Evaluation of the Characteristics and Plasticity of Bone Marrow and Limbus derived Mesenchymal Stem Cells

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

To

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500 046
INDIA

By

Naresh Polisetti
Hyderabad Eye Research Foundation
L.V. Prasad Eye Institute
Hyderabad-500 034
January 2010
Enrolment No. 05LAPH01

Dedicated to beloved ZEEVE sir
and
my grandparents

University of Hyderabad
(A central university established in 1974 by an act of parliament)
Hyderabad-500046, INDIA

DECLARATION

I hereby state that the work embodied in this thesis entitled **"Evaluation of the Characteristics and Plasticity of Bone Marrow and Limbus derived Mesenchymal Stem Cells"** has been carried out by me under the supervision of Dr. Geeta K. Vemuganti and Prof. P. Prakash Babu and that this has not been submitted for any degree or diploma of any other university earlier.

Dr. Geeta K. Vemuganti
Supervisor _____

Prof. P. Prakash Babu
Co-Supervisor _____

Naresh Polisetti
Research Student _____

University of Hyderabad
(A central university established in 1974 by an act of parliament)
Hyderabad-500046, INDIA

CERTIFICATE

This is to certify that Mr. **Naresh Polisetti** has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis entitled **"Evaluation of the Characteristics and Plasticity of Bone Marrow and Limbus derived Mesenchymal Stem Cells"** for submission for the degree of Doctor of Philosophy of this University.

Dr. Geeta K. Vemuganti
Supervisor _____

Prof. P. Prakash Babu
Co-Supervisor _____

Head
Department of Animal Sciences _____

Dean
School of Life Sciences _____

ACKNOWLEDEGEMENTS

First of all, I would like to acknowledge **Dr. Geeta K Vemuganti** for giving me this opportunity to do my Ph.D under her valuable guidance. I thank her very much for being supportive in establishing my career as a stem cell biologist and encouraging me to bring out my best at every step.

I take this opportunity to thank **Prof. P. Prakash Babu** who graciously agreed to register me as his Ph.D student. I also thank him for his valuable suggestions during my work.

I extend my heartfelt gratitude to **Prof. D. Balasubramanian**, Director of Research, LV Prasad Eye Institute. It is an opportunity of a lifetime to have worked with a research group headed by such an eminent scientist. I thank him for all the encouragement and the space he provided me to work at LV Prasad Eye Institute.

I extend my heartfelt thanks to **Dr. G.N. Rao**, Chairman, LVPEI for letting me work at this amazing institution – L V Prasad Eye Institute. Only a visionary like him could have shaped this institution of excellence in our country and it gives me immense pleasure to be a part of his team, contributing in whatever little manner I could.

I thank the other faculty members, **Dr. Chitra Kannabiran, Dr. Savitri Sharma**, **Dr. Shubharata Chakrabarti, Dr. Indumathi Mariappan and Dr. Inderjeet Kaur** for their support and help during my stay at L V Prasad Eye Institute.

I thank to **Dr. S.G.A. Rao**, Ragavendra Biotechnologies PVT LTD. for his guidance and valuable suggestions during the bone marrow isolation and colony forming assays.

I specially thank **Prof. Paturu Kondaiah** from IISc, for his support and guidance in doing the microarray experiments.

I also thank **Dr. Narender K Dhingra** from NBRC, for his support and valuable suggestions in doing the animal experiments.

I thank my seniors **Dr. Anees Fatima, Mr. Purushotham Reddy, Mr. Guru Prasad, Dr. Venu, Dr. Srilatha, Dr. Rajeswari, Dr. Kalyan, Dr. Hardeep and Dr. Kiran who** taught me so many things in the lab and made me feel comfortable.

Special thanks to my friends from LVPEI (**Sagar, Soumya, Surya, Savithri, Subhash, Shuba, Vidya, Saritha, Pullarao, Subbarao, Neeraja, Nageswarao, Ganeshwarao, Gayathri, Haribabu**), University of Hyderabad (**Anand, Gangadhar, Dr. Padmini, Chaitanya, Suraj**), roommates **(Satya, Seshu, Jeebula, Sunil, Pardha, Kishore, Jany, Sha**) and all LVPEI staff members who made my tenure at LVPEI memorable.

I extend my warmest gratitude to **Mr. Chenchu Naidu** and **Mr. Shridhar Rao** Ophthalmic Pathology Services for their unconditional help and support that they offered during the times of need. It was impossible for me to finish my benchwork without their help, especially with tissue sectioning.

I thank the staff from various faculties of L V Prasad Eye Institute. **Mr. Aravind, Mr. Srinivas, Mr. Venkat** from Eye Bank for providing corneo scleral rims, **Mr. Venkatesh, Mr.Rayees, Mr. Shesha, Ms. Kavita** from Jhaveri Microbiology Centre for being extremely helpful whenever approached, **Mrs. Banu and Mr. Venkat** from Library for all the help they provided in the friendliest manner apart from regular service, **Mr. Chary and Mr. Jengaih** from Photography Dept. for the

photography work, **Mr. Koteswara, Mr. Shiva** and **Mr. Kumar** from the Information Systems Dept. for all those wonderful posters, they made for me for presentations at various conferences. I extend my warmest gratitude to **Ganesh, Uma, Elizabeth and Amina** who were of extreme help to me.

The financial support received from **Council of Scientific and Industrial Research (CSIR)** (Research Fellowship), **Department of Biotechnology, India,** is greatly acknowledged.

My heartfelt thanks to the woman I value the most **'Bangaram' (Yamini)** for, she has been the pillar of my emotional and moral strength during my Ph.D.

Last but not the least, the source of my existence, my building and my sheer presence – '**My grand parents and Parents'**. I thank them profusely for standing by me through thick and thin, encouraging and supporting me.

(Naresh Polisetti)

ABBREVIATIONS

α	: Alpha
µ	: Micron
µg	: Micro Gram
µl	: Micro Litre
µM	: Micro Molar
α-MEM	: Alpha- Minimal Essential Media
β	: Beta
Ab	: Antibody
Ag	: Antigen
ASC	: Adult Stem Cells
bFGF	: Basic Fibroblast Growth Factor
BMSC	: Bone marrow Mesenchymal Stem Cells
bp	: Base Pair
BSA	: Bovine Serum Albumin
CD	: Cluster of Differentiation
CFU-F	: Colony Forming Unit-Fibroblast
cm	: Centimeter
CO_2	: Carbon Dioxide
DMEM	: Dulbecco's Minimal Essential Medium
DMSO	: Dimethyl Sulphoxide
DNA	: Deoxyribose Nucleic Acid
dNTPs	: Deoxyribose Neucleoside Triphosphate
EDTA	: Etlylene Diamine Tetra-acetic cid
EGF	: Epidermal Growth Factor
EGF-R	: Epidermal Growth Factor Receptor
ESC	: Embryonic Stem Cells
EtBr	: Ethidium Bromide
FACS	: Fluorescence Activated Cell Sorting
FCS	: Fetal Calf Serum

FITC	: Flourescein Isothiocyanate
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
H&E	: Hematoxylin-Eosin
HCE	: Human Corneal Epithelial Medium
HSCs	: Hematopoietic Stem Cells
IRB	: Institutional Review Board
LSC	: Limbal stem Cells
LSCD	: Limbal Stem Cell Deficiency
LSCM	: Laser Scanning Confocal Microscope
LVPEI	: L V Prasad Eye Institute
mAb	: Monoclonal Antibody
MSCs	:Mesenchymal Stem Cells
MSCs	: Marrow Stromal Cells
ml	: Milliliter
mm	: Millimeter
PBS	: Phosphate Buffered Saline
PI	: Propidium Iodide
RNA	: Ribose Nucleic Acid
rpm	: Revolutions Per Minute
SC	: Stem Cells

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 2

ISOLATION, CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF RAT BONE MARROW STROMAL CELLS

CHAPTER 3

ISOLATION, CHARACTERIZATION OF HUMAN BONE MARROW STROMAL CELLS AND THEIR DIFFERENTIATION POTENTIAL

Quadri	Four	Mesenchymal progenitor cell	Cartilage cells, fat cells, stromal cells, bone forming cells
Tri	Three	Glial restricted precursor	2 types of astrocytes, oligodendrocytes
Bi	Two	Bipotential precursor from murine fetal liver	B cells, macrophages
Uni	One	Mast cell precursor	Mast cells
Nullipotential		None	Terminally differentiated cell e.g. Red blood cell

Those from the inner cell mass of blastocyst have been attributed with a '**pluripotent**' potential and therefore with the capacity to generate all or most cell lineages derived from the three embryonic germ layers: ectoderm (skin and neural lineages), mesoderm (blood, fat, cartilage, bone and muscle) and endoderm (digestive and respiratory systems) (Gardner and Beddington 1988, Li M *et al.*, 2001). During development, ESC divides and originates distinct subpopulations, including non-self regenerating progenitors that undergo terminal differentiation. The embryonic germ cells or the primordial germ cells, the embryonal carcinoma cells show the pluripotency as well. The fetal tissue stem cells, the cord blood and placental stem cells, the adult stem cells are all showing either **pluripotency** or **multipotency** depending on their function and physiological states. The properties of embryonic stem cells are illustrated in Table 1.2.

Table 1.2: Properties of ES cells

Tissue Origin	Derived from the inner cell mass/epiblast of the blastocyst
Long-term self-renewal	Capable of undergoing an unlimited number of symmetrical divisions without differentiating
Karyotype	Exhibit and maintain a stable, full (diploid, normal complement of chromosomes), normal complement of chromosomes
Potentiality	Pluripotent ES cells can give rise to differentiated cell types that are derived from all the three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm). Capable of integrating into all fetal tissues during development. Mouse ES cells maintained in culture for long periods can still generate any tissue when they are reintroduced into an embryo to generate a chimeric animal. Capable of colonizing the germ line and giving rise to egg or sperm cells.
Clonogenicity	A single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell. EC cells express the transcription factor Oct-4, which then activates or inhibits a host of target genes and maintains ES cells in a proliferative, non-differentiating state.
Cell fate	Can be induced to continue proliferation or to differentiate.
Cell cycle	Lack the G1checkpoint in the cell cycle. ES cells spend most of their time in the S phase of cell cycle, during which they synthesize DNA. Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.

1.5.2. Germinal Stem Cells

Early in embryogenesis a few cells are designated to become germinal cells (Meachem *et al*., 2001). These cells migrate into primitive gonad (genital ridge) and differentiate into the female or male germ cell precursors, depending on the presence of two X chromosomes (female) or one X and one Y chromosome (male). They can be recognized by expression of the transcription factor Oct4 and of alkaline phosphatase (Anderson *et al.,* 2000). Studies beginning in the 1970s involving the transplantation of germinal cells clearly demonstrated the totipotency and tumorogenicity of the germinal cells.

1.5.3. Stem Cells from Umbilical Cord Blood:

The blood that remains in the umbilical cord is a plentiful and potentially exhaustible source of pluripotent stem cells that can be used in a number of cell therapies. The cord blood is collected after a baby is born and the cord has been clamped and cut. The collection is painless and safe and families can choose to save and store or donate blood (www.cordblood.org). These are primitive cells with clinical potential matching that of the far more controversial embryonic stem cells ESC. They appear to be much more versatile than “adult stem cells” such as those found in bone marrow which repair damaged tissue during life.

There is an abundance of clinical applications using human umbilical cord blood (HUCB) as a source for stem cell populations. Other than haematopoietic progenitors, there are mesenchymal, endothelial stem cells and neuronal precursors, in varying quantities, which are found in human umbilical cord blood. These may be useful in diseases such as immune deficiency and autoimmune disorders. Considering issues of safety,

availability, transplant methodology, rejection and side effects, it is contended that a therapeutic stem cell transplant, utilizing stem cells from HUCB, provides a reliable repository of early precursor cells that can be useful in a great number of diverse conditions. Drawbacks of relatively smaller quantities of mononucleated cells in one unit of cord blood can be mitigated by *in-vitro* expansion procedures, improved *in-vivo* signalling, and augmentation of the cellular milieu, while simultaneously choosing the appropriate transplantation site and technique for introduction of the stem cell graft (Ghen *et al*., 2006).

Tissue-engineered living blood vessels (TEBV) with growth capacity represent a promising new option for the repair of congenital malformations TEBV with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, blood-derived progenitor cells obtained before or at birth may enable the clinical realization of tissue engineering constructs for pediatric applications (Schmidt *et al*., 2006).

Mesenchymal stem cells (MSC) could be isolated from human umbilical cord Wharton's Jelly. They were capable of differentiating into nerve-like cells using beta-mercaptoethanol. The induced MSC not only underwent morphologic changes, but also expressed the neuron-related genes and neuronal cell markers. They may represent an alternative source of stem cells for central nervous system cell transplantation (Ma *et al*., 2005).

1.5.4. Adult Stem Cells:

Adult stem cells, somatic stem cells, or organ-specific adult stem cells are small subpopulations of quiescent slow- cycling-undifferentiated resident cells, with high proliferative and pluripotent potentiality and the ability to self-

renew and to originate daughter cells, which finally differentiate into functionally mature cells, regenerating all the cell types of the tissue where they are located. Their proliferative reserve exceeds an individual lifetime. These adult stem cells present few organelles and a large nuclear cytoplasmic ratio and may express specific antigens, (Spangrude *et al*., 1988, Welm *et al.,* 2002), integrins (Collins *et al*., 2001). Table 1.3 showing the properties of adult stem cells.

Table 1.3: Properties of Adult SC.

Tissue Origin	Present in many tissues
Long-term	Capable of maintaining homeostatic of SC
Self-renewal	Compartment for the entire life time of the organism
Karyotype	Exhibit and maintain a stable, full (diploid, normal complement of chromosomes)
Potentiality	The large majority of adult stem cells are not pluripotent, like ES, since they have a limited differentiation capacity. They can be multipotentent, such as hematopoietic SC or unipotent such as skin SC. Experimental evidence suggests that the only exception are MAPc since these can give rise to differentiated cells of all the three types of primary germ layers of the embryo (endoderm, mesoderm and ectoderm).
Clonogenicity	A single adult SC, *in vitro* can only give rise to a colony of differentiated cells lacking the properties of the original cell. The molecular mechanisms that maintain adult SC in

	a proliferative, non-differentiating state are almost completely unknown.
Cell fate	Can be induced to differentiate
Cell cycle	The large majority of adult SC are in a quiescent state. Adult SC requires an external stimulus from the microenvironment to enter the cycle and initiate DNA replication (Stem cell niche).
Plasticity	Adult SC may have the ability to generate specialized cells of other tissues. The mechanism is still debated (Cell fusion? Transdifferentiation?)

There are various sources of adult stem cells including bone marrow, peripheral blood, liver, kidney. Adult SC has been identified in many animal models and human tissues. The list of adult tissues reported to contain SC is growing and included bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelium of the skin and digestive system, cornea, retina, liver, pancreas, heart and the CNS (Table 1.4).

Table 1.4: Adult stem cells from various tissues

Type of adult stem Cell	Source	Reference
Hematopoieitic stem cells	Bone marrow	Zhao *et al.,* 2003`
Mesenchymal Stem cells	Bone marrow,amniotic fluid, peripheral blood, adipose tissue, dermis, articular	Friedenstein *et al*.,1974, Pittenger *et al.,* 1999, Zvaifler *et al.,* 2000, Campagnoli *et al.,* 2001,

	sinovium, compact bone, muscle and brain	De Bari *et al*., 2001, Jiang *et al.,* 2002, Zuk *et al* 2002, Javazon *et al.,* 2004
Blood Monocytes	Peripheral blood	Zhao *et al.,* 2003
Epidermal Stem Cells	Skin	Alonso and Fuchs *et al*., 2003
Hair follicle Stem Cells	Hair follicle	Hoffman *et al*., 2006
Corneal epithelial stem cells	Limbus	Schermer *et al*., 1986, Lavker *et al*., 2004
Respiratory Tract stem cells	Respiratory Tract	Delplanque *et al*., 2000, Kotton *et al*., 2001
Dental Stem Cells	Dental Pulp	Shi *et al*., 2005
Gastrointestinal Tract Stem Cells	Neck/isthmus region	Modlin *et al.*, 2003
Hepatic Stem Cells	Intraheptic biliary tree of liver	Thorgeirsson and Grisham, 2003
Pancreatic Stem Cells	Duct cells of pancreas	Bonner-Weir *et al*., 2000
Salivary Gland stem cells	Intercalated duct of salivary glands	Kishi *et al*., 2006
Renal Stem Cells	Renal papilla of kidney	Oliver *et al*., 2004
Mammary gland Stem Cells	Mammary gland ducts	Stingl *et al*., 2006
Prostatic Stem Cells	Proximal region of ducts of prostate	Tsujimura *et al*., 2002
Myogenic Progenitors	Adult skeletal and Cardiac Muscle	Beauchamp *et al*., 2000,

Among tissues identified to harbor stem cells throughout postnatal life, bone marrow has been studied for many years. There are two major types of SC

found in the BM: HSC which generate blood cells, and MSC that support hematopoiesis.

1.5.4.1. Mesenchymal Stem Cells:

MSCs reside in the stromal fraction of the bone marrow, which provides the cellular microenvironment supporting hematopoeisis. Mesenchymal stem cells were first described as bone-forming progenitors from the stromal fraction of rats by Friedenstein and Petrakova in 1966 (Friedenstein *et al*., 1966) and Friedenstein went on to pioneer *in vitro* culture methods for the isolation and differentiation of MSCs (Friedenstein *et al*., 1987). MSCs have subsequently been shown to differentiate into a number of mesenchymal cell types including osteoblasts, chondrocytes and adipocytes (Pittenger *et al*., 1999) (Figure 1.5).

