

Leukemic cell lines, human acute promyelocytic HL60 (M3) and Tamm-Horsfall Protein 1(THP1) were purchased from National Center of Cell Sciences (NCCS, PUNE, INDIA). The leukemic cells were maintained in RPMI-1640 culture media supplemented with 10% FBS, L-Glutamine (100x) and Penicillin/Streptomycin (100x). Media change was done for every 3 to 4 days for a week by centrifugation at 300g for 5 minutes. The flasks were maintained at 37^oC and 5% of CO_2 incubator.

3.5. Lentiviral vectors

The lentiviral vectors used for the study were based on pHRcPPT SIEW Sin vector with IRES regulating eGFP reporter gene. The vector contains spleen focus forming virus (SFFV)-long terminal repeats (LTR)- promoter and WPRE (Woodchuck Hepatitis Virus) element for post-transcriptional processing (Jaganathan BG et, al.2007). RHOA constitutively active V14 (RHOAV14) and dominant negative N19 (RHOAN19) and control SIEW plasmids were used without insert. Along with expression vectors we used packaging plasmid cauliflower mosaic virus 8.74 (pCMVR8.74), which contain *gag* and *pol* gene and envelope plasmid pMD.G.2-VSV-G for *env* gene.

3.6. Plasmid extraction reagents

P1 Solution: 50 mM Tris (pH8.0), 10 mM EDTA, 100 µg/ml RNAase

P2 Solution: 200 mM NaOH, 1% SDS (Sodium Dodecyl Sulphate)

P3 Solution: 3M Potassium Acetate (pH5.5)

Methods

3.7. Cell biology techniques

3.7.1. ECM coating

For preparation of matrices, the tissue culture plates were coated with collagen (COL), fibronectin (FBN) or poly-l-lysine (PLL). The required concentration of COL (2 ug/cm^2), PLL (100 ng/cm^2) or FBN (100 ng/cm^2) was used. The required concentration of COLwas diluted in PBS and plates were coated at 37ºC for 3hr and tissue culture plates were exposed to UV for 1hr after unbound COL taken from plate. For FBN and PLL coating, required concentration of FBN or PLL was plates were coated at 37ºC for 1hr. The unbound FBN or PLLwas washed with PBS and all the coated plates were used either immediately or stored at 4ºC for 24-48hr before use.

3.7.2. Cell adhesion assay

The MSCs were seeded on control surface or COL, FBN, PLL with the seeding density of 5000 cells/cm^2 and allowed to adhere either for 2hr or 12hr. The non-adherent cells were gently removed by washing with PBS and adherent cells in each condition was counted**.**

3.7.3. Cell cycle

Cell cycle analysis was done by staining the DNA of the cells with PI. The cells were seeded on different substrates with the seeding density of 3000 cells/cm^2. The cells were harvested by trypsinization, washed with PBS and fixed with ice-cold ethanol (70%) for 30 minutes at 4ºC. The cells were washed with an ice cold PBS and treated with RNAase A, stained with PI and analysed by flowcytometry.

3.7.4. MTT assay

MTT assay was performed as per the manufacturer's instructions (Himedia Laboratories). The MSCs were seeded in 96 well plates (which were coated with matrix proteins), with the seeding density of 500 cells/well. MTT reagent was added and incubated for 4hr at 37ºC. Formation of formazan precipitate was solubilized with solubilization reagent and absorbance was measured at 570nm. Each sample was analysed in triplicates and average value was taken for plotting the graph**.**

3.7.5. Reactive oxygen species (ROS) analysis

Superoxide production in the cells cultured on different substrates was determined using mitosox red mitochondrial superoxide indicator kit (Life Technologies) according to manufacturer'sinstructions. Cells were cultures on different matrices with the seeding density of 5000 cells/cm^2 and serum starved for 48hr and mitosox superoxide indicator was added to cells for 30 minutes. The cells were trypsinized and washed with warm PBS. MitoSOX red fluorescence was analysed by flow cytometry.

3.7.6. Adipogenic and Osteogenic differentiation

MSCs were subjected to differentiate into adipocytes and osteocytes. The MSCs were seeded with the 5000 cells/cm^2 seeding density on coated with different substrates in 96 well plates and induced by addition of osteogenic media for 21-35 days in DMEM containing 10% FBS. Osteogenic differentiation, at the end of 21-35 days cells were

washed with PBS and fixed with 10% formalin for 1hr at room temperature. The fixed cells were washed with PBS and stained for calcium with Alizarin Red S (ARS) for 15 minutes at room temperature. The stained cells were washed with distilled water and PBS and images were taken by using microscope. Quantification of ARS was done with cetylpyridinium chloride (CPC), which elutes the ARS stain, diluted and measured absorbance at 562nm.

For adipogenic differentiation, MSCs were seeded with 20000 cells/cm^2 on ECM coated 96-well plate. Differentiation was carried out in adipogenic media for 21-30 days. At the end of differentiation, cells were washed with PBS and fixed with 10% formalin for 1hr at room temperature. The fixed cells were rinsed with 60% isopropanol and stained with Oil Red O (ORO) for 20 minutes at room temperature. The stained cells were washed with distilled water and counted under the microscope and images were taken using microscope. Extraction of ORO by isopropanol, from ORO positive cells and quantification was done by absorbance measurement at 500 nm.

3.7.7. Actin staining

MSCs were seeded 3000 cells/cm^2 in 96 well plates, which was coated with different matrices. Cells were washed twice with PBS and fixation was done with 4% paraformaldehyde for 20 minutes at room temperature twice. Paraformaldehyde solution washed with PBS for 5 times and 5 minutes each. After washing, cells were permealized with 0.1% triton-x-100 in PBS for 15 minutes at room temperature. Washed with PBS for 5 minutes and incubated with 5% FBS for 1 hr at room temperature and cover with aluminum foil. The cells were stained with phalloidin-TRITC (1:2000 / 1:3000) in 2% FBS in PBS and incubate the cells at 4^{o}C overnight in dark. Cells were washed with PBS for 5 times and 5minutes each. The cells were stained with phalloidin-TRITC to visualize filamentous-actin (F-actin) which was excited with 488 nm laser. F-actin was visualized by staining with TRITC conjugated phalloidin. Nucleus was stained with DAPI and the cells were documented using Zeiss Axio observer and CCD camera (Zeiss).

3.7.8. Total internal reflection microscopy imaging

MSCs were seeded 3000 cells/cm^2 on glass coverslips (1.5 mm thickness) coated with different substrates. Cells were washed twice with PBS and fixation was done with 4%

paraformaldehyde for 20 minutes at room temperature. Paraformaldehyde solution removes and washes the cell layers with PBS for 5 times and 5 minutes each. After washing, cells were incubated with 0.1% triton-x-100 in PBS for 15 minutes at room temperature. Wash with PBS for 5 minutes and incubate with 5% FBS for 1hr at room temperature and cover with aluminum foil. The cells were stained with phalloidin-TRITC (1:2000 / 1:3000) in 2% FBS in PBS and incubate the cells at 4ºC overnight in dark. Cells were washed with PBS for 5 times and 5minutes each. The cells were stained with phalloidin-TRITC to visualize F-actin which was excited with 488nm laser. Objective type-total internal reflection (OTIR) fluorescence microscopic analysis was performed as reported earlier to identify the cell-surface contact points. The fluorescence signal was collected through a long-working distance water immersion objective (63x) using a camera-based detection system (Zeiss).

3.7.9. Wound healing assay

Ten thousand cells/cm^2 were seeded in a 12-well plate coated with different substrates and the MSCs were allowed to attach for 24-36hr or until they reached confluence. A scratch was made in the cell monolayer and cell migration was observed and documented microscopically at regular intervals until the wound closed. The migration speed of the cells was calculated by measuring the distance covered by the cells at each time point by using TScratch software. In order to negate the effect of cell proliferation on MSCs migration, cells were serum starved for 12hr before wound healing assay was to be performed.

3.7.10. Phenotype

The cell surface protein expression was analysed by flow cytometry. The cells were trypsinised and washed with PBS and incubated with anti-human fluorescent conjugated antibodies against CD13, CD29, CD49a, CD49b, CD49d or CD49e, CD73, CD44, CD90 and CD95 for 30 minutes at 4ºC. The cells were washed with 2% FBS, resuspended in buffer containing PI and analysed with FACS caliber (BD biosciences).

3.7.11. RHOA activity analysis

The active level of RHOA was determined by RHOA GLISA assay (Cytoskeleton) following the manufacturer's instructions. Briefly, ice-cold lysis buffer was added to the cell layer and cell lysate was collected by scraping. The protein concentration of the cell

lysates were measured by Bradford assay. Equal amount of protein was used for analysis of active RHOA levels by GLISA.

3.7.12. Apoptosis

MSCs or leukemic cells were treated with chemotherapeutic agents for 48hr. Cells were collected and washed with ice cold PBS and incubated with FITC labeled AnnexinV and PI, analyzed by flowcytometry.

3.7.13. Mitochondrial staining

Cells were seeded on COL, FBN, PLL and uncoated surface with the seeding density of 3000 cells/cm^2. To visualize active mitochondria inside the cell, and MSCs were incubated with TMRE (100 nM) at 37°C for 30 minutes. The cells were washed with PBS, fresh media added and nucleus was stained with DAPI. Mitochondrial distribution and nucleus was visualized using Zeiss Axio Observer Z1 inverted microscope (Zeiss, Goettingen, Germany).

3.7.14. Transfection of packaging cells

The cells were transfected by the polyethylene imine (PEI) method. Transfection mix was prepared by adding 100 µl of incomplete DMEM-HG with adding 0.7 µg ENV plasmid (pMD2-VSV-G), 1.5µg Packaging plasmid (CMVΔR8.74), and 2 µg of plasmid containing the gene to be expressed together per 6-well plate. After mixing properly added 12 µl of PEI and mix without bubbles and incubate for 10 minutes at room temperature. After incubation added 600 µl of complete media and incubated for 2 -3hr in incubator. After incubation added complete media to make 2 ml of total volume. Replaced the transfection mix with normal DMEM-HG media within 24hr.

3.7.15. Transduction

Lentiviral particles were generated by transfecting the transfer plasmid into 293 T cells with the packaging plasmids and envelope pMD.G. Viral supernatants were collected 48 and 72hr after transfection and concentrated by centrifugation. Collected supernatant and added to the target cells. Polybrene (4 µg/ml) was added for concentrating the virus. Replaced the transduction media with normal growth medium within 24hr and checked for green fluorescent protein (GFP) expression within 48 to 72hr of post transduction.

3.7.16. Chemotherapeutic drugs treatment

Cells were treated with cytarabine (CYT) or daunorubicine (DAU) or vincristine (VIN) for 48hr. The required concentration of CYT (1 µM), DAU (0.1 µM) or VIN (0.1 µM). Colchicine (1 µM) and Nocodazole (0.1 µM) treated for 24hr and 1 week during differentiation.

3.8. Molecular Biology techniques

3.8.1. RNA isolation

All the material which is required for robinucleic acid (RNA) isolation and reverse transcription, which include centrifuge tubes, microtips were treated with di Ethyl Pyro Carbonate (DEPC) water and sterilized by autoclave. After adding TRIZOL, the cell pellet was mixed gently by vortexing and incubated for 5 minutes at room temperature. Chloroform was added, mixed gently by vortexing and incubated for 3 minutes at room temperature. Centrifugation was done with the speed of 12000g for 15 minutes at 4^{o}C. After centrifugation, separated aqueous phase from the other layers and added 100% isopropanol and glycogen and mixed by vortexing. Centrifugation was performed with the speed of 12000g for 10 minutes at 4^{o}C. Discarded supernatant; the cell pellet was washed with 70% ethanol and centrifuge with the speed of 7500g for 5 minutes. After centrifugation the pellet was air dried for 10 to 15 minutes and suspended in DEPC water. The 1% agarose gel was ran for RNA and gel was visualized by staining with Ethidium Bromide (EtBr). Isolated RNA was stored at -80^{o}C until use.

3.8.2. Reverse transcription (cDNA Synthesis)

Complementorydeoxy ribonucleic acid (cDNA) synthesis was done from reverse transcription of RNA by using cDNA reverse transcription kit (Invitrogen). For making 2x master mix, 10x reverse transcriptase (RT) Buffer, 24x deoxy nitrotriphospahates (dNTP) mix, Oligodeoxythymidines (dT) primers, RT, RNAase inhibitor and nuclease free water were added to make final volume. For 15 µl reaction, 10 µl of RNA was added to 5 µl of 2X master mix into PCR tubes. Reverse transcription was performed by using Veriti Thermal Cycler PCR machine (Applied Biosystems,USA). The conditions used for cDNA synthesis are as follows: 25^{o}C for 10 minutes (Holding), 37^{o}C for 120

minutes (Extension), 85^{o}C for 15 minutes (Holding) and 4^{o}C at α time (Holding). Synthesised, cDNA was stored at -80^{o}C until use.

3.8.3. Real-time PCR analysis

Gene expression analysis was performed by using SYBR Green master mix (Invitrogen). For 10 µl of reaction mixture contains, 2x SYBR green PCR master mix, cDNA, Primer mix and final volume made with nuclease free water. Real-time PCR was performed using Power SyBr Green reagents (Invitrogen) in an ABI 7500 (ABI) real-time PCR machine. The gene expression levels in each sample were normalized to their GAPDH expression levels.

3.8.4. Preparation of competent cells

An overnight 10ml culture of *E.coli* DH5α bacterium was prepared. The overnight culture was inoculated in 50 ml Luria Broth for 2hr at 37°C. The culture was centrifuged for 10 minutes at 5500 rpm at 4°C, the medium completely removed, the pellet re-suspended in 25 ml ice cold 0.1M $CaCl_2$ and incubated for 30 minutes on ice. The solution was then centrifuged for 10 minutes at 5500 rpm at 4°C, the supernatant thoroughly removed and the pellet is re-suspended in 5ml ice-cold 0.1M $CaCl_2$/20% glycerol in $CaCl_2$. The competent cells are subjected to shock freeze in liquid nitrogen are stored at -80°C.

3.8.5. Transformation of expression vectors

100 µl competent cells which were stored in -80°C freezer were thawed on ice. 1 µg of DNA was added to the competent cells and kept on ice for 30 minutes. Cells were subjected to heat shock in a water bath at 42°C for 45 seconds. Cells were placed on ice immediately to recover for 2 minutes. 700 µl of super optimal broth with catabolite repression (SOC) medium was added and the cells were incubated for 60 minutes at 37°C and under shaking conditions. Cells were placed on Luria Broth agar plate with appropriate antibiotic. The plates were kept overnight at 37°C.

3.8.6. Plasmid extraction by alkaline lysis method

5 ml overnight culture was centrifuged at 12000 rpm for 5 minutes and the supernatant was discarded. The pellet was suspended in 300 µl P1 and kept at room temperature for 5 minutes. 300 µl P2 was added, mixed by gently tipping the tube over and incubated at

room temperature for 3 minute. 400 µl P3 was added and kept on ice for 10 minutes. The tubes were centrifuged at 13000 rpm for 10 minutes. The entire supernatant was transferred to a new tube. 400 µl 3M Sodium Acetate and 400 µl Iso-propanol were added to the supernatant and mixed well gently. The mixture was incubating for 30 inues on ice. After incubation, centrifuged at 13000 rpm for 20 minutes. The supernatant was removed, centrifuged again at 13000 rpm for 20 minutes after adding 750 µl Ethyl alcohol. The supernatant were removed completely, pellets were dried for 5 minutes and resuspended in 100 µl sterile water.

3.8.7. Digestion of the RHOA mutant present in the expression vector

To confirm the insert present in the expression vector, we make a mixture which contain the 0.5 µg of expression vector and 1 µl of Bam H1 10x Buffer and made volume to 10 µl with sterile water and incubated for 37°C for 1hr. To confirm the result we ran the agarose gel (0.8%) which shows the release of insert, hence it confirms the insert.

3.9. Data analysis

The flowcytometric data was analyzed using FCS6 Express software. Migration was analyzed using t-Scratch software. Statistical analysis was performed with student t-test using Microsoft excels. Values of $p<0.05$ were considered statistically significant.

Chapter4

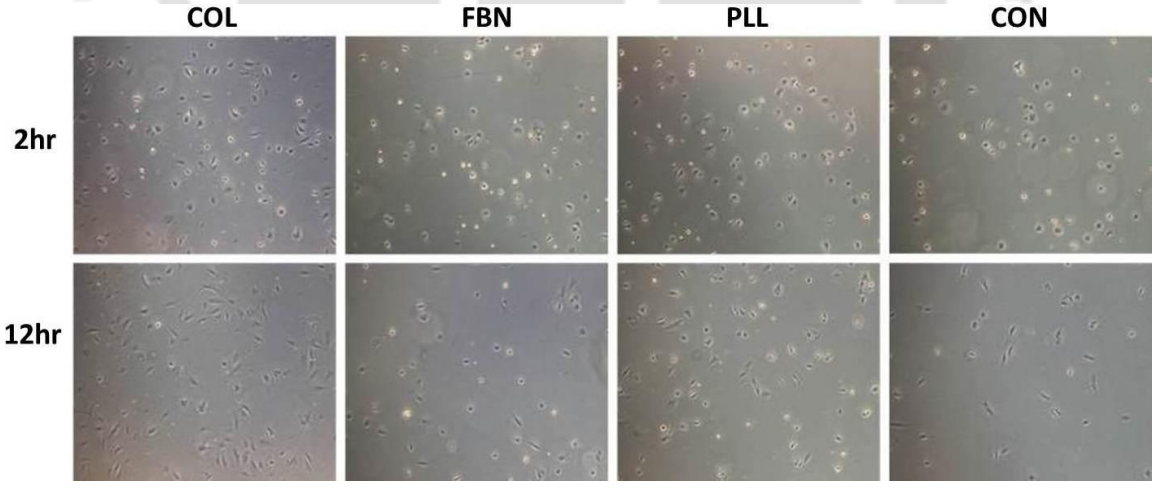
Results

4.1. Effect of ECM and RHOA on MSCs characteristics

Cell adhesion, survival and proliferation

Cells attach to their cell surface to maintain survival, proliferation and differentiation. Extracellular matrix (ECM) proteins have been found to be important in regulating the cell adhesion, proliferation and differentiation of mesenchymal stem cells (MSCs). In this study, MSCs were grown on surfaces coated with ECM proteins collagen (COL), fibronectin (FBN) and poly-l-lysine (PLL) and their changes in properties were studied to identify the suitable ECM that can be utilized for tissue engineering applications. Equal number of MSCs were seeded on ECM surface coated with COL, FBN, PLL and control surface (CON) which is tissue culture treated surface and the percentage of cells that adhered to the surface at 2hr and 12hr were calculated for each surface. COL promoted highest cell adhesion at 2hr and 12hr although the adhesion percentage did not vary between 2hr and 12hr. However, the percentage of cells adhered to FBN increased significantly over time where high cell adhesion was seen after 12hr of seeding compared to 2hr. PLL also promoted MSCs adhesion whereas lowest cell adhesion was observed in CON surface (Fig.4.1A,B). Thus, COL facilitated high cell adhesion of MSCs.

A



B

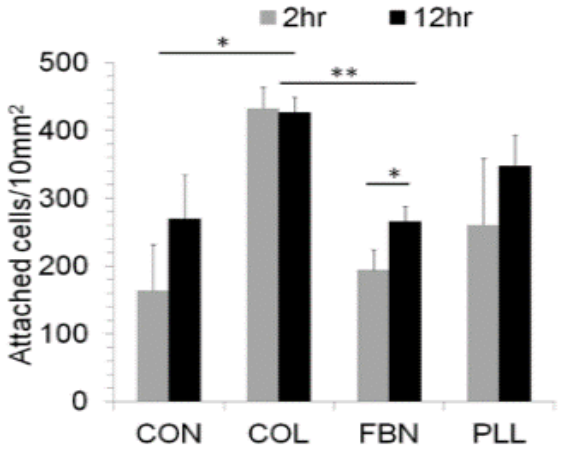


Fig.4.1. Effect of ECM on cell adhesion. Equal numbers (5000 cells/cm2) of MSCs were seeded on COL, FBN, PLL and CON surfaces and adherent cells were counted after 2 or 12hr. Microscopic images of MSCs cultured on COL, FBN, PLL and CON (A). Adherent cells were counted microscopically (B). *p<0.05, **p<0.005. n= 3 samples.

In addition to cell adhesion, cell survival under stress conditions is important. Cells *in vivo* are subjected to oxidative as well as nutritional stress and we determined the effect of different matrices on the cell survival under stress conditions. MSCs cultured on different matrices were subjected to serum starvation, to simulate the nutrient stress and cell death percentage was calculated. While cell proliferation was negligible during serum starvation, cells grown on different matrices did not show any significant changes in the cell survival (Fig.4.2A). Further, MSCs on COL, FBN, PLL and CON were treated with H_2O_2 under serum starvation conditions to cause oxidative stress and cell death percentage was analysed. There was a significant reduction in the cell death percentage in cells cultured on COL surface compared to FBN, PLL and CON surfaces. No difference in cell death was observed between FBN, PLL or CON surfaces during H_2O_2 treatment (Fig.4.2B).

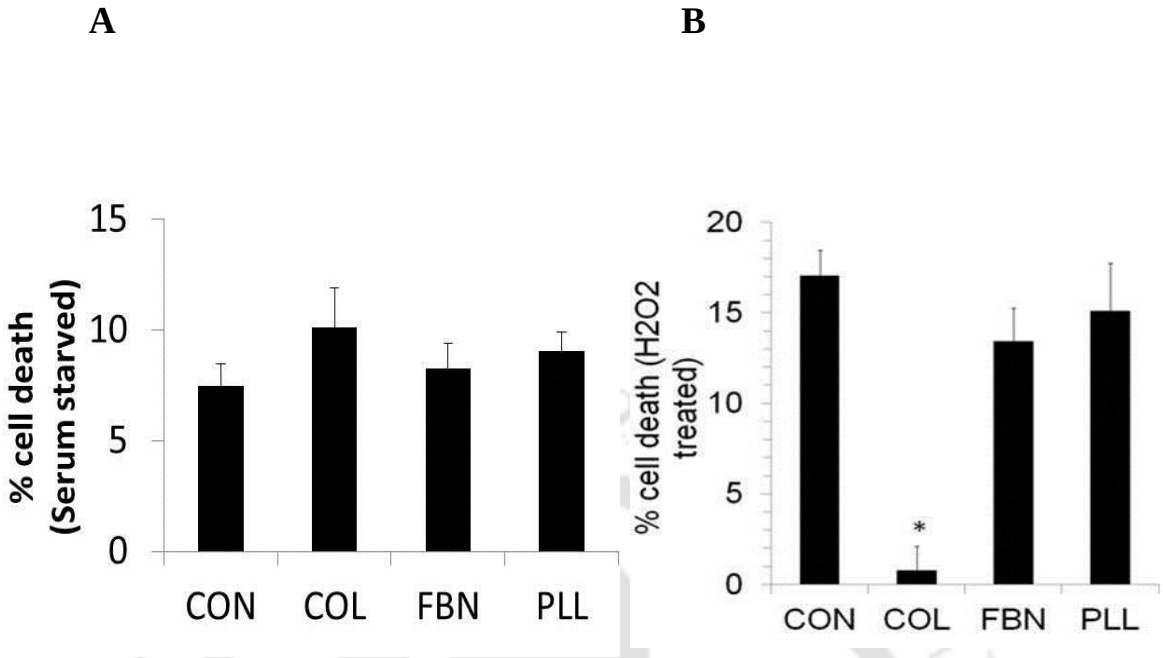


Fig.4.2. Effect of ECM on cell survival. Dead cell counting of MSCs cultured under serum starvation conditions (0.1% FBS) (A). Dead cell counting of MSCs treated with H_2O_2 on COL, FBN, PLL and CON (B). Cell death percentage of MSCs during H_2O_2 (400 μM) and low serum conditions (0.1% FBS). *$p<0.05$. n= 3 independent experiments.

Reactive oxygen species (ROS) levels negatively impact cell survival and differentiation and so, the amount of mitochondrial ROS produced was determined on MSCs cultured on different matrices. During serum starvation, cells cultured on all the different matrices showed ROS production, however, it was significantly higher in cells cultured on PLL (Fig.4.3A,B).

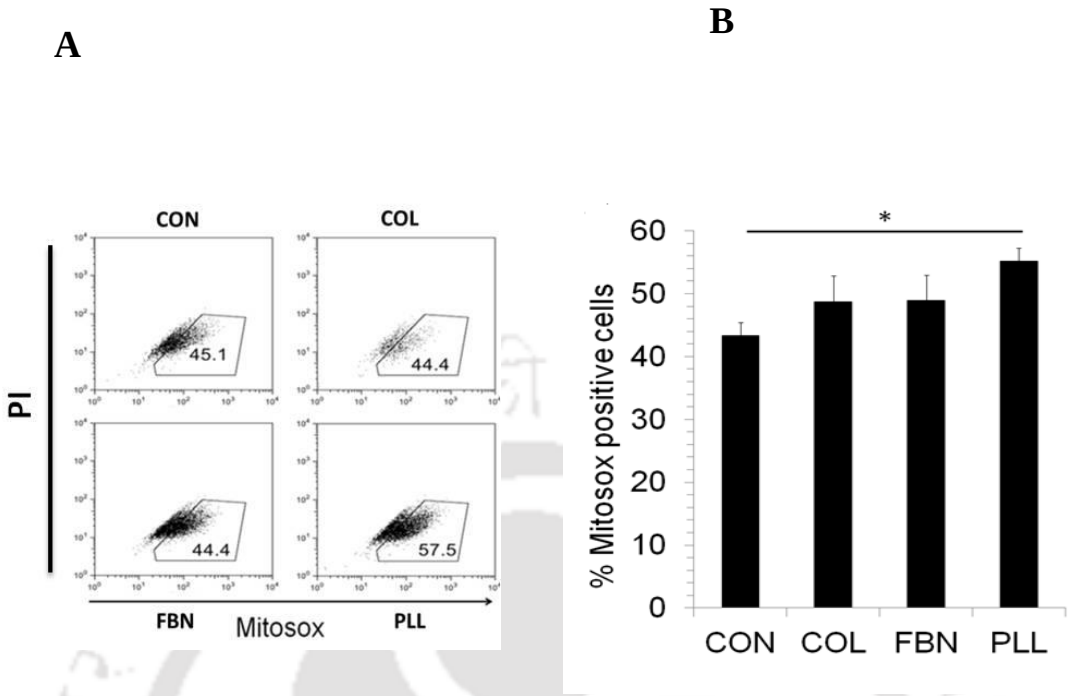


Fig: 4.3. Effect of ECM on ROS production. MSCs grown on COL, FBN, PLL and CON matrices were serum starved for 48hr. Representative mitosox flow cytometric analysis (A). Mitosox percentage on COL, FBN, PLL and CON (B). *p<0.05. n= 3 independent experiments.

When MSCs are subjected to stress conditions in the *in vivo* system, they might have increased levels of inflammatory cytokines which might affect their cell survival. For this, the level of IL6 mRNA in MSCs cultured on different matrices was determined. Cells cultured on CON had significantly high expression of IL6 transcript levels, whereas it was low in cells cultured on COL, FBN or PLL (Fig.4.4).

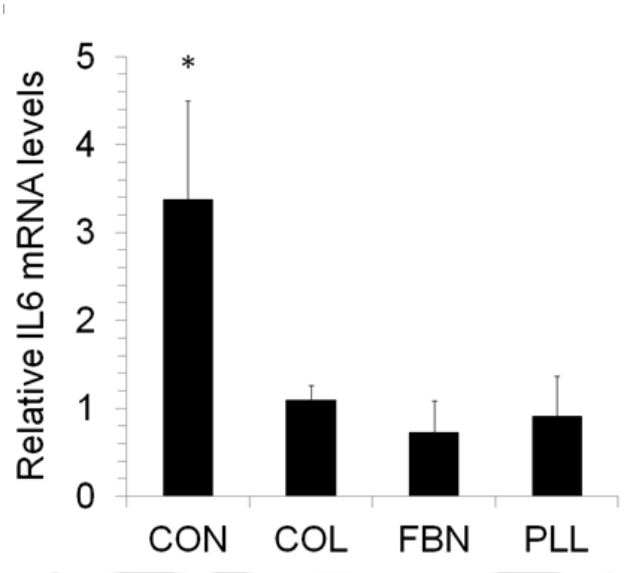
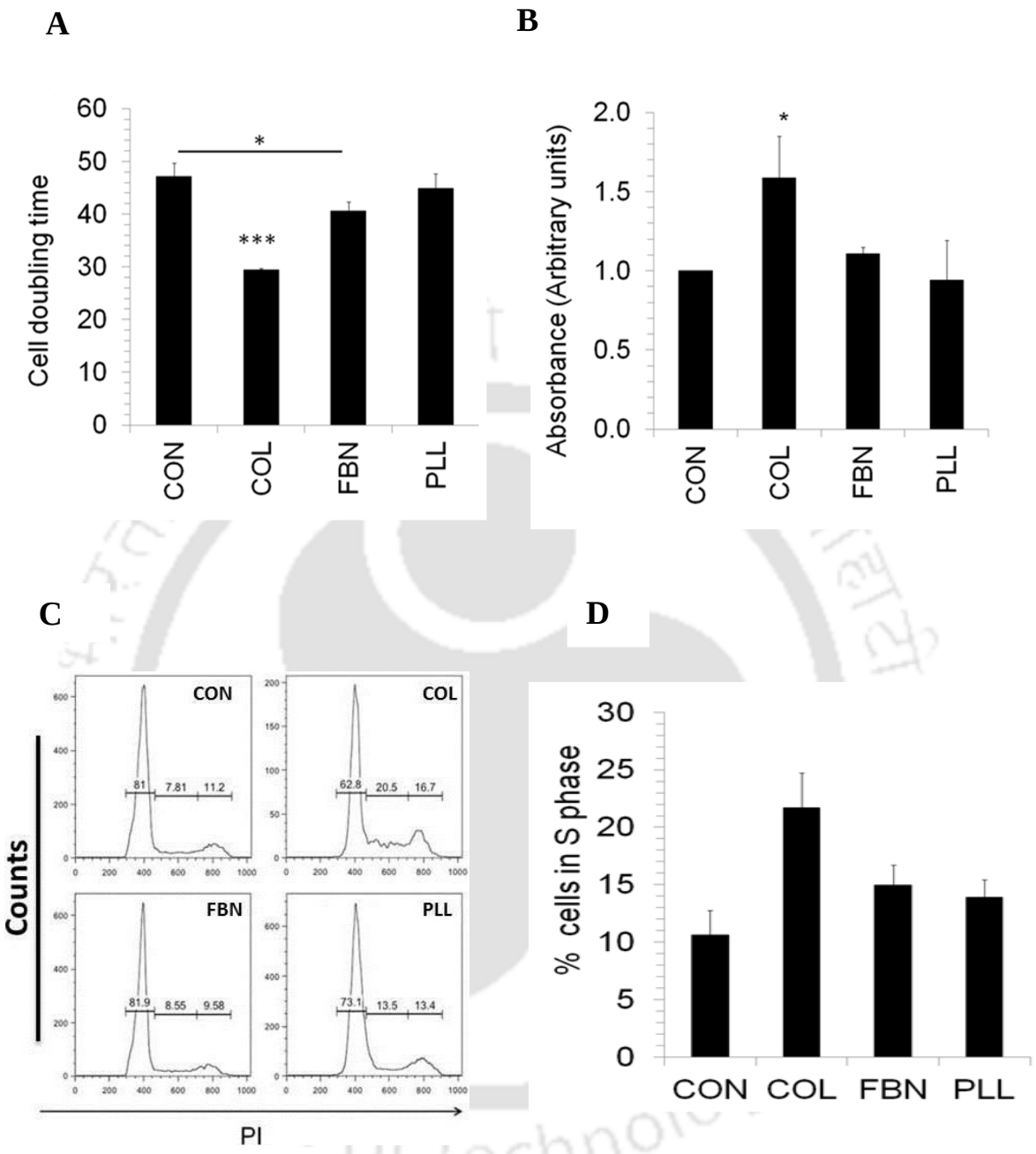


Fig.4.4. Effect of ECM on IL-6 gene expression. MSCs grown on COL, FBN, PLL and CON surfaces. Semi-quantitative real-time PCR data for IL6 expression levels. *p<0.05. n= 5 samples.

Cell proliferation is essential for *in vitro* expansion of cells for tissue engineering applications. In order to identify the suitable matrix for MSCs culture, MSCs was cultured on COL, FBN, PLL and CON surfaces and after 3 days, the cells were counted to check proliferation and representative microscopic images were taken. There was a significantly high proliferation of MSCs cultured on COL compared to other matrices (Fig.4.5B). Accordingly, MSCs on COL showed significantly reduced doubling time (Fig.4.5A) and cell cycle analysis showed higher percentage of cells in S phase (Fig.4.5C,D). Doubling time was also significantly less in cells grown on FBN compared to CON surface (Fig.4.5A). In all the different matrices, MSCs showed spindle shaped morphology (Fig.4.5E).



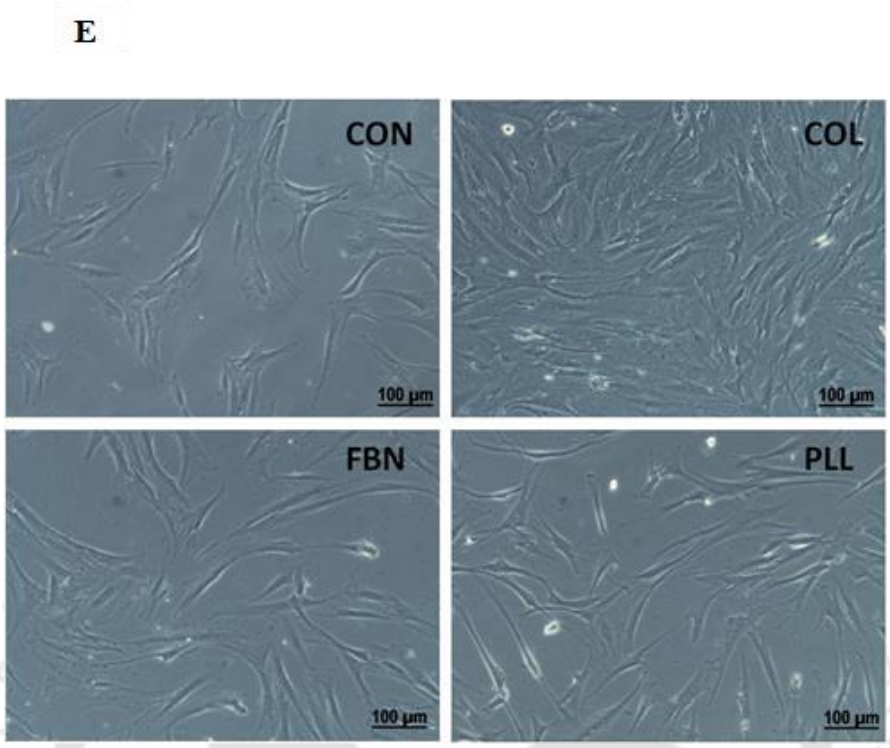


Fig.4.5. Effect of ECM on cell proliferation. MSCs were cultured on COL, FBN, PLL and CON surfaces for 48–72hr. Doubling time (A) and MTT assay of MSCs (B). Histograms of MSCs cell cycle (C) and percentage of MSCs in S phases of cell cycle (D). Microscopic images of MSCs cultured on ECM substrates (E). *p<0.05, ***p<0.0005. n= 3 independent experiments.

Actin cytoskeleton

Actin acts as mechano sensor and is responsible for signal transduction from extracellular environment, which in turn might regulate the cell shape, differentiation and cell migration (Hayakawa K et, al. 2013). To understand the cell shape and actin arrangement, MSCs cultured on COL, FBN, PLL and CON were stained with phalloidin to visualize the F-actin. MSCs cultured on COL, FBN, PLL or CON surface showed a comparable actin arrangement. The cells cultured on all the surfaces showed a regular, parallel F-actin arrangement, however, the cells were more polygonal in shape while proliferating on PLL and uncoated surfaces (Fig.4.6A,B).

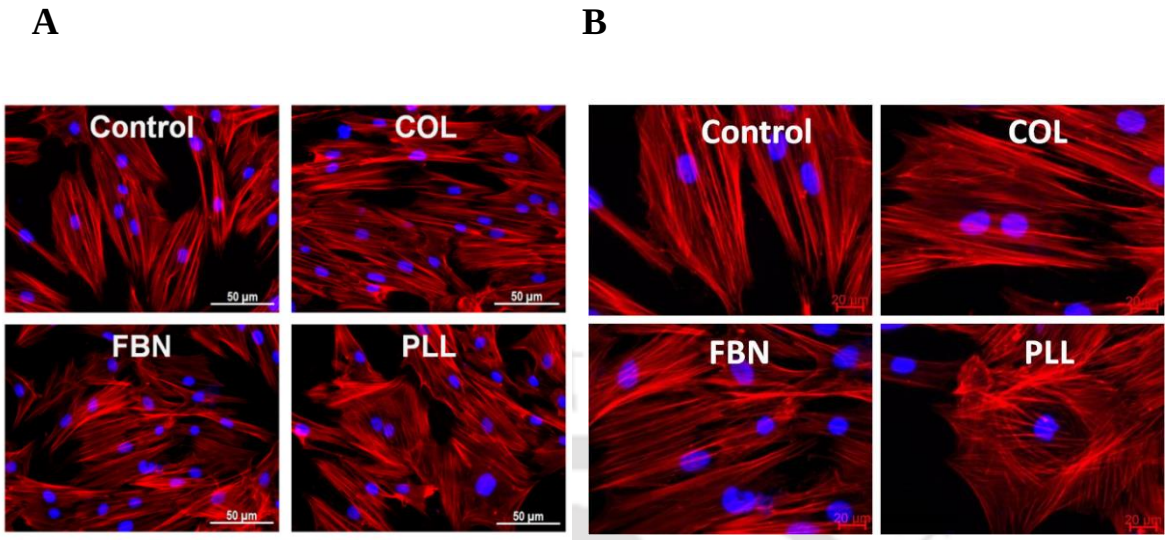


Fig.4.6. Effect of ECM on actin cytoskeleton modification. MSCs cultured on COL, FBN, PLL and Control surfaces. F-actin was stained phalloidin-TRITC (red) and nucleus was stained with DAPI (blue). Magnifications represent 20x (A) and 40x (B). n= 5 samples.

The cells attach to the ECM through integrins and actin mediates the connection between the integrins and other proteins inside the cell to regulate signal transduction. In order to understand that, the number of cell-surface contact points of MSCs cultured on different matrices was examined with TIRF microscopy. MSCs were stained with phalloidin-TRITC to visualize the F-actin and to understand the cell to surface adhesion (Fig.4.7B). Several cells to surface contact points were observed in MSCs cultured on COL, FBN and PLL matrices, however, very few contact points were seen on CON surface. The cell surface contact points were highest when MSCs were cultured on FBN matrix followed by COL and PLL (Fig.4.7A,B).

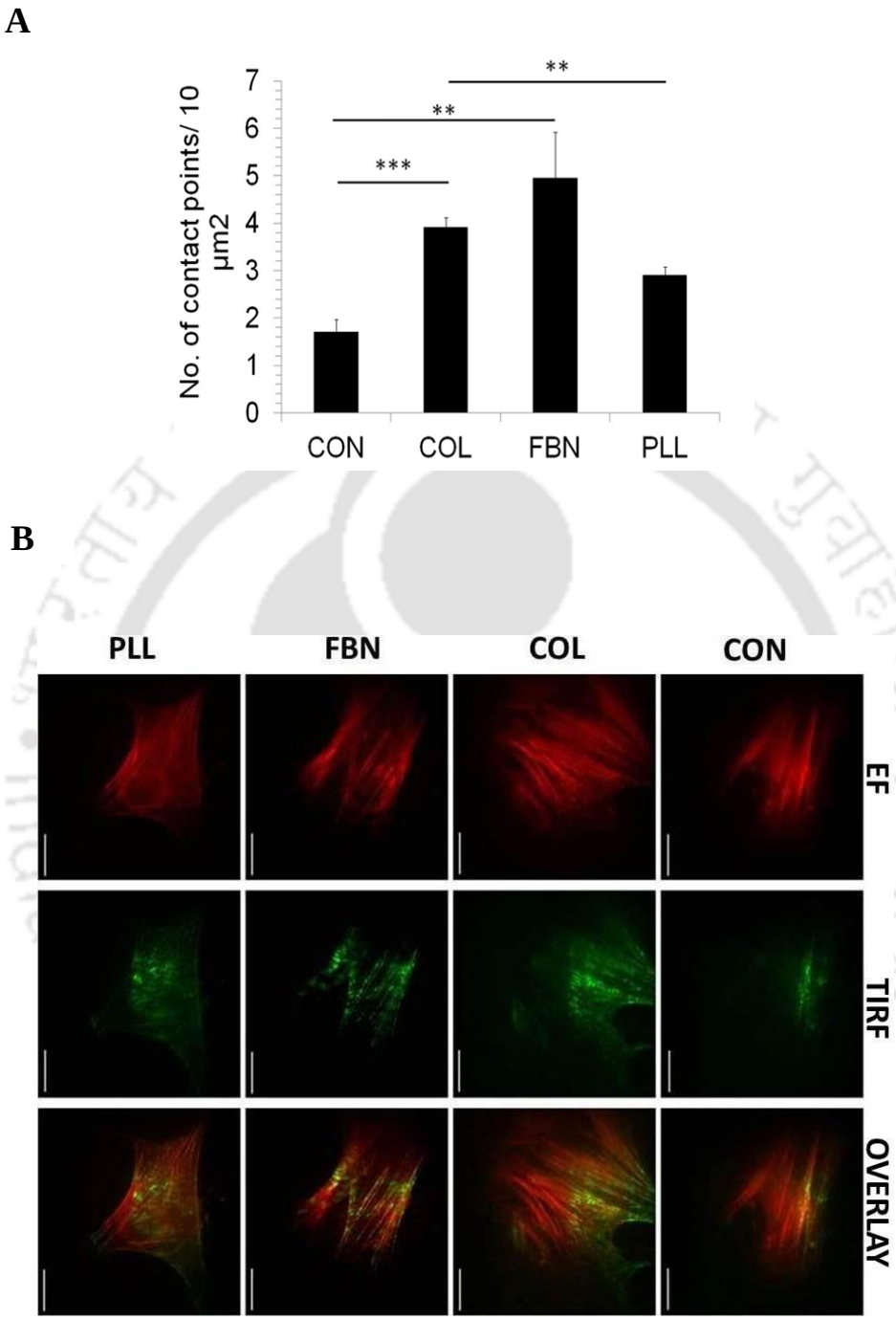


Fig.4.7. Effect of ECM on cell contact points. MSCs cultured on COL, FBN, PLL and CON surfaces and the cell-contact points of MSCs (A) with different substrates was examined with total internal reflection fluorescence microscopy (TIRF) in cells stained with phalloidin-TRITC cultured on glass coverslips (B). **p<0.005. ***p<0.005. n= 3 independent experiments.

Integrins play a role in cell migration and differentiation as they cross talk with the intracellular proteins mediated by actin. For this, expression levels of integrins CD29 (integrin beta 1), CD49D (integrin alpha 4) and CD49E (integrin alpha 5) were assessed on cells grown on different ECM. MSCs cultured on all the matrices expressed CD29 and CD49E at high levels and a low CD49D expression was observed. The expression levels of CD29, CD49D were unaffected in MSCs cultured on different matrices (Fig.4.8A) whereas a significantly increased CD49E expression was seen in cells cultured on COL compared to CON, FBN or PLL (Fig.4.8B) and the expression of CD49E did not vary much between CON, FBN or PLL.

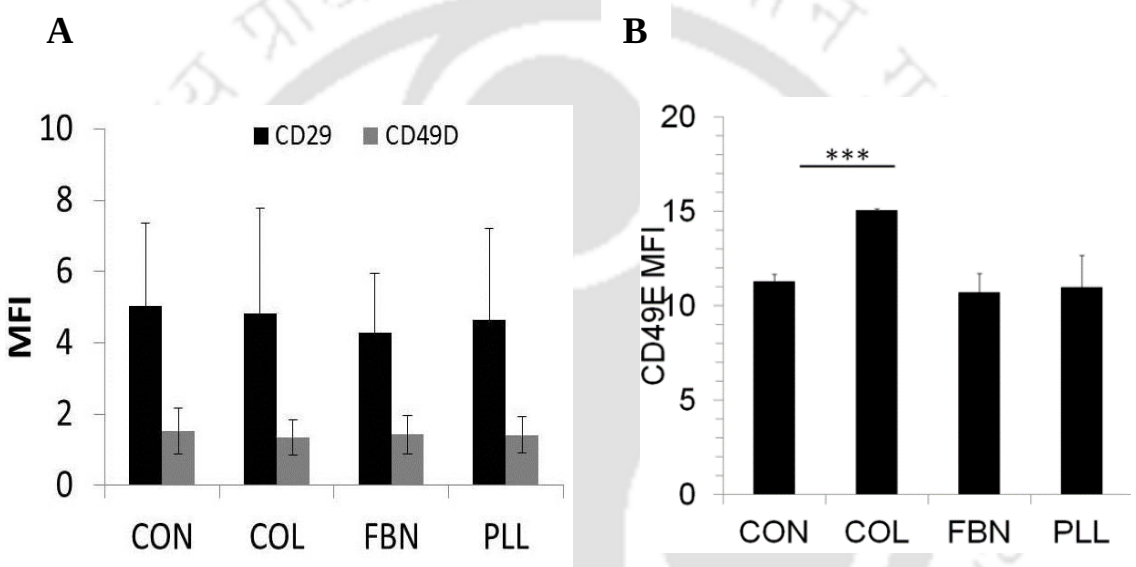


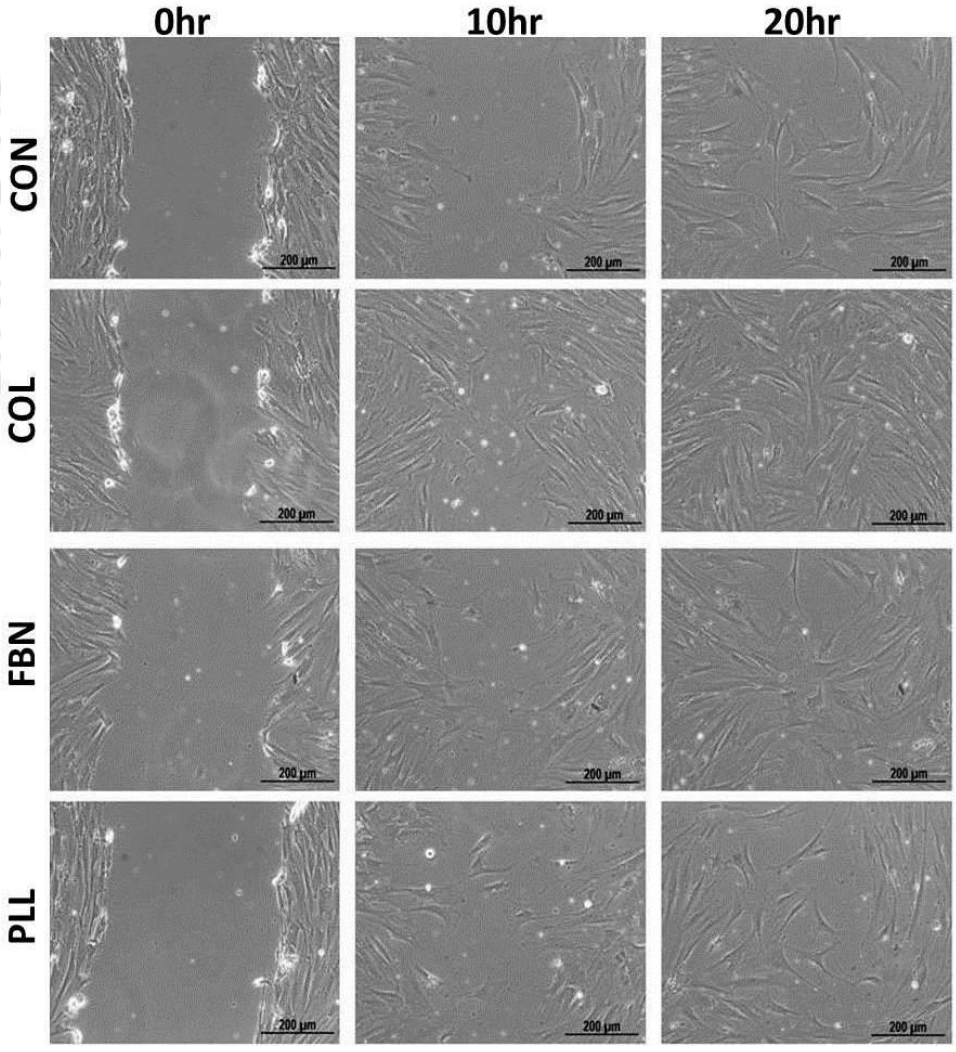
Fig.4.8. Effect of ECM on Integrin expression. The expression levels of CD29, CD49D (A) and CD49E (B) were determined in MSCs cultured COL, FBN, PLL and CON surfaces for 48hr. ***p<0.0005. n= 5 samples.

Cell migration

Active migration of cells in the scaffold is a required feature for proper functioning of the engineered tissue or organ. To understand the migration facilitated by different matrices, migration of MSCs was analyzed by wound healing migration assay. Equal number of cells seeded on CON, COL, FBN and PLL coated surfaces were allowed to migrate until the wound closed completely. The cells were documented microscopically at regular intervals to calculate the average migration speed (Fig.4.9A). Cells migrated at a higher speed on COL surface and closed the wound faster compared to other matrices.

The cells migrated with least speed on the CON surface with a 2.5 fold reduced speed compared to COL. The average migration speed observed on COL was 8.8µm/hr whereas in TC was 3.5µm/hr. While the wound closed within 24hr on COL, it took more than 48hr for the cells to close the wound on CON (Fig.4.9B,C). Cells on FBN and PLL showed similar migration speeds of 5.9µm/hr and 6µm/hr respectively and it was significantly higher than the CON surface (Fig.4.1.9B,C). Since cell proliferation was higher on COL, FBN and PLL compared to CON, the cells were serum starved for 12hr prior to the migration assay to negate the effect of cell proliferation in accelerating the wound healing.

A



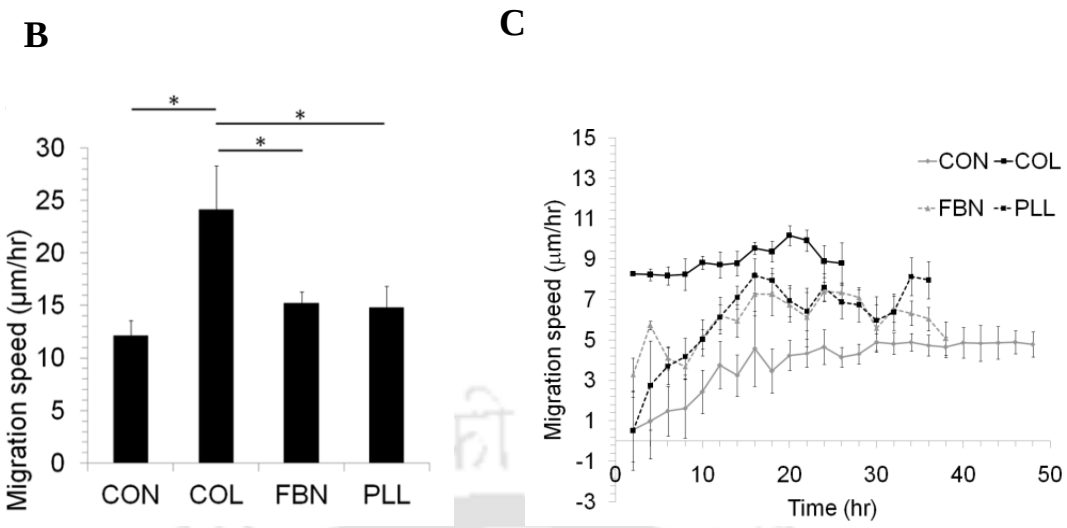


Fig.4.9. Effect of ECM on cell migration. MSCs were seeded on COL, FBN, PLL and CON surfaces. Cell migration was documented at regular intervals of time by microscopically (A). Average migration speed was calculated from experiments (B,C). *p<0.05. n= 3 samples.

Sub cellular mitochondrial distribution

Varum et al (Varum S et, al. 2011) has reported that mitochondrial distribution inside a cell predicts the stemness and so we determined the mitochondrial distribution in MSCs cultured on different matrices. In ES cells, the mitochondria were arranged in the perinuclear fashion when they were in stem cell state whereas the arrangement was altered into cytoplasmic distribution when the cells started differentiation (Varum S et, al. 2011). The active mitochondria distribution in MSCs cultured on different matrices was identified by TMRE staining. The cells cultured on COL and FBN showed predominantly peri-nuclear distribution whereas the cells grown on CON as well as PLL had high cytoplasmic mitochondrial distribution pattern (Fig.4.10A,B,C). Thus, MSCs might be maintained in a more stem-cell like state in COL and FBN compared to cells cultured on CON surface or PLL.

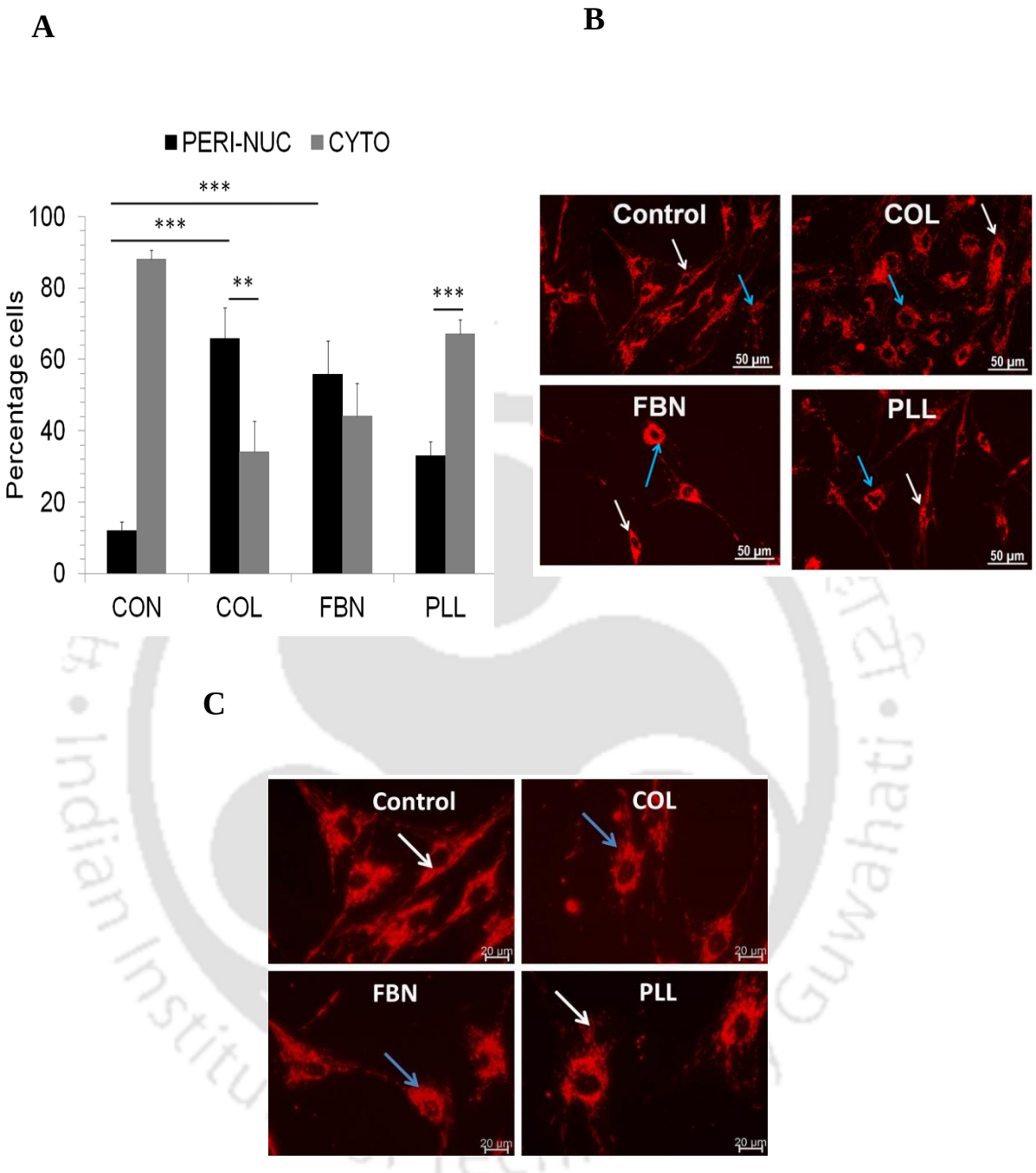


Fig.4.10. Effect of ECM on sub cellular mitochondrial distribution. MSCs cultured on COL, FBN, PLL and CON surfaces were stained for active mitochondria with TMRE. The percentage of peri-nuclear and cytoplasmic distribution of active mitochondria (A). The peri-nuclear staining was represented with blue arrow and white arrow points represent cytoplasmic staining of active mitochondria (B,C). Lower magnification (20X) (B) Higher magnification (40X) (C). **p<0.005, ***p<0.0005. n = 3 samples.

Effect of ECM on MSCs differentiation

An efficient and directed differentiation is desirable during tissue repair and MSCs are capable of differentiation into various cell types such as adipocytes, osteocytes and chondrocytes. Several factors affect their differentiation ability and here we analysed whether the ECM proteins themselves that are used to culture the MSCs could prime MSCs into either adipogenic or osteogenic lineage. MSCs were cultured on COL, FBN, PLL and CON surfaces and transcript levels of *OSTEOCALCIN* (OCN) and *PPAR gamma* (PPARG) was analysed before they were induced with the differentiation media. While the expression levels of PPARG was very low in uninduced MSCs, cells cultured on CON showed significantly high levels of PPARG compared to other matrices (Fig.4.11), suggesting a bias in the differentiation potential of CON surface expanded cells. The expression level of OCN in uninduced MSCs was comparable in all the matrices tested (Fig.4.11).

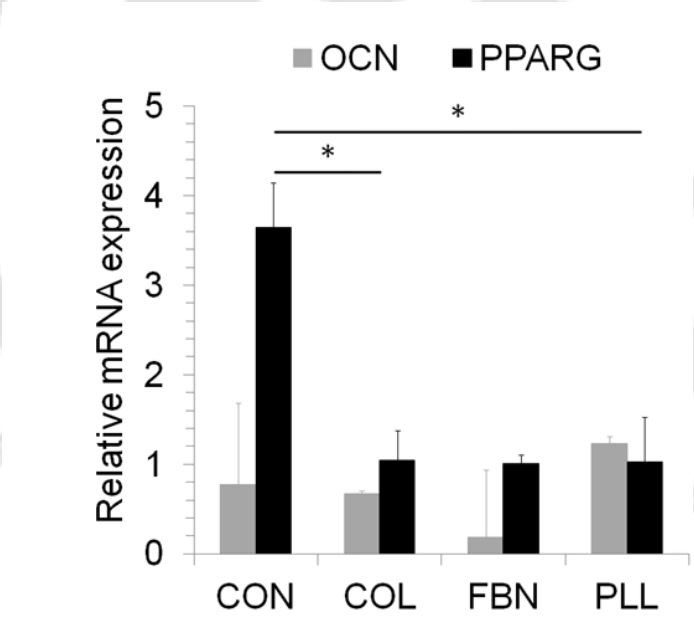


Fig.4.11. Effect of ECM on osteocalcin and PPAR gamma gene expression. MSCs were cultured on COL, FBN, PLL and CON surfaces. The semi quantitative PCR expression levels of OCN and PPARG. *p<0.05. n= 3 samples.

To test how the different ECM matrices regulate the differentiation of MSCs, MSCs were initially expanded on CON surface and seeded to COL, FBN, PLL and CON in the presence of adipogenic and osteogenic induction media. Cells differentiated on COL showed the highest osteogenic differentiation whereas cells cultured on CON had the least osteogenic differentiation. Osteogenic differentiation was higher in PLL differentiated cells than FBN, and both PLL, FBN had higher osteogenic differentiation than CON surface (Fig.4.11A,B).

Similarly, adipogenic differentiation was determined in CON expanded MSCs seeded on different matrices. In line with the increased PPARG expression on CON surface grown MSCs, adipogenic differentiation was highest in CON surface compared to the other matrices. Higher adipogenic differentiation was also observed in cells on COL matrix compared to PLL and FBN (Fig.4.13C,D).

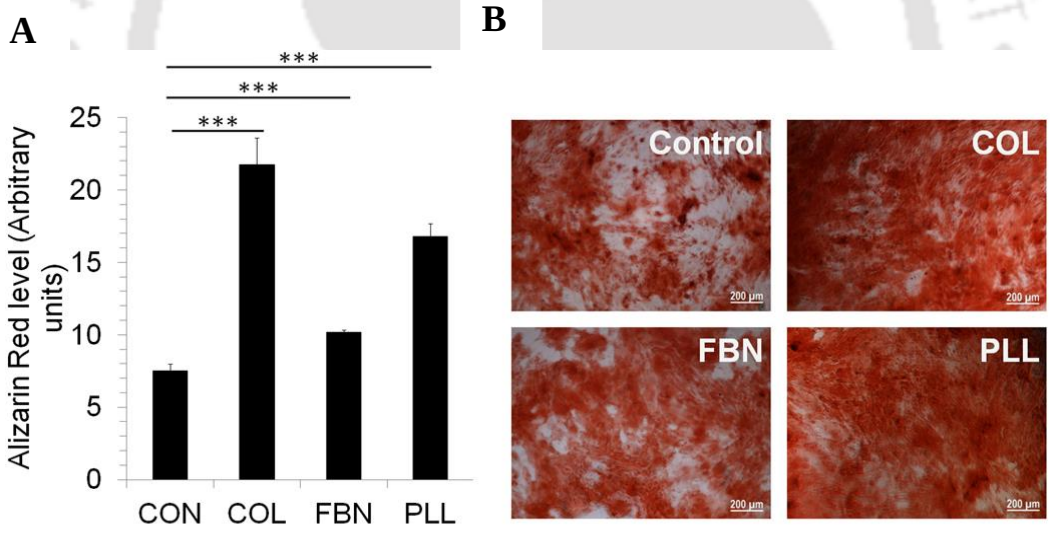


Fig.4.12. Effect of ECM on osteogenic differentiation. MSCs were differentiated into osteocytes on CON or coated surfaces. Differentiated cells were stained by Alizarin Red S (ARS) after 21-35 days of induction. Quantification of ARS was done by absorbance at 562 nm (A). Representative micrographs of ARS stained osteocytes (B). ***p<0.0005. n= 6-12 samples.