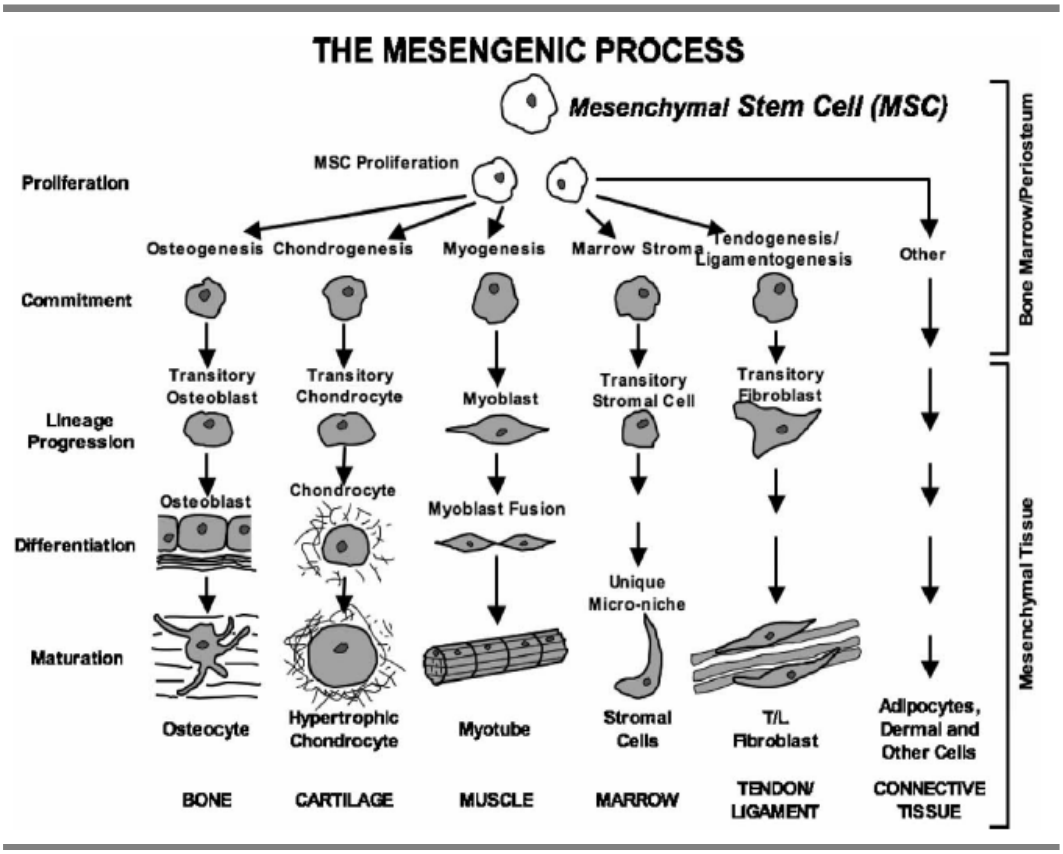

Figure 1.5: The mesengenic process of MSC

1.5.4.1.1 Sources of Primary MSC

MSCs are typically isolated from the stromal fraction of adult bone marrow. In fresh bone marrow, MSCs account for only 0.01-0.0001% of nucleated marrow cells (Dazzi *et al*., 2006). Murine MSCs are classically obtained from the femurs and tibias of mice by flushing the marrow out of the bones with culture medium and transferring the resultant cell suspension in culture. Human MSCs can be similarly obtained from healthy volunteers by taking aspirates of bone marrow from the iliac crest and expanding on tissue-culture plastic (Risbud *et al*., 2006). Over recent years, MSC-like cells have also been identified in a number of different tissues (Friedenstein *et al*., 1987). Cells exhibiting MSC morphology and cellular characteristics have been isolated from adult peripheral blood (Zvaifler *et al*., 2000), adipose tissue (Zuk *et al*., 2001) skin tissue (Chunmeng *et al*., 2004), trabecular bone (Sottile *et al*., 2002) as well as fetal blood, liver, bone marrow (Campagnoli *et al*., 2001), lung (in ‘t Anker *et al*., 2003) and even in exfoliated deciduous teeth (Miura *et al*., 2003) Further MSC-like populations have been discovered in umbilical cord blood (Erices *et al*., 2000) and within the chorionic villi of the placenta (Igura *et al*., 2004) Amniotic fluid has also been cited as a source of MSCs, with potential far-reaching implications for such areas as prenatal diagnosis and gene therapy (In 't Anker *et al*., 2003).

1.5.4.1.2 Isolation of Primary MSC

It is notable that, contrary to most biological systems, human MSCs are better characterized than animal MSCs. This is probably due to the fact that MSCs are easily isolated and expanded from adult human tissue collected from

healthy volunteers. MSCs have nevertheless been isolated from a number of other species. Along with human MSCs, the better characterized cultures are those of rat (Santa Maria *et al*., 2004) and mouse (Baddoo *et al*., 2003) origin, although therapeutic potential in large animal models has been investigated with MSCs from horse (Smith *et al*., 2003), cow (Bosnakovski *et al*., 2005), pig (Moscoso *et al*., 2005) dog (Silva *et al*., 2005), sheep (Rhodes *et al*., 2004) and baboon (Devine *et al*., 2001).

Three main approaches have been described for the isolation of MSCs and can either be used independently or combined together to obtain a more homogeneous culture. The traditional isolation method relies on the fact that MSCs selectively adhere to plastic surfaces, whereas hematopoietic cells do not and can therefore be removed through medium changes (Luria *et al*., 1971). Whilst this eliminates most contaminating cells, the remaining heterogeneity of the culture progressively decreases by serial passaging and after a number of passages the culture is enriched in the self-renewing fraction, the stem cells. Another published isolation protocol involves centrifugation over a Percoll gradient, which separates cell populations based on their density and allows the enrichment of nucleated cells (Dazzi *et al*., 2006). However, both methods are quite nonspecific and an approach that is now increasingly being used, resorts to sorting of bone marrow populations by flow cytometry (FACS), based on MSC reactivity to a number of antibodies. This can either be achieved by positively selecting for expressed antigens or by a process of immunodepletion of cells expressing hematopoietic and/or other lineage antigens. For instance, antibodies against CD34, a surface marker found on hematopoietic cells, are frequently used to identify and

remove nonmesenchymal cells from a marrow culture (Pittenger *et al*., 1999). Since there is no single specific marker available to unequivocally identify the MSC, different groups have opted for a variety of marker combinations. MSCs appear relatively stable as primary cultures (Mareschi *et al*., 2006, Bernardo *et al*., 2006) although spontaneous transformation events have been observed in long-term cultures (Rubio *et al*., 2005).

1.5.4.1.3 Surface Markers on MSC

There are various markers used by different groups to identify the MSC fraction from human bone marrow include, but are not limited to, CD13, CD29, CD31, CD44, CD54, CD63, CD73, CD105, CD106, CD140b, CD166 and Stro1 (Pittenger *et al*., 1999, Bruder *et al*., 1998, Gronthos *et al*., 1994, Vogel *et al*., 2003, Mitchell *et al*., 2006, Covas *et al*., 2003). Comparisons of the various combinations used by different groups show that the majority of subsets include either CD29, CD105 or both. Although various groups have used these markers, there is still no general consensus on the optimal marker combination for MSCs. Some of this conjecture may be due to variations in sample origin, culture techniques and media composition among laboratories or differences in the age of the donors from which the MSCs were obtained and used for immunophenotyping. Because different antibody subsets are likely to selectively isolate slightly different cell types, comparison and evaluation of published data arising from different groups can be difficult. For instance, some groups report a degree of heterogeneity in their cultures after isolation and purification, with occasional description of a subset of small rounded cells among the more common fibroblast-like phenotype (Colter *et al*., 2000). This ambiguity begs the question of what MSCs are: do they purely

represent the proliferating fibroblastic-like progenitors from the bone marrow stroma or do they include all cells capable of forming mesenchymal tissue? In the absence of a specific cell marker, MSCs may well incorporate a number of different cell populations all potentially variable in their phenotypic and growth characteristics, with mesenchymal differentiation as a common denominator.

1.5.4.1.4 Basic Biology and Function of MSC

1.5.4.1.4.1 Basic Biology

Human MSCs are known to constitute a heterogeneous population of cells and their properties and functionality depend on the environmental characteristics. MSCs can be expanded in culture were they give rise to fibroblastic colonies (CFU-F). The CFU-F units are well documented to possess an extended proliferative potential *in vitro* (Dazzi *et al*., 2006). Studies in rodents with 3[H]-thymidine labelling demonstrated that CFU-F are essentially in a noncycling state *in vivo* (Zvaifler *et al*., 2000). The number of colonies obtained from bone marrow aspirates differs among species, as well as throughout the culture conditions used in each individual experiment. Colony formation by MSCs derived from adult human BM is feeder cell independent, while the rodent cells require a source of irradiated feeder cells to achieve maximal plating efficiency (Prockop *et al*., 1997, Bruder *et al*., 1998). The cultures of MSCs are, however, not completely explored. Former studies claimed that MSCs isolated from bone marrow comprise a single phenotypic population forming symmetric, spindle-shaped colonies (homology up to 98%) (Pittenger *et al*., 1999). More recent studies, however, indicate that single-cell derived colonies are morphologically heterogeneous, containing at least two different cell types: small spindle shaped cells and

large cuboidal or flattened cells (Bruder *et al*., 1998, Im *et al*., 2005). In terms of proliferative potential, the cells have been also described as small rapidly-renewing, and large slowly-renewing (Reyes *et al*., 2001). Contrastingly, the work performed by Colter *et al.* (Colter *et al*., 2000) describes the population of small and agranular cells (RS-1) within stationary culture of MSCs with a low capacity to generate colonies and non-reactive to the cell cycle-specific antigen Ki-67. That cell subpopulation was shown, however, to be responsible for the capacity of the whole population of MSCs to expand in culture. Furthermore, it was speculated that RS cells may cycle under stimulation by factors secreted by the more mature MSCs. These cells were, thus, proposed to represent an *ex vivo* subset of recycling uncommitted mesenchymal stem cells (Colter *et al*., 2000). Nevertheless, the latest findings show that MSC colonies contain as much as three types of cells. The third fraction was described to be composed of very small rapidly self-renewing cells (Colter *et al*., 2001), which are reported as the earliest progenitors and possess the greatest potential for multilineage differentiation. The examination of these cells revealed that they were about 7 μm in diameter and had a high nucleus to cytoplasm ratio. They could be also distinguished from more mature cells by the presence of specific surface epitopes and expressed proteins, like vascular endothelial growth factor receptor-2, tyrosine kinase receptor, transferrin receptor and annexin II (lipocortin 2). Some of the rapidly renewing cells contained also other markers, like c-kit (CD117), multidrug resistance epitope and epithelial membrane antigen. Interestingly, these cells were negative for STRO-1, an antigen originally considered as a marker for MSCs (Dennis *et al*., 2002)

1.5.4.1.4.2 Function of MSC:

MSCs play a significant role in bone marrow microenvironment. The major function of these cells is to create a tissue framework, which assures a mechanical support for hematopoietic cell system. They secrete a number of extracellular matrix proteins, including fibronectin, laminin, collagen and proteoglycans. Moreover, MSCs produce hematopoietic and non-hematopoietic growth factors, chemokines and cytokines, thereby participating in the regulation of hemopoiesis. MSCs secrete: IL-1a (Interleukin), IL-1b, IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-SCF), leukemia inhibitory factor, stem cell factor (SCF), fetal liver tyrosine kinase-3, thrombopoietin and hepatocyte growth factor (HGF) (Boiret *et al*., 2005, Colter *et al*., 2001, Dazzi *et al*., 2006, Gronthos *et al*., 2003, Katz *et al*., 2005). Some of these proteins are produced by quiescent cells, whereas the others after stimulation. The involvement of MSCs in hematopoiesis is additionally consolidated by their presence in fetal liver and bone marrow just prior to the onset of definitive hemopoiesis at those sites (Campagnoli *et al*., 2001). An animal model study confirmed that human MSCs marked with GFP and transplanted into the tibia of NOD/SCID mice, integrated into the functional components of hematopoietic microenvironment and actively participated in the hematopoietic cell development (Muguruma *et al*., 2006). During 4 to 10 weeks after transplantation, GFP-MSCs differentiated into pericytes, myofibroblasts, stromal cells, osteocytes and endothelial cells. This led to the increase in the number of functionally and phenotypically primitive human hematopoietic cells in murine bone marrow microenvironment. The

engrafted cells supported human hematopoiesis via secreted factors and by physical interactions with primitive hematopoietic cells. Other studies showed that cotransplantation of human MSCs and HSCs resulted in increased chimerism or/and accelerated hematopoietic recovery in animal models and in humans (Fibbe *et al*., 2003, Koc *et al*., 2000, Lane *et al*., 1999). Moreover, MSCs are known to produce a variety of cytokines that are involved in homing (stromal derived factor-1 - SDF-1) or proliferation and differentiation of hematopoietic cells (GM-CSF, SCF, IL-6) (Hoffman A *et al*., 2002). It has been proposed that several chemokine axes are involved in maintaining bone marrow homeostasis, and that some chemokines, which MSCs possess the receptors for, like CCR9 and CXCR4 may operate in an autocrine manner, similarly as it is in case of HSCs (Honczarenko *et al*., 2006).

Among other well-known biological activities of MSCs, it is worth to emphasize their immunomodulatory functions. These cells are able to inhibit responses of alloreactive T lymphocytes. They express neither MHC class II molecules nor costimulatory receptors (CD80, CD86) on their surface, therefore they do not exhibit antigen-presenting cell activities (Angoulvant *et al*., 2004, Fibbe *et al*., 2003]. The addition of interferon-γ (IFN- γ) to the cultures of MSCs enhances the expression of MHC class I and triggers the expression of MHC class II, but not of the costimulatory molecules (Fibbe *et al*., 2003). It has been well established that MSCs from various species can exert profound immunosupression by inhibiting T-cell responses to polyclonal stimuli (Di Nicola *et al*., 2002) and to their cognate peptide (Krampera M *et al*., 2003). The inhibition did not seem to be antigen specific and targeted both primary and secondary T cell responses (Krampera et al., 2003). The

inhibitory effect was shown to be directed mostly at the level of cell proliferation. T cells stimulated in the presence of MSCs were arrested in the G1 phase as a result of cyclin D downregulation (Glennie *et al*., 2005). The suppression, however, was not apoptotic and could be reversed. In the absence of MSCs and with appropriate stimuli, T cells continue to proliferate (Di Nicola *et al*., 2002). The precise mechanism by which MSCs modulate immunological response is still to be clarified, but overall data suggest that soluble factors as well as cell contact mediated mechanisms are involved. Blocking experiments with the use of neutralizing monoclonal antibodies against transforming growth factor-α (TGF-α) and HGF suggest that these factors are at least in part responsible for the inhibitory effects caused by MSCs (Di Nicola *et al*., 2002). Moreover, MSCs can affect other cells participating in immune response like B cells (Glennie *et al*., 2005) and dendritic cells (Jiang *et al*., 2005).

1.5.4.1.5 Circulation and Niche of MSCs

Little is known about the nature and localization of undifferentiated multipotent MSCs. These cells may be found in various tissues in special places called 'stem cell niches', which serve as stem cells reservoirs. They remain quiescent and possess the capacity for self-renewal after an injury, disease or aging (Pittenger *et al*., 1999). The stem cell niche hypothesis for the bone marrow cells (Figure 1.6) was developed by Schofield, who suggested that certain microenvironmental conditions of the marrow stroma could maintain the stem cells in a primitive, quiescent state (Shofield *et al*., 1978). The investigation of anatomical distribution of MSCs within bone marrow revealed that the cells are located in a close association with endosteum (Gronthos *et*

al., 2003). Such places, therefore, could be regarded as potential niches for MSCs. The findings are, however, based on the STRO-1+ stromal cell population, and the identification of MSCs expressing other specific markers, may change this picture. The question how MSCs maintain their undifferentiated state within the niche is not completely resolved. However, there are some findings indicating that MSC decision to differentiate or to stay quiescent is regulated by Wnt family members, which support undifferentiated state of MSCs, as well as their inhibitors, like: Dickkopf-1 (Dkk1), Frizzled b-1 (Frzb-1) or sFRP1 (Sato N *et al*., 2004). Wnt signaling is known to prevent differentiation process by inducing high levels of oct-3/4, rex-1 and the homeodomain transcription factor Nanog (Sato N *et al*., 2004). Apart from Wnt- and Dkk1-mediated signaling, also Notch, Hedgehog and BMP-pathways play a role in proliferation and differentiation of stem cells. Therefore, it can be speculated, that at least some of these factors are also important for MSCs growth in their niche. After particular stimuli, a stem cell may leave its niche and circulate in blood (Fernandz *et al*., 1997). The cell must be afterwards attracted to another site, where under specific microenvironmental circumstances is able to enter its differentiation program (Watt *et al*., 2000).

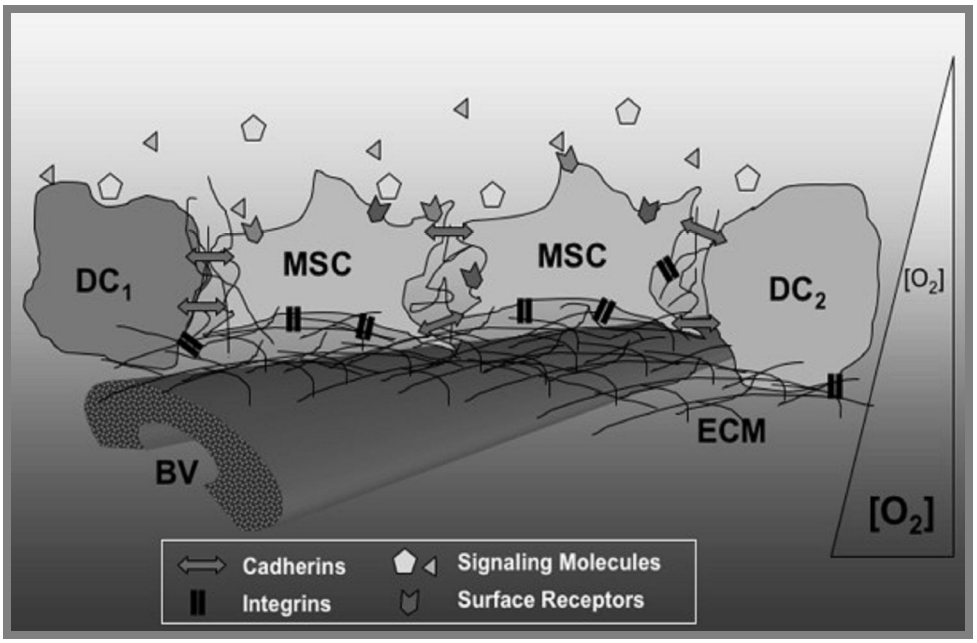

Figure 1.6: Mesenchymal stem cell niche. MSCs are shown in their putative perivascular niche (BV, blood vessel), interacting with (1) various other differentiated cells (DC_1, DC_2, etc.) by means of cell-adhesion molecules, such as cadherins, (2) extracellular matrix (ECM) deposited by the niche cells mediated by integrin receptors, and (3) signaling molecules, which may include autocrine, paracrine, and endocrine factors. Another variable is O_2 tension, with hypoxia associated with MSCs in the bone marrow niche. (Source: Kolf *et al.,* 2007)

The study on MSC homing indicates that the expression of chemokine receptors, as quoted previously, help them in trafficking to various tissues, including bone marrow (Lee *et al*., 2006). Among them, a pivotal role is played by CXCR4, the receptor for SDF-1, which, *inter alia*, is produced by stromal cells. Many findings confirm the extensive multi-organ homing ability of MSCs. In murine model, circulating mesenchymal progenitors, detected in bloodstream, were able to migrate and colonize various tissues (Gao *et al*., 2001). Similar results were obtained in humans (Reading *et al*., 2000). Moreover, these cells were present in the blood of breast cancer patients after growth factor-induced mobilization of hematopoietic stem cells. These data suggest that adequate stimuli may mobilize and release quiescent MSCs

residing in a tissue. Additionally, a subset of quiescent cells (5-10%) was identified in cultures of mesenchymal cells isolated from cord blood, suggesting that uncommitted mesenchymal progenitors circulate during gestation, and travel from fetal sites into other tissues early during development (Makino *et al*., 1999). As another example, MSCs were described to locally migrate to injured sites, to support the regeneration process. Such cases were documented in cartilage repair (Caplan *et al*., 1997), muscle (De Bari *et al*., 2003) and heart (Shake *et al*., 2002) regeneration, migration throughout forebrain and cerebellum (Kopen *et al*., 1999) and differentiation into osteoblasts in regenerating bone (Horwitz *et al*., 2002). The homing capacity of MSCs may decrease after extensive culturing *in vitro*. A study based on syngenic mouse model revealed that primary bone marrow derived MSCs were able to home efficiently to the bone marrow and spleen, whereas culture-expanded MSCs had lost this capacity after 24-48 hours in culture (Fibbe *et al*., 2003). It might be speculated, therefore, that *in vitro* propagation of bone marrow-derived MSCs dramatically decreases their homing to bone marrow and spleen.

1.5.4.1.6 Differentiation:

Cell differentiation proceeds from unspecialised cells to tissue specific cells through selective environmentally induced protein expression. Almost all organs and tissues have a pool of progenitor cells that can respond to normal cells turnover demands, or during injury or damage response. To efficiently function in the organ or tissue where progenitor cell differentiation is required, differentiation must result in a phenotypically matched cell. Therefore through targeting specific genes, many signaling possibilities exist that can

differentially control cell phenotype. Several *in vitro* studies have been conducted to assess the differentiation potential of MSCs, as well as to set up culture conditions, differentiation stimuli, and methods for the identification of each differentiated phenotype. These are supported by *in vivo* studies demonstrating that bone marrow-derived MSCs develop into terminally differentiated phenotypes, like those forming bone (Goshima *et al*., 1991; Kadiyala *et al*., 1997), cartilage (Kadiyala *et al*., 1997), tendon (Young *et al*., 1998), muscle (Ferrari *et al*. 1998), neural (Parr *et al*., 2007), and adipose tissues (Mauney *et al*., 2007).

1.5.4.1.7 Clinical Applications of MSC

The availability of autologous MSCs, which are easily accessible from patients, makes them a promising source of cells for many clinical applications in the evolving field of regenerative medicine. As well as providing the scaffolding (stromal) fraction of the bone marrow for HSCs to proliferate on, MSCs are thought to play a role in hematopoiesis itself. (Dazzi *et al*., 2006). MSCs have been shown to significantly improve hematopoietic recovery in patients receiving high-dose chemotherapy when compared with autologous blood stem cell transfusion alone (Koc *et al*., 2000). Koc and coworkers (Koc *et al*., 2000) co-infused culture-expanded MSCs with autologous blood stem cells in breast cancer patients and observed accelerated hematopoietic recovery.

Furthermore, MSCs represent an advantageious cell type for allogenic transplantation as evidence suggests that MSCs are immune-previleged with low MHC (Major Histocompatibility Complex) I and no MHCII expression, (Uccelli *et al*., 2006) therefore reducing risks of rejection and complications for

transplantation. In utero transplantation of human MSCs into sheep have shown that grafted cells could integrate a variety of host tissues without any specific immune response (Liechty *et al*., 2000). MSCs have also been found to be immunosuppressive, through a mechanism thought to involve paracrine inhibition of T- and B-cell proliferation (Di Nicola *et al*., 2002) as such have been used in trails investigating their effect on autoimmune diseases and GVHD (Le Blanc *et al*., 2004). Co-infusion of donor-derived MSCs together with HSCs has been shown to reduce the incidence and severity of GVHD is sibling allografts (Lazarus *et al*., 2005). It was reported that a nine-year-old patient suffering from progressive severe GVHD that was unresponsive to classical therapy was treated with a MSC intravenous transplant from his mother and demonstrated a complete recovery (Le Blanc *et al*., 2004). The hypo-immunogenic properties of MSCs are considered by some to be sufficient to allow transplantation even between individuals who are not HLA-compatible (Le Blanc *et al*., 2003).

(i) Potential Application of MSC for Osteochondral repair:

One of the fields for MSC use in regenerative medicine is the treatment of bone defects. The osteogenic potential of MSCs has been utilised to treat cases of defective fracture healing, both alone and in combination with scaffolds to repair large bone defects with a high degree of success (Quarto *et al*., 2001). MSCs have also been used for cartilage repair. Autologous MSCs were expanded *ex vivo*, embedded in a collagen gel and reimplanted into areas of articular cartilage defect in osteoarthritis patients (Wakitani *et al*., 2002). In this study, formation of hyaline cartilage-like tissue was improved in the experimental group compared to control. Although most applications for

tissue repair involve local transplantations of MSCs has been in place for a long time hematopoietic stem cell transplants. Recently, children suffering from osteogenesis imperfecta were treated systemically with allogenic MSCs. Transplanted MSCs were shown to migrate to the bone and produce collagen, thus providing a new and efficient route to alleviate the debilitating consequences of this genetic condition (Horwitz *et al*., 1999).

(ii) Potential Application of MSC for Myocardial Repair

Current clinical trials are investigating the potential of MSCs for the treatment of myocardial infarction (Stamm *et al*., 2003). As previously discussed, a number of groups have reported MSC differentiation into cardiomyocytes *in vitro*. The current *in vivo* approach consist of injecting undifferentiated MSCs or whole bone marrow directly into the heart and although the underlying mechanisms remains to be elucidated, significant improvement has been detected (Wollert *et al*., 2004, Fuchs *et al*., 2003). The report by Chen and coworkers (Chen *et al*., 2004) demonstrated a significant and sustained improvement in global left-ventricular ejection fraction, suggesting that MSC infusion triggers the formation of new cardiomyocytes and neoangiogenesis in the human heart (Nagaya *et al*., 2004). It is still unclear whether MSCs act directly by in situ differentiation or fusion with resident myocytes (Lee *et al*., 2005) or indirectly through secretion of pro-myogenic factors promoting endogenous myocardial repair, such as VEGF and FGF (Xu *et al*., 2006).

(iii) Potential Applications of MSCs for Neurological Disorders

Promising results have been also obtained when using MSCs in neuronal lesion treatment. Previous studies showed that MSC transplantation

improves recovery after stroke or traumatic brain injury (Chopp *et al*., 2002). Additionally, in *in vitro* co-cultures of MSCs and neural stem cells, preferential neuronal differentiation has been observed (Lou *et al*., 2003). Moreover, grafts of MSCs in animal models have been shown to promote remyelination (Akiyama *et al*., 2002) as well as partial recovery of function (Chopp *et al*., 2000). After direct injection of MSCs into rodent brain, the cells migrated within the brain and differentiated into GFAP+ glial populations (Azizi *et al*., 1998). The transplantation of MSCs into infracted brain led to the reduction of cell death and the increase in cell proliferation. Moreover, MSCs were demonstrated to be able to produce even myelinating schwann-like cells, with the typical spindle- shaped morphology and the expression of specific markers, such as LNGFR, Krox-20, CD104 and S100 (Keilhoff *et al*., 2006). Testing these cells *in vivo*, by means of transplantation to autologous muscle conduit with 2 cm gap in rat sciatic nerve, showed their capacity to colonize the lesion site and regenerate the damaged nerve. The cells were able to myelinate more than one axon in some cases, similarly as it is in CNS (Keilhoff *et al*., 2006). In a different set of experiments, MSCs transplanted into a subtotal cervical hemisection in adult female rats, were able to integrate efficiently into the injury site. Moreover, immunohistochemical analysis showed marked axonal growth, indicating that these cells enhance axonal growth after spinal cord injury. Interestingly, the recovery levels strongly depended on the human donor and even varied from lot to lot of MSCs isolated fraction (Neuhuber *et al*., 2005).

The list of reports indicating that MSCs contribute to tissue repair *in vivo* enlarges. There are examples of MSC utilization in the repair of kidney

(Herrera *et al*., 2004) muscle (De Bari *et al*., 2003) and lung (Oritz *et al*., 2003). The cells were also found to promote angiogenesis (Hernigou *et al*., 2002) and were used in chronic skin wound treatment (Badiavas et al., 2003). The implantation of MSCs together with occlusive dressing and subsequent epidermal grafts significantly accelerated wound healing and decreased the risk of amputation in endangered patients (Yamaguchi *et al*., 2005). Clinical trials based on MSCs can omit many of the limitations associated with the use of embryonic stem cells (ES). Unlike ES, MSC are not immunogenic, when used autologically, they do not induce immune rejection and are also less probable to trigger teratoma formation, not to mention the ethical concerns. Unfortunately, there are also some drawbacks concerning the use of MSCs. Firstly, according to some observations MSCs fused with endogenous differentiated cells and formed tetraploid cells *in vivo*, although such an event seems to be extremely rare (Spees *et al*., 2003). Secondly, MSCs were shown to permit tumor growth in allogenic recipients (Djouad *et al*., 2003) in animal models. A further question arises, whether the grafted MSCs can maintain their undifferentiated state, thus supporting the therapeutic effect on a long term basis.

1.5.4.2 Limbal Stem Cells

1.5.4.2.1 The Cornea

Cornea is the main structure for refraction of light penetrating the eye; hence its transparency is essential for vision. The cornea is made up of five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium (Figure 1.7). The corneal epithelium, which represents 10% of the total corneal thickness, and is responsible for protecting the eye from

foreign material as well as absorbing oxygen and nutrients. A population of adult stem cells, known as limbal epithelial stem cells (LESC) are responsible for regenerating the corneal epithelium through out life in response to normal wear and tear and following injury (Daniels *et al*., 2001). It is thought that stem cells reside in a "niche" or microenvironment surrounded by tissue, cells, and substrates, which control the self-renewal and differentiation potential of stem cells (Spradling *et al*., 2001). The cornea is an ideal model system in which to study adult stem cells because unlike other sources of stem cells in the human body e.g., the bone marrow, the cornea is readily accessible and transparent. Hence, cornea has the capacity to enhance our general knowledge of human adult stem cell regulation and function.

1.5.4.2.2 Limbal Epithelial Stem Cells

All self-renewing tissue must contain a stem cell pool, which provides an unlimited supply of proliferating cells. This is true for the corneal epithelium with a large body of research indicating these cells reside in the limbal basal region and are aptly named limbal epithelial stem cells (LESC; Figure 1.8). LESC share a number of features with other adult somatic stem cells. These include having small cell size (Romano *et al*., 2003), the lack expression of differentiation markers such as cytokeratin 3/12 (Schermer *et al*., 1986) and high nuclear to cytoplamsic ratio.

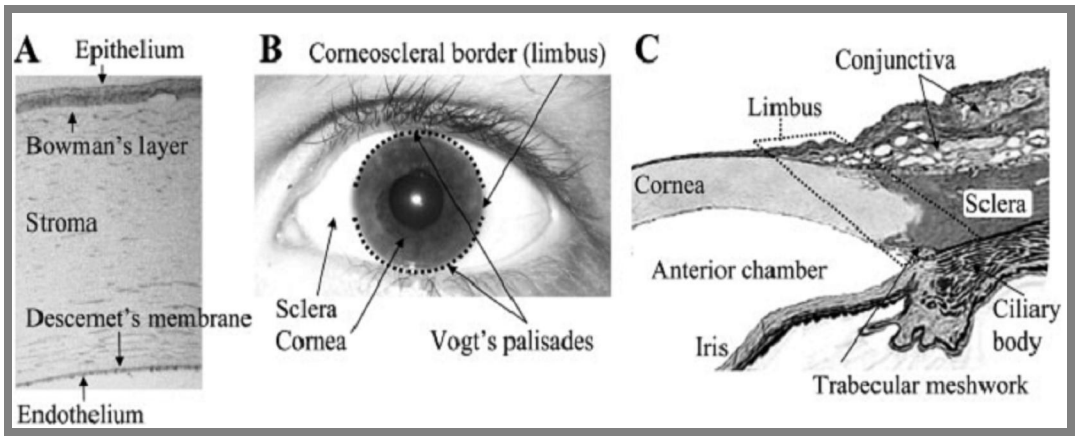

Figure 1.7: Localization of corneal stem cells. A: Histological section and tissue layers of cornea. B: The corneal limbus is localized to the corneoscleral border. The upper and lower regions most protected by the eyelids contain the Vogt's palisades that apparently host most the corneal epithelial stem cells. C: Cross-section of the corneoscleral transition. The corneal epithelium is contiguous with the conjuctiva, the corneal stroma transits into the sclera, whereas the corneal endothelium is linked with the trabecular meshwork. These transitional zones together contain the majority of stem cells in the adult cornea.

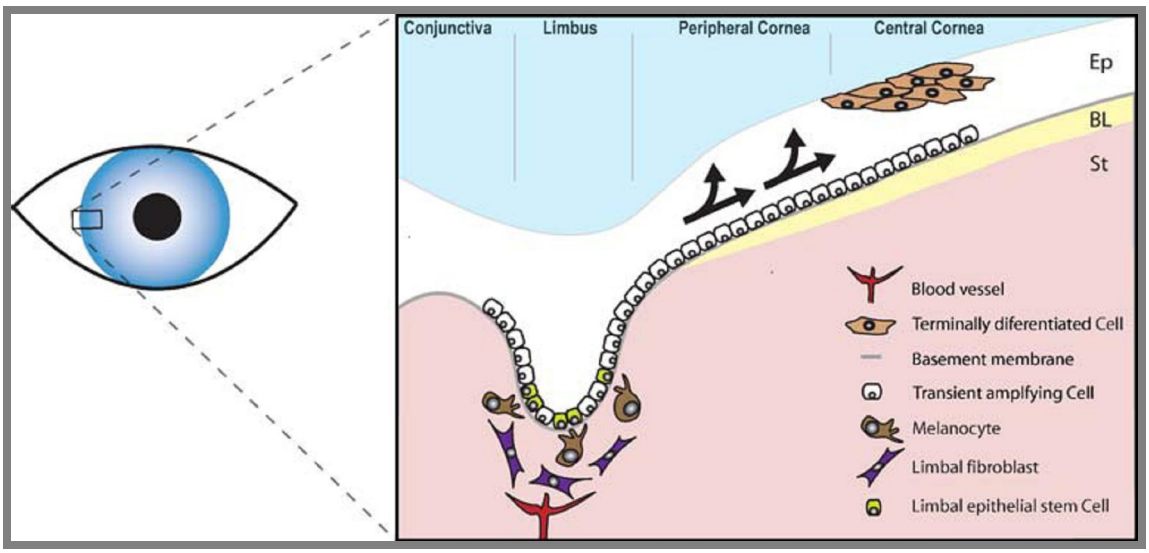

Figure 1.8: A cross-sectional diagram of the human corneal limbus. Limbal epithelial stem cells reside in the basal layer of the epithelium (Ep), which undulates at the limbus. Daughter transient amplifying cells divide and migrate towards the central cornea (arrowed) to replenish the epithelium, which resets on Bowman's layer (BL). The stroma (St) of the limbal epithelial stem cell niche is populated with fibroblasts and melanocytes and also has a blood supply.

LESC are considered to be primitive cells as they are slow cycling and therefore label retaining under normal conditions but have the ability to be highly proliferate in response to injury (Cotsarelis *et al*., 1989, Lavker *et al*., 2003). Stem cells have the ability to divide asymmetrically to repopulate the stem cell pool. Barbaro *et al*., (2007) found expression of C/EBP in a subset of LESC both *in vivo* and *in vitro*, and have suggested it is involved in the regulation of self-renewal and cell cycle length of LESC. Other pathways have been linked to stem cell renewal, such as Notch-1. Corneal specific inducible ablation of Notch1 demonstrated differentiation of LESC into hyperplastic, keratinised skin like epithelium (Vauclair *et al*., 2007). Furthermore LESC express progenitor markers, including, p63 (Pellegrini *et al*., 2001), ABCG2 (Watanabe *et al*., 2004) and more recently N-cadherin.