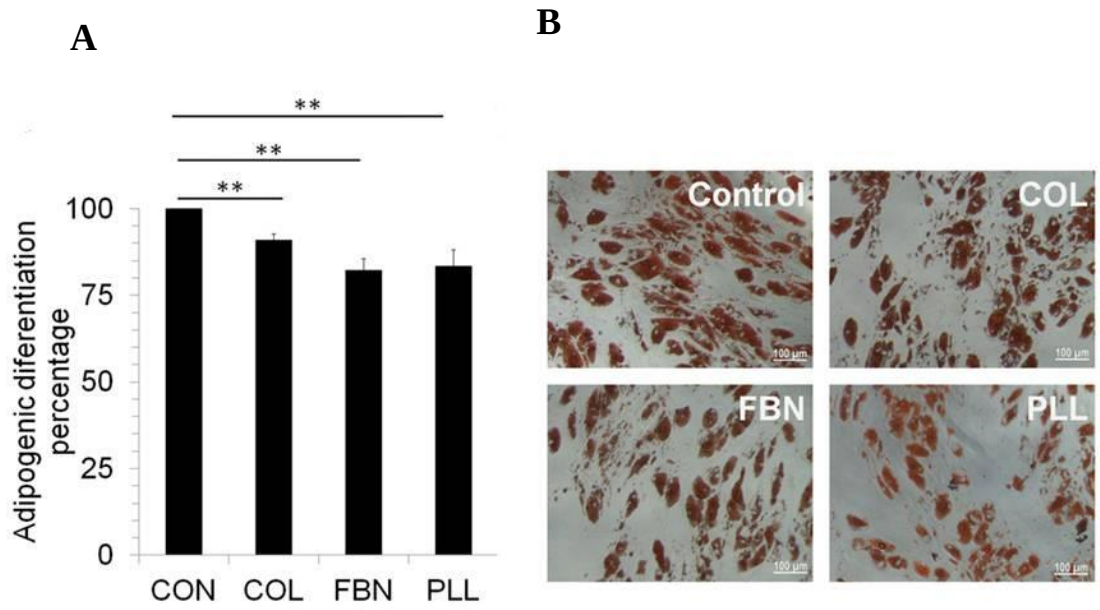
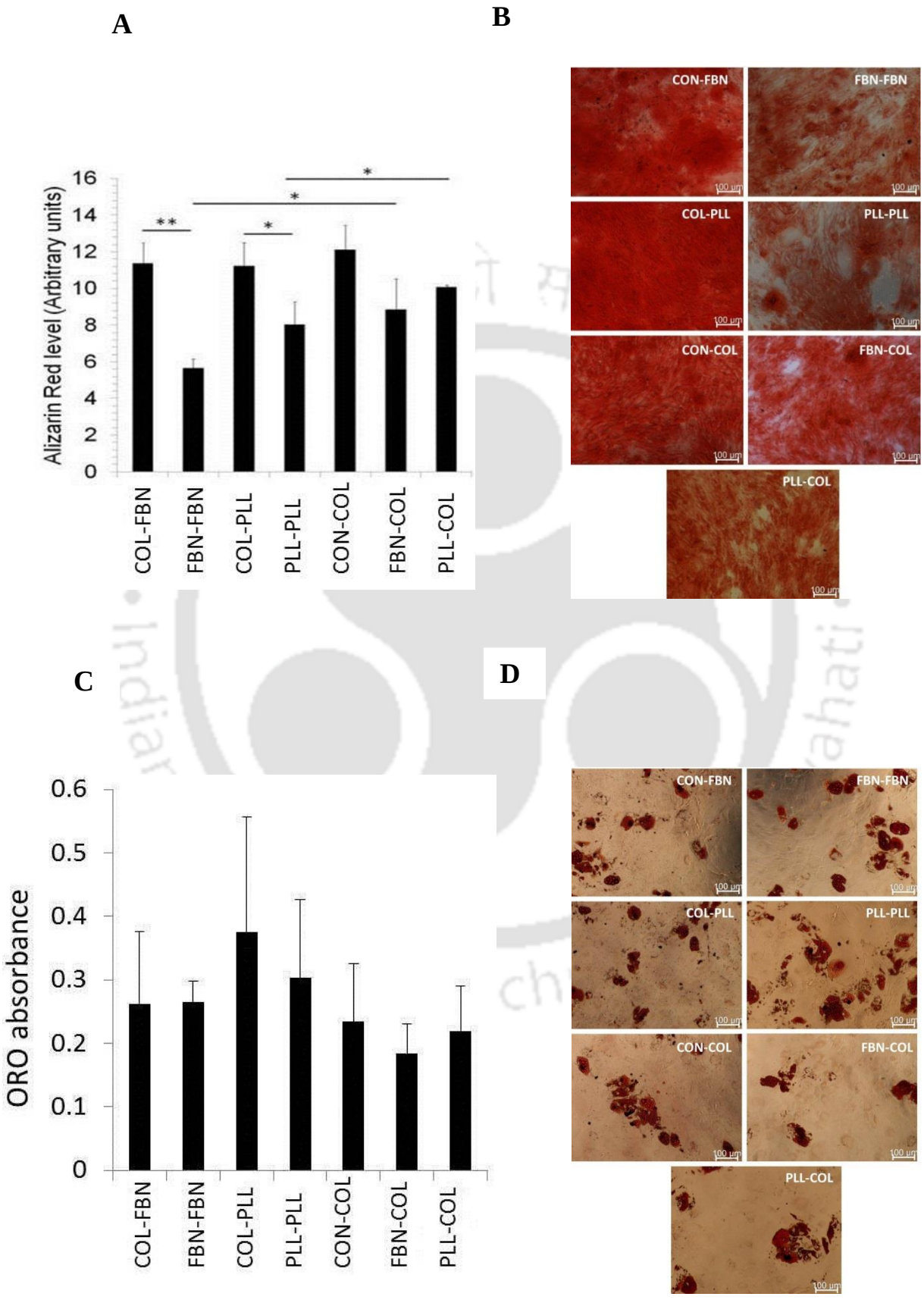


Fig.4.13. Effect of ECM on adipogenic differentiation. MSCs were differentiated into adipocytes on COL, FBN, PLL and CON surfaces. Differentiated cells were stained by Oil red O (ORO) after 21-35 days of induction. Percentage of ORO positive cells (A). Representative micrographs of ORO stained adipocytes (B). ***p<0.0005. n= 6-12 samples.

To understand the effect of COL on MSCs differentiation further, MSCs were cultured on COL matrix for a week and seeded on FBN, PLL, CON, COL and adipogenic, osteogenic differentiation was tested. Similarly, MSCs cultured on other matrices FBN, PLL and CON were seeded onto COL surface to test their adipogenic and osteogenic differentiation. When MSCs were seeded on COL matrix for osteogenic differentiation, after expanding the cells on FBN, PLL or CON, there was a significant increase in osteogenic differentiation compared to the cells differentiated on the same expansion matrices (Fig.4.14A,B). Interestingly, a significantly high osteogenic differentiation was observed, when cells were expanded on COL matrix prior to differentiation induction on FBN or PLL surface (Fig.4.14A,B). This suggests that COL matrix in the presence of differentiation factors has a synergistic effect in promoting high osteogenic differentiation.

Adipogenic differentiation was unaffected on COL expanded or COL differentiated cells although higher adipogenic differentiation was observed in cells differentiated on CON surface (Fig.4.14C, D).



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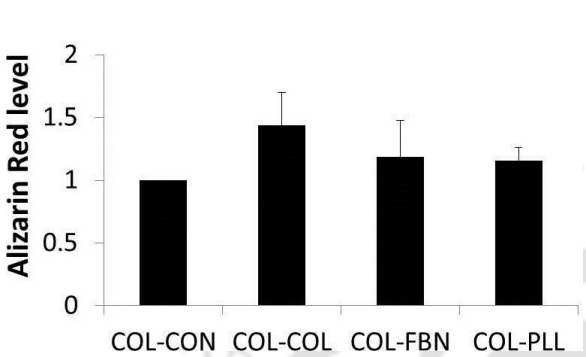

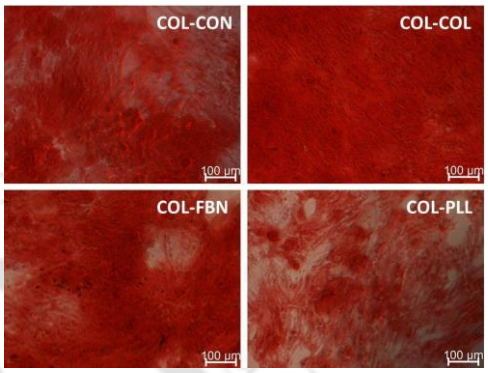

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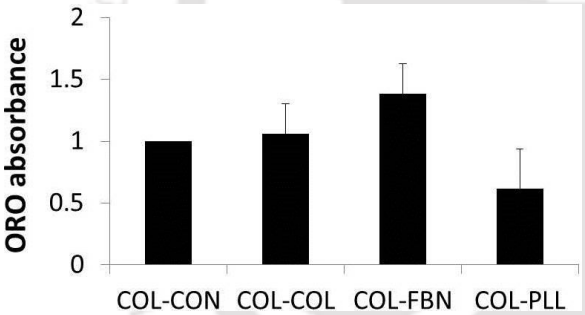

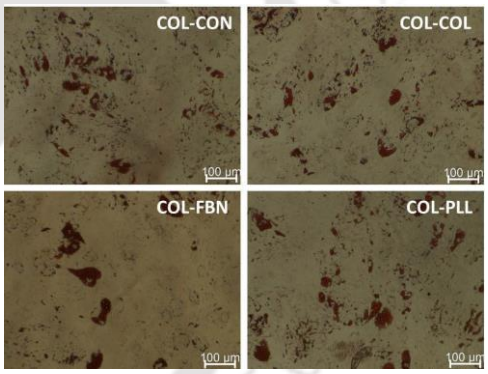

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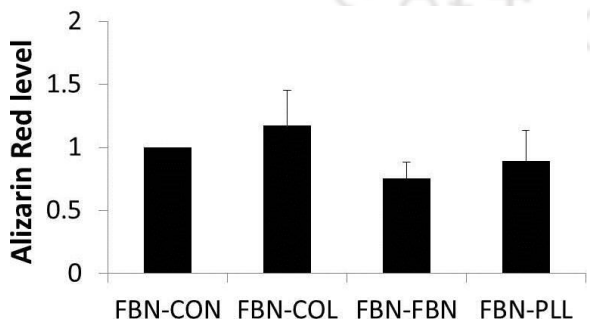

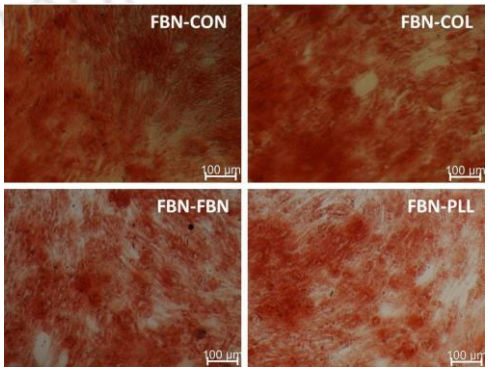

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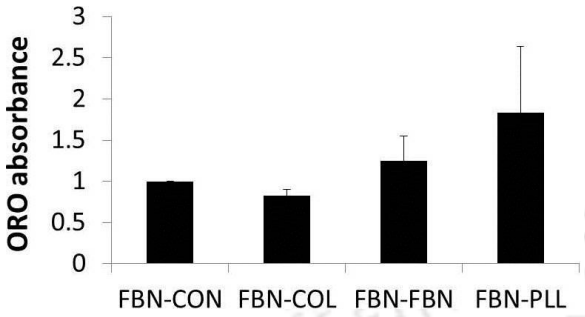

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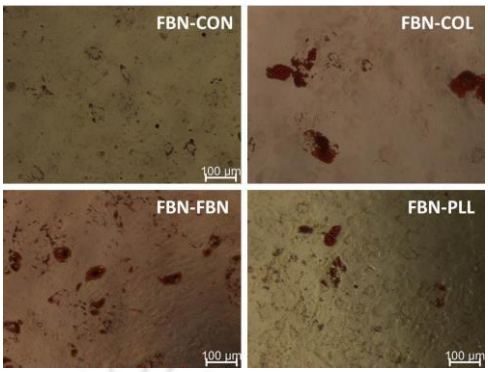

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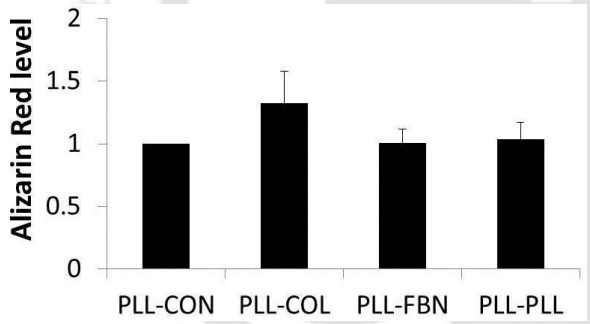

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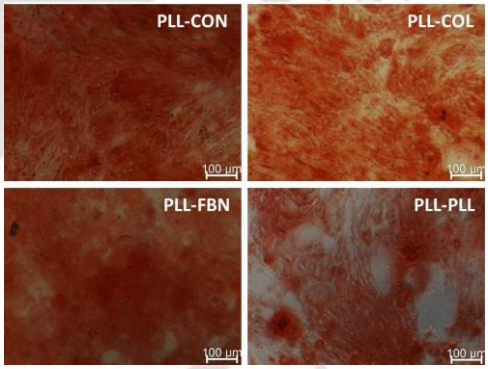

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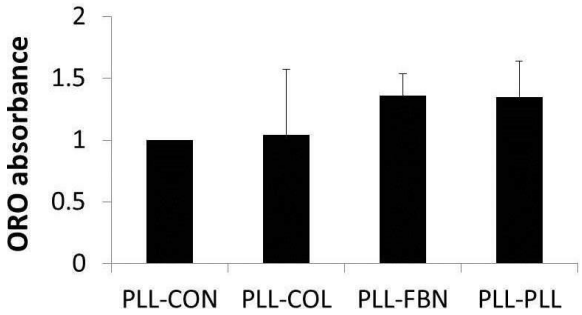

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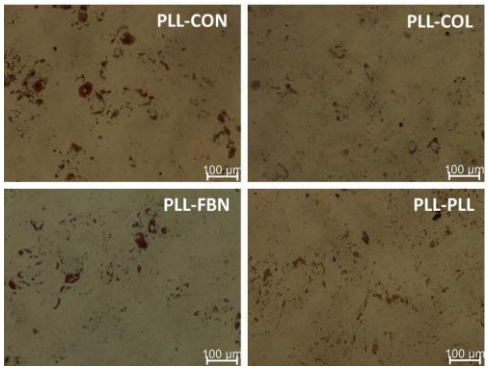


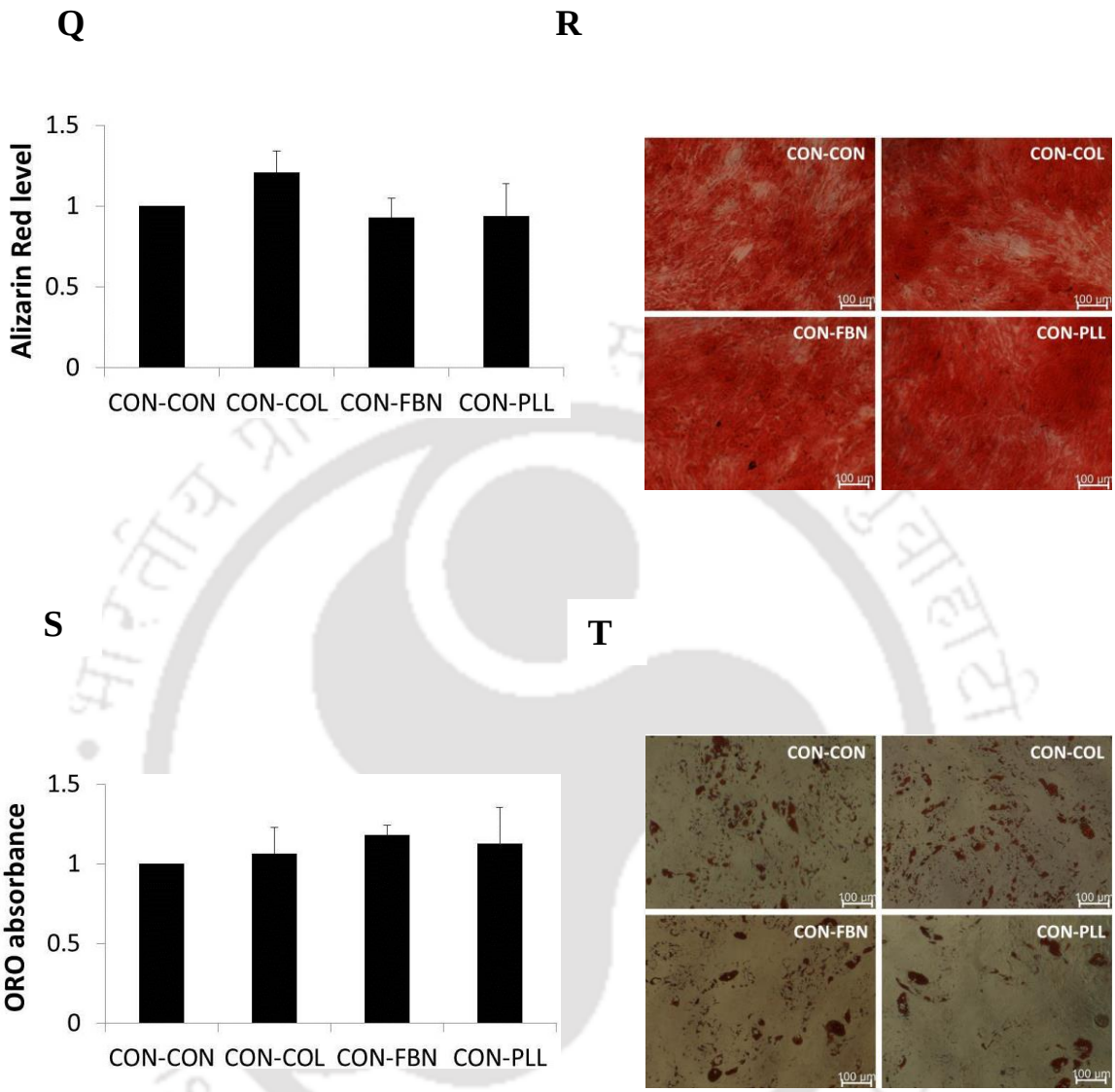
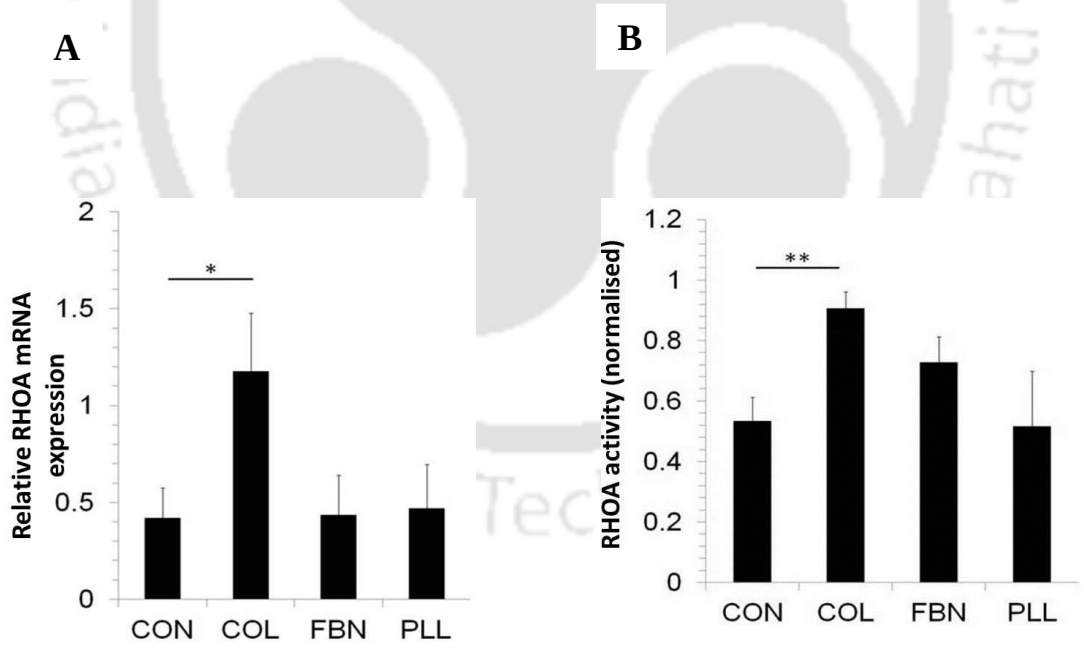


Fig.4.14. ECM switching differentiation. MSCs were grown on COL, FBN, PLL and CON surfaces for 1 week and seeded for osteocytes or adipocytes on COL, FBN, PLL and CON surfaces. Differentiated cells were stained by Alizarin Red S (ARS) for osteocytes or Oil red O (ORO) for adipocytes after 21-35 days. ARS quantified by absorbance at 560nm (A,E,I,M,Q). Representative micrographs of ARS stained osteocytes (B,F,J,N,R). ORO absorbance was quantified at 500nm (C,G,K,O,S). Representative micrographs of ORO stained adipocytes (D,H,L,P,T). *p<0.05, **p<0.005. n= 3 independent experiments.

RHOA Expression

RHOA is an important regulator of actin cytoskeleton, cell migration and MSCs differentaition (Jaganathan BG et, al. 2007) (McBeath R et, al. 2004). Active RHOA was reported to promote osteogenic differentaition whereas inhibition of RHO promoted adipogenic differentiation. To test, how the different ECM contribute to RHOA activation, MSCs cultured on COL, FBN, PLL and CON were tested for their RHOA expression levels through real-time PCR. A significantly high RHOA levels were seen in cells cultured on COL matrix compared to others (Fig.4.15A). However, RHOA activity is regulated at the protein level where they switch between active GTP bound state and inactive GDP bound state and downstream signaling is promoted by GTP bound RHOA (Van Aelst L et, al.1997). Thus, the active or GTP bound RHOA level in MSCs on COL, FBN, PLL and CON was determined through GLISA assay. Higher active RHOA levels were observed in cells cultured on COL and FBN and it was highest on COL grown MSCs compared to other conditions (Fig.4.15B).



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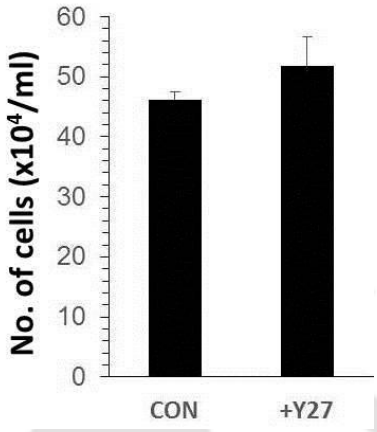


Fig.4.15. Effect of ECM on RHOA expression. MSCs were cultured on COL, FBN, PLL and CON surfaces. RHOA mRNA expression levels (A). Active RHOA GTP levels on different ECM (B). MSCs were seeded on COL surface and treated with RHO inhibitor (+Y27) for 24hr and cell count was determined. Cell count in untreated (CON) and Y27 treated (+Y27) is shown (C). $p<0.05$, $^{**}p<0.005$. n= 3 samples.

However, inhibition of RHOA in MSCs by treatment with Y27632 on COL matrix did not affect its proliferation rate (Fig.4.15C). Since RHOA is an important signaling molecule and to understand its role further, MSCs were transduced with constitutively active (RHOAV14) and dominant negative (RHOAN19) RHOA and change in MSCs characteristics was studied. GLISA analysis showed that MSCs transduced with RHOAV14 had significantly high GTP bound RHOA and RHOAN19 transduced MSCs had low GTP bound RHOA thereby diminishing RHOA downstream signaling (Fig.4.16).

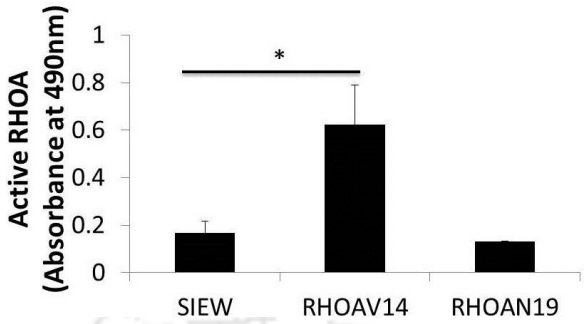


Fig.4.16. RHOA activity in RHOA transduced MSCs. Active GTP bound RHOA levels in cells transduced with RHOA Plasmids was determined by RHOA G-LISA activation assay. *p<0.05. n= 3 samples.

To understand the effect of RHOA further, the role of RHOA in modifying MSCs characteristics was studied. Active RHOA significantly altered the morphology of MSCs, where the cells lost their spindle shape and acquired flattened morphology (Fig.4.17A,B). MSCs expressing RHOAN19, however, retained their spindle shaped morphology (Fig.4.17A,B).

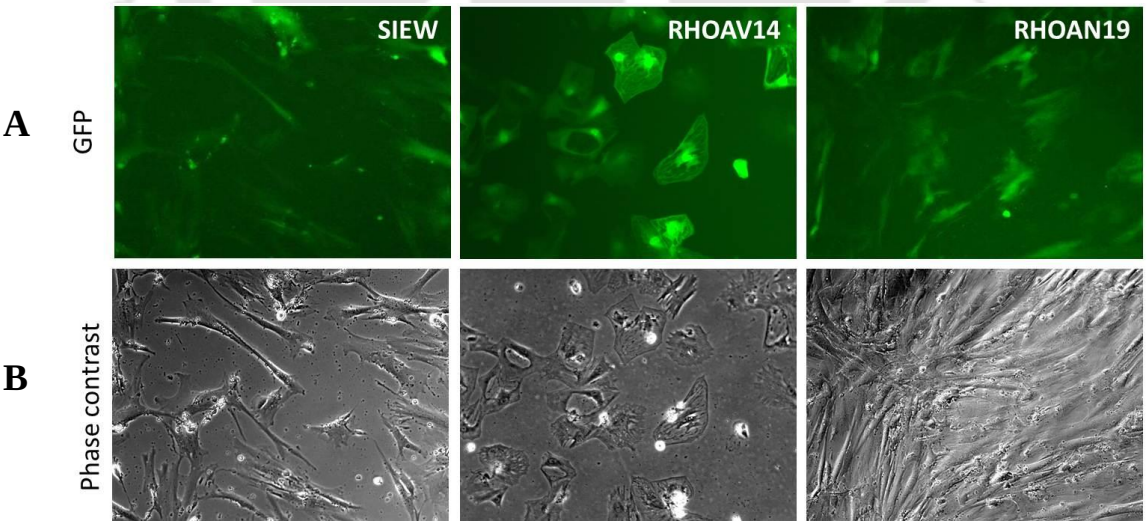


Fig.4.17. Effect of RHOA on MSCs morphology. MSCs were transduced with lentiviral vectors. Representative microscopic images showing GFP expression of lentiviral transduced MSCs (A) and phase contrast images of transduced MSCs (B).

MTT assay was performed to check the changes in proliferation due to RHOA modification in MSCs. There was a significant decrease in proliferation when RHOA was activated through RHOAV14 transduction in MSCs whereas down regulating RHOA activity through RHOAN19 expression did not affect the proliferation (Fig.4.18).

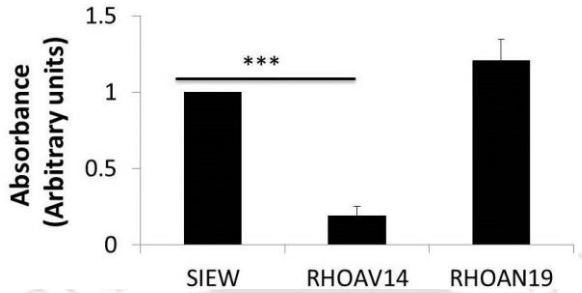


Fig.4.18. Effect of RHOA on MSCs proliferation. Transduced MSCs were seeded in equal density for 48hr and proliferation was analyzed by MTT assay. Representative graph plotted by average values of absorbance. ***p<0.0005. n= 3 samples.

RHOA is important in regulating the actin cytoskeleton and to test the effect of RHOA modification in MSCs, MSCs transduced with control, RHOAV14 and RHOAN19 were stained with phalloidin-TRITC to visualize F-actin. As reported by others (Jaganathan BG et, al. 2007) MSCs transduced with active RHOA showed increased stress fibers arranged in a criss-cross pattern whereas in control MSCs, the F-actin showed parallel and regular arrangements through the cell. In RHOAN19 transduced cells, there was a reduction in the F-actin amount suggesting decreased actin polymerization due to RHOA inactivation in the cells (Fug.4.19)

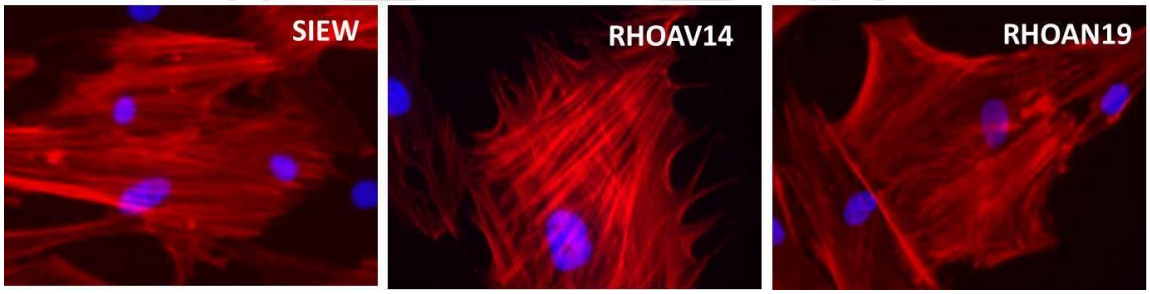
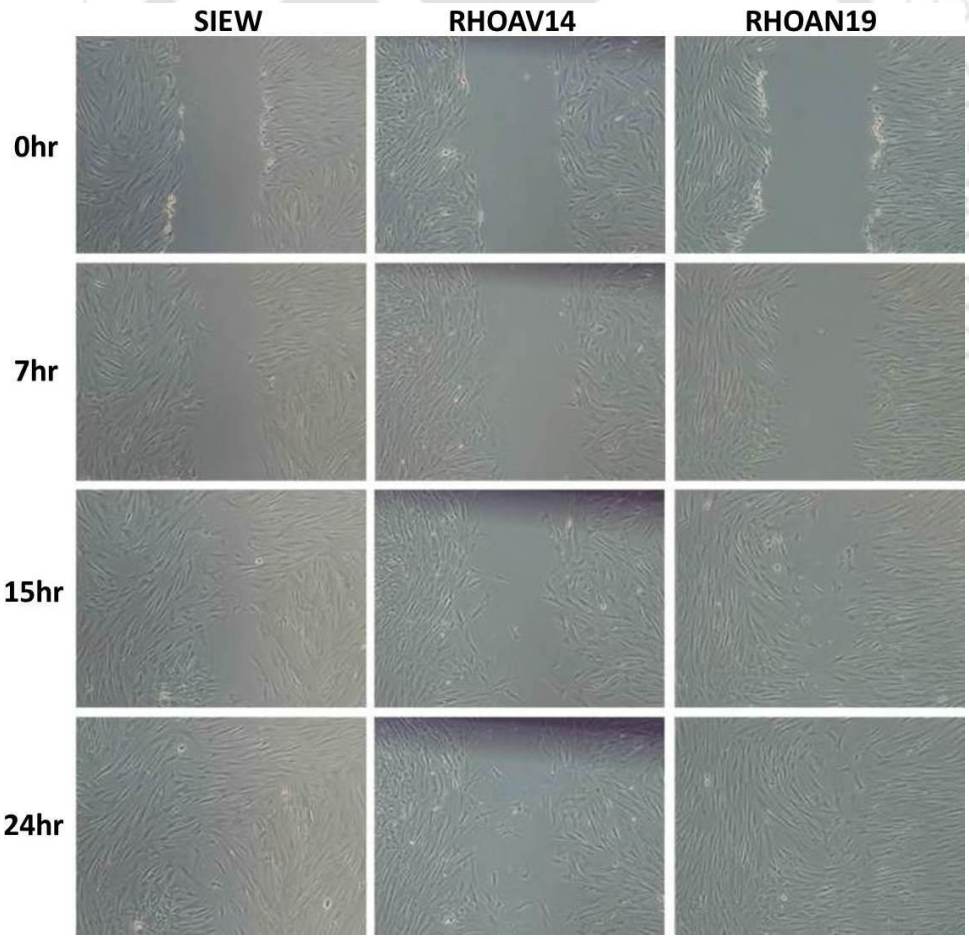


Fig.4.19. Effect of RHOA on MSCs actin cytoskeleton modification. SIEW, RHOAV14 and RHOAN19 transduced MSCs were stained for actin cytoskeleton with phalloidin -TRITC and nuclear stain with DAPI and visualized by fluorescence microscope.

By regulating actin polymerization and focal adhesion, RHOA also regulates cell migration. To check the effect of RHOA on MSCs migration, RHOA transduced MSCs were subjected to wound healing migration assay. The cells were observed and documented at regular intervals until the wounds closed (Fig.4.20A). There was a significant decrease in migration in MSCs when RHOA was activated in the cells through RHOAV14 expression. The average migration speed in control vector transduced cells was 13.10 µm/hr whereas RHOAV14 transduced cells had an average migration speed of 4.91 µm/hr (Fig.4.20B,C). Although not significant, there was an increase in the migration speed of RHOAN19 transduced MSCs compared to the control cells with an average migration speed of 14.59 µm/hr (Fig.4.20B,C).

A



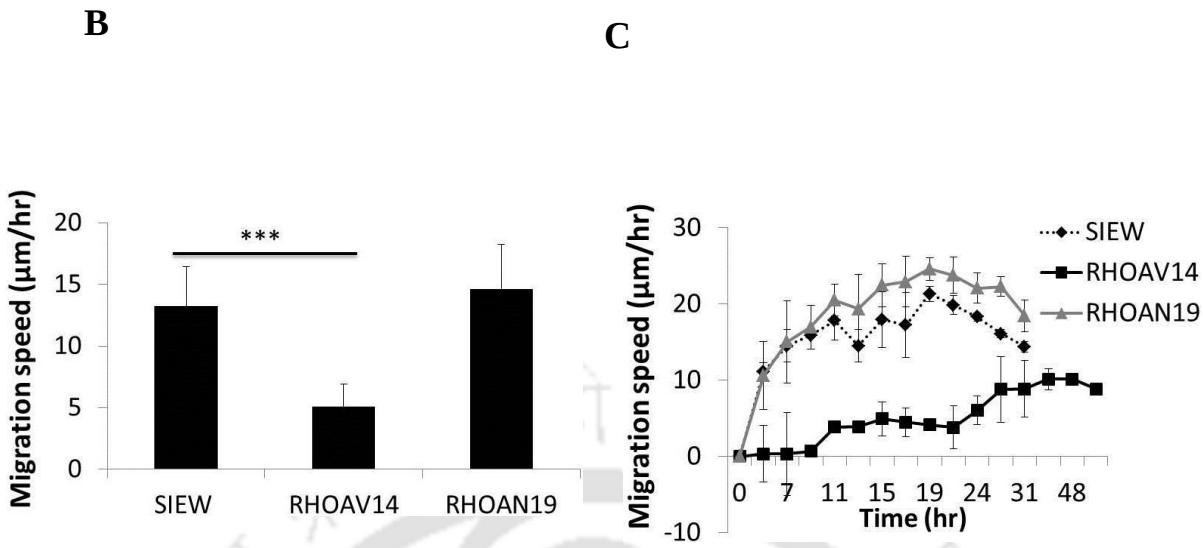


Fig.4.20. Effect of RHOA on MSCs migration. MSCs were transduced with SIEW, RHOAV14 and RHOAN19 vectors and the migration speed was analysed every 2hr until the wound closed. Cell migration was documented at regular intervals microscopically (A). Average migration speed was calculated (B,C). ***p<0.0005. n= 3 samples

Since RHOA was significantly higher in MSCs on COL matrix which also showed higher migration and osteogenic differentiation, we tested the adipogenic and osteogenic differentiation of RHOA modified MSCs. As reported by others (Jaganathan BG et, al. 2007) (McBeath R et, al. 2004) RHOAV14 transduced MSCs showed significantly higher osteogenic differentiation as seen by increased alizarin red staining in the differentiated cells whereas there was a reduction in osteogenic differentiation in MSCs transduced with inactive RHOA, RHOAN19 (Fig.4.21A,C). On the other hand, RHOAN19 transduced MSCs showed significantly increased adipogenic differentiation whereas RHOAV14 expressing MSCs had significantly reduced adipogenic differentiation compared to the control cells (Fig.4.21B,D). Thus, RHOA expression altered the balance between the adipogenesis and osteogenesis in MSCs. However, constitutively increased RHOA activity in MSCs did not promote high proliferation or migration.

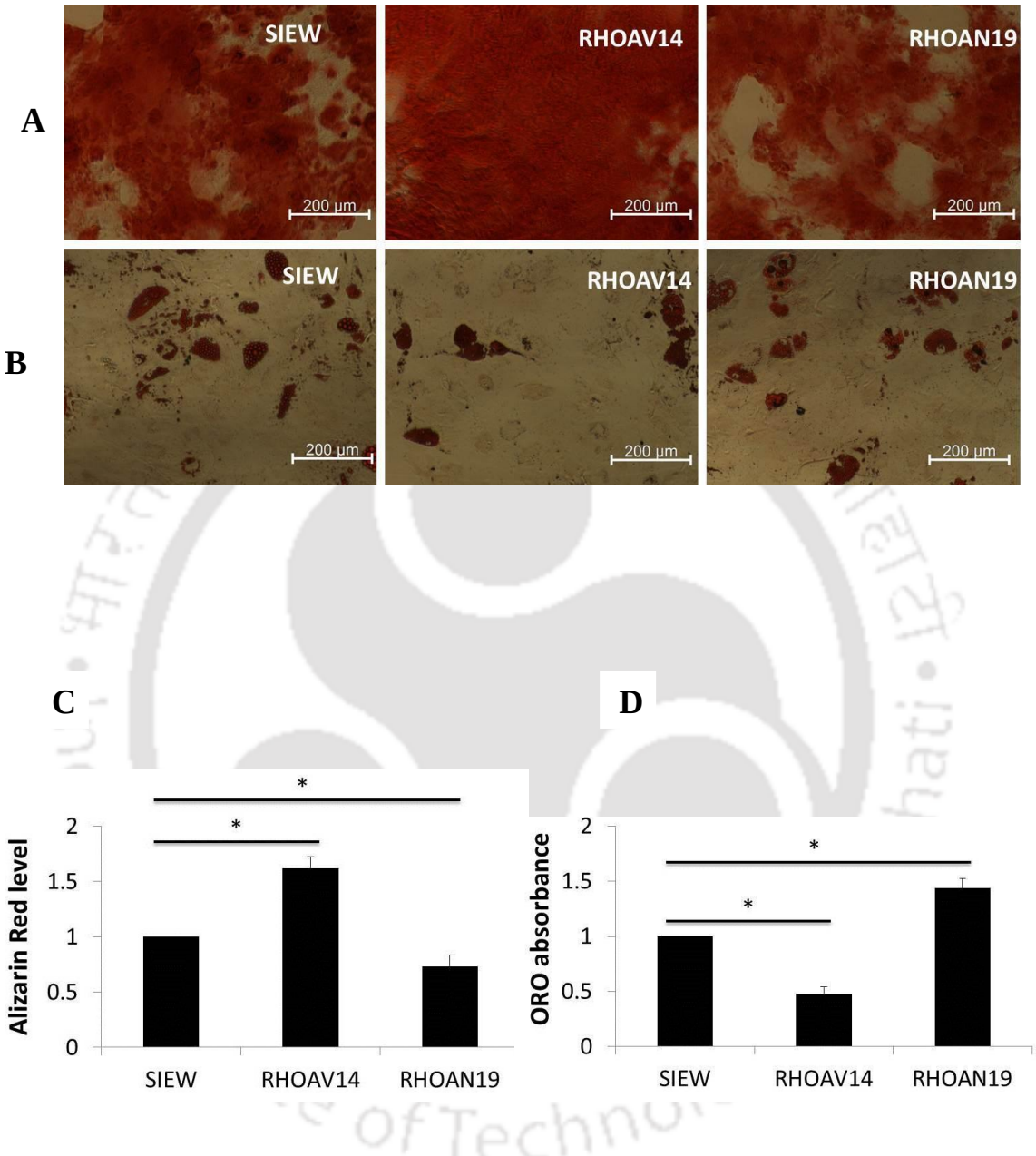


Fig.4.21. Effect of RHOA on MSCs differentiation. Transduced MSCs were differentiated into either adipocytes or osteocytes. After 21days of induction with respective media osteocytes were stained with Alizarin Red S (ARS) (A) and Oil red O (ORO) for adipocytes (B). Quantification of ARS (C) and ORO (D). *$p<0.05$, n= 8 wells.

4.2. Properties of MSCs on interaction with leukemic cells

Bone marrow is an important source of MSCs but several reports show that leukemic affects the bone marrow microenvironment where the MSCs reside. Leukemia cells also affect the secretary profile of the stromal cells to make them supportive of the leukemia cells proliferation. So, it is important to understand how the leukemic cells affect the MSCs phenotype and differentiation potential to determine its use for cell therapy applications.

Leukemic cells interact with MSCs through various adhesion molecules, integrins and cell surface receptors. To mimic the *in vivo* leukemic conditions MSCs were co-cultured with primary leukemic cells from the patient bone marrow or leukemic cell lines HL60 and THP1 for 1 week and properties like surface marker expression and differentiation potential was studied (Fig 4.22).

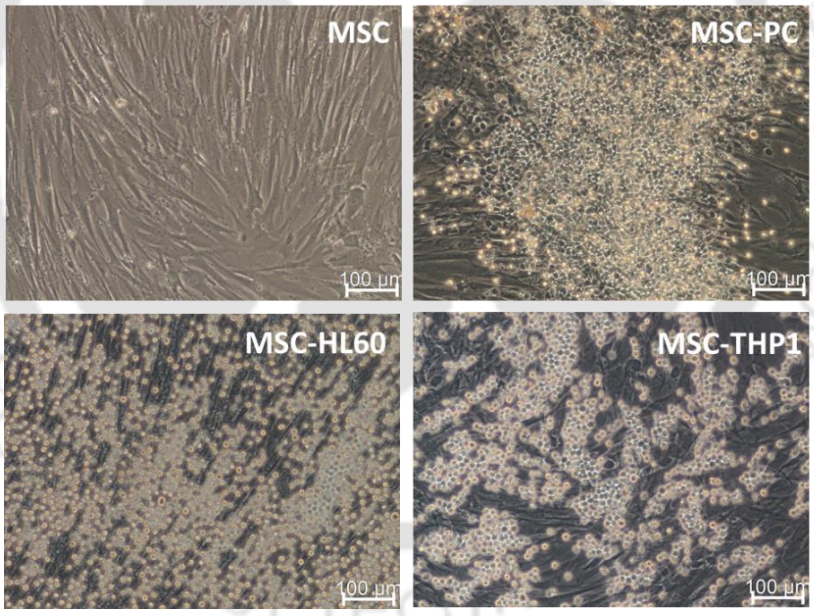


Fig.4.22. Co-culture of MSCs with leukemic cells. Phase contrast images of control MSCs, during co-culture with primary leukemic cells (PC) or HL60 or THP1.

Cell surface expression levels of CD13, CD29, CD49E, CD73 and CD90 on MSCs were screened following co-culture of MSCs with leukemic cells. A decrease in CD73 and CD90 expression levels was observed in MSCs interacting with primary leukemic cells

(Fig.4.23A,B). However, no significant difference was observed in surface marker expression levels in MSCs during co- culture with THP1 or HL60 cells (Fig.4.23C,D).

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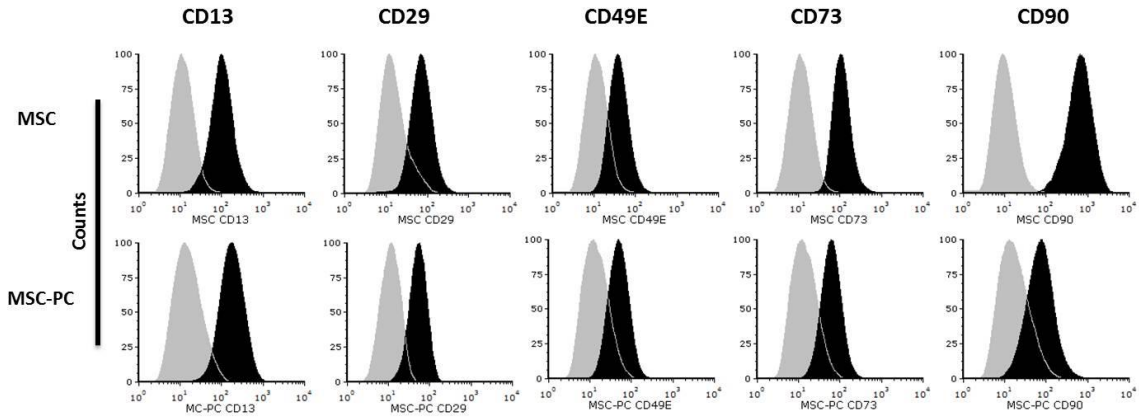

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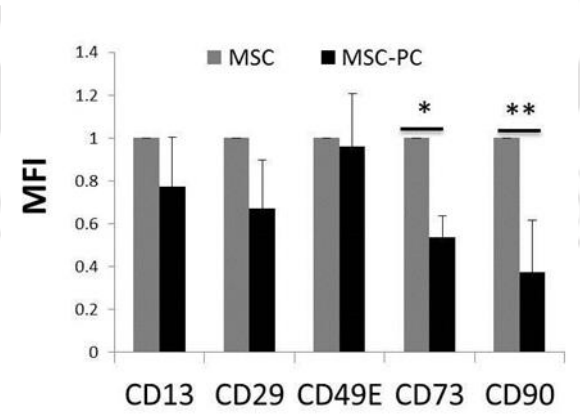

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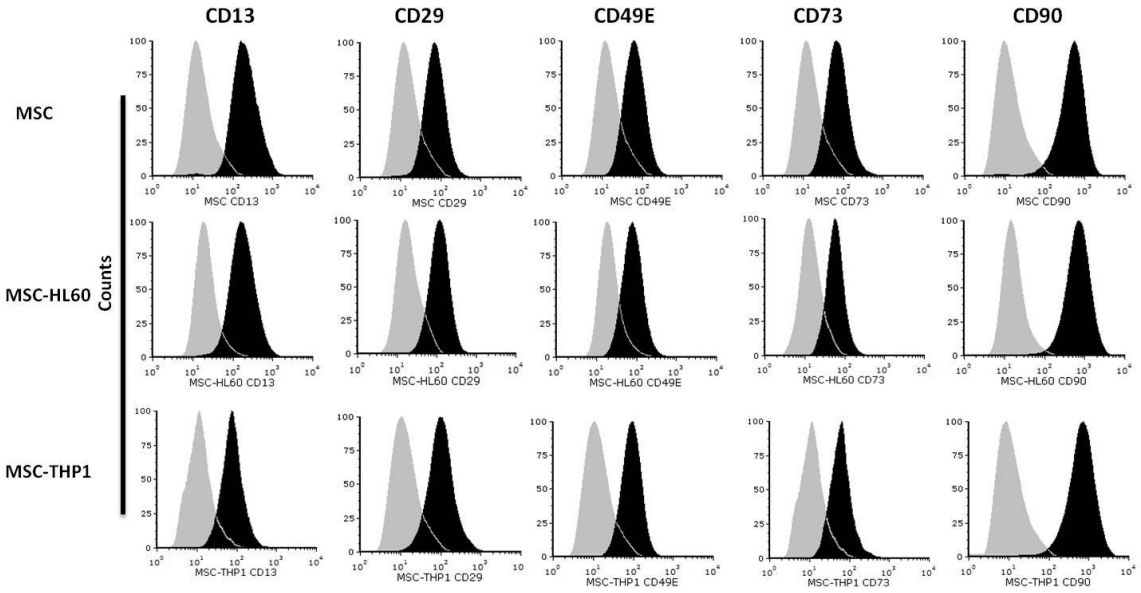

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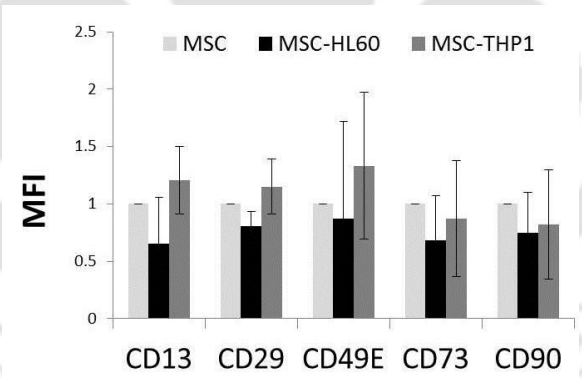


Fig.4.23. Surface marker expression of MSCs during leukemic cell co-culture. MSCs were co-cultured with primary leukemic cells or HL60 or THP1 for 1 week and the surface marker expression was analyzed by flow cytometer. Representative histograms of surface marker expression of primary leukemic cells (PC) (A) and HL60 or THP1 co-cultured MSCs (C). MFI of CD13, CD29, CD49E, CD73, CD95 and CD90 expression in PC (B) HL60 or THP1 co-cultured MSCs (D). *p<0.05, **p<0.005. n= 3 samples.

Co- cultured MSCs were induced to differentiate into adipocytes and osteocytes with respective induction media for 28 days and stained with Oil Red O (for adipocytes) and Alizarin Red S (for osteocytes). MSCs co-cultured with leukemic cells showed reduced adipogenic potential (Fig 4.24C, D, E) whereas, no change was observed in osteogenic differentiation (Fig 4.24A, B).

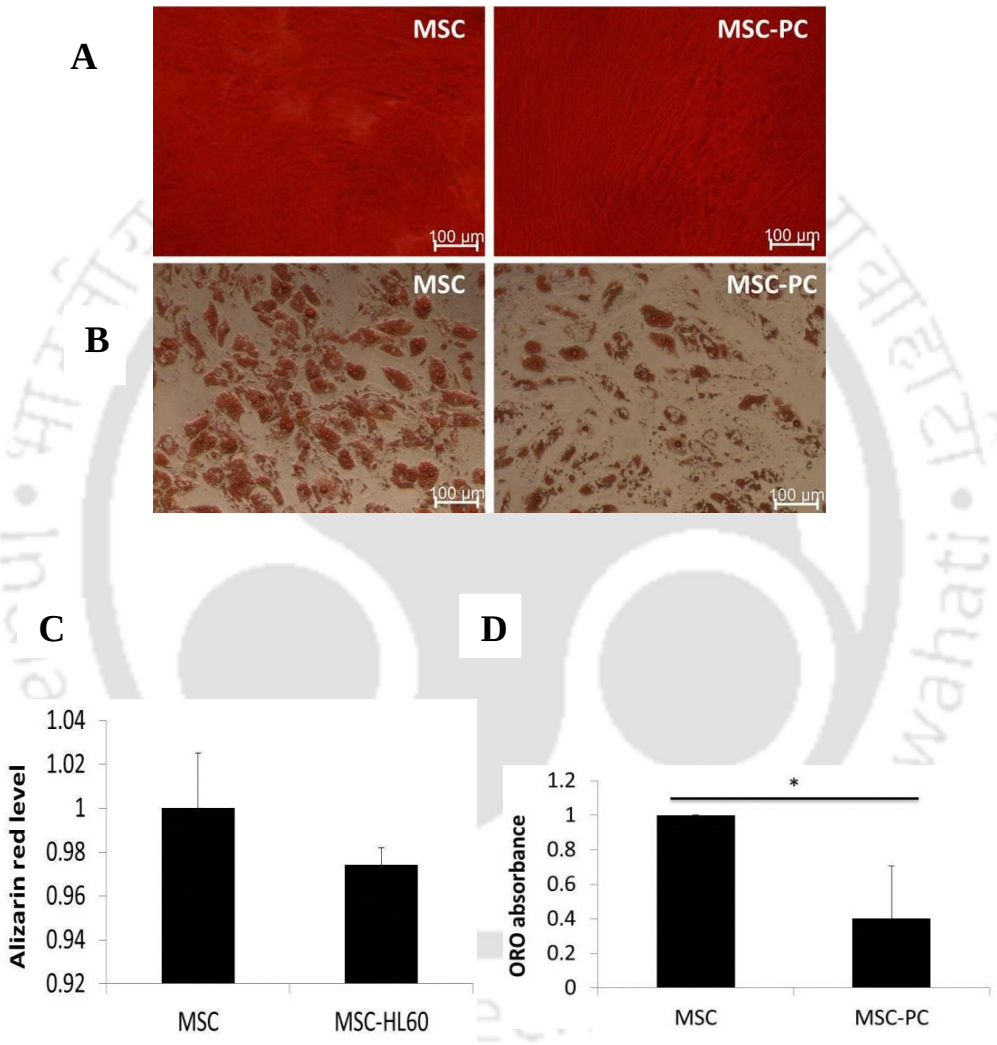


Fig.4.24. Differentiation of MSCs during primary leukemic cells co-culture. MSCs were co- cultured with primary leukemic cells (PC) and differentiated into either adipocytes or osteocytes. After 21days of induction with respective media osteocytes were stained with Alizarin Red S (ARS) (A) and Oil red O (ORO) for adipocytes (B). Quantification of ARS (C) and ORO (D). *$p<0.05$, n= 10 wells.

To check further, MSCs were co-cultured with leukemic cell line HL60 for 1 week. Co-cultured MSCs were induced to differentiate into adipocytes and osteocytes with respective induction media for 28 days and stained with Oil Red O (for adipocytes) and Alizarin Red S (for osteocytes). Co-cultured MSCs showed reduced adipogenic potential (Fig 4.25B,D) whereas, no change was observed in osteogenic differentiation (Fig 4.25A,C) as seen with the primary cells co-culture. Thus, leukemic cells affect the phenotype and differentiation potential of MSCs when they were in contact with each other.

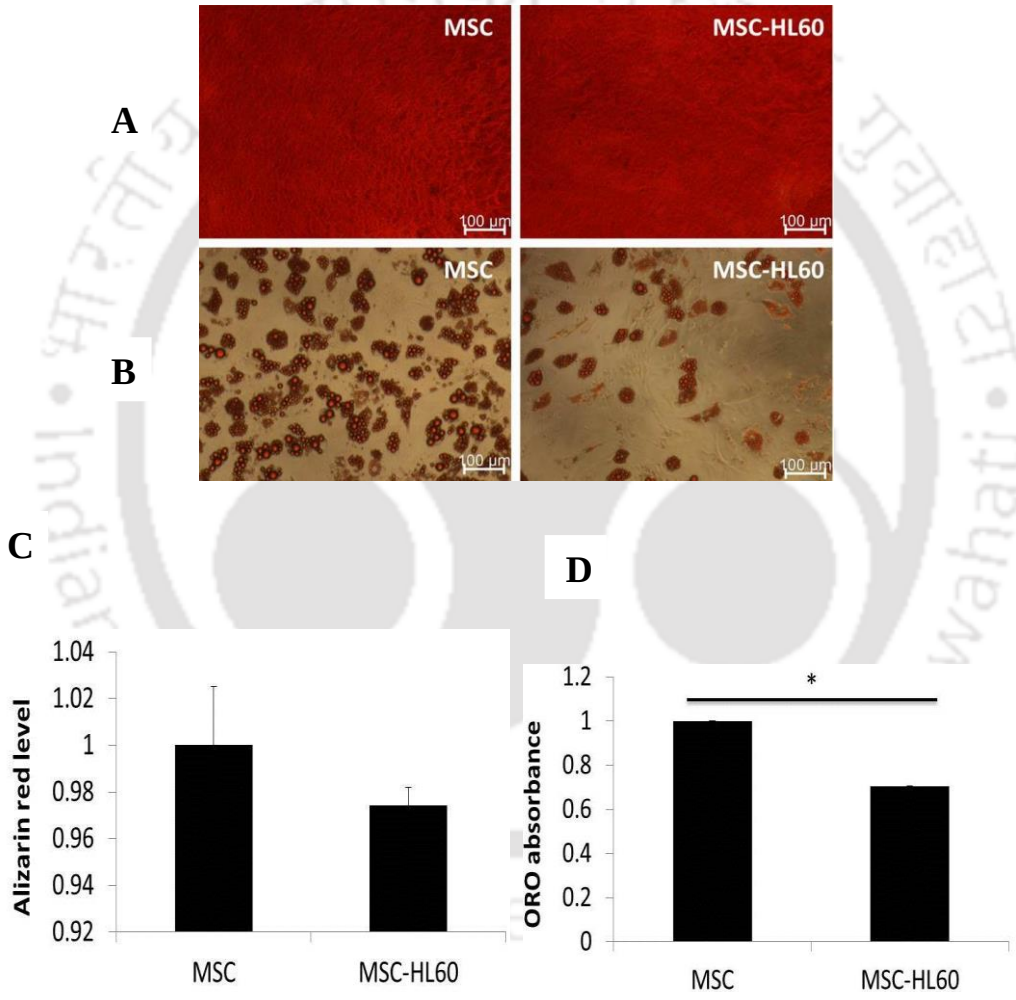


Fig.4.25. Differentiation of MSCs during HL60 co-culture. MSCs were co- cultured with HL60 and differentiated into either adipocytes or osteocytes. After 21days of induction with respective media osteocytes were stained with Alizarin Red S (ARS) (A) and Oil red O (ORO) for adipocytes (B). Quantification of ARS (C) and ORO (D). *$p<0.05$, n= 4 wells.

4.3. Effect of chemotherapeutic drugs on MSCs properties

The first line of therapy for leukemia is chemotherapy with drugs in different combinations based on the FAB type and the severity of the disease. Several chemotherapy drugs such as cytarabine (CYT), daunorubicin (DAU) and vincristine (VIN) are used for treatment. The chemo drugs not only target the leukemic cell population but also destroy the healthy cells present in the niche. To test whether MSCs isolated from chemotherapy treated individuals could be used for cell therapy, chemotherapy induced changes in morphology, phenotype and differentiation was studied.

To study the effect of chemo drugs on MSCs morphology, equal number of MSCs were treated with CYT (1 μM), DAU (0.1 μM) and VIN (0.1 μM) for 48hr. VIN induced a marked change in cell shape, cells converted from spindle shape to round cells, whereas, MSCs showed flat morphology in DAU treatment. No change in morphology was observed when treated with CYT (Fig. 4.26A). Pretreated MSCs recovered from CYT or DAU showed mixed population of cells with irregular morphology. However VIN treated cells changed from round or spherical morphology to spindle shape after 1 week of chemo therapeutic drug withdrawal (Fig.4.26B).

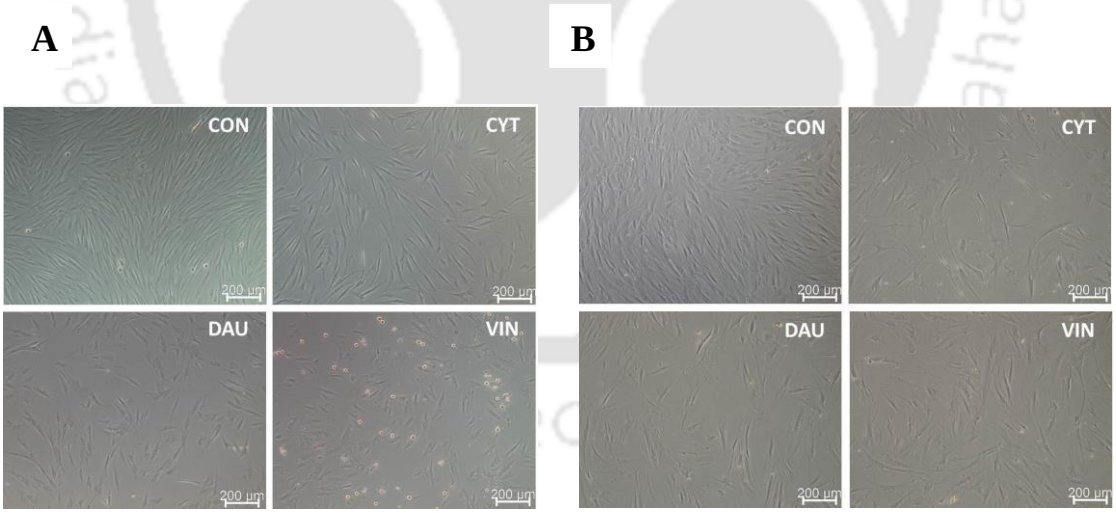


Fig.4.26. Effect of chemotherapeutic drugs on MSCs morphology. MSCs were treated with CYT (1 μM), DAU (0.1 μM) and VIN (0.1 μM) for 48hr (A). Microscopic images showing morphology of MSCs cultured for 48hr in expansion medium (CON) or media containing chemotherapeutic drugs (A) and MSCs recovered from chemo therapeutic agents cultured for 1 week in expansion medium after chemotherapeutic drugs treatment (B).