1.5.4.2.3 Evidence for the Location of LESC to the Limbus:

The first experimental evidence for the location of LESC to the limbus was the movement of pigment from the limbal region towards an epithelial defect in rabbit corneas following wounding (Mann *et al*., 1944). Some years later Davanger and Evenson (Davanger *et al*., 1971) observed a similar migration of pigment from limbus to central cornea and proposed that the Palisades of Vogt (PV) situated in the corneal limbus provided the source of LESC (Huang *et al*., 1991). This movement from limbal to central cornea has been described as centripetal migration. This was demonstrated by gradual replacement of donor epithelium with host cells following lamellar keratoplasty, by looking at the dilution of sex chromatin using a female donor graft in a male recipient in rabbits (Kinoshita *et al*., 1981). Furthermore, the complete removal of the

limbus results in impaired corneal function, neovascularisation and conjunctival in growth (Huang *et al*., 1991).

As stem cells are slow cycling they divide occasionally and therefore can be identified as label retaining cells (LRCs) (Bickenbach *et al*., 1986). Exposing cells to DNA precursors such as tritiated thymidine and bromodeoxyuridine followed by a chase period of 4–8weeks, the slow cycling stem cells retain this label whereas the more differentiated transient amplifying cells (TAC) undergo dilution of the label through multiple divisions. Through the use of tritiated thymidine, Cotsarelis *et al*., found slow cycling or LRCs located in the limbal basal region of the mouse cornea and postulated that 10% of limbal basal cells were stem cells. This population of limbal basal cells phenotypically appear to be more primitive as they are small and round (Romano *et al*., 2003).

The limbal basal region has areas lacking in differentiation markers. For example, the 64kDa cytokeratin 3 (CK3) was found in all layers of the corneal epithelium and the suprabasal layers of the limbal epithelium, however it was absent from the limbal basal cells and the adjacent conjunctiva (Scheremer *et al*., 1986). A similar pattern was found with the corneal specific 55kDa protein, cytokeratin 12 (CK12) (Chaloin-Dufau *et al*., 1990). Furthermore, there is a lack of markers such as connexin 43 (Matic *et al*., 1997) and involucrin (Chen *et al*., 2004), both associated with cells destined for differentiation. Interestingly, the limbal basal region expresses progenitor cell markers such as the transcription factor p63 (Pellegrini *et al*., 2001), especially the ΔNp63α isoform (Di Iorio *et al*., 2005), the ATP-binding

cassette transporter Bcrp1/ABCG2 and more recently N-cadherin (Hayashi *et al*., 2007).

In vivo and *in vitro* studies have found that limbal basal cells have a higher proliferative potential when compared to peripheral and central cornea. Large epithelial wounds in rabbits have been shown to heal faster than smaller central defects, implying that the proliferative capacity of the peripheral cornea is greater than that of the central (Lavker *et al*., 1991). In the human, limbal explant cultures have a greater proliferative potential when compared to central explants (Ebato *et al*., 1987). Based on human epidermal studies (Barrandon *et al*., 1987), supporting clonogenicity studies found cells isolated from the limbus produced the larger holoclones (stem cell derived) compared to the less clonogenic meroclones and paraclones found elsewhere in the cornea (Pellegrini *et al*., 1999). Furthermore, LESC proliferation is resistant to inhibition by tumour-promoting phorbol esters (Kruse *et al*., 1993, Lavker *et al*., 1998).

Supplementary to experimental studies the clinical evidence also points toward the limbus as the location of corneal stem cells. In normal corneal maintenance, the limbal epithelial cells are thought to act as a barrier to the conjunctival epithelial cells (Tseng SC, 1989). Ambati and coworkers has recently shown that soluble vascular endothelial growth factor receptor 1 (sFlt1) plays an important role in corneal avascularity (Ambati *et al*., 2006). Further to this, expression of sFlt1 was found in the corneal epithelium of normal individuals with less seen in vascularised patients (Ambati *et al*., 2007). When the limbus is non-functional, the conjunctiva can invade the corneal epithelium leading to chronic inflammation, neovascularisation and

corneal opacity. This phenomenon is known as limbal stem cell deficiency and can be attributed to both hereditary and acquired conditions. Further clinical evidence suggesting the location of LESC was shown by Kenyon and Tseng, where they transplanted two limbal explants taken from the contralateral healthy eye of patients onto the other damaged eye. This resulted in re-epithelisation of the cornea and regression of persistent epithelial defects and neovascularisation (Kenyon *et al*., 1989). This initial work has lead to the use of expanded LESC grown on amniotic membrane (Tsai *et al*., 2000) and the use of autologous mucosal epithelial cell grafts (Nakamura *et al*., 2003).

1.5.4.2.4 The LESC Niche

The surrounding microenvironment or niche of a stem cell, which consists of cellular and extracellular components, is hypothesised to prevent them from differentiating and thus determines their fate (Schofield *et al*., 1983, Watt *et al*., 2000). Once a stem cell divides asymmetrically and leaves its niche it enters a differentiation pathway under the influence of different environmental stimuli. Interestingly, the mechanism by which this occurs still remains unclear. This theory is in keeping with the LESC niche as it differs from the remaining corneal stroma both anatomically and functionally.

The hypothesized presence of a limbal niche is supported by the following reported *in vivo* studies:

a) The stromal support for the limbal epithelium determines the final phenotypic outcome of the epithelium proven the fact that when embryonic (Coulombre and Coulombre, 1971) or adult (Ferraris *et al*., 2000) rabbit corneal epithelium is recombined with embryonic murine dermis and subsequently transplanted into nude mice, there is a change in expression

of the corneal epithelial specific keratin pair CK3/CK12 to the epidermis-specific keratin pair CK1/CK10 with the formation of hair follicles and sweat glands. This process of transdifferentiation from corneal to epidermal phenotype is controlled by the embryonic dermal stroma

b) Espana *et al*. (2003) have shown in the rabbit corneal epithelium that the limbal or corneal stroma can modulate stem cells and transient amplifying cells by influencing epithelial differentiation and lineage commitment. They showed that the limbal stroma promotes less epithelial differentiation and protects the epitheial cells from apoptosis, while the corneal stroma promotes increased epitheial differentiation and apoptosis. Like other stem cell niches it is proposed that soluble factors, matrix components and cell adhesion molecules probably mediate these process within the limbal stem cell niche.

c) Clinically, the limbal 'niche' theory is supported by the fact the pathological conditions that affect the limbal stroma (e.g. inflammation, neurotrophic disorders, hormonal deficiencies and developmental anomalies such as pax6 gene mutations) can lead to limbal stem cell deficiency (Puangsricharern and Tseng, 1995).

d) The presence of a specific limbal stem cell niche that functions to maintain these cells in a quiescent state while communicating differently with their non-stem cell neighbours is supported by three studies: i) Espana and colleagues (Espana *et al*., 2002) showed that the limbal stroma enhances corneal epithelial stem cells survival; ii) the fact that the basement membrane beneath the limbal basal is distinct in terms of matrix composition (Ljubimov *et al*., 1995) and iii) the work of Stepp MA *et al.* that

showed integrins were differentially expressed at the limbus (Pajoohesh-Gangi *et al*., 2004).

1.5.4.2.4.1 Factors that Maintain the Limbal Stem Cell Niche (Figure 1.9):

A) Intrinsic Factors

1) **Asymmetrical Division** – It can be hypothesized that limbal epithelial stem cells like other stem cells probably undergo asymmetric cell division, giving rise to a determined transient amplifying cell and stem cells to maintain and preserve the longevity of the epithelial population through the entire life span of the individual. It has still not been proven that the corneal epithelial stem cells can asymmetrically divide.
2) **Positional** – The unique position of limbal stem cells within the undulation of the limbal palisades makes the corneal stem cells respond better to signals from the neighboring conjunctival cells, transient amplifying cells, limbal fibroblasts, limbal capillaries and to cytokines and growth factors made available by the vascular tissue.
3) **Interactions of Proteoglycans and Matrix Molecules** - Proteoglycans and matrix molecules within the limbal basement membrane react with the various cytokines released from the neighboring tissue to initiate stem cell division and their subsequent differentiation.
4) **Cell Receptors and Protein Expression** – Strong expression of the TrkA receptor in basal limbal epithelial cells suggests that the nerve growth factor (NGF) signaling is involved in the control of limbal stem cell compartment (Touhami *et al*., 2002).

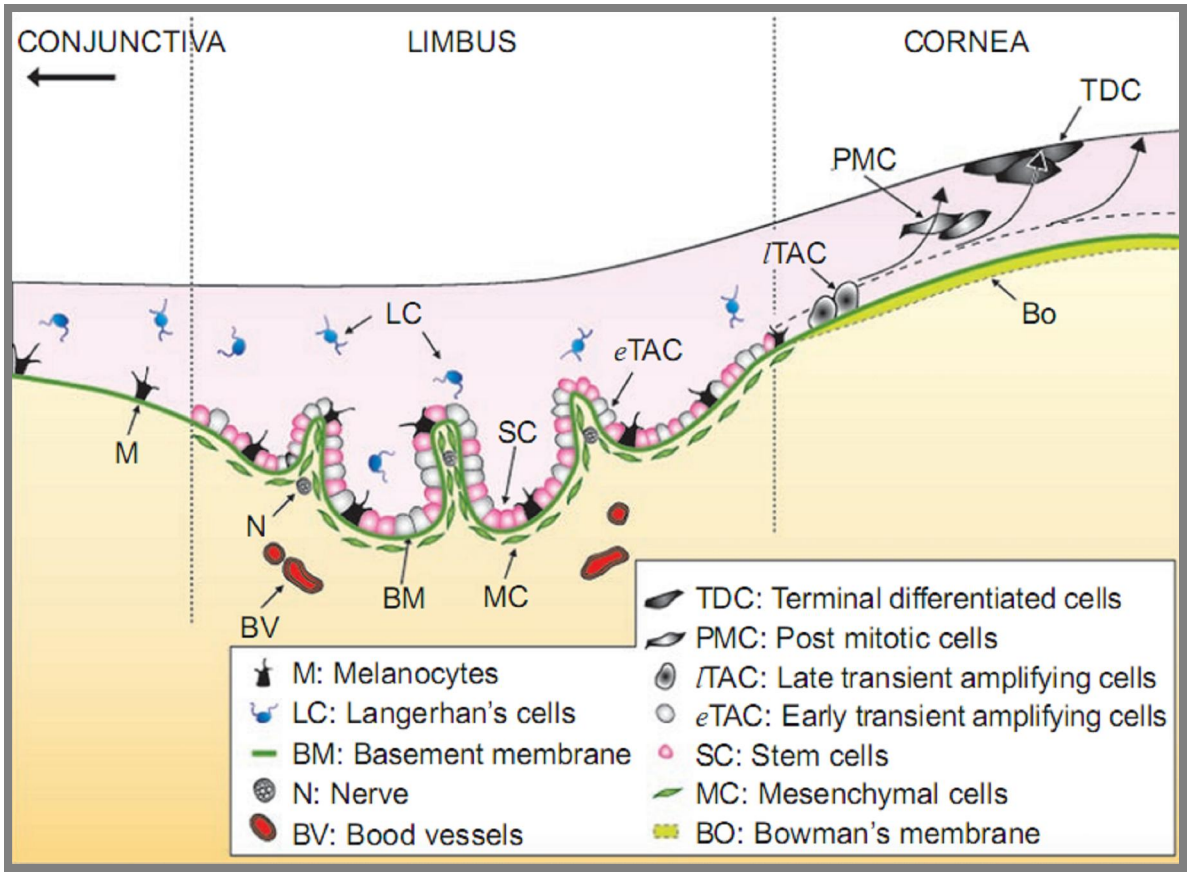

Figure 1.9: Limbal stem cell niche. Limbal epithelial stem cells (SC) are located at the limbal basal layer. In this epitheial level, there are several other cell types in the vicinity such as the immediate progeny, i.e., early transient amplifying cells (eTAC), melanocytes (M), and Langerhan's cells (LC). It remains to be determined whether these cell types act as niche cells. It is believed that eTAC will be destined for progeny production by differentiating into late TACs (/TAC) located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiating cells (TDC). The limbal basement membrane (BM) separating the epithelium from the underlying stroma has several unique components. The subjacent limbal stroma contains mesenchymal cells (MC), which may also serve as niche cells. Because the limbal stroma is highly innervated and vascularized, the respective role of nerves (N) and blood vessels (BV) in niche remains to be defined. (Li W *et al*., 2007)

B) Extrinsic Factors

The microenvironment or niche of stem cells maintains "stemness" (Schofield, 1983). Tissue culture studies have shown that limbal epithelial cells lines

senesce with time, indicating that stem cells are maintained by factors other than intrinsic properties alone. The therapeutic success of transplanted cultured limbal epithelial cells may be due to the persistence of TACs, rather than stem cells alone, and long-term follow-up is required to determine the duration over which this presumed "stemness" is maintained (Lindberg *et al*., 1993). The limbus acquired its blood supply from the palisades of Vogt, which provide nutrition and a greater scope for interaction with blood-borne cytokines (Gipson, 1989; Zieske, 1994).

1) **Extracellular matrix role in functioning as the niche** – Integrins have been postulated to regulate the onset of differentiation and morphogenesis in the strarified epidermis. High levels of β1 integrin have been postulated to maintain the stem cells in their presumed niche via the mitogen activated protein kinase (MAPK) pathway (Zhu *et al*., 1999). Such a role of integrins may also be important in the limbal epithelium.
2) **Contribution of stem cells to an establishment of their own niche** – Stem cells can create their own microenvironment. Fuschs and coworkers on grafting individually cultured epidermal stem cells onto the skin of mutant hairless mice reported the regeneration of new hair follicles and supporting cells, by creating a niche de-novo (Blanpain *et al*., 2004). This role is yet to be investigated in the limbal stem cells.
3) **Epithelial-mesenchymal cytokine interactions in the limbal stem cell niche** - Stem cell behaviour is determined by interactions of the corneal epithelium with its underlying stroma via extracellular matrix, cell membrane associated molecules and cytokines. Li *et al*., have

implicated different patterns of cytokine cross-talk between the epitheial cells and stromal keratocytes by showing that: a) epithelial cells produce: TGFα (transforming growth factor), IL-1β (interleukin), and PDGF-B (platelet derived growth factor), b) epithelial and stromal cell produce: IGF-1, TGF-β1, TGF β2, LIF and bFGF, and c) stromal fibroblasts produce KGF and HGF (Li and Tseng, 1997). KGF, produced by limbal stromal fibroblast modulates stem cells proliferation by a mitogenic effect. HGF, produced by central corneal fibroblasts initiates differentiation and migration signals in epithelial cells. KGF production by limbal fibroblasts can be stimulated by IL-1β and inhibited by TGF-α, PDGF-B and IL-1β, all of which are expressed by corneal epithelial cells (Brazzell *et al*.,1991). IL-1β, produced by epithelial cells at times of cellular stress, stimulates limbal stromal fibroblasts to release KGF, which in turn stimulates limbal epithelial cell proliferation.

4) **Contribution by conjunctival epithelial cells** – Besides producing various defence factors that help protect the corneal epithelium, the conjunctival epithelium expresses much lower Id1, Id3 and Id4 (inhibitor of differentiation proteins), when compared to the limbal epithelium. This may indicate that there are genes like the Id group that are involved in domain segregation and determination and/or phenotype maintenance of the distinct lineages such as the corneal and the conjunctival epithelium (Wolosin *et al*., 2004). These finding were reported during the development stages of these two epithelial lineages.

5) **Contribution by the vascular endothelial cells in the niche** – The vascularized limbal stroma provides the limbal epithelium with nutrition and with a scope for epitheial interaction with blood and fibroblast derived cytokines (Kruse and Tseng 1993b; Kruse and Volcker, 1997). The limbus acquires its blood supply from the capillaries within the Palisades of Vogt.

6) The melanocyte-epithelial unit in the limbal niche – The dendritic melanocytes with the limbal basal epithelium forms a melanocyte epithelial unit, that functions in: i) protecting the limbus against UV light and ii) anti-oxidative activity (Prota, 1980), which assists in quenching UV-induced oxidant formation in the corneal epithelium.

1.5.4.2.5 Stromal Stem Cells:

In 2005, isolation of murine and bovine corneal stromal stem cells by sphere forming assay was reported by two independent groups (Du Y *et al*., 2005, Yoshida *et al.*, 2005). In the same year, isolation of stromal stem cells from human cornea was also reported (Du Y *et al*., 2005). In this latter study, some stromal cells have shown ABCG2 positivity. Based on this observation, the side population was selected by digestion with collagenase and hyaluronidase. In culture, these side population cells showed clonal growth and could be differentiated to express keratocyte, chondrogenic and neurogenic markers (Du Y *et al*., 2005). The same group has concomitantly showed that while these undifferentiated corneal stromal cells predominantly expess stem cell related genes (Bmi-1, kit, Notch-1, Six2, Pax-6, ABCG2, Spag10, p62/OSIL) in adherent cultures, when passaged in suspension in serum free medium with FGF2 and insulin, they from spheroid pellets, in

which keratocyte-like cells secrete an ordered ECM and express mRNAs of known (keratocan, PTGDS, ALDH3A1) and potential (FLJ30046/SLAIN, CxAdR, PDK4, MTAC2D1, F13A1) keratocyte markers (Du Y *et al*., 2007).