Cell counting was done for MSCs treated with chemotherapeutic drugs for 48hr and recovered after withdrawal of the drugs. There was a significant decrease in the cell number when MSCs were treated with CYT, DAU and VIN compared to the control cells (Fig.4.27A). When chemotherapeutic drug treated MSCs were allowed recover for one week after the treatment, the cells gained their proliferation capacity after VIN treated whereas CYT and DAU treated cells had significantly reduced proliferative capacity (Fig.4.27B).

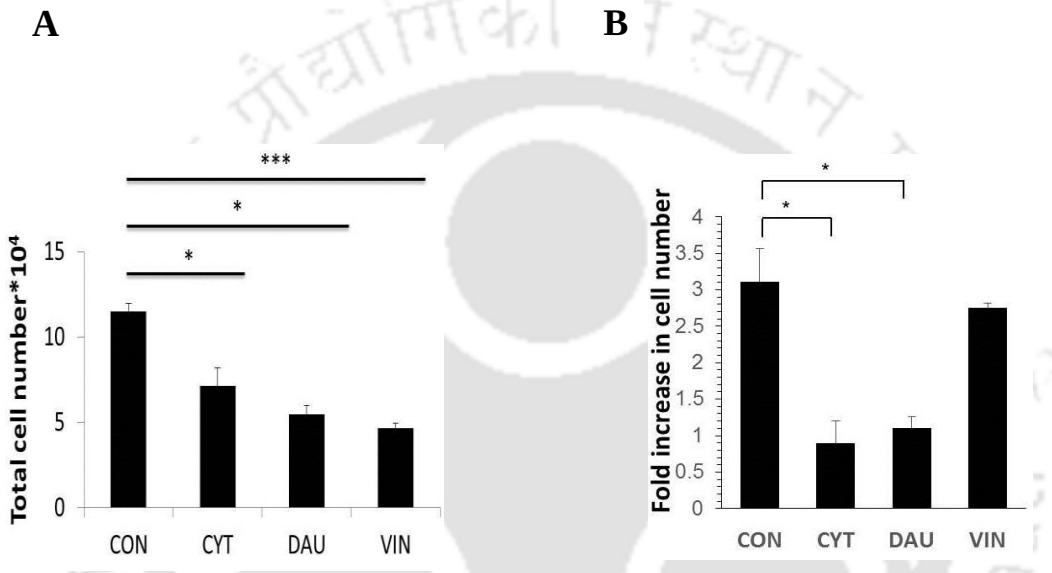
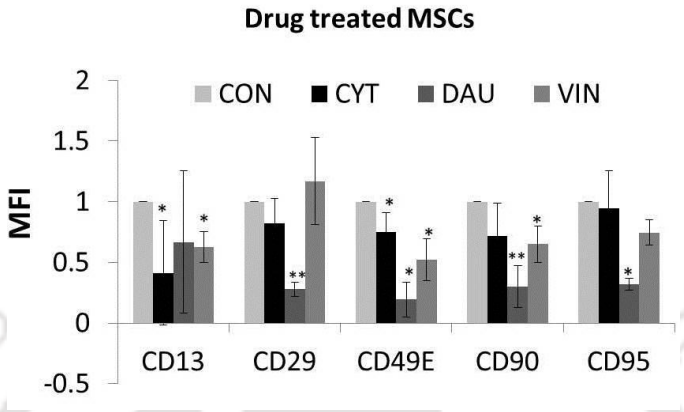


Fig.4.27. Effect of chemotherapeutic drugs on MSCs survival. Viable cell number was determined after treatment with drugs (A). The fold increase in cell number after culturing the drug treated cells in drug free media for 1 week (B). *p<0.05, ***p<0.0005. n= 3 samples.

We checked for alterations in MSCs cell surface marker expression, upon treatment with drugs. CD13, CD49E expressions was reduced upon CYT treatment. Whereas, upon DAU treatment, expression of all markers were downregulated. CD13, CD49E and CD90 expression was decreased upon treatment with VIN (Fig.4.28A, C). In order to understand the long term effect of these drugs, MSCs were exposed to chemo drugs for 48hr. Fresh media without drugs was added for one week and screened for expression of CD13, CD29, CD49E, CD90 and CD95 markers. VIN treated cells could completely regain their cell surface marker expressions. Whereas, CD13, CD29 and CD90 expression was decreased in DAU treated cells (Fig.4.28B, D).

A



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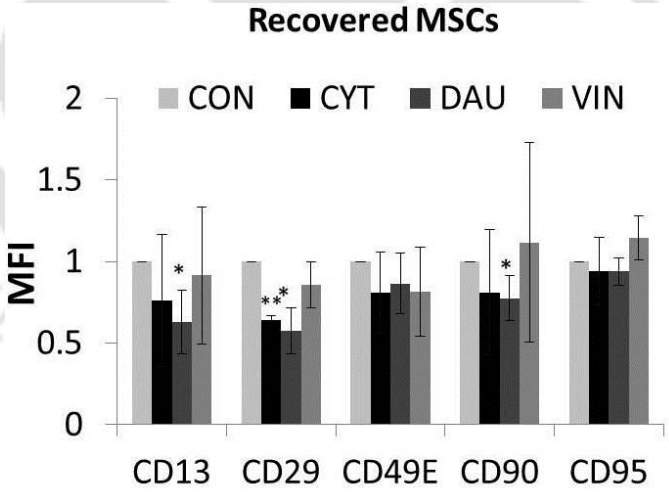
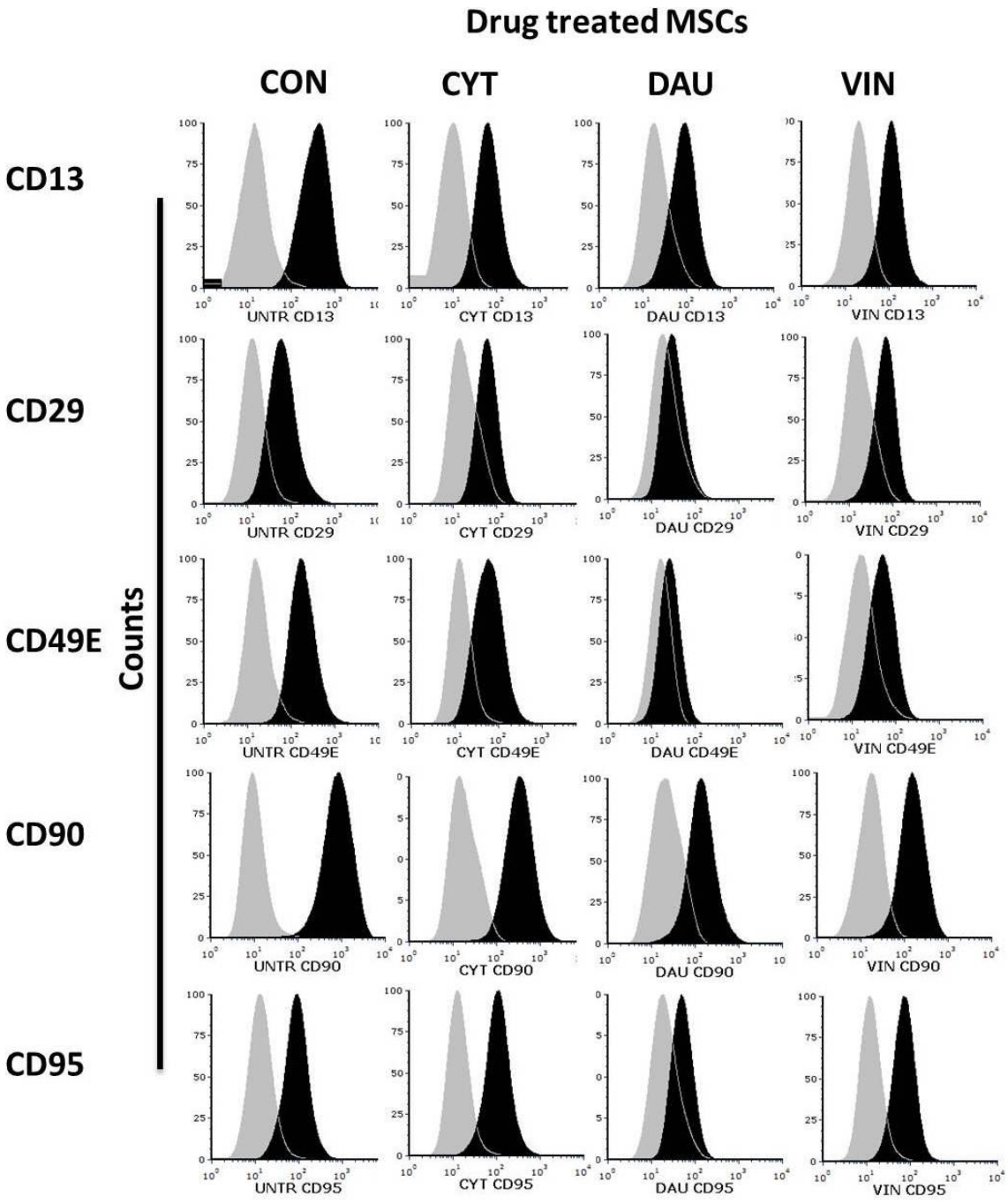


C



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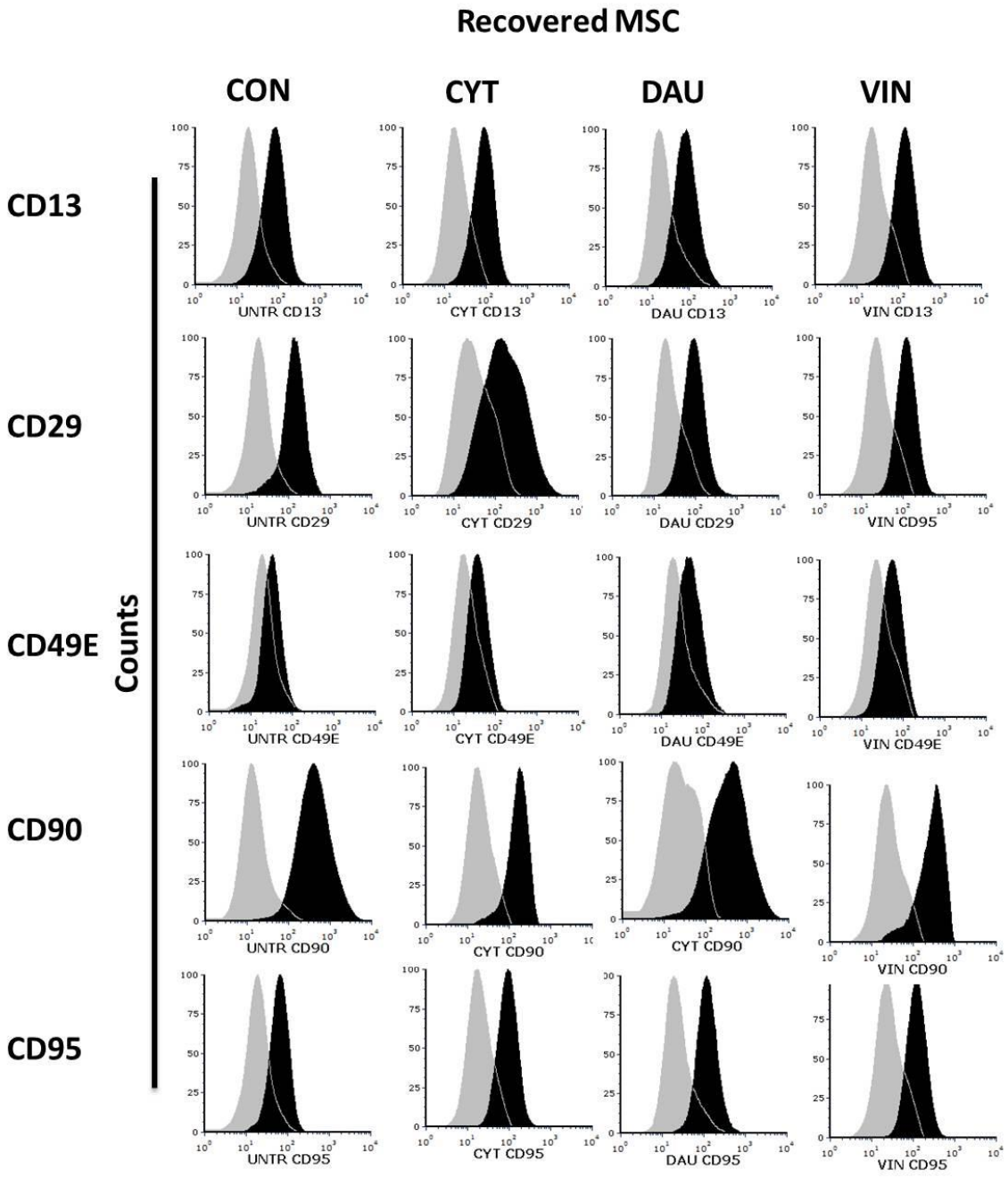


Fig.4.28. Effect of chemotherapeutic drugs on surface marker expression of MSCs. MSCs were screened for the expression of CD13, CD29, CD49E, CD95 and CD90 by flow cytometry (C) **:** MSCs were treated with chemo drugs (CON, CYT, DAU and VIN) (A) and grown in normal media for 1 week (B). Representative histograms of drug treated (C) and recovered MSCs phenotype (D). *p<0.05, **p<0.005. n= 3 samples.

To evaluate the effect of chemo drugs on differentiation potential, MSCs were treated with drugs for 48hr and induced to differentiate into osteocytes. Osteocyte differentiated MSCs were stained with Alizarin Red S (ARS) on 28th day of induction (Fig.4.29B). Significantly reduced osteogenic potential was seen in drug-treated MSCs (Fig.4.29A, B).

To evaluate the long-term effect of chemo drugs treatment on differentiation potential, MSCs were treated with drugs for 48hr and the cells were cultured in normal media without the drug for 1 week. The recovered cells were induced to differentiate into osteocytes by osteogenic induction media. Although drug treated MSCs still showed a significantly reduced osteogenic differentiation compared to the control cells, it was higher than that observed immediately after the drug treatment. Culturing in the normal media helped the MSCs to regain their differentiation potential after CYT and VIN treatment whereas the differentiation potential was still significantly less after DAU treatment (Fig.4.30A, B).

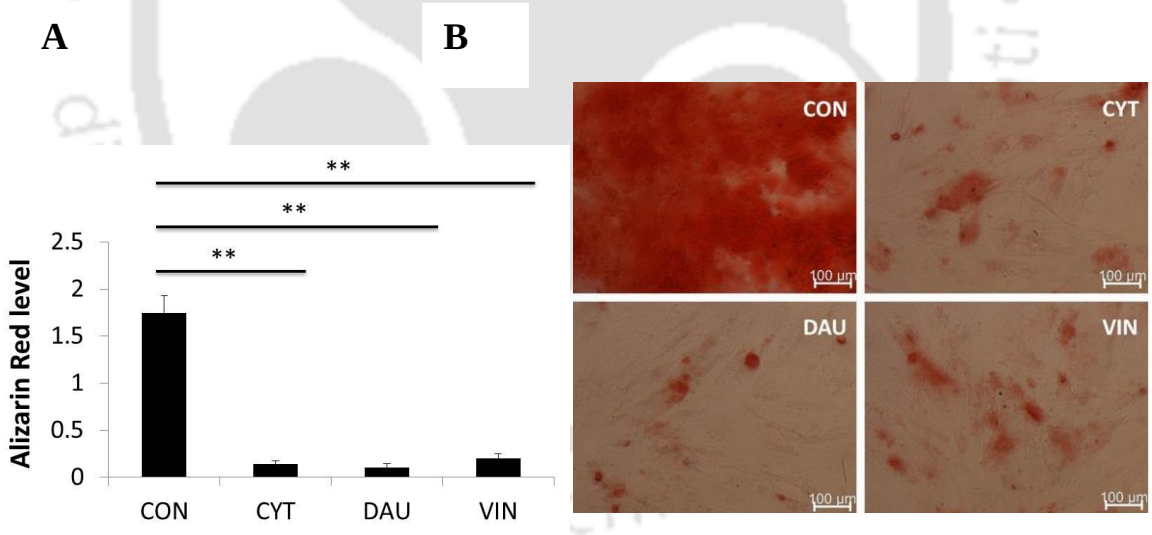


Fig.4.29. Osteogenic differentiation of chemotherapeutic drug treated MSCs. MSCs were treated with chemo drugs (CON, CYT, DAU and VIN) and differentiated into osteocytes. After 21-35 days of induction osteocytes stained for Alizarin Red S (ARS). Quantification of ARS (A). Representative micrographs of ARS stained osteocytes (B). **p<0.005. n= 3 samples.

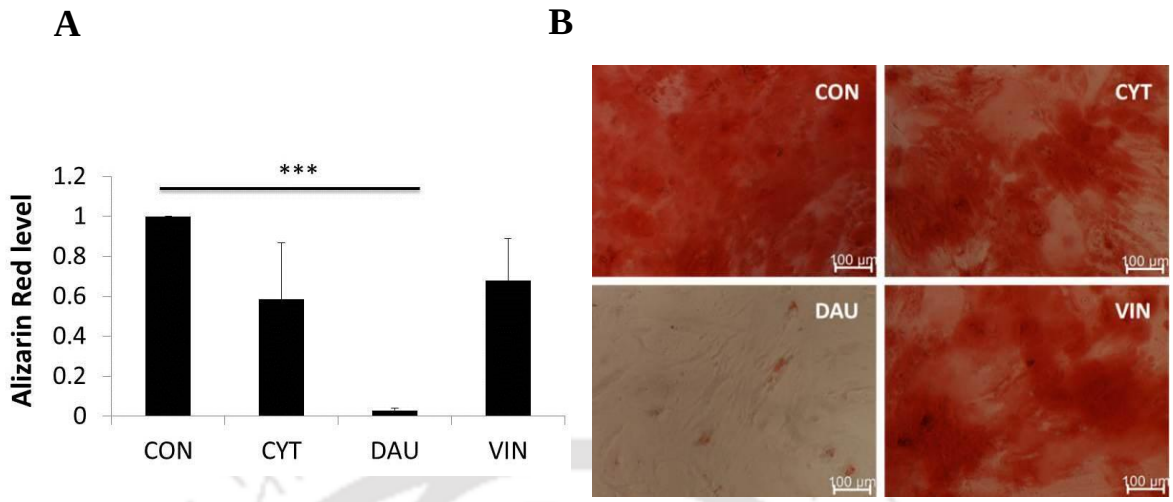


Fig.4.30. Osteogenic differentiation of recovered pretreated MSCs. MSCs were pretreated with chemo drugs (CON, CYT, DAU and VIN) and differentiated into osteocytes. After 21-35 days of induction osteocytes stained for Alizarin Red S (ARS). Quantification of ARS (A). Representative micrographs of ARS stained osteocytes (B). ***p<0.0005. n= 3 samples.

To evaluate the effect of chemo drugs on adipocyte differentiation potential, MSCs were treated with drugs for 48hr and induced to differentiate into adipocytes by adipogenic induction media. Oil Red O (ORO) staining performed on day 28 of adipogenic differentiation showed positive for adipocytes on all the conditions (Fig.4.31C). Drug treated MSCs showed less adipogenicity compared to untreated (Fig.4.31A, B, C).

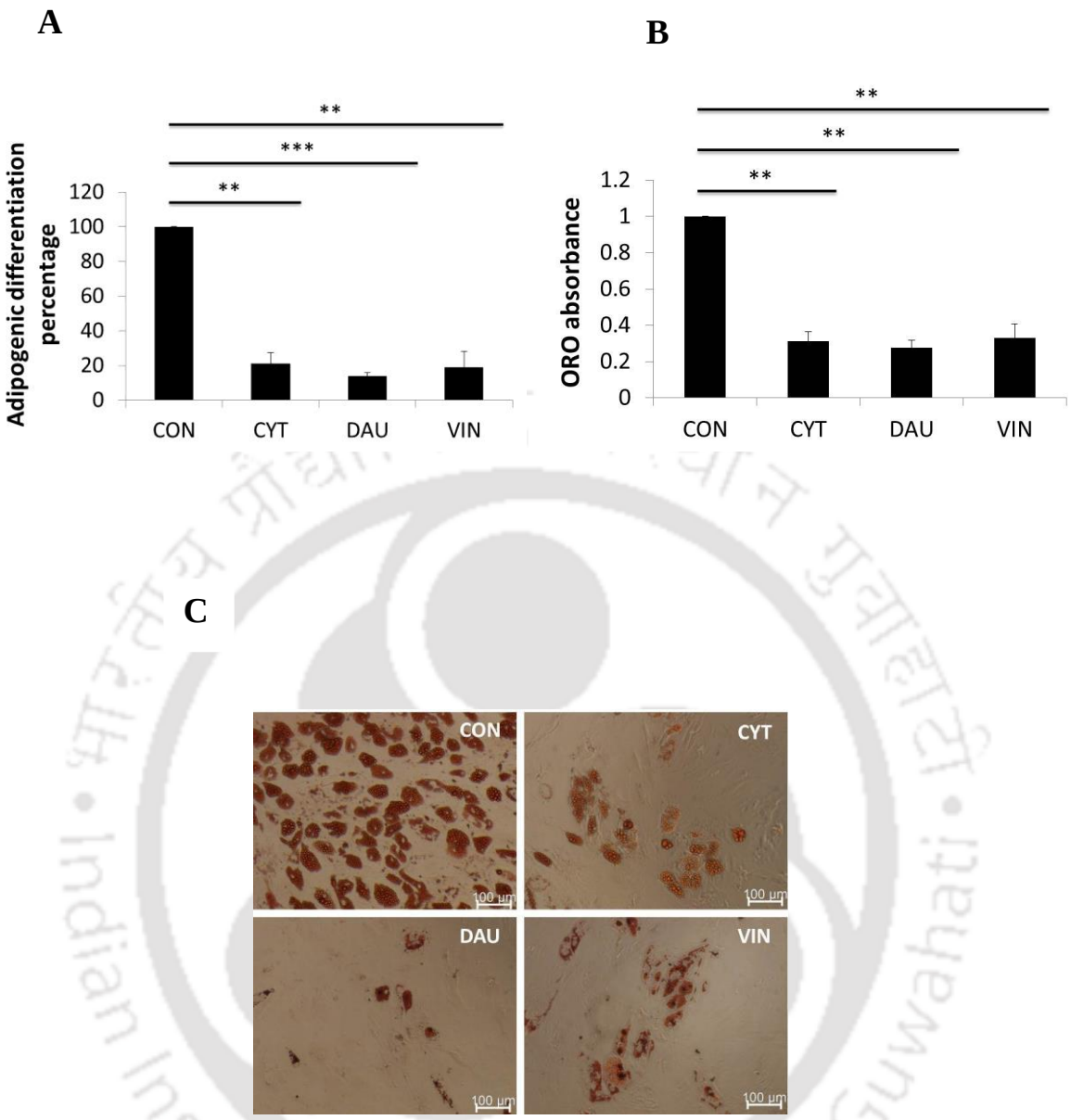


Fig.4.31. Adipogenic differentiation of chemotherapeutic drug treated MSCs. MSCs were treated with chemo drugs (CON, CYT, DAU and VIN) and differentiated into adipocytes. After 21-35 days of induction adipocytes were stained with Oil red O (ORO). Percentage of ORO positive cells (A) and quantification of ORO (B). Representative micrographs of ORO stained adipocytes (C). **p<0.005, ***p<0.0005. n= 3 samples.

Next, MSCs were treated with drugs for 48hr and induced to differentiate into adipocytes after culturing for 1 week in normal media. Similar to the osteogenic differentiation, drug removal resulted in recovery of adipogenic differentiation after CYT and VIN treatment whereas the inhibitory effect of DAU was not reversible. CYT and VIN treated MSCs almost fully recovered their adipogenic differentiation and it was similar to the control untreated cells (Fig.4.32A, B, C).

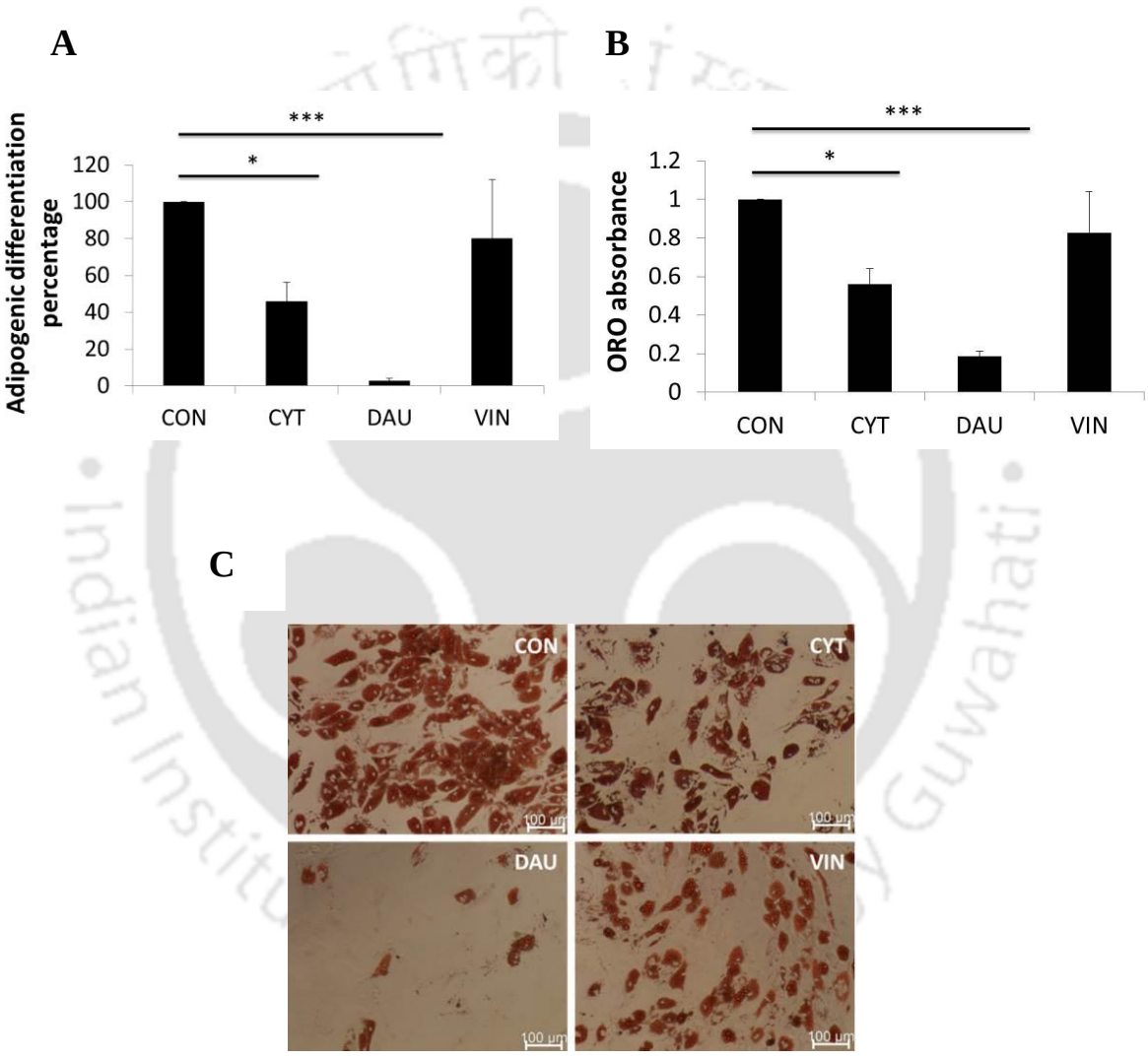
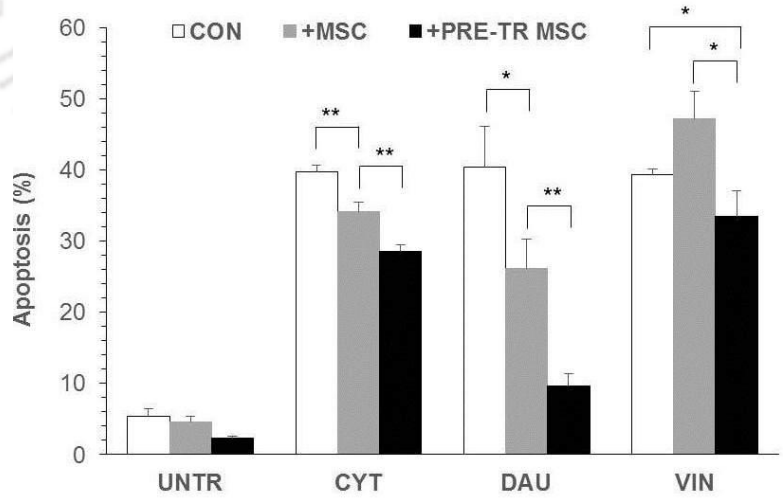


Fig.4.32. Adipoogenic differentiation of recovered pretreated MSCs. MSCs were pretreated with chemo drugs (CON, CYT, DAU and VIN) and differentiated into adipocytes. After 21-35 days of induction adipocytes were stained with Oil red O (ORO). Percentage of ORO positive cells (A) quantification of ORO and (B). Representative micrographs of ORO stained adipocytes (C). *p<0.05, ***p<0.0005. n= 3 samples.

Drug treated MSCs on leukemia cell proliferation and support

Since chemotherapeutic drug treatment affects MSCs properties significantly, we studied whether the changes acquired during chemotherapeutic treatment could affect ability of MSCs to support the leukemic cells. To study the effect of drug treated MSCs on chemo protection of leukemic cells, leukemic cell lines HL60 were co-cultured with control MSCs or CYT, DAU, VIN pre-treated MSCs and treated further with CYT, DAU and VIN and their apoptosis percentage was analyzed. CYT, DAU and VIN treatment induced similar percentages of apoptosis in HL60. Co-culture with MSCs significantly reduced the apoptosis induced by CYT and DAU in HL60 cells (Fig.4.33A, B). VIN induced apoptosis percentage was similar in both control and MSCs co-cultured HL60 cells. However, when HL60 were co-cultured with DAU pre-treated MSCs, there was a significant reduction in the apoptosis percentage compared to control cells or HL60 co-cultured with control MSCs. There was a higher chemo protection offered by DAU treated MSCs to the leukemic cells when they were treated with DAU again (Fig.4.33A, B). Control MSCs or VIN pre-treated MSCs did not chemo protect the leukemic cells from VIN treatment.

A



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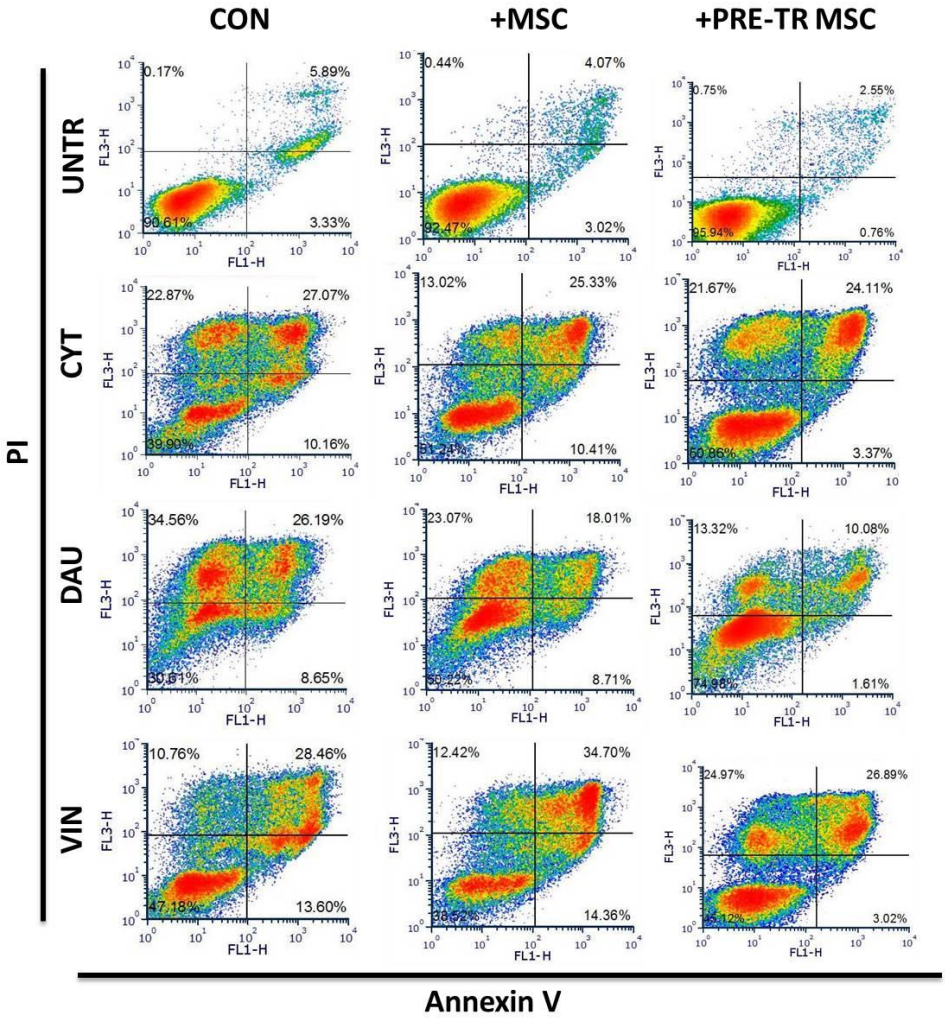


Fig.4.33. Drug treated MSCs on leukemia cell proliferation and support. HL60 was cultured alone (CON) or with MSCs (+MSC) or with pretreated MSCs (+PRE-TR MSC) for 48hr. Leukemic cells were treated with chemo drugs (CYT, DAU and VIN) or left untreated (UNTR) for 48hr. Apoptotic percentage was determined by annexin V (A). Representative flow cytometry dot plots for annexin V and propidium iodide (PI) in HL60 cells representing conditions in A (B). *p<0.05, **p<0.005. n= 3 samples.

Thus, MSCs chemo protected leukemic cells from chemotherapeutic agents and leukemic cells had high survival during chemotherapeutic drug treatment when co cultured with chemotherapeutic drug treated or untreated MSCs.

4.4. Effect of antimitotic drugs on MSCs properties

In some instances, anti-mitototic drugs were combined with other chemotherapeutic agents to treat cancers. So, the effect of anti-mitotic drugs colchicine and nocodazole was tested on MSCs. When MSCs were treated with the colchicine and nocodazole, the spindle shaped morphology changed to flattened shape due to the cell cycle arrest (Fig.4.34).

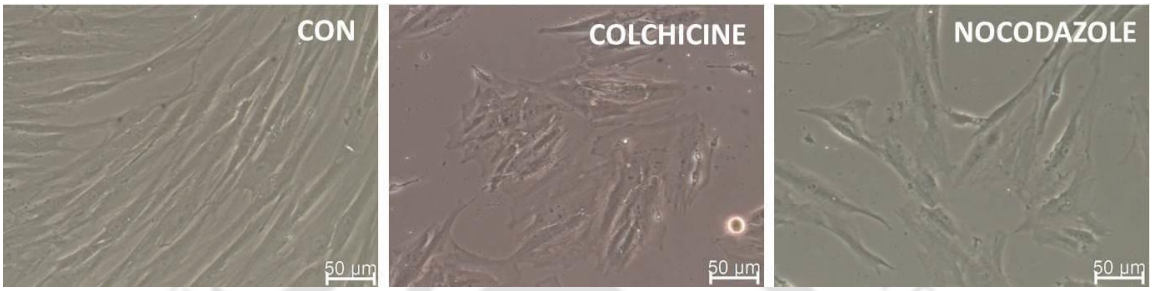
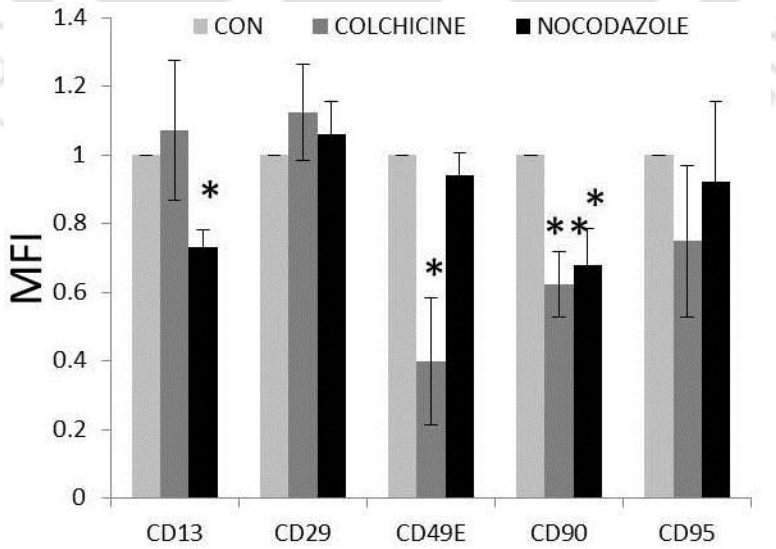


Fig.4.34. Effect of colchicine and nocodazole on MSCs morphology. Microscopic images showing MSCs were treated with colchicine and nocodazolefor 48hr.

CD13, CD29, CD49E, CD90 and CD95 surface marker expression levels were screened for colchicine and nocodazole treated MSCs by flowcytometry. Our results have shown that CD49E and CD90 expression decreased in colchicine treated cells whereas, nocodazole treated cells showed reduction in CD13 and CD90 levels (Fig.4.35A, B).

A



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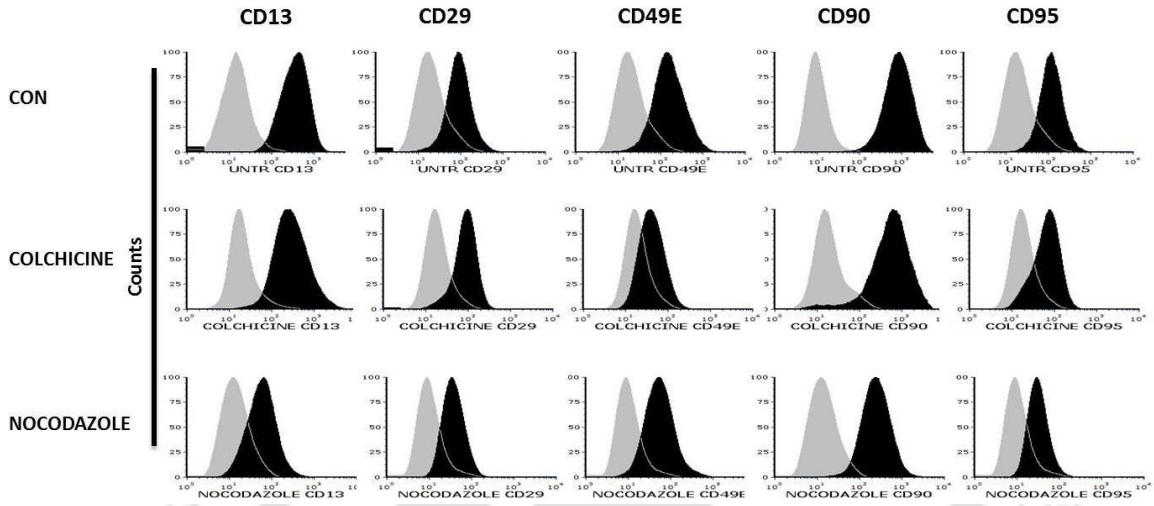

Fig.4.35. Effect of colchicine and nocodazole on MSCs surface marker expression. Histograms of surface markers (A). MSCs were treated with drugs (colchicine and nocodazole) and screened for surface marker expression (B). *p<0.05, **p<0.005. n= 3 samples.

To evaluate the effect of colchicine and nocodazole treatment on MSCs differentiation, we treated MSCs with colchicine and nocodazole during osteogenic differentiation for initial 24hr or 1 week. After treatment, fresh differentiation media (without drugs) were added and incubation was continued for 28 days. Differentiated cells were stained with ARS (for osteocytes) (Fig.4.36B). Here we observed that MSCs showed reduced osteogenic potential on colchicine treatment for 24hr or 1week. Nocodazole treatment for 1 week also showed similar result. No difference was seen after 24hr of nocodazole treatment (Fig.4.36A,B).

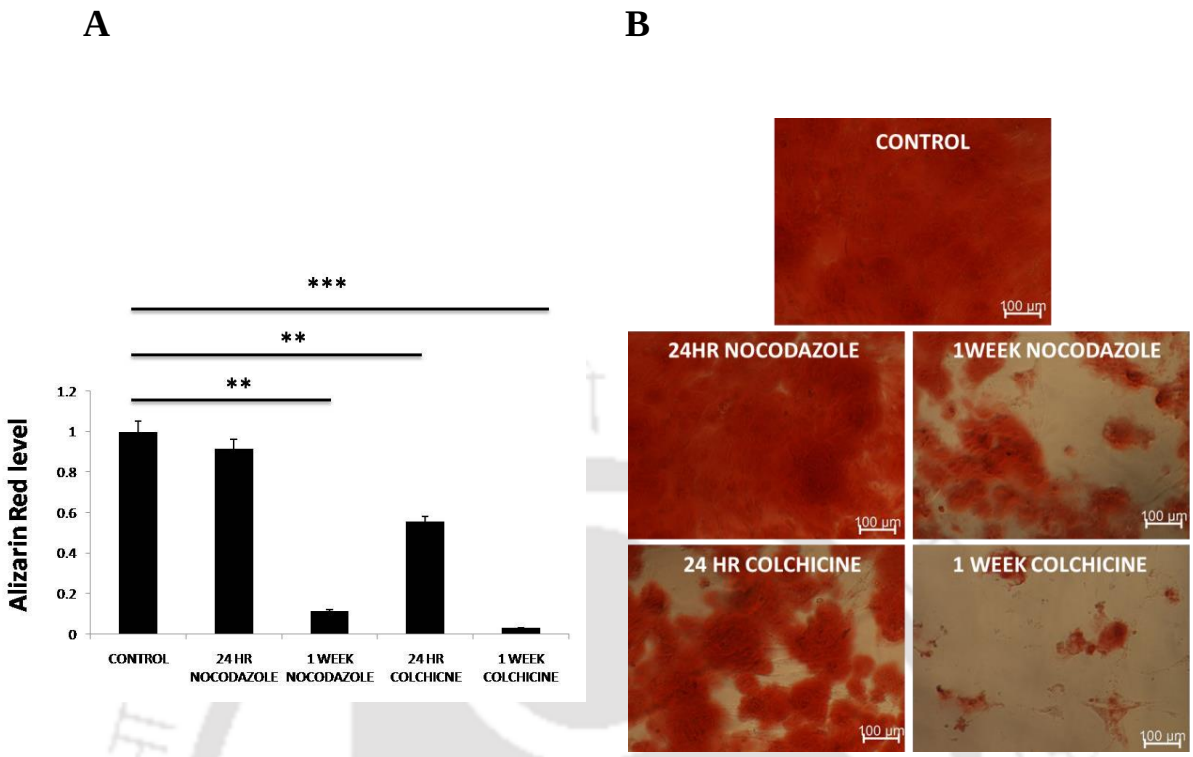

Fig.4.36. Effect of colchicine and nocodazole on MSCs otseogenic differentiation. MSCs were treated with (Nocodazole and Colchicine) and differentiated into osteocytes. After 21-35 days of induction osteocytes were stained with Alizarin Red S (ARS). Quantification of ARS (A). Representative micrographs of ARS stained osteocytes (B). **p<0.005, ***p<0.0005. n= 3samples.

Next, we treated MSCs with colchicine and nocodazole in adipogenic differentiation media for 24hr and 1 week. After treatment, fresh differentiation media (without drugs) were added to the respective MSCs samples and incubation was continued for 28 days. Differentiated cells were stained with ORO (for adipocytes). MSCs showed reduced adipogenic potential on colchicine treatment for 24hr and 1week. Nocodazole treatment for 1 week also showed similar result. No difference was seen after 24hr of nocodazole treatment (Fig.4.37A, B, C).

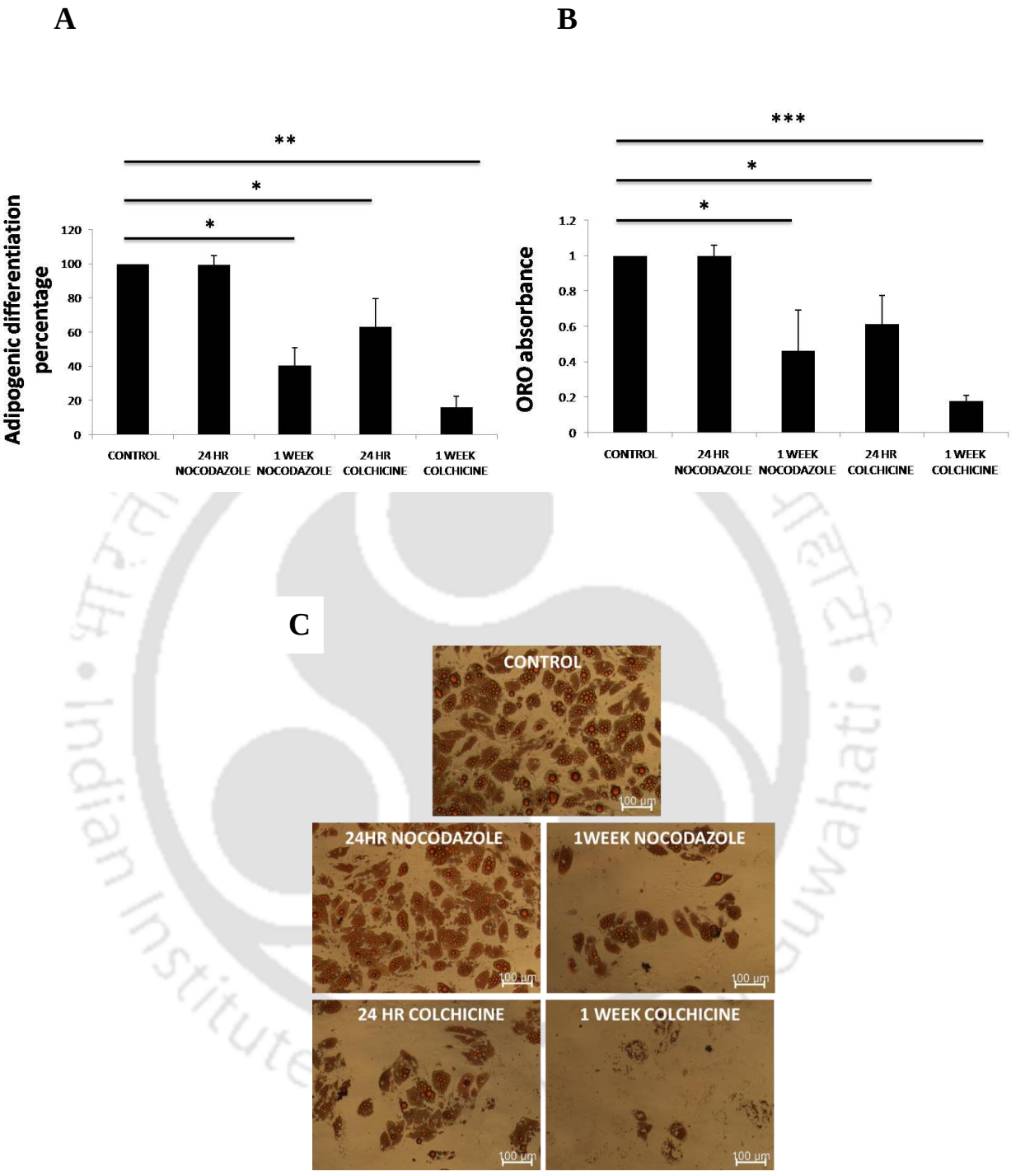

Fig.4.37. Effect of colchicine and nocodazole on MSCs adipogenic differentiation. MSCs were treated with (Nocodazole and Colchicine) and differentiated into adipocytes. After 21-35 days of induction osteocytes were stained with Oil red O (ORO). Percentage of ORO positive cells (B). Quantification of ORO (B). Representative micrographs of ORO stained adipocytes (C). *p<0.05, **p<0.005, ***p<0.0005. n= 3samples.

Thus leukemic cells affected the MSCs cell surface expression and differentiation. Chemotherapeutic drugs and antimitotic drugs had negative effect on MSCs differentiation. However, MSCs recovered their cell surface expression and differentiation potential after removal of chemotherapeutic drugs and thus might be suitable for cell therapy.

Chapter5

Discussions

5. Discussions

5.1. Effect of ECM and RHOA on MSCs characteristics

Mesenchymal stem cells (MSCs) have the potential to differentiate into various types of soft and solid tissues *in vi*tro and are a suitable candidate for cell therapy. Several reports have already demonstrated that MSCs differentiate into specific lineages upon induction (Majumdar *et, al.* 2000; Chun-mao et, al. 2007; Procop. 1997; Pittenger et, al. 1999). In addition to bone marrow (BM), MSCs have been identified in other tissues like umbilical cord blood, umbellical cord matrix, adipose tissues, peripheral blood, post-partum placenta and non-hematopoietic bone marrow (Suva et, al. 2004; Erices et, al. 2000). MSCs isolated from different sources show comparative expression of surface marker expression, proliferation, differentiation protein and gene expression (Majumdar et, al. 1998; Banfi et, al. 2010; Wanger et, al. 2005). MSCs are commonly isolated from the mixture of cells by adherence selection. Isolation of MSCs depends on the tissue availability, donor age, tissue of origin, *ex vivo* isolation and expansion method (Wanger et, al. 2005; Kretlow et, al. 2008). Furthermore, *in vitro* expansion of MSCs for several passages is associated with loss of proliferation and differentiation potential (Kretlow et, al. 2008). In order to retain stemness, cell interaction with associated extracellular matrix (ECM) proteins during *in vitro* maintenance of MSCs is important (Kalpan et, al. 2002; Volloch et, al. 2002).

The role of ECM proteins in cell adhesion, proliferation and differentiation has been well studied (Reilly et, al. 2010). This is the first detailed study about cell adhesion, survival, proliferation, actin cytoskeleton modification, migration, differentiation, RHOA gene expression and mitochondrial distribution of MSCs on different ECM proteins. COL, FBN and PLL are among the ECM proteins used for our studies.

Adherent cells to perform its functions have to attach to a particular substrate. The cell or tissue architecture is dependent on the adhesion mechanisms triggered by cell-cell and cell-ECM interactions. Most of the muscle tissues in well-organized animals are surrounded by ECM proteins composed of COL, proteoglycans and adhesive matrix proteins. Reports have shown that attachment of cells to matrix proteins down-regulates integrin molecules on surface of the cells. HSCs adhesion to the ECM protein FBN, which is secreted by stromal cells is required for the proliferation and differentiation.

Earlier reports have shown that biomaterials which were coated with COL increase cell adhesion (Munisamy et, al. 2008). Higher cell adhesion and spreading was observed in cells which were cultured on ECM proteins like FBN, vitronectin and laminin (Wittmer et, al. 2007; Carlsson et, al. 1981).

In our study, in order to identify the suitable scaffold for cell therapy and tissue engineering, regulation of MSCs properties by ECM was analyzed. To find out the surface which promotes rapid and more adhesion strength, maximum adhesion was observed within 2hr on COL, PLL and control surface and no further increase in cell adhesion was observed until 12hr low area cell adhesion percentage significantly increased on FBN from 2hr to 12 hr.

During cell therapy, MSCs enter into different microenvironment and are exposed to stressful conditions (oxygen and nutrient deprivation). MSCs injected into injured or inflammatory regions, have to survive and perform their effective function. Earlier reports have suggested that cell-ECM interaction increase the cell survival and reduces the anoikis process (Fiona M. Watt et, al. 2011).

To mimic the oxidative stress and serum starvation, cells were treated with H_2O_2 in serum starved conditions. Very minimal cell death was observed on COL grown MSCs (1%) and compared to CON (17%), FBN (14%) and PLL (15%) cultured MSCs during 400 μM H_2O_2 in 0.1% serum starved conditions for 3hr. Serum starvation is known to induce the ROS production and significant increase in mitosox positive cells was observed on PLL (57.5%) cultured cells. We hypothesized that inflammatory cytokine may be involved in cell survival and significantly increased IL6 (2.2 fold) found on cells cultured on CON compared to other matrices.

Cell proliferation is essential for *in vitro* expansion of cells during tissue engineering applications. Since, the number of MSCs isolated from BM and other sources are very small, MSCs expansion *in vitro* for is necessary for clinical applications. It has been reported that MSCs cultured on laminin showed lower proliferation compared to COL and FBN when cultured in different serum conditions (Ode A et, al. 2010). Higher proliferation of fibroblasts was reported on ECM proteins like COL and FBN (Tipton DA et, al. 1997).

In order to identify the suitable matrix for expansion, MSCs were cultured on COL, FBN, PLL and tissue cultured treated plastic (CON) surfaces. COL grown MSCs showed less time to double (30hr), whereas more doubling time was seen on CON (47hr), FBN (40hr) and PLL (44hr) cultured cells. This was further confirmed when high cell viability was seen on COL grown MSCs in compared to others. In addition, cell cycle analysis showed high percentage of cells in S phase of the cell cycle in COL (20 %) compared to CON (11%), FBN (13%), and PLL (12%) cultured cells. Even though PLL surface showed high cell attachment it also showed a high percentage of ROS production and high oxidative stress. From our study, we can conclude that MSCs cultured on COL showed high cell adhesion, high survival and more proliferation capacity, hence can be used as a coating on scaffold for in vivo administration to protect MSCs from oxidative and nutrient stress.

Actin cytoskeleton plays an important role in regulating cellular properties. It responds to external stimuli and shows its action by modulating cell shape. ECM regulates cell migration by altering the cytoskeleton structure.

Focal adhesions formed by integrin cluster and associated complexes communicates between cells and ECM (Grinnell F. 1994; Burridge K et, al. 1996; Tomasek, J. J. et, al. 2002). Cell contact points are the regions where cells interact with ECM molecules via integrins or adapter proteins and activate the signaling pathways linked to actin cytoskeleton. Topographical studies suggested that MSCs differentiate into osteogenic cells by increasing the formation of contact points (Biggs MJ et, al. 2009). Activation of FAK promotes cell contact points formation and increased osteogenic potential were reported in human osteoblast like cells on titanium substrates and hydroxyapatite (Keselowsky BG et, al. 2007; Okumura A et, al. 2001). It has also been shown that extracellular signal-related kinase 1 and 2 (ERK 1/2) pathway also promotes osteogenesis by increasing the cell contact points of MSCs cultured on hydroxyapatite surface (Lin L et, al. 2009). Runt-related transcription factor (Runx2) also directly regulates the osteogenesis of MSCs by formation of cell contact points (Salasznyk RM et, al. 2007). MSCs derived from adipose tissue which were cultured on nano pillars also suggested that increase in contact points formation directly correlated with osteogenic potential (Hye Sung Kima et, al. 2015). Other studies have also reported that increased RHOA activity promoted the formation of contact points and switch towards osteogenic potential in MSCs (McBeath R et, al. 2004). MSCs grown on COL, PLL and FBN shows

many contacts points but in case of tissue cultured surface very few contact points were present. Which also correlated with their osteogenic differentiation in our study.

Cell migration is essential for tissue repair, regeneration, tissue engineering and diseases. Silencing of FAK leads to inhibition of cell contact points resulting in decreased MSCs migration under fluid flow conditions (Riehl BD et, al. 2014). Also formation of contact points increases the migration potential of keratinocytes and endothelial cells (Gates RE et, al. 1994; Song Li et, al. 2001). Thus in our study, where high number of contact points were observed on COL, the cells also migrated faster.

Cells interact with ECM proteins with the help of cell surface receptors and regulate migration and differentiation. Interaction is possible, between ECM and surface receptors. Multiple ECM proteins were found to interact with single cell surface receptor (α5β3 can bind to COL, FBN and vitronectin) or single ECM can interact with multiple receptors (FBN can interact with α5β1 and α5β3) (Miranti C.K. et, al. 2003). Reports have suggested that integrin regulates the OCN gene expression in embryonic chicken calvaria osteoblastic cells when cultured on different ECM proteins (Carvalho RS et, al. 2003). Osteo differentiation of MSCs isolated from dental bud also found to be induced by integrins when cultured on ECM proteins (Di Benedetto A et, al. 2005).

Activation of integrin αvβ3 led to increase in leucocyte cell migration (Kiosses WB et, al. 2001). It has also been reported that activation of protein kinase A by integrin's also induce the cell migration (Lim CJ et, al. 2008). We observed increased CD49E levels in MSCs cultured on COL surface, which is known to be involved in migration of hematopoietic stem cells (Sahin AO et, al. 2012). It has also been reported that increased CD49E levels correlated with the high osteogenic potential (Hayakawa K et, al. 2013). Also, studies have reported that inhibition of CD49E expression leads to reduced cell adhesion potential in cells (Linhares-Lacerda L et, al. 2011).

Stem cells have the ability to differentiate into particular lineages. In order to improve the differentiation capacity for tissue repair applications, MSCs were subjected to differentiation on different ECM. Cells gorwn on CON tissue culture treated surface showed 2.6 fold increases in *PPARgamma* (PPARG) an adipocytes lineage commitment marker compared to other ECM suggesting a pre-commitement to adipocyte lineage. This correlated with high adipogenic differentiation on CON surface. COL treated plate showed highest calcium deposition during osteogenic differentiation compared to other

ECM proteins FBN and PLL. CON surface showed least calcium deposition than all the ECM.

To achieve the better performance, modification of ECM surface by cross linking with other ECM proteins was done. Modification might change the biophysical properties and stiffness of the ECM scaffolds. Earlier reports have shown that changes in the biophysical properties of the ECM scaffold regulate the lineage commitment of the stem cells (K. Saha et, al. 2008). Modification of ECM protein led to increase in neuronal differentiation by altering the biophysical and structural properties of the scaffold. (Sébastien Sart et, al. 2016). Also, synergic effect was reported between FBN and laminin on osteogenic differentiation compared to MSCs differentiated individually on FBN or laminin surfaces (Fan Yang et, al. 2010).

In order to understand the molecular mechanism, we cultured and expanded MSCs on CON surface and allowed them to grow on COL, FBN and PLL for a particular time period. Significant differences in osteogenic differentiation was seen when MSCs switched from other matrix to COL surface. COL promoted high osteogenic differentiation. However, no difference was seen in adipogenic potential during ECM switching.

In addition, COL grown MSCs showed significant increase in RHOA transcript levels and high RHOA activity compared to others. RHOA pathway regulates cell proliferation, cytoskeletal changes and stress fibers formation (Maekawa M et, al. 1999; Arnsdorf EJ et, al. 2009; Pellegrin S et, al. 2007). Earlier studies have also demonstrated that RHO GTPases regulate the cell shape and differentiation of MSCs into cartilage, adipocytes and osteocytes (Arnsdorf EJ et, al. 2009). RHO GTPases are also linked with integrins and cell junctions (Chiquet M et, al. 2009).

Genetically modified MSCs with high RHOA activation led to high osteogenic differentiation but low adipogenic differentiation as reported by others. Increased actin polymerization with criss cross arrangement of actin fibers were observed in RHOAV14 MSCs. In comparision, parallel arrangements of actin fibers were observed in control MSCs.

According to previous reports, RHOA member of the RHO GTPase family (Etienne-Manneville et, al. 2002; Hall et, al. 2002), is a key player in actin cytoskeleton

modification, it regulates stress fibers and stabilizes actin cytoskeleton (Darling et, al. 2008). RHOA is also involved in the focal adhesion formation by integrin cluster and associated molecules (Darling et, al. 2008). Various reports demonstrated that RHOA plays an important role in cell migration, reduced migration was seen in MSCs by RHOA inhibition (Lee et, al. 2008; Raheja et, al. 2011). Also overexpression of RHOA led to reduced migration (Jaganathan et, al. 2007). While higher expression of RHOA has been correlated with high osteogenic and lower adipogenic potential (McBeath et, al. 2004).

Mitochondria play an important role in physiological properties of the cells. Mitochondrial distribution in the cells is very important to distinguish between differentiated and undifferentiated state. The active mitochondria distribution in MSCs cultured on ECM were identified by TMRE staining. MSCs grown on COL majorly showed perinuclear distribution whereas the cells cultured on PLL and CON surface showed high cytoplasmic distribution.

Although the matrix rigidity and elasticity were not tested in our study, the results show that differential mechano-sensing and signaling was promoted in cells cultured on different matrices. Other studies have shown the usefulness of COL and FBN as suitable extracellular matrix for osteogenic differentiation of MSCs (Bernhardt A et, al. 2008; Huang Y et, al. 2013; Wang J et, al. 2012; Zhang WH et, al. 2006; Linsley C et, al. 2012; Thibault MM et, al. 2007; Kang Y et, al. 2015). The stiffer matrix which mimics muscle cells induced MSCs into myocytes like cells (Garcia AJ et, al. 2005). Other reports on stiffer matrix which mimic crosslinking of COL promoted osteoblast like cells (Yarlagadda P et, al. 2005). Stiffness of ECM was also shown to guide the differentiation of MSCs into osteoblasts, skeletal and neuronal lineages (Engler AJ et, al. 2006). Micro patterned islands regulate the stem cells fate. Especially, cell spreading favoured osteogenesis and rounding promoted adipogenesis (McBeath et, al. 2004). Further investigation showed that, geometry of ECM also regulates differentiation capacity (Kilian et, al. 2012). Elasticity of ECM decides the lineage commitment; stiff matrix favours osteogenesis, whereas soft substrates favour adipogenesis (Engler AJ et, al. 2006). Substrate modulus direct the neuronal differentiation, softer gel promotes neuronal, whereas stiffer gels favors glial cells differentiation in the presence of serum.