Multipotent, fibroblast-like cells were isolated from limbal stroma by other groups as well (Dravida *et al*., 2005). In the earlier study, after enzymatic digestion of de-epithelized stroma of limbal explants, stage specific embryonal antigen 4 (SSEA-4) positive cells were sorted by magnetic activator cell sorter (MACS). The isolated multipotent fibroblast like cell showed a unique marker profile (CD34, CD45, CD123, CD14, CD106, $HLADR^-$ /CD31, SSEA4, CD73, $CD105^+$), different from that of bone marrow mesenchymal (Jung *et al*., 2009) or other adult stem cells but similar to that of embryonic stem cells (Oct-4, Sox-2, Tra1-60, $Tra1\text{-}80^+$) (Dravida *et al*., 2005). This marker profile is quite similar to that of very small embryonic-like stem cells of the adult humans (Zuba-Surma *et al*., 2009).

The presence of bone-marrow derived cells in the cornea was shown to when irradiated wild type mice were transplanted with bone marrow or hematiopoietic stem cells of GFP expressing transgenic mice. Most of these cells differentiated into antigen presenting cells in the host's cornea and only a small percentage of BM derived cells represented other (unidentified) cell types (Sosnova *et al*., 2005). Bone marrow derived cells formed approximately half of the pericytes but none of the endothelial cells of new vessels in a mouse model of experimental corneal neovascularization (Ozerdem *et al*., 2005). Recently, bone marrow derived progenitor cells were shown to promote wound healing and re-epithelization in alkali injured rabbit corneas (Ye *et al*., 2006).

Yoshida and coworkers (Yoshida *et al*., 2005) isolated a subset of cells termed neural crest derived corneal precursors (COPs) from stromal cells of adult mice. These cells showed side population characteristics, were multipotent, clonogenic (sphere forming), and expressed various adult stem cell markers (nestin, notch-1, musashi-1, ABCG2,). Experiments with transgenic mice proved that limbal bone marrow derived cells and COPs are two distinct cell populations and that COPs have a neural crest origin, which as also confirmed by the expression of the embryonic neural crest markers Twist, Snail, Slug and Sox-9. COPs expressed surface markers Sca-1 and CD34 and were negative for CD45 and c-kit.

Altogether, these results indicate that bone marrow derived cells mainly act as enhancers of wound healing and neovascularizaton, and take part in the immunological defence of the cornea. On the other hand, corneal stromal stem cells and COPs may serve as stem cells in the maintenance of the mesenchyma-derived parts of the cornea. As both cell types are located mainly in the peripheral cornea. As both cell types are located mainly in the peripheral cornea, interactions between them are possible. Understanding these interactions, as well as elucidating the behaviour of these cell types under physiological and pathological conditions will greatly increase our knowledge on corneal wound healing and regeneration.

1.6. Scope and Aim of the Study

1.6.1. Scope of the Study

The field of stem cell biology is gaining a lot of importance in therapeutics and the role of these cells in regenerative medicine is being explored in a number of clinical trails worldwide. Various sources of cells that are being evaluated

include: embryonic cells, fetal cells, bone marrow cells, ciliary body cells, muller and retinal pigment epithelial cells and induced pluripotent stem cells. With the belief that adult autologous cells have a better acceptance in clinical trials, we explored the potential of bone marrow derived stromal cells to transdifferentiate into neuronal lineage. Our initial aim was to establish and characterize the BMSCs of rat and human origin and explore their stemness and plasticity. Around the same time, our lab has reported a new observation of finding stromal cells in limbal cultures, which showed features similar to BMSC. So I pursued the objective of comparing the phenotype of these mesenchymal cells by various techniques.

1.6.2. The Focus of the Thesis

1. **Rat Bone Marrow Stromal Cells** - Isolation, characterization and differentiation of rat bone marrow stromal cells
2. **Human Bone Marrow Stromal Cells** – Isolation, characterization and differentiation of human bone marrow stromal cells
3. **Limbal Stromal Cells** – Isolation, characterization and differentiation of limbal stromal cells.
4. **Gene Expression Profile** – Gene expression profile of limbal explant culture derived cells in comparison to bone marrow derived mesenchymal stem cells.

ISOLATION, CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF RAT BONE MARROW STROMAL CELLS

2.1 Introduction:

Bone marrow is a complex tissue containing stem cells with hematopoietic properties. The hematopoietic stem cells, which are the primary source of blood cells in the adult body, are regulated within a microenvironment of stromal cells in the bone marrow (Hunt P *et al*., 1987; Aubin JE *et al*., 1999; Chen ZZ *et al*., 1991, Colter DC *et al*., 2001, Deans RJ *et al*., 2000). The stromal cells exert their effects on the hematopoietic cells through direct cell-cell interactions as well as by the release of soluble factors (Yanai N *et al*., 1994; Ryan DH *et al*., 1991; Dittel BN *et al*., 1993). Stromal cells isolated from bone marrow (BMSC) are heterogeneous and fibroblastic in appearance (Prockop *et al.,* 1997). In 1974, Friedenstein *et al.,* isolated fibroblastoid cells in bone marrow by plastic adherence. Fibroblastoid cells make up 0.001-0.01% of bone marrow cells and display a colony forming unit (CFU-F). They were initially named plastic-adherent cells or colony-forming-unit fibroblasts and subsequently referred to as either marrow stromal cells or mesenchymal stem cells (MSC), due to their potency to differentiate into various connective tissue lineages including adipocytes, osteoblasts, chondrocytes or myoblast (Pittenger MF *et al*., 1999; Jiang *et al*., 2002).

Bone marrow derived MSCs have been isolated from a variety of species, including mouse (Peister A *et al*., 2004), rat (Javazon EH *et al*., 2001), rabbit (Johnstone B *et al*., 1998) and human subjects (Colter DC *et al*., 2001). Although MSCs from different species have similar characteristics in part, some data

suggest that variations occur among species. MSCs from human bone marrow are relatively easy to harvest and to expand in culture (Sekiya *et al*., 2002a), whereas rodent MSCs have proven more difficult (Friedestein AJ *et al*., 1974; Simmons DJ *et al*., 1991; Rennick D *et al*., 1987), although this is not without controversy (Javazon EH *et al*., 2001). The technical difficulties in preparing MSCs from rodent bone marrow have limited the number of experiments, because animal transplantation models are required for preclinical studies. The selection of suitable cell populations is apparently crucial for the outcome of *in vivo* experiments with MSCs.

Although there are many methods to isolate MSCs from the bone marrow, no optimal method is available. The methods include plastic adherence (Dexter *et al*., 1981), gradient density centrifugation (Chen ZZ *et al*., 1991) and immunomagnetic selection (Dezawa M *et al*., 2004; Jia L *et al*., 2002). Different methods have different defects and virtues. Plastic adherence is an easy method of obtaining such cells on the basis of their plastic adherence characteristics, but it is difficult to get pure stromal cells. Gradient density centrifugation depends on the relative density of MNCs to separate MSCs. Immunomagnetic selection uses the principle of separating the MSC based on the immune recognition of the surface antigens by the use of appropriate antibodies. Extensive experimentation has defined the conditions for the isolation, propagation, and differentiation of MSCs *in vitro* and *in vivo*. In our study we have isolated and established bone marrow stromal cells by the simple and reliable method of combining density

gradient centrifugation with plastic adherence and differentiated them to adipocytes, osteocytes and neuronal like cells.

2.2. Hypothesis:

We hypothesize that rat bone marrow mesenchymal stem cells can be isolated by simple and reliable method

2.3. Aim

1. Isolation and characterization of rat bone marrow mononuclear cells
2. Establishment and characterization of stromal of cell cultures
3. Differentiation of stromal cells into adipocytes, osteocytes and neural lineage

2.4. Material and Methods

The protocol was approved by the Institutional Review Board (IRB) at L.V.Prasad Eye Institute.

2.4.1. Preparation of Chemicals

All the chemicals and culture media were prepared as described in the appendix I.

2.4.2. Sterility Check for Chemicals & Media:

Following the filter sterilization the media and chemicals were kept for sterility check. A few drops of media/chemicals were inoculated on chocolate agar and in thioglycolate broth to screen for both aerobic and anaerobic microorganisms. Inoculated media were then incubated in a bacterial incubator at 37^0C for about 7 days, before the media/chemicals are approved for tissue culture use.

2.4.3. Source of Animals

Wistar rats (12 weeks old) were used. All protocols followed for the use of animals were approved by the Institutional ethical committee and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

2.4.4 Isolation of mononuclear cells:

Wistar rats were sacrificed by cervical dislocation, and then placed in 70% alcohol for 10 min. Both femurs from one rat were taken and stripped of adherent muscles of the knee end. A needle was inserted into the bone and cells were aspirated followed by several flushes through the bone using a 1 ml syringe filled with culture medium, until all the bone marrow was flushed out of the bone. A similar procedure was performed from other end of the bone as close to the tip as possible. The marrow thus obtained was suspended by pipetting the large marrow cores through a 1 ml pipette. The medium containing the cells was layered on HISTOPAQUE – 1077 (Sigma) and centrifuged on 400x*g* for 30min. Mononuclear cells were removed from the gradient interface and washed with PBS. The suspension was then centrifuged at 200x*g* for 5min. The pellet thus obtained was dissolved in 1ml of PBS; the cell count was done in a Neubauer chamber and tested for viability by the Trypan Blue elution test. The mononuclear cells were resuspended in growth medium (see below), and plated in 25cm^2 tissue-culture flasks made of polystyrene plastic (Nunclone) at a density of 1x10^{6}cells/ml. Nonadherent cells were removed after 48h, replacing the media every 2-3 days.

2.4.5 Cell culture conditions:

The adherent cells were cultured in the growth medium containing Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% Fetal bovine serum (FBS; SIGMA) 250KU/L Penicillin, 1.25mg/L Amphotericin-B, 100mg/L Streptomycin, 50µl/L Gentamycin and 1.2g/L Sodium bicarbonate. The cultures were maintained at 37^0 C in a humidified 5% CO_2 incubator. When the cells reached 80-90% confluency, cultures were harvested with Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA; Sigma).

2.4.6 Colony-forming assays:

For these assays, 2 cells per cm^2 at passage 0 were plated and cultured for 14 days in 75 cm^2 tissue culture flasks. The cells were fixed with methanol and stained with Giemsa. Colonies less than 2 mm in diameter and those that were only faintly stained were ignored.

2.4.7 Characterization of mononuclear and stromal cells

2.4.7.1 Immunophenotyping (Immunocytochemistry)

Immunophenotyping is a technique for identifying cellular or tissue constituents (antigens) by means of Ag-Ab (Antibody) reactions, the site of Ab binding being identified either by direct labelling of the Ab or by use of a secondary labelling method.

MSC's (3^{rd} passage) were seeded into 24 well plates and cultured up to confluency. The cells were fixed with 4% Paraformaldehyde in 0.1 M phosphate buffer (PH 7.2) for 20 min. and then processed for immunocytochemistry. Non-specific reactions were blocked with 5% fetal calf serum for 30 min at room

temperature. The fixed cells were then incubated for one hour with primary antibodies (Table 2.1). After three washes cells were incubated with FITC-conjugated secondary antibody for an hour. They were washed three times and counter-stained with Propidium Iodide (PI) to detect the cell nuclei. Cells were photographed by confocal Laser Scanning microscopy (LSM510; Carl Zeiss) with a fluorescent light source (excitation wavelength 480 and 540 nm)

Table 2.1: Antibodies used for immunocytochemistry

S.No.	Antibody	Dilutions	Company
1	CD90	1:200	Millipore
2	CD45	1:200	Millipore
3	CD11a	1:200	Millipore
4	CD18	1:200	Millipore
5	CD34	1:200	Millipore
6	CD31	1:200	Millipore
7	FITC- Conjugated 2^0 Antibody	1:250	Millipore
8	Vimentin	1:250	Dako Cyomation

2.4.7.2 Flowcytometry:

Flow cytometry is a useful technique due to the fact that the cells can be monitored, providing sensitive and specific information about each single cell. In relation to the optics of flow cytometry, when the light source hits a cell, amount of light scattered to the side is detected by the size and shape of the cell. Flow cytometers use lasers as their source to excite cells. The excitation from the lasers must be equivalent to the absorption wavelengths of flurochromes used. The argon laser is the most commonly used since it produces several lines in the

UV, and can excite fluorescein, which is a common fluorochrome. The other parameter detected is forward scatter and it provides information about the surface properties, complexicity of the cells and can determine how granualated the cells are. Various population of cells can be distinguished from the information provided by side and forward scatter following acquisition of samples. In addition, antibodies have fluorescent attached enabling the surface expression of specific cell markers.

2.4.7.2.1 Preparation and staining of MNC's and MSC's

Mononuclear cells were obtained as described above. MSC's were harvested from the tissue culture flasks after passage 3 *in vitro* and centrifuged at 200x*g* for 5 min at room temperature. The cells were washed and counted in a Neubauer Chamber. A single cell suspension of 0.5 to 1x 10^6 cells were placed in 50µL of buffer (PBS, 0.1% sodium azide, 2% FBS). The cells were incubated with primary antibody for 40 min with saturating concentrations of monoclonal antibodies (Table 2.2). After the cells were washed three times in buffer and centrifuged at 200xg for 5 min, they were resuspended in ice cold PBS and incubated with the FITC-labelled or TRITC-labelled secondary antibody for 30 minutes in the dark at 4^0C. Cell fluorescence was evaluated by flow cytometry in an FACS Calibur instrument (Becton Dickinson) and the data were analyzed using Cell Quest software (Becton Dickinson). An isotype control was included in each experiment, and specific staining was measured from the cross point of the isotype with a specific antibody graph.

Table 2.2: Antibodies used for flow cytometry

S.No.	Antibody	Dilutions	Company
1	CD90	1:50 (10µl)	Millipore
2	CD45	1:50 (10µl)	Millipore
3	CD11a	1:50 (10µl)	Millipore
4	CD18	1:50 (10µl)	Millipore
5	Fibronectin	1:50 (10µl)	Millipore
6	CD31	1:50 (10µl)	Millipore
7	FITC or TRITC- Conjugated 2^0 Antibody	1:50 (10µl)	Millipore
8	Vimentin	1:25	Dako Cyomation
9	IgG1	1:25 (10µl)	Millipore
10	IgG2a	1:25 (10µl)	Millipore

2.4.7.3 Reverse Transcription PCR (RT PCR)

2.4.7.3.1 Isolation of Total Cellular RNA (Trizol-Method):

The Isolation of RNA from cultured cells involves the following steps:

I) Homogenization, II) Phase Separation, III) RNA precipitation, IV) RNA wash

V) Resuspension of the RNA pellet

I) Homogenization: Rat bone marrow stromal cells were dislodged using trypsin. Cells were counted on Neubaur's counting chamber. One ml TRIzol was added to the cells and passed several times through a pipette for homogenization.

II) Phase Separation: Homogenized samples were incubated for 5mins at 15-30^{o}C. 0.2ml of chloroform was added for each 1 ml TRIzol reagent added. Tubes

were shaken vigorously for 15-20 seconds, incubated at 15-30^{o}C for 2-3mins. Centrifuged at 12,000x*g* for 15mins at 2-8^{o}C. After centrifugation the lower red phenol-chloroform phase, an interphase, and an upper aqueous phase is formed.

III) RNA Precipitation: The aqueous phase containing (organic phase - for DNA, Protein isolation) the RNA was transferred to a fresh tube. RNA was then precipitated by isopropyl alcohol (0.5 ml/1ml TRIzol) and incubated at 15-30^{o}C for 10mins. The sample was then centrifuged at 12,000x*g* for 10mins at 2-8^{o}C. The RNA gets precipitated, and forms a gel like pellet at the bottom of the tube.

IV) RNA Wash: The supernatant was removed and RNA pellet was washed in 75% ethanol (1ml/1ml of TRIzol). The sample was mixed by vortexing and centrifuged at 7.500x*g* for 5mins at 2-8^{o}C.

V) Resuspension of the RNA pellet: RNA pellet was then dried (air dried) and dissolved in RNase-free water and stored at -70^{o}C.

2.4.7.3.2 Reverse Transcription [Synthesis of cDNA (1st Strand) using Oligo-d (T) primers]:

cDNA strand was synthesized using reverse transcriptase from Moloney-Murine Leukemia Virus (M-MLV). The total cellular RNA (1-2μg) was used for cDNA synthesis. For the first strand synthesis, two master mix were prepared

Master Mix 1: 1 μg of Total RNA + 500ng of oligo - (dT) primer

Master Mix 2: 1X RT buffer (10X stock), 200U of RT enzyme (200U/μl) were added for a 50 μl reaction, RNase free water.

Master mix 1 was denatured at 65^{o}C for 10minutes, followed by immediate chilling on ice to avoid renaturation. Master mix 2 was then added to master mix 1 and a PCR amplification reaction was carried out under following conditions.

PCR Conditions:

25^{o}C for 10mins (Primer Annealing)

42^{o}C for 1hour (Amplification)

70^{o}C for 30mins

Synthesis of 2nd strand using gene specific primers:

The second strand was synthesized using gene specific primers (Table 2.3) for the following genes – Collagen type 1alpha 1 and Vimentin. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as internal control.

Table 2.3: Rat primer sequences used for RT PCR

Gene	Primer Sequence	Fragment Length	Accession Number	Annealing Temperature
Vimentin	F:AATTGCAGGAGCTGAATGAC R:AATGACTGCAGGGTGCTCTC	301bp	NM_031140	55
Collagen type 1 alpha 1	F:ACAGACCAACAACCCAAACTC R:GTAAGGTTGAATGCACTTTTGG	379bp	XM_213440	55
GAPDH	F: CCCACGGCAAGTTCAACGGCA R: TGGCAGGTTTCTCCAGGCGGC	606bp	NM_017008	55

All the primers were obtained from published literature (Chen *et al* 2004).

PCR conditions

PCR for all the three sets of primers was performed for 35 cycles with the reaction mixtures and conditions shown in table 2.4

Table 2.4: Reaction mixture for various primer sets

Reagents	Vimentin	Collagen type alpha 1	GAPDH
cDNA	2 µl	2 µl	2 µl
1X PCR buffer	2.5 mM	2.5 mM	2.5 mM
Magnesium chloride	1.5 mM	1.5mM	2.5 mM
dNTPs	200 µM	200 µM	200 µM
Forward primer	5.0 pm	5.0 pm	5.0 pm
Reverse primer	5.0 pm	5.0 pm	5.0 pm
Taq polymerase	1U	1U	1U
Total volume	25 µl	25 µl	25µl

PCR conditions for Vimentin, collagen type 1 alpha 1 and GAPDH primers

1. Initial denaturation at 94^{o}C for 3 minutes
2. Denaturation at 94^{o}C for 30 seconds
3. Annealing of primers at 55.5^{o}C for 30 seconds
4. Extension at 72^{o}C for 45 seconds. The steps 2-4 were repeated for 35 cycles.
5. Final extension step at 72^{o}C for 5 minutes.

2.4.7.3.3 Quality of PCR products

PCR products were run on 1.5% agarose gel to determine their quantity and quality.

Gel Preparation

1. Required amount of agarose was added to electrophoresis buffer (1X Tris Borate EDTA- TAE) in a glass flask.
2. Agarose was boiled in microwave and the flask was swirled to ensure even mixing.
3. Melted agarose was cooled to a tolerable temperature (~55^{o}C).
4. Ethidium bromide (EtBr-5µg/mL) was added and the flask was swirled for even mixing of EtBr.
5. The melted gel was poured in the casting apparatus with an inserted comb.
6. The gel was allowed to stand till it got solidified.
7. The comb was removed gently from the gel plate after solidification.

Gel loading and running

1. The gel plate was placed in the electrophoresis tank
2. 1X TAE buffer was poured to cover the wells
3. PCR products were mixed with 1µl of loading buffer (6X) (MBI Fermentas) on parafilm and were loaded with a micropipette into the wells along with the marker (100-bp ladder) (MBI Fermentas) to determine their size
4. The gel was run for approximately half an hour at a voltage supply of 10V/cm till bromophenol migrated atleast half the distance through the gel
5. The gel was removed from the tank and was placed on UV transilluminator (UV tec) and the amplification was documented in a gel doc system

2.4.8 Differentiation potential

2.4.8.1 Adipogenic differentiation

Passage 2 cells were seeded on cover slips in 6 well plates and cultured in complete medium upto confluency. At confluency, the cells were switched to an adipogenic induction medium (Appendix 1) and further cultured up to 21 days with the medium being changed on every alternate day.

2.4.8.1.1 Oil red O staining

After 21 days, the adipogenic cultures were fixed in 4% paraformaldehyde for atleast 1hr and fixative was carefully aspirated and cultures were rinsed three times with PBS. Then washed twice with water. Three ml of fresh 0.3% oil Red-O solution was added and incubated for 2hr at room temperature. After incubation, the oil red O solution was removed and washed thrice with water. Then counterstained with haematoxylin for 5 to 15 minutes.

2.4.8.2 Osteogenic differentiation

Passage 2 cells were seeded on cover slips in 6 well plates and cultured in complete medium upto confluency. The medium was then replaced with a calcification medium (Appendix 1) and incubated for 21 days. After incubation these cover slips were stained with fresh 0.5% alizarin red solution.

2.4.8.2.1 Alizarin Red staining

Alizarin Red S, an anthraquinone derivative, may be used to identify calcium in tissue sections. The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere, but these elements usually do not occur in sufficient concentration to interfere with the staining.

Calcium forms an Alizarin Red S-calcium complex in a chelation process, and the end product is birefringent.

1. Coverslips were fixed with methanol for 5 min.
2. Coverslips were stained with alizarin red solution (2%) for 30 seconds to 5 minutes
3. Shakeoff excess dye and blot sections
4. Dehydrated in acetone (20 dips). Then in acetone-xylene (1:1) solution (20 dips)
5. Clear in xylene and mount in a mounting media

2.4.8.3 Neural differentiation: Passage 2 cells were used for neural differentiation. At confluency, the cells were switched to DMEM+ ITS for 24 hrs. After 24 hrs, the neurogenic induction medium (Appendix 1) was added. After 6-7 days the cultures were terminated and processed for immunocytochemistry and RT-PCR analysis.

2.5 Results:

2.5.1 Isolation and culturing of MSCs:

2.5.1.1 By plastic adherence: The cell suspension containing both stromal and hematopoietic were seeded in tissue culture flasks using DMEM with 10% FCS. At the end of two days, many of the round and spindle shaped cells had attached to the base of the tissue culture flask. The rounded cells remained adherent even after subsequent media change (Figure 2.1a).

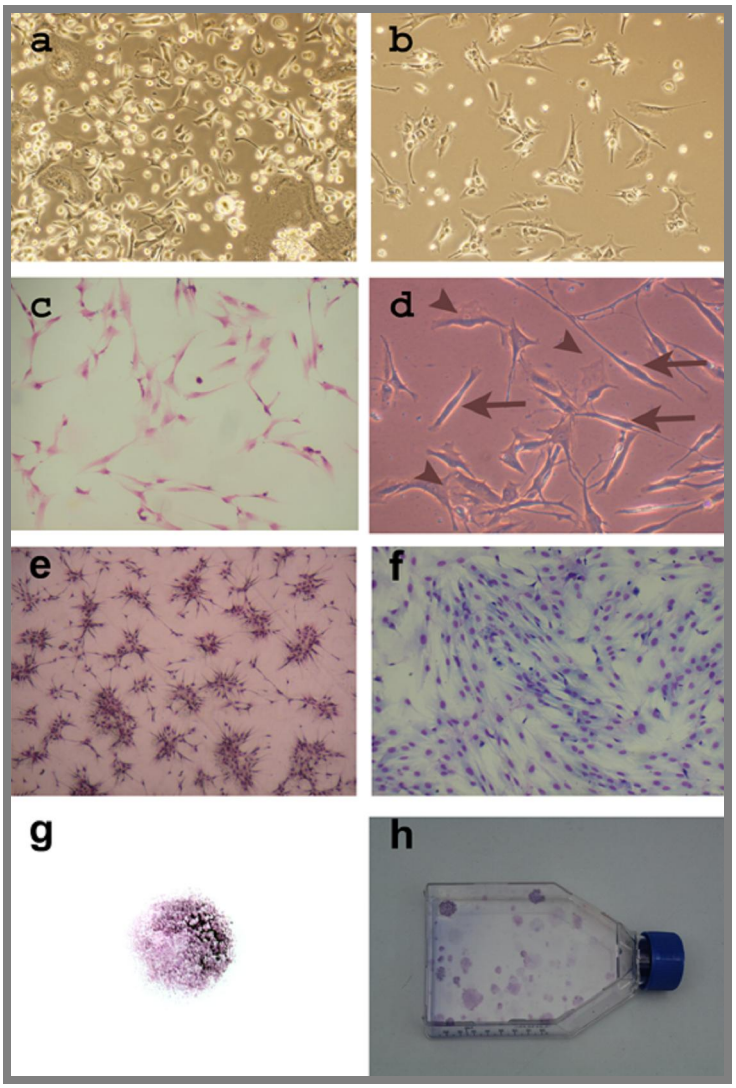

Figure 2.1: Bone marrow stromal cells by plastic adherence (a) and combined density gradient and plastic adherence (b-h). The bone marrow stromal cells (BMSCs) showing spindle like shape in morphology (c) by adherence to the plastic. The BMSC showed diverse morphologies including spindle shaped cells (indicated by arrow) and broad flattened cells (indicated by arrowheads) (d). These cells are forming colonies on day 4 (e) and growing the colonies more densely distributed (f). Cells formed colonies after 14 days of culture, when seeding on low density stained with methylene blue. g – Single colony; h – T75 flask.

2.5.1.2 Ficoll hypaque separation and plastic adherence (Combination Method): The cell suspension was layered on hypaque and was subsequently seeded in tissue culture flask. The spindle shaped cells attached to the bottom of

the flask while the round cells remain suspended in the medium and were mostly eliminated from the culture with subsequent media changes. (Figure 2.1b). Majority of adherent cells displayed a spindle like shape (Figure 2.1c). These cells began to proliferate at about day 4, and gradually grew to form small colonies (Figure 2.1d). By day 7, the number of cellular colonies of different sizes had obviously increased. In large colonies, cells were more densely distributed and showed a spindle shape (Figure 2.1e). As cells continued to grow, colonies gradually expanded in size and reached confluency by day 10. Passaged MSCs behaved similarly to those in primary cultures. However, the cells were larger and more heterogeneous in morphology and growth properties. Grossly, the MSCs in subcultures could be divided into two types, spindle shaped and broad flattened cells (Figure 2.1f). The flattened cells seldom proliferated and were gradually surrounded by the spindle shaped cells, which replicated faster. It seemed that the spindle-like MSCs gradually transformed into broad flattened cells with further passages. When seeding on low density the cells form colonies. The colony forming efficiency was counted as 10%.

2.5.2 Characterization

2.5.2.1 Flow Cytometry: The mononuclear cells expressed CD45, CD11a, CD18 and CD31 (Figure 2.2) suggesting hematopoietic lineage. The adherent marrow stromal cells expressed Vimentin, Fibronectin and CD90 (Figure 2.3). They expressed neither hematopoietic lineage markers such as CD45, CD11a, CD18 nor an endothelial related antigen CD31 (Figure 2.3). The lack of expression of CD45, CD11a, CD18 and CD31 suggests that cell cultures were

depleted of hematopoietic cells during sub cultivation. Table 2.5 summarizes the expression of markers by stromal cells isolated by solo density gradient centrifugation, solo plastic adherence, and combination of both.

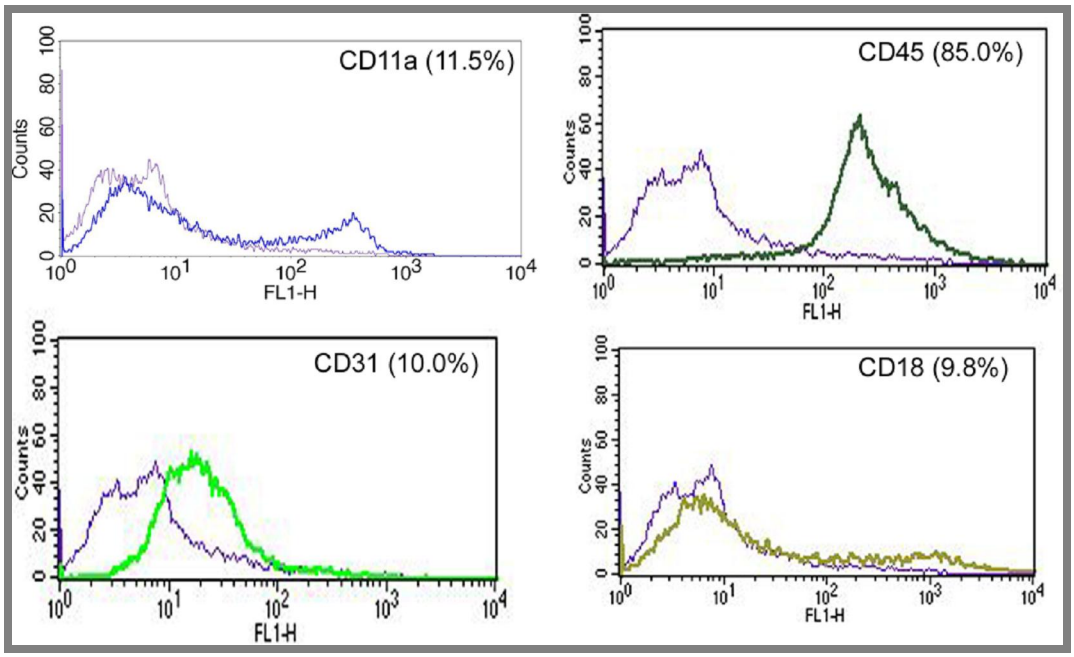

Figure 2.2: Flow cytometry analysis of rat bone marrow mononuclear cells: Bone marrow mononuclear cells expressed the markers of CD45, CD31, CD11a and CD18 and the percentage of positivity was mentioned in brackets. The purple line indicates the isotype matched antibody serving as a control, x-axes intensity log values, y-axes cell counts.

Table 2.5: Flow cytometry analysis of rat marrow stromal cells were isolated by different techniques and the percentage of expression of each marker was measured by flow cytometry analysis.

Marker	**Ficoll density gradient centrifugation ($1x10^6$ cells)**	**Plastic Adherence ($1x10^6$ cells)**	**Ficoll density gradient centrifugation and plastic adherence ($1x10^6$ cells)**
CD90	1.2%	24.4%	84%
Fibronectin	0.9%	19.3%	77%
CD45	85.0%	15.4%	6.7%

CD11a	11.5%	3.7%	1.5%
CD31	10.0%	0.5%	0.4%
CD18	9.8%	30.9%	2.7%
Vimentin	1.4%	24.1%	87.8%

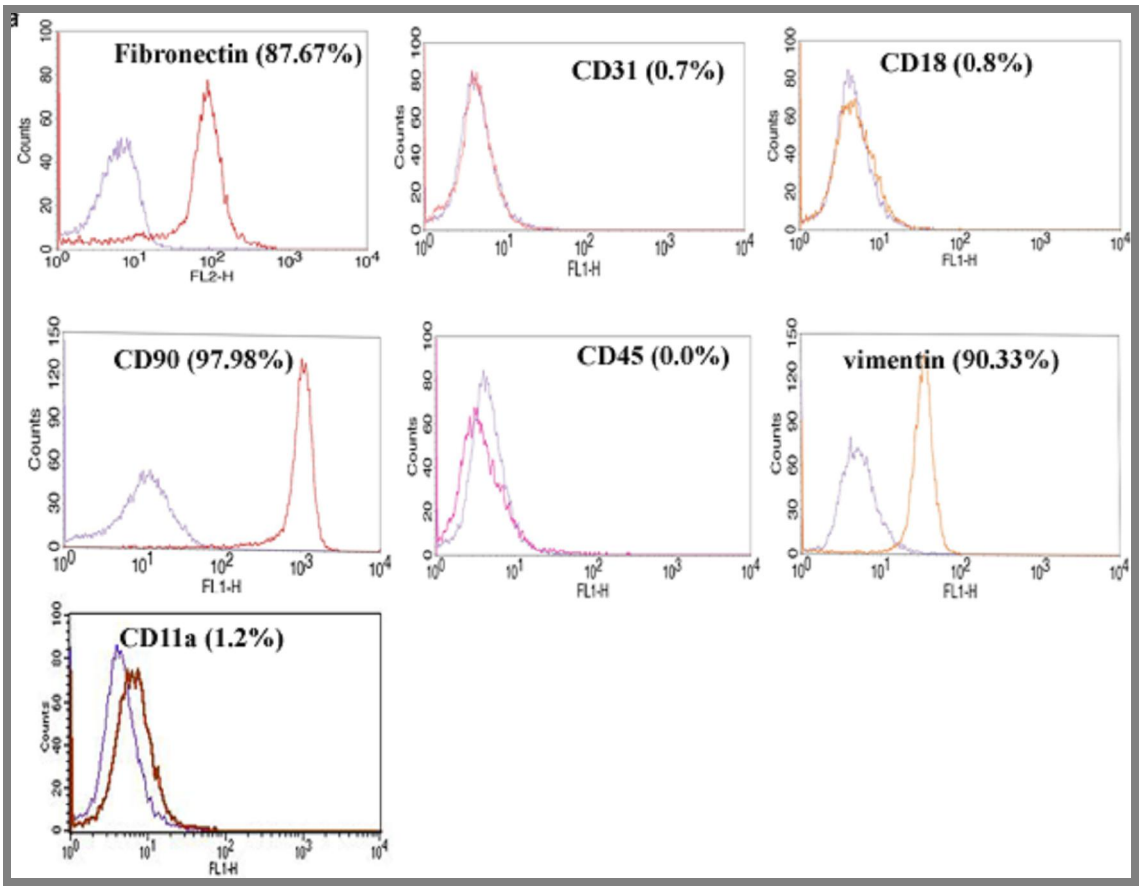

Figure 2.3: Flow cytometry analysis of bone marrow stromal cells expressed the markers of CD90, fibronectin and vimentin and negative for CD31, CD45, CD11a, CD18 and the percentage of positivity was mentioned in the brackets. The purple line indicates the isotype matched antibody serving as a control, x-axes intensity log values, y-axes cell counts

2.5.2.2 Immunocytochemistry: The mononuclear cells showed a high nucleus to cytoplasmic ratio on giemsa staining (Figure 2.4). Immunocytochemistry examination clearly detected the localization of CD34, CD45, CD11a, CD18 and

CD31 on bone marrow mononuclear cells (Figure 2.4). Rat bone marrow stromal cells were expressing the markers of Vimentin and CD90 but negative for CD45, CD11a, CD18, and CD31 (Figure 2.5).