The ability of progenitor cells to attach to a scaffold surface during early stages of differentiation is important in the development of new tissue structures. The initial contact of the cells with the implant surface is an important event for adhesion of the bone cells. It influences osteointegration and involves the precoating of implants with ECM protein. This precoating enables cell-ECM interactions and the use of growth factors facilitates the differentiation of osteoblasts.

According to our results we can recommended that COL might be used as a matrix protein for *ex vivo* expansion of MSCs for maintaining osteogenic differentiation potential. It can be used as a coating on scaffold for in vivo transplantation for supporting self-renewal, cell adhesion, migration and survival during oxidative and nutrient stress conditions.

5.2. Effect of leukemic cells and chemotherapeutic drugs on MSCs characteristics

MSCs play an important role in maintenance and support of hematopoiesis in bone marrow microenvironment (Majumdaret et, al. 1998, 2000). Qualitative and quantitave damages to MSCs due to chemotherapy reduces engraftment of HSCs (Schwartzet et, al. 1998; Banfiet et, al. 2001). After HSCs transplantation, damages to stromal cell population could not be repaired by donor MSCs (Devine et, al. 2000). Even though sufficient number (up to $10 \bullet 10^6$cells/kg) of cells were injected, it failed to repopulate allogeneic MSCs in the recipient system (Kocet et, al, 2002). Several reports have suggested that enhancing capacity of MSCs enhance hematopoietic engraftments in recipient (Fibbe et, al. 2003).

Apart from this, our next aim was to find out, whether MSCs from leukemia patients can be used for therapeutic purposes. Our co-culture studies demonstrated a decrease in cell surface expression of CD73 and CD90 in MSCs co -cultured with primary AML leukemic cells. Furthermore, we evaluated the effect of leukemic cells on MSCs differentiation potential. Our results demonstrated that decrease in adipogenesis was seen in MSCs co cultured with primary leukemic cells and leukemia cell line HL60. Several reports suggested that stromal cell functions were altered by leukemic cells (Krevvata M et, al. 2014). Human and murine models also demonstrated that MSCs properties were modulated by leukemic cells (Huan J et,al. 2015). Studies also reported that healthy

MSCs proliferation and differentiation potential of healthy MSCs were altered during treatment with condition media derived from leukemic cell lines (Geyh et, al. 2015).

Cytotoxic damages were seen in MSCs, after chemotherapeutic drug treatment (Li J et, al. 2004; Domenechet et, al. 1998). Animal studies have also shown that CYT causes damages to stromal cell function (Ben Ishay et, al. 2001) and irreversible damages were seen with chemotherapeutic agents (Carlo-Stella et, al. 1997).

MSCs play an important role in the bone marrow during hematopoiesis (Majumdar et, al. 1998; Divine et, al. 2000; Majumdar et, al. 2000). Conflicts in results regarding sensitivity of MSCs towards chemotherapeutic drugs exists and it is essential to understand the exact role of MSCs during drug treatment (Nicolay NH et, al. 2013; Nicolay NH et, al. 2014; Nicolay NH et, al. 2016; Jing Li et, al. 2011; Wei Liang et, al. 2011). During chemotherapy, a combination of two or three drugs is used, but the effect of individual chemo drug is not clear. We evaluated the effect of individual drugs on MSCs *in vitro* (CYT, DAU and VIN) at clinically relevant concentrations. The MSCs were sensitive to the drugs and showed suppression of proliferation after treatment with CYT, DAU and VIN for 48hr. We also determined whether the reduction in proliferation by chemo drugs was due to apoptosis since prior reports have shown that reduced cell proliferation was seen in CYT, DAU and VIN treated cells (Li J et, al. 2004; Wei Liang et, al. 2011; Li J et, al. 2010). Using AnnexinV staining, our data suggested that chemo drugs can induce apoptosis in MSCs. The MSCs treated with chemo drugs showed a change in surface marker expression. The decrease in CD13 and CD49E expression was seen in MSCs treated with CYT. Furthermore, DAU treated MSCs showed reduced expression of CD13, CD29, CD49E, CD90 and CD95. Earlier reports show that MSCs treated with doxorubicin showed reduced CD73 and CD90 cell surface expression (Maira Souza Oliveira et, al. 2014). However, CD13, CD49E, and CD90 cell surface expression decreased in VIN treated MSCs. The effects of chemo drugs on adipo and osteogenic differentiation potential of MSCs were also determined. Our study showed decrease in adipogenic and osteogenic potential in CYT, DAU and VIN treated MSCs. Previous reports indicated a decrease in MSCs number (Galotto et, al. 1999) during chemotherapy and a dose-dependent toxicity to bone marrow osteo progenitors and bone marrow stromal cells (A Banfi et, al. 2001; Francis Corazza et, al. 2004).

In addition, our results show that the cells pretreated with DAU have decreased CD13 and CD29 expression. However, complete recovery was seen in surface marker expression of VIN pretreated MSCs. MSCs recovered from CYT and VIN showed normal differentiation potential whereas MSCs recovered from DAU had low differentiation potential. Earlier studies reported slow recovery in CYT treated cells, whereas fast recovery was seen in VIN pretreated cells (Li J et, al. 2004; Wei Liang et, al. 2011).

MSCs are sensitive to chemotherapeutic agents (Castells M et, al. 2013; Bruno Corrêa Bellagamba et, al. 2016). But once the drug was removed from the culture, most of the studies showed that MSCs regained all its properties (Bruno Corrêa Bellagamba et, al. 2016; Li J et, al. 2004). Adipose derived MSCs recovered from chemotherapeutic agents showed that normal morphology, proliferation, surface marker expression and normal differentiation capacity (Mueller LP et, al. 2006).

MSCs isolated from a chemotherapy treated individual can be used for autologous cell transplantation. Damages to MSCs including alterations in properties like surface marker expression, differentiation and proliferation potential occured during DAU treatment and even after the cells were recovered from DAU treatment. Though, MSCs treated with CYT showed damages in proliferation, differentiation and surface marker expression during treatment, once the drug was removed, they showed normal differentiation potential and such MSCs can be used for cell therapy. MSCs treated with VIN showed normal spindle shape with increased cell survival, and with restored surface marker and differentiation potential after removal of the drug and thus can be used for cell therapy.

Earlier studies also showed that VIN treatment induces morphological changes in MCF-7 cells but the cells recovered when cells were cultured in drug free media. Also, studies on HL60 treated with VIN, showed damage in cell morphology and recovery once drug was removed from the media. The reason is a VIN binds directly to the microtubules and does neither form any complex nor form any copolymerization to form microtubules. Thus VIN binding to microtubule is a reversible process.

We also studied the effect of antimitotic drugs, colchicine and nocodazole on MSCs. Both drugs are known to depolymerize the microtubules in fibroblasts (Stargell LA et, al. 1992), P388D1macrophages, human polymorpho nuclear leucocytes (Michael Kuhn. 1998; H.U.Keller et, al. 1989), oviduct (EB-Ulrich et, al. 1989) and inhibit microtubule

synthesis. In our studies, we demonstrated that effect on differentiation potential of MSCs exposed to antimitotic drugs is time and dose dependent. The 24hr and 1-week colchicine treated MSCs showed decrease in adipose and osteogenic potential. 24hr colchicine treatment was sufficient to reduce differentiation capacity of MSCs. 24hr nocodazole-treated MSCs when removed from the drug, which showed normal differentiation. It correlates with the earlier report, which showed that colchicine was still bound to the mammalian KB cells even after the drug was removed from the media. The metaphase blockage by colchicine was irreversible. Most of the cells blocked in metaphase did not recover or regenerate spindle fibers when the external colchicine was removed from the media. Bound form of colchicine was present in the cells and the slow release took place when cells were culture in colchicine free media (Edwin W. 1965). Neverthless, treatment with of nocodazole for 1 week showed decrease in differentiation capacity of MSCs. Our results suggest that, in contrast to colchicine, nocodazole drug treatment was a reversible process; earlier reports have also demonstrated that nocodazole action on microtubule was reversible in contrary to colchicine treatment (De Brabander Me et, al. 1980; Zeive G.W et, al. 1981; H.U.Keller et, al. 1989).

Colchicine binds to beta subunit of the colchicine domain of microtubules. Colchicine in free form does not bind directly to microtubules. It first binds tubulin, which in soluble form induces conformational changes and form weak, reversible tubulin-colchicine complexes then copolymerize into the ends of the microtubules from small to high numbers. The complex becomes very tight and reduces the dissociation of tubules from the complex.

Thus, we can conclude that MSCs isolated from patients who underwent chemotherapy treatment with VIN and CYT can be used for autologous transplantation.

Chapter6

Conclusions

6. Conclusions

In conclusion, our study demonstrates that extracellular matrices induce differential cell behavior of MSCs by altering their cell proliferation, migration and differentiation. MSCs can be pre-differentiated on COL to obtain high number of osteocytes or on uncoated tissue culture treated plastic to obtain adipocytes based on the therapy requirement. Increased proliferation can be achieved by coating the cell growth surface with COL and when used in tissue engineering, COL coated scaffolds will also promote high cell migration, proliferation, survival and osteogenic differentiation. In addition, leukemic cells and chemotherapeutic treatment affect the bone marrow MSCs. However, MSCs recovered their cell surface expression and differentiation potential after removal of chemotherapeutic drugs and thus might be suitable for cell therapy.

Chapter7

References

A John Barrett, and Minoo Battiwalla. Relapse after allogeneic stem cell transplantation. Expert Rev Hematol. 2010. 3: 429–441.

A Koussounadis, S P Langdon, D J Harrison, and V A Smith. Chemotherapy-induced dynamic gene expression changes in vivo are prognostic in ovarian cancer. *Br J Cancer*. 2014. 110: 2975–2984.

Adams G.B, Chabner K.T, Alley I.R, Olson D.P, Szczepiorkowski Z.M, Poznansky M.C, Kos C.H, Pollak M.R, Brown E.M, Scadden D.T. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*. 2006. 439: 599–603.

Ahmed Talebian, Razieh Moazam Goudarzi, Mahdi Mohammadzadeh, and Azadeh Sadat Mirzadeh. Vincristine-Induces Cranial Neuropathy. *Iran J Child Neueol*. 2014. 8: 88-68.

Alice E. Chen and Douglas A. Melton. Derivation of Human Embryonic Stem Cells by Immunosurgery. *J Vis Exp*. 2007. 10: 574.

Almeida-Porada G, Flake AW, Glimp HA, and Zanjani ED. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. *Exp Hematol.* 1999. 27: 1569–1575.

Alsberg E, von Recum HA, and Mahoney MJ. Environmental cues to guide stem cell fate decision for tissue engineering applications. *Expert Opin Biol Ther.* 2006. 6: 847-866.

Annette K. Brenner, Ina Nepstad,and Øystein Bruserud. Mesenchymal Stem Cells Support Survival and Proliferation of Primary Human Acute Myeloid Leukemia Cells through Heterogeneous Molecular Mechanisms. *Front Immunol.* 2017. 8: 106.

April L. Blajeski, Vy A. Phan, Timothy J. Kottke, and Scott H. Kaufmann. G1 and G2 cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *J Clin Invest.* 2002. 110: 91–99.

Arielle Glatman Zaretsky, Julie B. Engiles, and Christopher A. Hunter. Infection-induced changes in hematopoiesis. *J Immunol.* 2014. 192.

Arnsdorf EJ, Tummala P, Kwon RY, Jacobs CR. Mechanically induced osteogenic differentiation--the role of RhoA, ROCKII and cytoskeletal dynamics. *J Cell Sci.* 2009. 122: 546-53.

Aryan HE, Meltzer HS, Lu DC, Ozgur BM, Levy ML, Bruce DA. Management of pilocytic astrocytoma with diffuse leptomeningeal spread: two cases and review of the literature. *Childs Nerv Syst*. 2005. 21: 477-81.

Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells.* 2007. 25: 1384-92.

Ball LM, Bernardo ME, Locatelli F, and Egeler RM. Potential role of mesenchymal stromal cells in pediatric hematopoietic SCT. *Bone Marrow Transplant.* 2008. 42: S60–S66.

Banfi, A., Podesta, M., Fazzuoli, L., Sertoli, M.R., Venturini, M., Santini, G., Cancedda, R. & Quarto, R. High-dose chemotherapy shows a dose-dependent toxicity to bone marrowosteoprogenitors: a mechanism for post-bone marrow transplanta-tion osteopenia.*Cancer*. 2001. 92: 2419–2428.

Barletta JP, Fanous MM, Margo CE. Corneal and conjunctival toxicity with low-dose cytosine arabinoside. *Am J Ophthalmol.* 1992. 113: 587-8.

Bashir J, Sherman A, Lee H, Kaplan L, Hare JM. Mesenchymal stem cell therapies in the treatment of musculoskeletal diseases. *PM R.* 2014.6: 61-9

Ben-Ishay Z, Barak V. Bone marrow stromal dysfunction in mice administered cytosine arabinoside. *Eur J Haematol.* 2001. 230-7.

Benoit Biteau, Christine E. Hochmuth, and Heinrich Jasper. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. *Cell Stem Cell.* 2011. 9: 402–411.

Berenika Plusa and Anna-Katerina Hadjantonakis. Embryonic stem cell identity grounded in the embryo. *Nat Cell Biol.* 2014. 16: 502–504.

Bernard Lo and Lindsay Parham. Ethical Issues in Stem Cell Research. *Endocr Rev.* 2009. 30: 204–213.

Bernhardt A, Lode A, Boxberger S, Pompe W, Gelinsky M. Mineralised collagen—an artificial, extracellular bone matrix—improves osteogenic differentiation of bone marrow stromal cells. *Journal of Materials Science-Materials in Medicine*. 2008. 19: 269–75.

Boisvieux-Ulrich E, Lainé MC, Sandoz D. In vitro effects of colchicine and nocodazole on ciliogenesis in quail oviduct. *Biol Cell.* 1989. 67: 67-79.

Boles JC, Williams JC, Hollingsworth RM, Wang JG, Glover SL, Owens AP 3rd, Barcel DA, Kasthuri RS, Key NS, Mackman N. Anthracycline treatment of the human monocytic leukemia cell line THP-1 increases phosphatidylserine exposure and tissue factor activity. *Thromb Res.* 2012. 129: 197-203.

Bruno Corrêa Bellagamba, Bianca Regina Ribas de Abreu, Ivana Grivicich, Carolina Franke Markarian, Eduardo Chem, Melissa Camassola, Nance Beyer Nardi, and Rafael Rodrigues Dihl. Human mesenchymal stem cells are resistant to cytotoxic and genotoxic effects of cisplatin *in vitro. Genet Mol Biol*. 2016. 39: 129–134.

Bryan A. Anthony, Daniel C. Link.Regulation of hematopoietic stem cells by bone marrow stromal cells.*Trends in immunology*.2013. 35: 32–37.

Burke JF Jr, Laucius JF, Brodovsky HS, Soriano RZ. Doxorubicin hydrochloride-associated renal failure. Arch Intern Med. 1977. 137: 385-8.

Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol.* 1996. 12: 463-518.

Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell.* 2004. 116: 167-79.

Byrd RL, Rohrbaugh TM, Raney RB Jr, Norris DG. Transient cortical blindness secondary to vincristine therapy in childhood malignancies. *Cancer*. 1981. 47: 37-40.

C Riether, C M Schürch and A F Ochsenbein. Regulation of hematopoietic and leukemic stem cells by the immune system. *Cell Death and Differentiation* 2015. 22: 187–198.

C. Campagnoli, I.A. Roberts, S. Kumar, P.R. Bennett, I. Bellantuono, N.M. Fisk.Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow.*Blood*. 2001. 98: 2396–2402.

Calvi L. M., Adams G. B., Weibrecht K. W., Weber J. M., Olson D. P., Knight M. C., Martin R. P., Schipani E., Divieti P., Bringhurst F. R., et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* .2003. 425: 841-846.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003. 425: 841-6.

Camera A, Andretta C, Villa MR, Volpicelli M, Picardi M, Rossi M, Rinaldi CR, Della Cioppa P, Ciancia R, Selleri C, Rotoli B. Intestinal toxicity during induction chemotherapy with cytarabine-based regimens in adult acute myeloid leukemia. *Hematol J*. 2003. 4: 346-50.

Camerino B, Palamidessi G. Per prior citation, the first publication. *Gazz Chim Ital*. 1960. 90: 1802–1815.

Carlo-Stella, C., Tabilio, A., Regazzi, E., Garau, D., La Tagliata, R.,Trasarti, S., Andrizzi, C., Vignetti, M. & Meloni, G. Effect of chemotherapy for acute myelogenous leukemia on hematopoietic and fibroblast marrow progenitors. *Bone Marrow Transplantation.*1997. 20: 465–471.

Castells M, Milhas D, Gandy C, Thibault B, Rafii A, Delord JP, et al. Microenvironment mesenchymal cells protect ovarian cancer cell lines from apoptosis by inhibiting XIAP inactivation. *Cell Death Dis.* 2013. 4: e887.

Catharina Melzer, Yuanyuan Yang,and Ralf Hasscorresponding. Interaction of MSC with tumor cells. *Cell Commun Signal*. 2016. 14: 20.

César Nombela-Arrieta, Jerome Ritz, and Leslie E. Silberstein. The elusive nature and function of mesenchymal stem cells. *Nat Rev Mol Cell Biol*. 2011. 12: 126–131.

Chen S, Liu Z, Tian N, Zhang J, Yei F, Duan B, et al. Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. *J Invasive Cardiol.* 2006. 18: 552–6.

Chiquet M, Gelman L, Lutz R, Maier S. From mechanotransduction to extracellular matrix gene expression in fibroblasts. Biochim Biophys Acta. 2009. 1793: 911-20.

Chowdhury F, Na S, Li D, Poh YC, Tanake TS, et al. Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. *Nat Mater*. 2009. 9: 82–88.

Chu SH, Armstrong SA. Stem cells: Dual response to Ras mutation. *Nature*. 2013. 504: 91-2.

Corazza F, Hermans C, Ferster A, Fondu P, Demulder A, Sariban E.Bone Marrow Stroma Damage Induced by Chemotherapy for Acute Lymphoblastic Leukemia in Children. *Pediatr Res*. 2004. 55: 152-8.

Covas DT, Siufi JL, Silva AR, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. *Braz J Med Biol Res*. 2003. 36: 1179-83.

Daniel A. Arber, Attilio Orazi, Robert Hasserjian, Jürgen Thiele, Michael J. Borowitz, Michelle M. Le Beau, Clara D. Bloomfield, Mario Cazzola and James W. Vardiman. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016. 127: 2391-2405.

David A. Corral,Michael Amling, Matthias Priemel,Evelyn Loyer,Sebastien Fuchs,Patricia Ducy,Roland Baron,‖ and Gerard Karsenty. Dissociation between bone resorption and bone formation in osteopenic transgenic mice. *Proc Natl Acad Sci U S A*. 1998. 95: 13835–13840.

David N.Haylock,Susan K Nilsson. Osteopontin: A Bridge Between Bone and Blood. *Br J Haematol* .2006. 134 : 467-474.

deMarinis M, Hendricks A, Stoltzner G. Nail pigmentation with daunorubicin therapy. *Ann Intern Med*. 1978. 89: 516-7.

Dennis E. Discher, David J. Mooney, and Peter W. Zandstra. Growth factors, matrices, and forces combine and control stem cells. *Science*. 2009. 324: 1673–1677.

Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol*. 2001. 29: 244-55.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. *Cytotherapy*. 2006. 8: 315-7.

Douglas Kinghorn, Young-Won Chin, and Steven M. Swanson. Discovery of Natural Product Anticancer Agents from Biodiverse Organisms. *Curr Opin Drug Discov Devel*. 2009. 12: 189–196.

Drenberg CD, Hu S1, Li L, Buelow DR1, Orwick SJ, Gibson AA, Schuetz JD, Sparreboom A1, Baker SD. ABCC4 Is a Determinant of Cytarabine-Induced Cytotoxicity and Myelosuppression. *Clin Transl Sci*. 2016. 9: 51-9.

Dykstra B, Olthof S, Schreuder J, Ritsema M, de Haan G. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med*. 2011. 208: 2691-703.

E Manara, G Basso, M Zampini, B Buldini, C Tregnago, R Rondelli, R Masetti, V Bisio, M Frison, K Polato, G Cazzaniga, G Menna, F Fagioli, P Merli, A Biondi, A Pession, F Locatelli and M Pigazzi. Characterization of children with FLT3-ITD

acute myeloid leukemia: a report from the AIEOP AML-2002 study group.*Leukemia*.2016. 31: 18–25.

Edwin W. Taylor. The mechanism of colchicine inhibition of mitosis. I. Kinetics of Inhibition and the Binding of H3-Colchicine. *J Cell Biol*. 1965. 25: 145–160.

el Saghir NS, Hawkins KA. Hepatotoxicity following vincristine therapy. *Cancer.* 1984. 54: 2006-8.

Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood.* 2005. 106: 4057-65.

Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006. 126: 677-89.

Eric M. Darling, Matthew Topel, Stefan Zauscher, Thomas P. Vail, and Farshid Guilak. Viscoelastic properties of human mesenchymally-derived stem cells and primary osteoblasts, chondrocytes, and adipocytes. *J Biomech.* 2008. 41: 454–464.

Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol.* 2000. 109: 235-42.

Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature*. 2002. 420: 629-35.

Feldmann RE Jr, Bieback K, Maurer MH, Kalenka A, Bürgers HF, Gross B, Hunzinger C, Klüter H, Kuschinsky W, Eichler H. Stem cell proteomes: a profile of human mesenchymal stem cells derived from umbilical cord blood. *Electrophoresis.* 2005. 26: 2749-58.

Fernández M, Simon V, Herrera G, Cao C, Del Favero H, Minguell JJ. Detection of stromal cells in peripheral blood progenitor cell collections from breast cancer patients. *Bone Marrow Transplant.* 1997. 20: 265-71.

Fibbe, W.E. & Noort, W.A. Mesenchymal stem cells and he- matopoietic stem cell transplantation.*Annals of the New York Academy of Sciences.* 2003. 996: 235–244.

Fiona M. Watt and Hironobu Fujiwara. Cell-Extracellular Matrix Interactions in Normal and Diseased Skin. *Cold Spring Harb Perspect Biol*. 2011. 3: a005124.

Fiona M. Watt and Ryan R. Driskell. The therapeutic potential of stem cells. *Philos Trans R Soc Lond B Biol Sci.* 2010. 365: 155–163.

Forghieri F, Luppi M, Morselli M, Potenza L. Cytarabine-related lung infiltrates on high resolution computerized tomography: a possible complication with benign outcome in leukemic patients. *Haematologica.* 2007. 92: e85-90.

Francesca Gattazzo,Anna Urciuolo, and Paolo Bonaldo. Extracellular matrix: A dynamic microenvironment for stem cell niche. *Biochim Biophys Acta.* 2014. 1840: 2506–2519.

Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol*. 1976. 4: 267-74.

Fukuda S, Bian H, King AG, Pelus LM. The chemokine GRObeta mobilizes early hematopoietic stem cells characterized by enhanced homing and engraftment. *Blood.* 2007. 110: 860-9.

G J Quigley, A H Wang, G Ughetto, G van der Marel, J H van Boom & A Rich. "Molecular structure of an anticancer drug-DNA complex: daunomycin plus d(CpGpTpApCpG)". *PNAS.* 1980. 77: 7204–7208.

Gao Z, Zhang L, Hu J, Sun Y. Mesenchymal stem cells: a potential targeted-delivery vehicle for anti-cancer drug, loaded nanoparticles. *Nanomedicine.* 2013. 9: 174-84.

Gaspar BH and Thrasher AJ. Gene therapy for severe combined immunodeficiencies. *Expert Opin Biol Ther.* 2005. 5: 1175-1182.

Geoffrey I. Shapiro and J. Wade Harper. Anticancer drug targets: cell cycle and checkpoint control. *J Clin Invest*. 1999. 104: 1645–1653.

George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development*. 1993. 119: 1079–91.

Geyh S, Rodríguez-Paredes M, Jäger P, et al. Functional inhibition of mesenchymal stromal cells in acute myeloid leukemia. *Leukemia*. 2016. 30: 683–691.

Ghosh K, Sivakumaran M, Murphy P, Chapman CS, Wood JK. Visual hallucinations following treatment with vincristine. *Clin Lab Haematol.* 1994. 16: 355-7.

Gordon Keller. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes & Dev.* 2005. 19: 1129-1155.

Grässel S1, Ahmed N. Use of bone marrow mesenchymal stem cells for ex vivo cartilage regeneration. *Orthopade.* 2007. 36: 227-35.

Grassinger J., Haylock D.N., Williams B., Olsen G.H., Nilsson S.K. 2010. Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. *Blood.* 2010. 116: 3185-96.

Gregory CA, Ylostalo J, Prockop DJ. Adult bone marrow stem/progenitor cells (MSCs) are preconditioned by microenvironmental "niches" in culture: a two-stage hypothesis for regulation of MSC fate. *Sci STKE.* 2005: pe37.

Grinnell F. Focal adhesion sites and the removal of substratum-bound fibronectin. *J Cell Biol.* 1986. 103: 2697-706.

H M Amin, Y Yang, Y Shen, E H Estey, F J Giles, S A Pierce, H M Kantarjian, S M O'Brien, I Jilani and M Albitar. Having a higher blast percentage in circulation than bone marrow: clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid leukemias. *Leukemia*. 2005. 19: 1567–1572.

H. William SchnaperHynda K. Kleinman. Regulation of cell function by extracellular matrix. *Pediatr Nephrol*. 1993 Feb;7(1):96-104. 1993. 7(1).pp 96–104.

Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature*. 2003. 423: 349-55.

Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol.* 2009. 54: 2277–86.

Harold E. Renis. Antiviral Activity of Cytarabine in Herpesvirus–Infected Rats. *Antimicrob Agents Chemother*. 1973. 4: 439–444.

Heasman SA, Zaitseva L, Bowles KM, Rushworth SA, Macewan DJ. Protection of acute myeloid leukaemia cells from apoptosis induced by front-line chemotherapeutics is mediated by haem oxygenase-1. *Oncotarget.* 2011. 2: 658-68.

Hee-Sook Jun and Eun-Young Park. Adult Stem Cells as a Renewable Source of Insulin-Producing Cells. *Int J Stem Cells.* 2009. 2: 115–121.

Hong-Sheng Zhou,Bing Z. Carter, and Michael Andreeff. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biol Med*. 2016. 13: 248–259.

Hopen G, Mondino BJ, Johnson BL, Chervenick PA. Corneal toxicity with systemic cytarabine. *Am J Ophthalmol*. 1981. 9: 500-4.

Horvat-Karajz K, Balogh Z, Kovacs V, Drrernat AH, Sreter L, et al. In vitro effect of carboplatin, cytarabine, paclitaxel, vincristine, and low-power laser irradiation on murine mesenchymal stem cells. *Lasers Surg Med*.2009. 41: 463–469.

Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A;International Society for Cellular Therapy. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy*. 2005. 7: 393-5.

Horwitz R. and Webb D. Cell migration. *Curr Biol*.2003. 13: 756.

Hotkar AJ, Balinsky W. Stem cells in the treatment of cardiovascular disease--an overview. *Stem Cell Rev*. 2012. 8: 494-502.

Howard SM, Theologides A, Sheppard JR. Comparative effects of vindesine, vinblastine, and vincristine on mitotic arrest and hormonal response of L1210 leukemia cells. *Cancer Res.* 1980. 40: 2695-700.

Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell*. 2002. 9: 789-98.

Huang Y, Niu XF, Song W, Guan CD, Feng QL, Fan YB. Combined Effects of Mechanical Strain and Hydroxyapatite/Collagen Composite on Osteogenic Differentiation of Rat Bone Marrow Derived Mesenchymal Stem Cells. *Journal of Nanomaterials*. 2013. 2013.

Hutchings H, Ortega N, Plouët J. Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. *FASEB J.* 2003. 17: 1520-2.

Hynes RO, Naba A. Overview of the matrisome--an inventory of extracellular matrix constituents and functions. *Cold Spring Harb Perspect Biol.* 2012. 4: a004903.

Ingmar Glauche, Kateri Moore, Lars Thielecke, Katrin Horn, Markus Loeffler, Ingo Roeder. Stem Cell Proliferation and Quiescence—Two Sides of the Same Coin. *PLoS Comput Biol*.2009. 5: e1000447.

Jaganathan BG, Ruester B, Dressel L, Stein S, Grez M, Seifried E, et al. Rho inhibition induces migration of mesenchymal stromal cells. *Stem Cells*. 2007. 25: 1966–74.

James W. Behan, Jason P. Yun, Marina P. Proektor, Ehsan A. Ehsanipour, Anna Arutyunyan, Ara S. Moses,1 Vassilios I. Avramis, Stan G. Louie,Anna Butturini, Nora Heisterkamp, and Steven D. Mittelman. Adipocytes impair leukemia treatment in mice. *Cancer Res.* 2009. 69: 7867–7874.

Jayesh Sagar, Boussad Chaib, Kevin Sales, Marc Winslet and Alexander Seifalian. Role of stem cells in cancer therapy and cancer stem cells: a review. *Cancer Cell International*. 2007. 7: 9.

Jian Huang, Michelle Nguyen-McCarty,Elizabeth O. Hexner, Gwenn Danet-Desnoyers,and Peter S. Klein.Maintenance of Hematopoietic Stem Cells through Regulation of Wnt and mTOR Pathways. *Nat Med.* 2012. 18: 1778–1785.

Jing Li, Helen K. W. Law, Yu Lung Lau and Godfrey Chi Fung Chan. Differential damage and recovery of human mesenchymal stem cells after exposure to chemotherapeutic agents. *Br J Haematol.* 2004. 127: 326-34.

Jing Li, Wilfred Hing-Sang Wong, Shing Chan, James Chor San Chim, Kenneth Man-Chee Cheung, Tsz-Leung Lee,Wing-Yan Au, Shau-Yin Ha, Albert Kwok-Wei Lie, Yu-Lung Lau,Raymond Hin-Suen Liang,and Godfrey Chi-Fung Chan. Factors Affecting Mesenchymal Stromal Cells Yield from Bone Marrow Aspiration. *Chin J Cancer Res.* 2011. 23: 43–48.

John Mann. Natural products in cancer chemotherapy: past, present and future. *Nature Reviews Cancer*. 2002. 2: 143-148.

Junke Zheng,Masato Umikawa, Shichuan Zhang, HoangDinh Huynh, Robert Silvany, Benjamin P.C. Chen,Lieping Chen,and Cheng Cheng Zhang. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. *Cell Stem Cell.* 2011. 9: 119–130.

K.A. Kilian, M. Mrksich. Directing stem cell fate by controlling the affinity and density of ligand-receptor interactions at the biomaterials interface. *Angew Chem Int Ed Engl*. 2012. 51: 4891–4895.