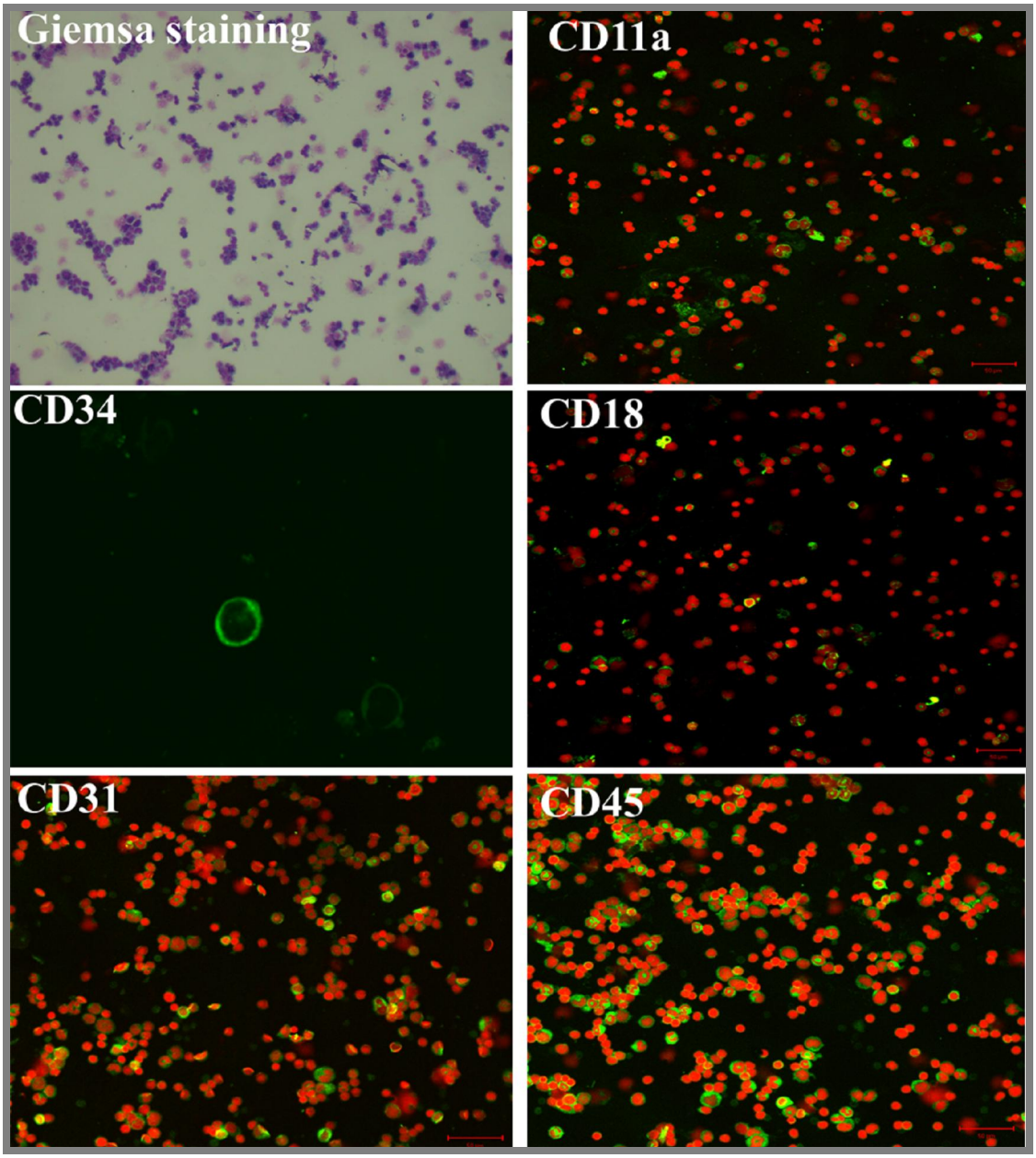

Figure 2.4: Characterization of rat bone marrow nuclear cells by immunocytochemsitry: Bone marrow mononuclear cells showing high nucleus to cytoplasmic ratio by giemsa staining. Immunofluoresence analysis showing the cells positive (green fluroscence) for CD11a, CD34, CD18, CD31 and CD45. Nucleus was counterstained with propidium Iodide (red).

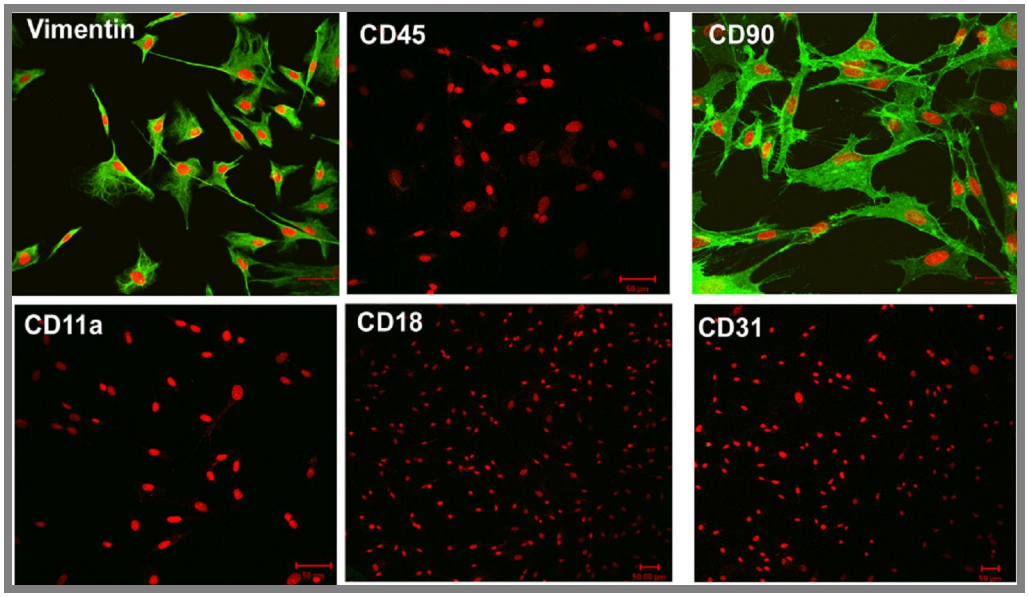

Figure 2.5: Immunofluroscence analysis of bone marrow stromal cells showing positive (green fluroscence) for vimentin, CD90 and negative for the CD45, CD11a, CD18 and CD31. Nucleus was counterstained with propidium Iodide.

2.5.2.3 RT-PCR: As the RT-PCR results showed expression of Vimentin and collagen type 1 alpha 1 in isolated BMSCs (Figure 2.6). This shows that the isolated cells are genuine marrow stromal cells with little or no contamination from other bone marrow cells such as hematopoietic cells.

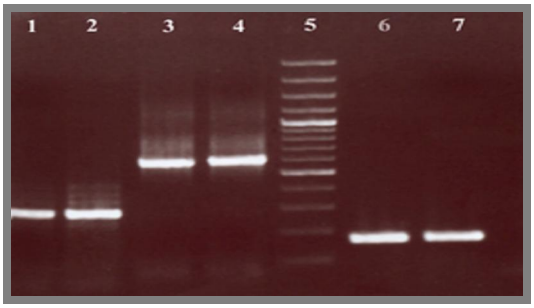

Figure 2.6: Gene expression during culture of marrow stromal cells by reverse transcription/polymerase chain reaction (RT-PCR). Samples were run in duplicates. Lane 1&2: Vimentin; Lane 3&4: GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase used as a internal control); Lane 5: 100bp ladder; Lane 6 and 7: Collagen type 1 alpha 1.

2.5.3 Differentiation

2.5.3.1 Adipocytic and Osteocytic differentiation: MSCs were differentiated in vitro using adipogenic, oesteogenic and chondrogenic induction media. Following 3 weeks of adipogenic induction, the cells stained Oil red 'O' positive showing lipid laden adipocyte phenotype (Figure 2.7). Similarly, when induced with oesteogenic induction medium for 2-3 weeks, these cells showed oesteogensis upon staining with alizarin red for calcium deposits (Figure 2.8).

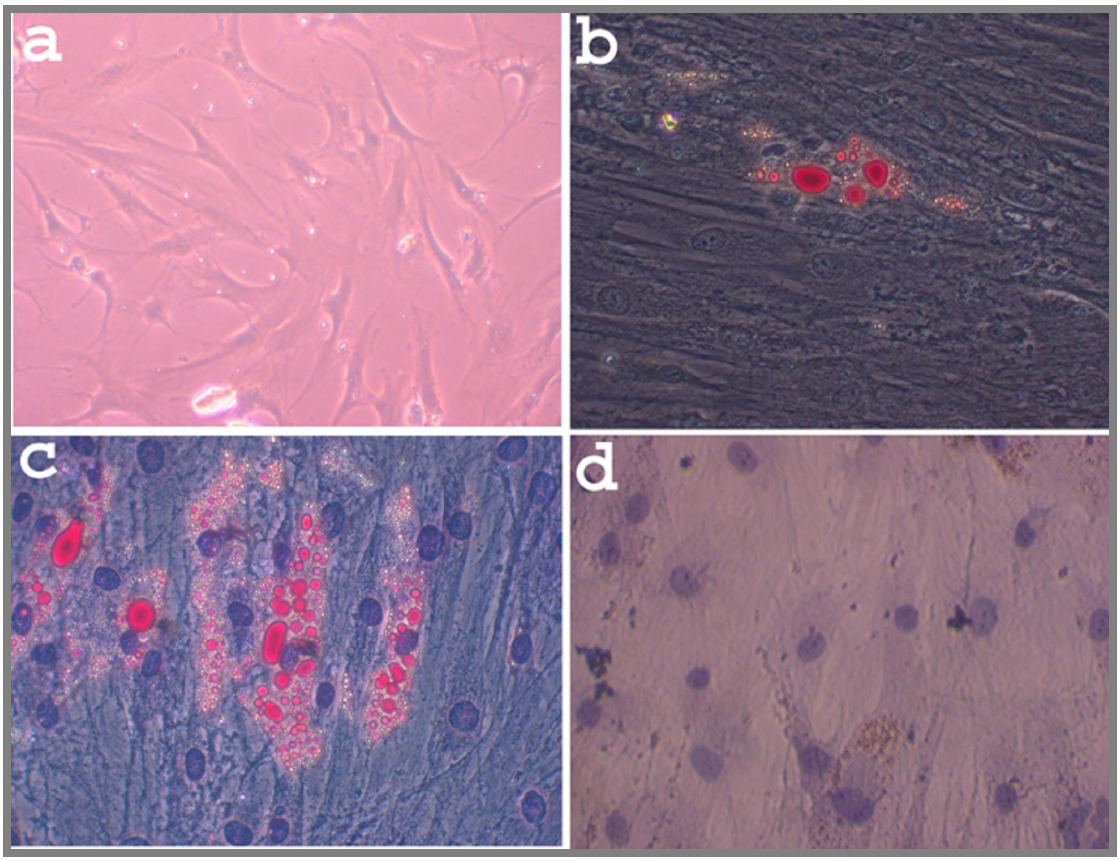

Figure 2.7: Adipocyte differentiation of rat marrow stromal cells. Upon induction with adipocyte induction media cells showed adipocyte globules on oil redo staining. a- before differentiation b- after differentiation with oil red o stain c- after differentiation with oil red o and counter stain with giemsa d- negative control (without induction media). (Magnificaton, 20X)

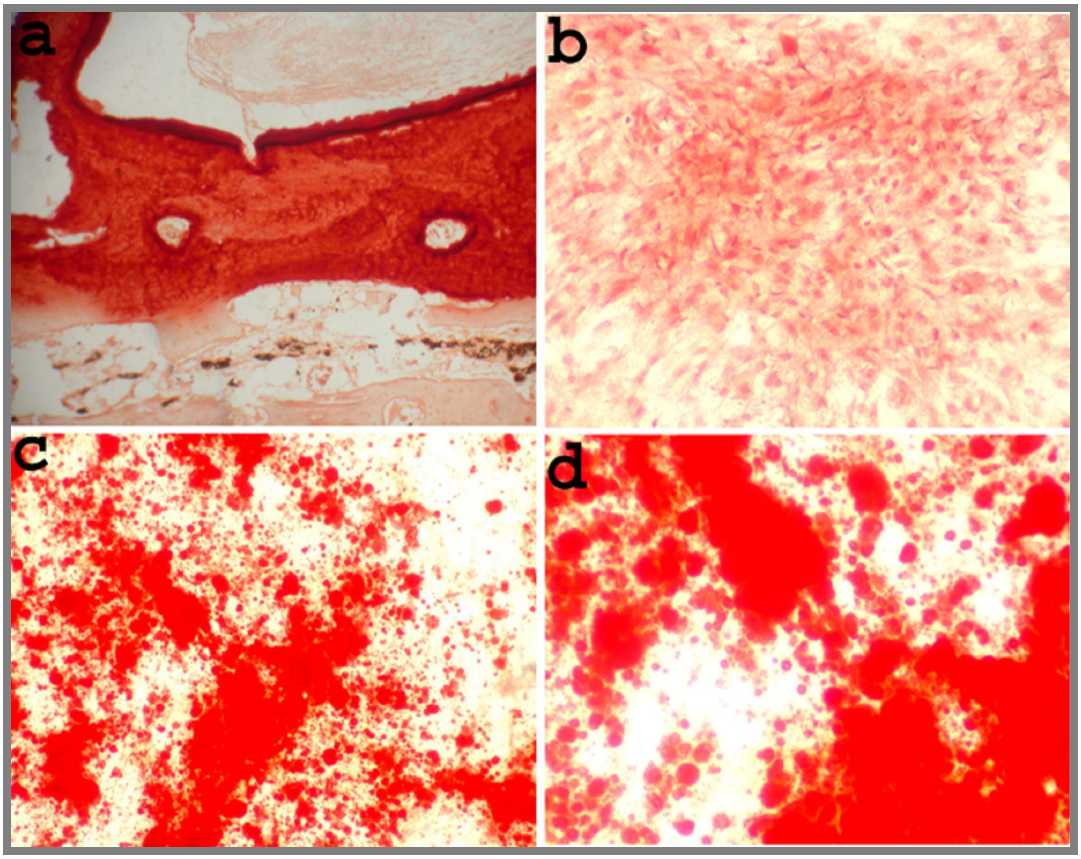

Figure 2.8: Osteogenic differentiation: Upon induction with osteogenic induction media cells showed calcium deposits on alizarin red staining. A- Positive control b- Negative control c and d - after differentiation with alizarin red stain. (Magnification, 20x)

2.5.3.2 Neural Differentiation: MSCs when induced with neural differentiation media for 8 days under serum-free conditions started showing neuron like morphology by day 4 with slender dendritic processes and characteristic aura around soma. Under the induction conditions provided in our lab, different types of neuron like cells were observed based on their morphology and axonal polarity like unipolar, bipolar, bipolar pyramidal etc (Figure 2.9). Apart from these, flat glial like cells were also seen extensively. Cultures induced beyond day 10 however gradually lost affinity to polystyrene and floated off the flask surface.

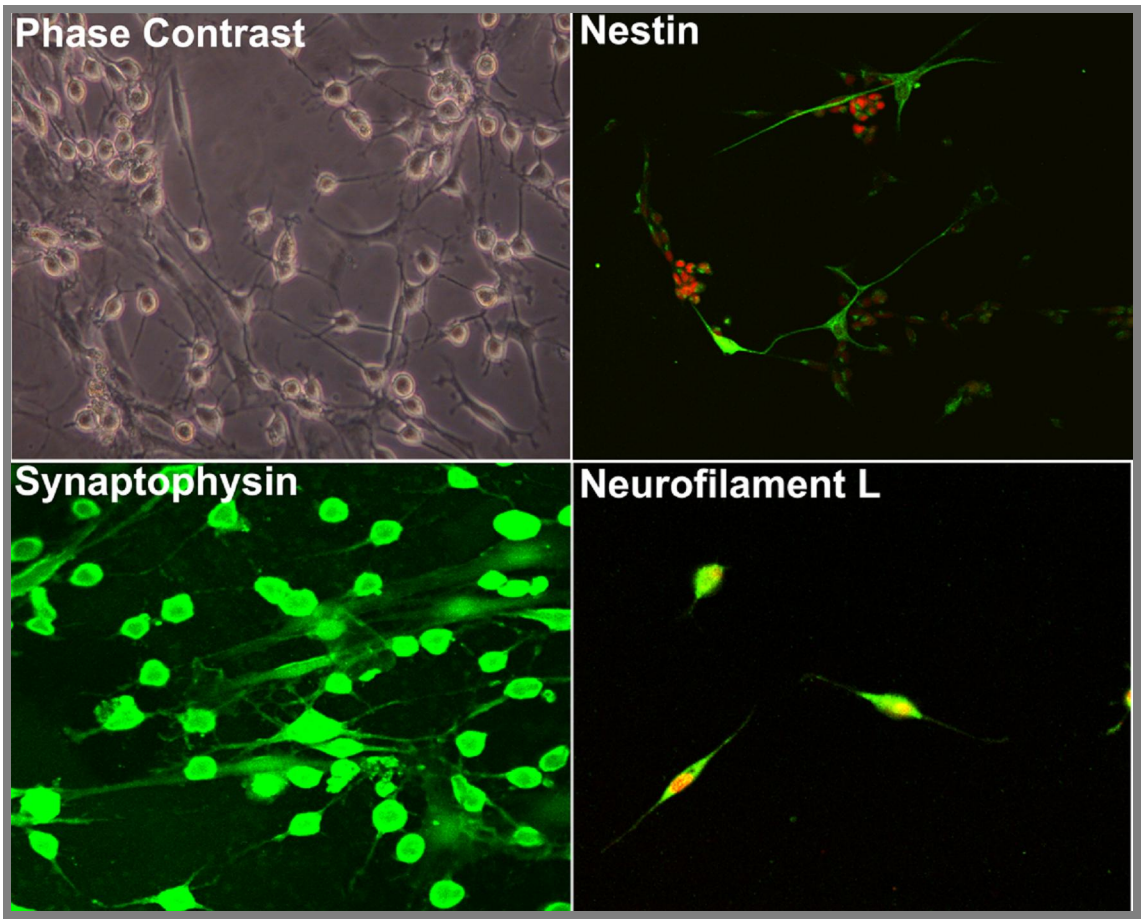

Figure 2.9: Neural Differentiation of Rat MSC. Neural differentiation of bone marrow stromal cells showed expressing the neural progenitor marker nestin and differentiated markers synaptophysin and neurofilament-H. (Magnification, 20x)

Immunocytochemistry was done on stromal cells differentiated into neuronal lineage. The cells were stained for neural specific markers using monoclonal antibodies. The differentiated neural cells stained positive for neural markers like nestin, Neurofilament, Synaptophysin (Figure 2.9).