K.E. Kadler, C. Baldock, J. Bella, R.P. Boot-Handford, Collagens at a glance. *J. Cell*

Kang Y, Georgiou AI, MacFarlane RJ, Klontzas ME, Heliotis M, Tsiridis E, et al. Fibronectin stimulates the osteogenic differentiation of murine embryonic stem cells. *J Tissue Eng Regen Med*. 2015.

Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006. 124: 407-21.

Katherine Y. King and Margaret A. Goodell. Inflammatory modulation of hematopoietic stem cells: viewing the hematopoietic stem cell as a foundation for the immune response. *Nat Rev Immunol.* 2011. 11: 685–692.

Keller HU, Naef A, Zimmermann A. Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. *Exp Cell Res*. 1984. 153: 173-85.

Kelly TM, Fishman LM, Lessner HE. Hyperpigmentation with daunorubicin therapy. *Arch Dermatol.* 1984. 120: 262-3.

Kenneth R. Hande. The Importance of Drug Scheduling in Cancer Chemotherapy: Etoposide as an Example. *The Oncologist*. 1996. 1: 234-239.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells.* 2006. 24: 1294-301.

Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005. 121: 1109-21.

Kikushige Y, Yoshimoto G, Miyamoto T, Iino T, Mori Y, Iwasaki H, Niiro H, Takenaka K, Nagafuji K, Harada M, Ishikawa F, Akashi K. Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. *J Immunol.* 2008. 180: 7358-67.

Kim HJ, Park SS, Oh SY, Kim H, Kweon OK, Woo HM, et al. Effect of acellular dermal matrix as a delivery carrier of adipose-derived mesenchymal stem cells on bone regeneration. *J Biomed Mater Res B Appl Biomater*. 2012. 100: 1645–53.

Kim J, Lee Y, Kim H, Hwang KJ, Kwon HC, Kim SK, Cho DJ, Kang SG, You J. Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. *Cell Prolif*. 2007. 40: 75-90.

Kirstetter P, Anderson K, Porse BT, Jacobsen SE, Nerlov C. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol.* 2006. 7: 1048-56.

Knittel T, Kobold D, Piscaglia F, Saile B, Neubauer K, Mehde M, Timpl R, Ramadori G. Localization of liver myofibroblasts and hepatic stellate cells in normal and diseased rat livers: distinct roles of (myo-) fibroblast subpopulations in hepatic tissue repair. *Histochem Cell Biol.* 1999. 11: 387-401.

Knittel T, Kobold D, Piscaglia F, Saile B, Neubauer K, Mehde M, Timpl R, Ramadori G. Localization of liver myofibroblasts and hepatic stellate cells in normal

and diseased rat livers: distinct roles of (myo-) fibroblast subpopulations in hepatic tissue repair. *Histochem Cell Biol.* 1999. 112: 387-401.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA.*1999. 96: 10711-10716.

Kopterides P, Lignos M, Mentzelopoulos S, Armaganidis A, Pappa V. Cytarabine-induced lung injury: case report. *Anticancer Drugs.* 2005. 16: 743-5.

Kraitchman DL, Tatsumi M, Gilson WD, Ishimori T, Kedziorek D, Walczak P, Segars WP, Chen HH, Fritzges D, Izbudak I, Young RG, Marcelino M, Pittenger MF, Solaiyappan M, Boston RC, Tsui BM, Wahl RL, and Bulte JW. Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. Circulation. 2005. 112: 1451-1461.

Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, Baggett LS, Mikos AG, Cao Y. Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. *BMC Cell Biol.* 2008. 9: 60.

Krevvata M, Silva BC, Manavalan JS, Galan-Diez M, Kode A, Matthews BG, et al. Inhibition of leukemia cell engraftment and disease progression in mice by osteoblasts. *Blood*. 2014. 124: 2834-46.

Kristine C. Rustad and Geoffrey C. Gurtner. Mesenchymal Stem Cells Home to Sites of Injury and Inflammation. *Adv Wound Care* (New Rochelle). 2012. 1: 147–152.

Kumar L, Dua H, Agarwal S, Singh S, Kochupillai V. Ocular toxicity of low dose cytosar. *N Z Med J.* 1987. 100: 361.

L Zanotti, R Angioni, B Calì,C Soldani,C Ploia1, F Moalli4, M Gargesha, G D'Amico,S Elliman, G Tedeschi, E Maffioli, A Negri,S Zacchigna,A Sarukhan,J V Stein and A Viola. Mouse mesenchymal stem cells inhibit high endothelial cell activation and lymphocyte homing to lymph nodes by releasing TIMP-1. *Leukemia*. 2016. 30: 1143–1154.

Laki.Our ancient Heritage in blood clooting and some of its consequences. Annals of the New York Academy of Sciences. 1972. 202: 297–307.

Lazennec Gwendala, Lam Paula Y. Recent discoveries concerning the tumor - mesenchymal stem cell interactions. Biochimica et Biophysica Acta (BBA) - *Reviews on Cancer*. 2016. 1866: 290–299.

Le Blanc K and Pittenger M. Mesenchymal stem cells: progress toward promise. *Cytotherapy*. 2005. 7: 36-45.

Le Blanc K, Götherström C, Ringdén O, Hassan M, McMahon R, Horwitz E, Anneren G, Axelsson O, Nunn J, Ewald U, Nordén-Lindeberg S, Jansson M, Dalton A, Aström E, Westgren M. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. *Transplantation*. 2005. 79: 1607-14.

Lee G, Kim HJ, Kim HM. RhoA-JNK Regulates the E-Cadherin Junctions of Human Gingival Epithelial Cells. *J Dent Res.* 2016. 95: 284-91.

Lee K, Majumdar MK, Buyaner D, Hendricks JK, Pittenger MF, and Mosca JD. Human mesenchymal stem cells maintain transgene expression during expansion and differentiation. Mol Ther. 2001. 3: 857-866.

Lee, S.T., Jang, J.H., Cheong, J.W., Kim, J.S., Maemg, H.Y., Hahn, J.S., Ko, Y.W. & Min, Y.H. Treatment of high-risk acute myelo-genous leukaemia by myeloablative chemoradiotherapy followed by co-infusion of T cell-depleted haematopoietic stem cells and culture-expanded marrow mesenchymal stem cells from a related donor with one fully mismatched human leucocyte antigen haplotype.*British Journal of Haematology*. 2002. 118: 1128–1131.

Levesque J. P., Helwani F. M., Winkler I. G. The endosteal 'osteoblastic' niche and its role in hematopoietic stem cell homing and mobilization. Leukemia.2010. 24: 1979-1992.

Li J, Lepski G. Cell transplantation for spinal cord injury: a systematic review. *Biomed Res Int.* 2013. 2013: 786475.

Li W, Ma N, Ong LL, Nesselmann C, Klopsch C, Ladilov Y, Furlani D, Piechaczek C, Moebius JM, Lutzow K, Lendlein A, Stamm C, Li RK, and Steinhoff G. Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. *Stem Cells.* 2007. 25: 2118-2127.

Liang W, Lu C, Li J, Yin JQ and Zhao RC. p73alpha regulates the sensitivity of bone marrow mesenchymal stem cells to DNA damage agents. *Toxicology*. 2010. 270: 49-56.

Linda J. Bendall, Kenneth F. Bradstock. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine & Growth Factor Reviews. 2014. 25: 355–367.

Linhares-Lacerda L, Ribeiro-Alves M, Nogueira AC, Mendes-da-Cruz DA, Magalhaes DA, Dardenne M, et al. RNA interference-mediated knockdown of CD49e (alpha5 integrin chain) in human thymic epithelial cells modulates the expression of multiple genes and decreases thymocyte adhesion. *BMC Genomics.* 2011. 5:S2.

Linsley C, Wu B, Tawil B. The Effect of Fibrinogen, Collagen Type I, and Fibronectin on Mesenchymal Stem Cell Growth and Differentiation into Osteoblasts. *Tissue Engineering Part A.* 2013. 19: 1416–23.

Lisa D. Muiznieks, Fred W. Keeley. Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective. *Biochimica et Biophysica Acta.* 2013. 1832: 866–875.

Lochhead J, Salmon JF, Bron AJ. Cytarabine-induced corneal toxicity. Eye (Lond). 2003. 17: 677-8.

Long G. Wang, Xiao M. Liu, Willi Kreis, Daniel R. Budman. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemotherapy and Pharmacology*. 1999. 44: 355–361

Lord B. I., Testa N. G., Hendry J. H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood*.1975. 46: 65-72.

M. M. Lalu, L. McIntyre, C. Pugliese, et al., "Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials.*PLoS ONE.* 2012. 7: e47559.

M.D. Shoulders, R.T. Raines, Collagen structure and stability. *Annu. Rev. Biochem.* 2009. 78: 929–958.

Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, Narumiya S. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science.* 1999. 285: 895-8.

Maira Souza Oliveiraa, , Juliana Lott Carvalhob, Ana Carolina De Angelis Camposb, Dawidson Assis Gomesb, Alfredo Miranda de Goesb, Marília Martins Meloa. Doxorubicin has in vivo toxicological effects on ex vivo cultured mesenchymal stem cells. *Toxicology Letters.* 2013. 224: 380-6.

Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. *J Hematother Stem Cell Res*. 2000. 9: 841-8.

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999. 103: 697-705.

Malhotra OP, Talwar KK, Chopra P, Kumar R. Erythroleukaemia and daunorubicin-induced cardiotoxicity in a young boy. *Br J Clin Pract.* 1990. 44: 633-4.

Mandel EM, Lewinski U, Djaldetti M. Vincristine-induced myocardial infarction. *Cancer*. 1975. 36: 1979-82.

Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med.* 2012. 209: 537-49.

Marcella Franquesa,M. J. Hoogduijn, O. Bestard,and J. M. Grinyó. Immunomodulatory Effect of Mesenchymal Stem Cells on B Cells. *Front Immunol.* 2012. 3: 212.

Mareddy S, Crawford R, Brooke G, and Xiao Y. Clonal isolation and characterization of bone marrow stromal cells from patients with osteoarthritis. *Tissue Eng.* 2007. 13: 819-829.

Mauney J, Olsen BR, Volloch V. Matrix remodeling as stem cell recruitment event: a novel in vitro model for homing of human bone marrow stromal cells to the site of injury shows crucial role of extracellular collagen matrix. *Matrix Biol*. 2010. 29: 657-63.

Mauney J, Volloch V. Progression of human bone marrow stromal cells into both osteogenic and adipogenic lineages is differentially regulated by structural conformation of collagen I matrix via distinct signaling pathways. *Matrix Biol.* 2009. 28: 239-50.

Mauney JR, Kirker-Head C, Abrahamson L, Gronowicz G, Volloch V, Kaplan DL. Matrix-mediated retention of in vitro osteogenic differentiation potential and in vivo bone-forming capacity by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion. *J Biomed Mater Res A.* 2006. 79: 464-75.

Mauney JR, Volloch V, Kaplan DL. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. *Tissue Eng*. 2005. 11: 787–802.

Maureen L. Condic. Totipotency: What It Is and What It Is Not. *Stem Cells Dev*. 2014. 23: 796–812.

Mayack SR, Wagers. Osteolineage niche cells initiate hematopoietic stem cell mobilization. *Blood.* 2008. 112: 519-31

McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell.* 2004. 6: 483-95.

Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010. 466: 829-34.

Michael Kuhn. The microtubule depolymerizing drugs nocodazole and colchicine inhibit the uptake of Listeria monocytogenes By P388D1 macrophages. *FEMS Microbiology Letters.* 1998. 60: 87-90.

Min-Wu Chao, Mei-Jung Lai, Jing-Ping Liou, Ya-Ling Chang, Jing-Chi Wang, Shiow-Lin Pan,corresponding author# and Che-Ming Teng. The synergic effect of vincristine and vorinostat in leukemia in vitro and *in vivo*. *J Hematol Oncol*. 2015.8: 82.

MIROSLAV HOLUB. Antibody Production by Lymphocytes after in vitro Contact with Bacterial Antigen and Transfer to New-born Rabbits. *Nature*.1958. 181: 122.

Moiseeva EP. Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovasc Res.* 2001.52: 372-86.

Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, Rottoli D, Angioletti S, Benigni A, Perico N, Alison M, Remuzzi G. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol.* 2004.15: 1794-804.

Morikawa S., Mabuchi Y., Kubota Y., Nagai Y., Niibe K., Hiratsu E., Suzuki S., Miyauchi-Hara C., Nagoshi N., Sunabori T., et al. 2009. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* 206: 2483–2496.

Morimichi Mizuno, Ryuichi Fujisawa, Yoshinori Kuboki. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. *Journal of Cellular Physiology*.2000. 184: 207-13.

Morrison SJ, Scadden DT.The bone marrow niche for haematopoietic stem cells. *Nature*. 2014. 505: 327-34.

Mueller LP, Luetzkendorf J, Mueller T, Reichelt K, Simon H and Schmoll HJ. Presence of mesenchymal stem cells in human bone marrow after exposure to chemotherapy: evidence of resistance to apoptosis induction. *Stem Cells*. 2006. 24: 2753-2765.

Mujagic H, Chen SS, Geist R, Occhipinti SJ, Conger BM, Smith CA, Schuette WH, Shackney SE. Effects of vincristine on cell survival, cell cycle progression, and mitotic accumulation in asynchronously growing Sarcoma 180 cells. *Cancer Res.* 1983. 43: 3591-7.

Myrto Raftopoulou, Alan Hall. Cell migration: Rho GTPases lead the way. *Developmental Biology.*2004. 265: 23–32.

Nakamura-Ishizu A, Suda T. Hematopoietic stem cell niche: an interplay among a repertoire of multiple functional niches. *Biochim Biophys Acta.* 2013. 1830: 2404-9.

Nauta AJ and Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood.* 2007. 110: 3499-3506.

Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood*. 2006. 108: 2114-20.

Naveiras O., Nardi V., Wenzel P. L., Hauschka P. V., Fahey F., Daley G. Q. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature*. 2009. 460: 259-263.

Neville AJ, Gilchrist KW, Davis TE. The chemotherapy of granulosa cell tumors of the ovary: experience of the Wisconsin Clinical Cancer Center. *Med Pediatr Oncol.* 1984. 12: 397-400.

Nicolay NH, Sommer E, Lopez R, Wirkner U, Trinh T, Sisombath S, Debus J, Ho AD, Saffrich R, Huber PE. Mesenchymal stem cells retain their defining stem cell characteristics after exposure to ionizing radiation. *Int J Radiat Oncol Biol Phys*. 2013. 87: 1171–8.

Nicolay NH, Sommer E, Perez RL, Wirkner U, Bostel T, Ho AD, Lahn M, Debus J, Saffrich R, Huber PE. Mesenchymal stem cells are sensitive to treatment with kinase inhibitors and ionizing radiation. *Strahlenther Onkol* .2014. 190: 1037–45.

Nils H. Nicolay, Ramon Lopez Perez, Alexander Rühle, Thuy Trinh, Sonevisay Sisombath, Klaus-Josef Weber, Anthony D. Ho, Jürgen Debus, Rainer Saffrich & Peter E. Huber. Mesenchymal stem cells maintain their defining stem cell characteristics after treatment with cisplatin. *Scientific Reports*. 2016 .6.

Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, Bertoncello I, Bendall LJ, Simmons PJ, Haylock DN. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*. 2005. 106: 1232-9.

Olle Lindvall and Zaal Kokaia. Stem cells in human neurodegenerative disorders — time for clinical translation? .*J Clin Invest.* 2010 .120: 29–40.

Olsen RJ, Chang CC, Herrick JL, Zu Y, Ehsan A. Acute leukemia immunohistochemistry: a systematic diagnostic approach. *Arch Pathol Lab Med.* 2008. 132: 462-75.

Openshaw H, Slatkin NE, Stein AS, Hinton DR, Forman SJ. Acute polyneuropathy after high dose cytosine arabinoside in patients with leukemia. *Cancer.* 1996. 78: 1899-905.

Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, Phinney DG. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A.* 2003. 100: 8407-11.

Oswald J, Boxberger S, Jørgensen B, Feldmann S, Ehninger G, Bornhäuser M, Werner C. Mesenchymal stem cells can be differentiated into endothelial cells *in vitro. Stem Cells.* 2004. 22: 377-84.

Ozmen S, Dogru M, Bozkurt C, Kocaoglu AC. Probable cytarabine-induced acral erythema: report of 2 pediatric cases. *J Pediatr Hematol Oncol.* 2013. 35: e11-3.

P.F. Seke Eteta, L. Vecchiob, A.H. Nwabo Kamdje. Signaling pathways in chronic myeloid leukemia and leukemic stem cell maintenance: Key role of stromal microenvironment. *Cellular Signalling.* 2012. 24: 1883–1888.

Pang B, de Jong J, Qiao X, Wessels LF, Neefjes J."Chemical profiling of the genome with anti-cancer drugs defines target specificities". *Nature Chemical Biology*. 2015. 11: 472–80

Pang B, Qiao X, Janssen L, Velds A, Groothuis T, Kerkhoven R, Nieuwland M, Ovaa H, Rottenberg S, van Tellingen O, Janssen J, Huijgens P, Zwart W, Neefjes J. "Drug-induced histone eviction from open chromatin contributes to the chemotherapeutic effects of doxorubicin". *Nature Communications*. 2013. 4: 1908.

Pankov R, Yamada KM. Fibronectin at a glance. *Journal of Cell Science.* 2002.115: 3861–3.

Park JH, Kim DY, Sung IY, Choi GH, Jeon MH, Kim KK, et al. Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. *Neurosurgery.* 2012. 70: 1238–47.

Partha Dutta, Friedrich Felix Hoyer, Lubov Grigoryeva, t Matthias Nahrendorf. Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. *Journal of Experimental Medicine*. 2015. 212: 497-512.

Peerani R, Bauwens C, Kumacheva E, Zandstra PW. Patterning mouse and human embryonic stem cells using micro-contact printing. *Methods Mol Biol.* 2009. 482: 21-33.

Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, Alon R, Zipori D, Lapidot T. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood.* 2000. 95: 3289-96.

Pellegrin S, Mellor H. Actin stress fibres. *J Cell Sci.* 2007.120: 3491-9.

Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. *J Cell Sci.* 2000 Jan; 113: 5-10.

Pierre A. Stryckmans, Joseph Manaster, Françoise Lachapelle, and Mireille Socqouet. Mode of Action of Chemotherapy In Vivo on Human Acute Leukemia. I. Daunomycin. *J Clin Invest*. 1973. 52: 126-133.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999. 284: 143-7.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999. 284: 143-7.

Prathibha Shetty, Khushnuma Cooper, and Chandra Viswanathan. Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. *Asian J Transfus Sci.* 2010. 4:14–24.

Prockop DJ. Marrow stromal cells as stem cells for continual renewal of nonhematopoietic tissues and as potential vectors for gene therapy. *J Cell Biochem Suppl.*1998. 31: 284-285.

Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 1997. 276: 71-4.

Pui C-H, Jeha S New Therapeutic Strategies for the Treatment of Acute Lymphoblastic Leukaemia Nature Review: *Drug Discovery.* 2007. 2007: 149-165.

R N Sanderson, P R E Johnson, A V Moorman, E Roman, E Willett, P R Taylor, S J Proctor, N Bown, S Ogston and D T Bowen. Population-based demographic study of karyotypes in 1709 patients with adult Acute Myeloid Leukemia. *Leukemia.* 2006. 20: 444–450.

Rahaman MN, Mao JJ. Stem cell-based composite tissue constructs for regenerative medicine. *Biotechnol Bioeng*. 2005. 91: 261-84.

Raheja LF, Genetos DC, Wong A, Yellowley CE. Hypoxic regulation of mesenchymal stem cell migration: the role of RhoA and HIF-1α. *Cell Biol Int.* 2011.35: 981-9.

Ravina, Enrique. The evolution of drug discovery: from traditional medicines to modern drugs. *Weinheim: Wiley-VCH.* 2011. 157–159.

Reinhard Lipowsky.The morphology of lipid membranes. *Current Opinion in Structural Biology.* 1995. 5: 531-540.

Reiser J, Zhang XY, Hemenway CS, Mondal D, Pradhan L, and La Russa VF. Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. *Expert Opin Biol Ther.* 2005. 5: 1571-1584.

Reya T, Morrison SJ, Clarke MF, Weissman IL.Stem cells, cancer, and cancer stem cells. *Nature.* 2001. 414: 105-11.

Rizo A, Vellenga E, de Haan G, Schuringa JJ. Signaling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche?. *Hum Mol Genet.* 2006. 2: R210-9.

Robert F. Klees, Roman M. Salasznyk,Karl Kingsley, William A. Williams,Adele Boskey,and George E. Plopper. Laminin-5 Induces Osteogenic Gene Expression in Human Mesenchymal Stem Cells through an ERK-dependent Pathway. *Mol Biol Cell.* 2005. 16: 881–890.

Rodrigo R, Libuy M, Feliu F, Hasson D. Oxidative stress-related biomarkers in essential hypertension and ischemia-reperfusion myocardial damage. *Dis Markers.* 2013. 35: 773–90.

Rosenberg RF, Caridi JG. Vincristine-induced megacolon. *Gastrointest Radiol.* 1983. 8: 71-3.

Rosova I, Dao M, Capoccia B, Link D, and Nolta JA. Hypoxic Preconditioning Results in Increased Motility and Improved Therapeutic Potential of Human Mesenchymal Stem Cells. *Stem Cells.* 2008. 26: 2173–2182.

Sahin AO, Buitenhuis M. Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adh Migr.* 2012. 6: 39–48.

Sanderson PA, Kuwabara T, Cogan DG. Optic neuropathy presumably caused by vincristine therapy. *Am J Ophthalmol.* 1976. 81: 146-50.

Santoro A, Vlachou T, Carminati M, Pelicci PG, Mapelli M.Molecular mechanisms of asymmetric divisions in mammary stem cells. *EMBO Rep.* 2016 .17: 1700-1720.

Sasportas LS, Kasmieh R, Wakimoto H, Hingtgen S, van de Water JA, Mohapatra G, Figueiredo JL, Martuza RL, Weissleder R, and Shah K. Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy. *Proc Natl Acad Sci.* 2009. 106: 4822-7.

Schaniel C., Sirabella D., Qiu J., Niu X, Lemischka I. R., Moore K. A. (2011). Wnt-inhibitory factor 1 dysregulation of the bone marrow niche exhausts hematopoietic stem cells. *Blood*.2011. 118: 2420-2429.

Schwartz, G.N., Warren, M.K., Rothwell, S.W., Zujewski, J., Halverson,D.C., Cowan, K.H., Tolcher, A., O'Shaughnessy, J. & Gress, R.E. Post-chemotherapy

and cytokine pretreated marrow stromal cell layers suppress hematopoiesis from normal donor CD34+ cells.*Bone Marrow Transplantation.*1998. 22: 457–468.

Sci. 2007. 120: 1955–1958.

Seiter K. Toxicity of the topoisomerase II inhibitors. *Expert Opin Drug Saf.* 2005. 4: 219-34.

Sewa S. Legha. Vincristine Neurotoxicity. *Medical Toxicology.* 1986. 1: 421–427.

Shengwen Calvin Li and Jiang F Zhong. Twisting immune responses for allogeneic stem cell therapy. *World J Stem Cells.* 2009. 1: 30–35.

Shigeo Takashima, David Gold,and Volker Hartenstein. Stem cells and lineages of the intestine: a developmental and evolutionary perspective. *Dev Genes Evol.* 2013. 223: 10.1007/s00427-012-0422-8.

Shihua Wang, Xuebin Qu, and Robert Chunhua Zhao. Clinical applications of mesenchymal stem cells. *J Hematol Oncol.* 2012. 5: 19.

Shurin SB, Rekate HL, Annable W. Optic atrophy induced by vincristine. *Pediatrics.* 1982 .70: 288-91

Singal PK, Iliskovic N.Doxorubicin-induced cardiomyopathy. *N Engl J Med.* 1998. 339: 900-5.

Sonowal H, Kumar A, Bhattacharyya J, Gogoi PK, Jaganathan BG. Inhibition of actin polymerization decreases osteogeneic differentiation of mesenchymal stem cells through p38 MAPK pathway. *J Biomed Sci.* 2013. 20: 71.

Stargell LA, Heruth DP, Gaertig J, Gorovsky MA. Drugs affecting microtubule dynamics increase alpha-tubulin mRNA accumulation via transcription in Tetrahymena thermophila. *Mol Cell Biol.* 1992. 12: 1443-50.

Stefania Montagnani, Maria A. Rueger, Toru Hosoda, and Daria Nurzynska. Adult Stem Cells in Tissue Maintenance and Regeneration. *Stem Cells International*. 2016.213: 5–25.

Stier S., Ko Y., Forkert R., Lutz C., Neuhaus T., Grunewald E., Cheng T., Dombkowski D., Calvi L. M., Rittling S. R., et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J. Exp. Med.* 2005. 201: 1781-1791.

Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity.* 2006. 25: 977-88.

Susanne Grassel, Nazish Ahmed. Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation.*Frontiers in Bioscience.* 2007. 12: 4946-56.

Suva D, Garavaglia G, Menetrey J, Chapuis B, Hoffmeyer P, Bernheim L, Kindler V. Non-hematopoietic human bone marrow contains long-lasting, pluripotential mesenchymal stem cells. *J Cell Physiol.* 2004. 198: 110-8.

Takada S, Chen SZ, Mori H. Toxicity of vincristine overdose in a patient with invasive mole. *Nihon Gan Chiryo Gakkai Shi.* 1989. 24: 2617-23.

Tanaka H, Monahan KD, Seals DR. Age-predicted maximal heart rate revisited. *J Am Coll Cardiol*. 2001. 37: 153-6.

Tefferi A, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia.* 2008. 22: 14-22.

Teti A. Regulation of cellular functions by extracellular matrix. *J Am Soc Nephrol.* 1992. 10: S83-7.

Thibault MM, Hoemann CD, Buschmann MD. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. *Stem Cells Dev.* 2007. 16: 489–502.

Thomas Graf , Matthias Stadtfeld. Heterogeneity of Embryonic and Adult Stem Cells. *Cell Stem Cell*. 2008. 3: 480–483.

Timothy P. O'Connor & Ronald G. Crystal. Genetic medicines: treatment strategies for hereditary disorders. *Nature Reviews Genetics*. 2006. 7: 261-276.

Ting Xie, Allan C. Spradling. A Niche Maintaining Germ Line Stem Cells in the Drosophila Ovary. *Science.* 2000. 2905490: 328-330.

Tomasek, J.J., G. Gabbiani, B. Hinz, C. Chaponnier, and R.A. Brown.Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell Biol.* 2002. 3: 349–363.

Tong Yin and Linheng Li. The stem cell niches in bone. *J Clin Invest.* 2006. 116: 1195–1201.

Torrente Y and Pollie E. Mesenchymal stem cell transplantation for neurodegenerative diseases. Cell Transplant. 2008. 17: 1103-1113.

Trigg ME, Finlay JL, Bozdech M, Gilbert E. Fatal cardiac toxicity in bone marrow transplant patients receiving cytosine arabinoside, cyclophosphamide, and total body irradiation. *Cancer.* 1987. 59: 38-42.

Tuan RS, Boland G, and Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther.* 2003. 5: 32–45.

Tzyy Yue Wong, Mairim Alexandra Solis, Ying-Hui Chen, and Lynn Ling-Huei Huang. Molecular mechanism of extrinsic factors affecting anti-aging of stem cells. *World J Stem Cells.* 2015. 7: 512–520.

U. Koch, E. Fiorini, R. Benedito, V. Besseyrias, K. Schuster-Gossler, M. Pierres, N.R. Manley, A. Duarte, H.R. Macdonald, F. Radtke.Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment.*J. Exp. Med.* 2008. 205: 2515–2523.

Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. Comparative characteristics of

mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol.* 2005. 33: 1402–16.

Wang J, Yang Q, Mao C, Zhang S. Osteogenic differentiation of bone marrow mesenchymal stem cells on the collagen/silk fibroin bi-template-induced biomimetic bone substitutes. *J Biomed Mater Res A.* 2012. 100: 2929–38.

Wei Liang, Hailong Xia, Jing Li, Robert Chunhua Zhao. Human adipose tissue derived mesenchymal stem cells are resistant to several chemotherapeutic agents. *Cytotechnology*. 2011.63: 523–530.

Wennerberg K, Der CJ. Rho-family GTPases: it's not only Rac and Rho (and I like it). *J Cell Sci.* 2004. 117: 1301-12.

Wu L, Van Kaer L. Natural killer T cells and autoimmune disease. *Curr Mol Med.* 2009. 9: 4-14.

Ying Cao, Zhao Sun, Lianming Liao, Yan Meng, Qin Han, Robert Chunhua Zhao. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization *in vivo*. *Biochemical and Biophysical Research Communications.* 2005. 332: 370–379

Yoko Tabe,Yue Xi Shi,Zhihong Zeng,Linhua Jin,Masato Shikami,Yasuhito Hatanaka,Takashi Miida,Frank J. Hsu,Michael Andreeff,Marina Konopleva. TGF-β-Neutralizing Antibody 1D11 Enhances Cytarabine-Induced Apoptosis in AML Cells in the Bone Marrow Microenvironment. *PLoS ONE*. 2013.8: e62785.

Yue Gao, Shuyun Liu, Jingxiang Huang, Weimin Guo, Jifeng Chen, Li Zhang, Bin Zhao, Jiang Peng, Aiyuan Wang, Yu Wang, Wenjing Xu, Shibi Lu, Mei Yuan, and Quanyi Guo. The ECM-Cell Interaction of Cartilage Extracellular Matrix on Chondrocytes. *BioMed Research International*. 2014. 4: 648459.

Zhang B, Li M, McDonald T, Holyoake TL, Moon RT, Campana D, Shultz L, Bhatia R. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-β-catenin signaling. *Blood.* 2013. 121: 1824-38.

Zhang WH, Sun HY, Mou YC, Jiang XX, Wei LJ, Wang QY, et al. Graphene Oxide-Collagen Matrix Accelerated Osteogenic Differentiation of Mesenchymal Stem Cells. *Journal of Biomaterials and Tissue Engineering.* 2015. 5: 257–66.

Zhang Y, Li CD, Jiang XX, Li HL, Tang PH, Mao N. Comparison of mesenchymal stem cells from human placenta and bone marrow. *Chin Med J (Engl).* 2004. 117: 882-7.

Zieve GW, NcIntosh JR. A probe for flagellar dynein in the mammalian mitotic apparatus. *J Cell Sci.* 1981. 48: 241-57.

Zuk PA, Elferink LA. Rab15 differentially regulates early endocytic trafficking. *J Biol Chem.* 2000. 275: 26754-26764.

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2. **Chinnapaka Somaiah,** Atul Kumar, Renu Sharma, Amit Sharma, Trishna Anand, Bhattacharyya, Damodar Das, Sewali Deka Talukdar, Bithiah Grace Jaganathan **Mesenchymal stem cells show functional defect and decreased anti-cancer effect after exposure to chemotherapeutic drugs.** Journal of Biomedical Science. 2018. 25:5.

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1. Poster presented on "**Study of Apoptosis in Mesenchymal Stem Cells**" at 3rd International conference for stem cells and cancer (ICSCC3)- New Delhi, India, 27-30 October 2012.
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Projects Completed:

Ph.D Thesis:

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