2.6 Discussion

There are many methods to isolate stromal cells from bone marrow, including plastic adherence (Dexter *et al*., 1981), gradient density centrifugation (Chen *et al.,* 1991) and Immunomagnetic selection (Dezawa *et al*., 2004; Jia *et al*., 2002).

Different methods have their own limitations and advantages. For example, plastic adherence is an easy method of obtaining such cells on the basis of their plastic adherence characteristics, but it is difficult to get pure stromal cells. Gradient density centrifugation depends on the relative density of the cells to separate MSCs. Immunomagnetic selection uses MSC receptors and antigens. Other methods have also been used to isolate MSCs (Silva *et al.,* 2003; Korkko *et al.,* 2001), but none of these has been found to be optimal. In this study we used the Ficoll (1.077g/ml) method to isolate MSCs from bone marrow aspirate. After centrifugation, we found many suspended cells in the medium for 72 hrs. This could be due to the density of cells, which was changed slightly in DMEM. Therefore we combined the density gradient centrifugation with plastic adherence and changed the medium three times to obtain a purer isolate of MSCs after the density gradient centrifugation. According to the results, this method is relatively simple and can easily be used to obtain pure MSCs.

MSCs were first described in 1968 by Friedenstein *et al*., who discovered that MSCs adhered to tissue culture plates, resembled fibroblast in their morphology, and formed colonies (Friedenstein *et al.,* 1976). These characteristics have been identified in MSCs from numerous species including human, rat, mouse, rabbit and monkey. However, the expandability of MSCs in vitro varied dramatically among different species and different methodologies for isolation and plating of the cells. In our study, the MSCs adhered to the plate and had a fibroblast spindle-shaped morphology, forming colonies when grown in the low plating density. A small number of MSCs have a broad flattened shape.

The *in vitro* cultures of bone marrow stromal cells serve as a useful system for the investigation of various aspects of these cells, including a) establishing reproducible ways of culturing them in different labs by different techniques and sorting out the different population of cells from the same source b) exploring the stem cell like characteristics of these cells c) identifying the common characteristics of all mesenchymal stromal cells obtained from different parts of adult tissue e.g., fat, muscle, limbus, etc. d) exploring the potential of plasticity *in vivo* and *in vitro* and e) investigating their ability to reduce immunological rejection when given along with solid organ transplantation.

Several groups have illustrated the multipotentiality of rat bone marrow MSCs and their usefulness as sources for cell therapy. For example, Woodbury *et al.,* (2000) stimulated rat MSCs to differentiate into neurons by plating rat MSCs at 8,000cells/cm^2 and growing them to confluency. Passage 6 cells were then used for neuron differentiation (Woodbury *et al.,* 2000). Hofstetter *et al.* (2002) implanted rat MSCs into the spinal cord. They plated rat MSCs at 5,000cells/cm^2 and grew them to conflluency. Passage 5 cells were used for implantation (Hofstetter *et al*., 2002). Dezawa *et al.* (2001) induced rat MSCs to differentiated into Schwann cells *in vitro* and implanted into the sciatic nerve. Rat MSCs were subcultured four times and used (Dezawa *et al*., 2001). None of the authors have noted the quantum of increase of rat bone marrow MSCs; however, the cells had to be replated more than 4 times to harvest enough cells for their purposes. In one report (Yoshimura *et al*., 2007) obtained 10^8 cells at passage 4 with the initial density of 6000 cells at passage 2. In our study, we started with

10, 000 cells in 75 cm^2 flasks at passage 1 and obtained 10^8 cells at passage 3. These cells seem to be sufficient for in vitro or transplantation analyses. These data indicate that the proliferation ability of our rat bone marrow MSCs compares favorably with those in previous reports. However it needs to be seen whether these cells consist of a single of mixed population of stromal cells

The stromal cells are non-hematopoietic in lineage (Dexter *et al*., 1981, Chen *et al*., 1991). To prove this concept we have performed Flow cytometric analysis for bone marrow mononuclear and stromal cells. Bone marrow mononuclear cells showed positive results for the CD45, CD11a, CD31 and CD18, indicating they are of haematopoietic lineage. Dezawa *et al.,* (2004) have stated that rat mesenchymal stem cells were positive for CD29, CD90 and negative for the CD11b/c, CD31, CD34 and CD45. After culturing, the mononuclear cells, the stromal cells that were adherent at passage 3 were negative for CD45, CD11a, CD31 and CD18, indicating the absence of any hematopoietic contamination in the culture. Additionally, these cultures have shown positive result for CD90 and Vimentin, and Fibronectin, indicating they are pure non-hematopoietic stromal cells.

Prior gene expression profile studies of BMSCs, including micro array analysis, have shown that certain genes such as Vimentin and collagen type 1 alpha 1 are selectively enriched in these cells (Jia *et al*., 2002; Silva *et al*., 2003; Korkko *et al*., 2001). Therefore we selected these genes that confirm the identity of isolated cells. As the RT-PCR results show, these transcripts are expressed in

isolated BMSCs. This indicates that the isolated cells are genuine, non-hematopoietic marrow stromal cells.

The phenotypic analysis shows that the marrow stromal cells do not express markers of hematopoietic lineage but do express markers of non-hematopoietic cells. Based on the phenotypic analysis by IF and FACS and RT-PCR analysis, particularly the presence of CD90, fibronectin and Vimentin antibodies, there is a strong suggestion these mesenchymal cells have stem cell like characteristics. The self-renewal capacity up to an average of 20 passages also points towards the increased potential for proliferation, and some of the cells so obtained may prove to possess stem cell properties.

As for many other adult stem cells, MSCs are traditionally considered to be capable of differentiating into cell types of their own original lineage, i.e. mesenchymal derivatives. This study supports the findings of many other groups (Muraglia A, 2000) in showing that MSCs are capable of forming osteoblasts, chondrocytes and adipocytes *in vitro*. The ability of clonally expanded cells to form these three distinct cell types remains the only reliable functional criterion available to identify genuine MSCs and distinguish it from preosteoblast, preadipocyte or prechondreocytic cells which each only give rise to one cell type (Halleux C *et al*., 2001).

In summary, by the simple principle of adhesion, it is possible to establish an efficient method of harvesting a fairly homogenous population of bone marrow stromal cells the phenotypic characteristics of which point towards the stem cell like features.

ISOLATION, CHARACTERIZATION OF HUMAN BONE MARROW STROMAL CELLS AND THEIR DIFFERENTIATION POTENTIAL

3.1 Introduction:

Human cells can be obtained from a small amount of bone marrow aspirate. They are relatively easy to expand in culture under conditions in which they retain some of their ability to differentiate into multiple cell lineages, including tissues of mesenchymal origin (e.g., adipocytes, osteoblasts, chondrocytes, tenocytes and myocytes) as well as tissues of both endodermal (hepatocytes) and ectodermal origin (neural cells) (Wang G *et al*., 2005; Brazelton TR *et al*., 2000; Mezey E *et al*., 2000), thus allowing speculation about their pluripotency.

Among the adult stem cells, bone marrow-derived MSCs (MSC-BM) are being explored extensively in the hope that they will lead the way to autologous stem cell-based replacement therapies as well as in treating graft versus host disease (Dezawa M *et al*, 2004; Awad HA *et al*., 1999). For this reason, the MSC is one of the most extensively studied adult stem cell type with respect to transdifferentiation potential (Ferrari G *et al.*, 1998, Awad HA *et al*., 1999; Bruder SP *et al.*, 1998; Kadiyala S *et al.*, 1997; Pittenger MF *et al.*, 1999). Of all the lineages, the particular interest is neural differentiation as it holds promise for developing therapeutics for neurodegenerative diseases (Sugaya K, 2003; Torrente Y *et al*., 2002; Chopp M *et al*., 2002). However, due to the lack of universally defined cell surface markers to characterize the MSC (Javazon EH *et al*., 2004; Baksh D *et al*., 2004; Devine SM, 2002), it remains enigmatic with regard to both its identity and qualification as a true stem cell (Javazon EH *et al*., 2004; Baksh D *et al.*, 2004).

Perhaps most interesting reports that under certain conditions MSCs can be made to form neural cells. Most studies showing the formation of both neuronal and glial cells from MSCs have been carried out *in vitro* (Hermann A *et al*., 2004; Alexanaian AR, 2005). A number of different approaches have been reported to trigger this apparent transdifferentiation *in vitro*. Some groups have used chemical treatments such as DMSO, (Suzuki H *et al*., 2004; Devine SM *et al*., 2001) whilst others have opted for the use of growth factors (Hermann A *et al*., 2004; Alexanian AR, 2005; Magaki T *et al*., 2005; Bossolasco P *et al.*, 2005). However, it appears that early positive results obtained using DMSO-based protocols are unreliable, as the neural-like morphology and gene expression displayed by MSCs after treatment were in fact due to toxicity. (Lu P *et al*., 2004; Neuhuber B *et al*., 2004). Regardless of the ongoing debate about the nature of this differentiation and the possibility of artifacts, MSC transdifferentiation has been widely used and reported (Wislet-Gendebien S *et al*., 2005; Hermann A *et al*., 2004; Keilhoff G *et al*., 2006).

In this study, we attempted to establish cultures of human MSCs and evaluated their phenotype using surface markers over time. We evaluated the potential of these cells to differentiate to mesenchymal and non-mesenchymal cell lineages, i.e., their potential for neural differentiation.

3.2. Hypothesis:

We hypothesize that un-stimulated, discarded diagnostic marrow taps are minimally invasive and consistently serve as reliable source of MSC-BM.

3.3. Aims

- Ø Isolation and characterization of Human bone marrow mononuclear cells
- Ø Establishment and characterization of stromal cell cultures
- Ø Differentiation of these cell to other mesenchymal and neural lineages

3.4. Material and Methods

The protocol was approved by the Institutional Review Board at L.V.Prasad Eye Institute.

3.4.1. Preparation of Chemicals

All the chemicals and culture media were prepared as described in the appendix I.

3.4.2. Sterility Check for Chemicals & Media:

Following the filter sterilization the media and chemicals were kept for sterility check. A few drops of media/chemicals were inoculated on chocolate agar and in thioglycolate broth to screen for both aerobic and anaerobic microorganisms. The inoculated media were then incubated in a bacterial incubator at 37^0C for about 7 days, before the media/chemicals are approved for tissue culture use.

3.4.3. Source of Bone Marrow

Bone marrow aspirates from human volunteers (15) and clinical marrow specimens of patients (15) were collected after seeking Institutional Review Board (IRB) approval and informed consent of the subjects.

3.4.4 Isolation of Bone Marrow Stromal Cells from Marrow Aspirates

3.4.4.1 Isolation of Bone Marrow Mononuclear Cells

1. Bone marrow (1-2cc) was collected in sodium heparin tubes from the upper iliac crest or sternum by using an aspiration needle
2. The aspirated bone marrow was diluted with PBS at a ratio of 1:3.
3. In 15 ml tarson tube 3ml of ficoll-hypaque was added.
4. Diluted bone marrow was then carefully layered on to the ficoll-hypaque without disturbing the density gradient.
5. The tubes were spun at 400x*g* for 30 min at 25^0 C.
6. After centrifugation, the upper layer was aspirated leaving the mononuclear cell layer undistrubed at the interphase (Buffycoat).
7. Carefully transferred the MNC-BM at the interphase to new 15ml tube.
8. Wash cells by adding PBS, mix gently and centrifuge at 200x*g* for 5 minutes at 25^0 C. Carefully removed supernatant completely.
9. Finally, the cell pellet was dissolved in 1ml of PBS and cell counting was done using hemocytometer.

3.4.4.2 Cell Counting:

The hemocytometer is a device originally designed for the counting of blood cells. It is now used to count other types of cells.

The cell suspension is then mixed with 1:1 ratio of trypan blue. The hemocytometer was cleaned first with alcohol and then wiping dry. The coverslip was positioned carefully over both the chambers. The diluted cell suspension was mixed thoroughly and then filled into the hemocytometer chambers with the

help of the micropipette. Total viable cells (trypan blue negative cells) were counted in 4 large squares (1 x 1 x 0.1 mm) or =100 cells. The cell count (cells per ml) was determined as follows:

Cell Count/ml = Average Cell Count/Square X Dilution Factor$X10^4$

3.4.5 Cell Culture Conditions:

The mononuclear cells were re-suspended in growth medium of DMEM supplemented with 10% FCS and plated in polysterene plastic T75 cm^2 tissue-culture flasks (Nunclone) at a density of $1x10^6$cells/ml. Non-adherent cells were removed after 48h. The cultures were maintained at 37^0 C in a humidified 5% CO_2 incubator. When the cells reached 80-90% confluency, the cultures were harvested with Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA; Sigma).

3.4.6 Population Doublings:

The population-doubling assay was performed on MSC from passages 0 through 6. $1X10^4$ MSC-BM were seeded at each passage and trypsinized after obtaining 75% confluency.

3.4.7 Colony-forming Assay:

For these assays, 2 cells per cm^2 at passage 0 were plated and cultured for 14 days in T75 cm^2 tissue culture flasks. The confluent cultures were fixed with methanol, and stained with giemsa. Colonies less than 2mm in diameter and faintly stained colonies were ignored, while counting.

3.4.8 Differentiation:

3.4.8.1 Adipocyte Differentiation:

Cell at passage 2 were seeded on cover slips in 6 well plates and cultured in DMEM with 10% FCS medium up to confluency. At confluency, the cells were switched to an adipogenic medium (Appendix 1) and further cultured up to 21 days with the medium being changed on every alternate day. After incubation, cells were fixed and stained with Oil red O staining (Chapter 2).

3.4.8.2 Osteocyte Differenentitation:

Cells at passage 2 were seeded on cover slips in 6 well plates and cultured in complete medium up to confluency. The medium was then replaced with a calcification medium (Appendix 1) and incubated for 21 days. After incubation these cover slips were stained with fresh 0.5% alizarin red solution (Chapter 2).

3.4.8.3 Neural Transdifferentiation

The stromal cells of the third passage were seeded on specially cut cover slips of 9x9mm area placed in 24 well plates. The cells were allowed to adhere to the coverslips overnight, supplemented with DMEM + 1X insulin transferring selenium (ITS) (Invitrogen) prior to neuronal induction. Later the serum free media were replaced with DMEM containing neural inducing growth factors viz., epidermal growth factor (EGF) 100ng/ml, basic fibroblast growth factor (bFGF) 10ng/ml, nerve growth factor (NGF) 50ng/ml, and platelet derived growth factor (PDGF) 10ng/ml and 1X ITS. Changing the induction media every alternative day for 8 days induced the cells. On day eight, the neural induction medium was withdrawn to which, DMEM with 1X ITS and Forskolin 10 μM/ml was added for

24 hours. Cells were later fixed with 4% paraformaldehyde (PFA) for immunocytochemistry. A similar induction protocol was used to obtain large-scale neural inductions with cells plated in T25 and T75 flasks for various other experiments.

3.4.9 Characterization of Cells

3.4.9.1 Immunocytochemistry: For immunofluoresence analysis, cells were fixed with acetone: methanol (1:1) washed with phosphate buffered saline (PBS) and incubated with the primary monoclonal and polyclonal antibodies (Table 3.1) for one hour at room temperature. The washed slides were then incubated in the dark with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody (FITC), (Dako, USA) for 30 min. Fluorescence was visualized using a Carl Zeiss Confocal microscope. The nucleus was counterstained with propidium iodide (PI).

Table 3.1: Antibodies used for immunocytochemistry

S.No.	Antibody	Dilutions	Company
1	CD90	1:150	Millipore
2	CD45	1:100	Millipore
3	CD11a	1:200	Millipore
4	CD29	1:250	Millipore
5	CD11c	1:300	Millipore
6	CD34	1:100	Millipore
7	FITC or TRITC-Conjugated 2^0 Antibody	1:200	Millipore

8	Vimentin	1:500	Dako Cytomation
9	HLA-ABC	1:250	Millipore
10	Neurofilament –L	1:150	Millipore
11	Growth associated protein 43	1:100	Millipore
12	Tau-1	1:200	Millipore

3.4.9.2 Flowcytometry:

MSCs were harvested from the tissue culture flasks after passage 2 *in-vitro* and centrifuged at 200x*g* for 5 min at room temperature. The cells were washed and counted using a Neubauer Chamber. A single cell suspension of 0.5 to 1x 10^6 cells in 100µL of buffer (PBS, 0.1% sodium azide, 2% FBS). The cells were incubated with primary antibody (Table 3.2) for 40min with saturating concentrations of monoclonal antibodies for the markers mentioned above. After washing the cells thrice in buffer and centrifuging at 200x*g* for 5 min, they were resuspended in ice cold PBS and then incubated with the FITC-labelled secondary antibody 30 minutes at 4^0C in the dark. Cell fluorescence was evaluated by flow cytometry in a FACS Caliber instrument (Becton Dickinson); data were analyzed using Cell Quest software (Becton Dickinson). An isotype control was included in each experiment, and specific staining was measured from the cross point of the isotype with specific antibody graph. A total of 10,000 events were acquired to determine the percentage positivity of the different cell surface markers used.

Table 3.2: List of antibodies used for flow cytometry

S.N0	Marker	Dilution (10 µl used for 100µl reaction)	Company
1	CD 34	1:50 (1mg/ml)	Millipore
2	CD45	1:50	Millipore
3	CD11a	1:50	Millipore
4	CD11c	1:50	Millipore
5	CD38	1:50	Millipore
6	CD138	1:50	Millipore
7	CD68	1:50	Millipore
8	CD106	1:50	Millipore
9	CD105	1:50	Millipore
10	CD90	1:50	Millipore
11	CD29	1:50	Millipore
12	CD71	1:50	Millipore
13	HLA-ABC	1:50	Millipore
14	HLA-DR	1:50	Millipore
15	CD31	1:50	Millipore
16	CD4	1:50	Millipore
17	CD8	1:50	Millipore
18	CD11b	1:50	Millipore
19	CD14	1:50	Millipore
20	IgG1	1:25 (0.5mg/ml)	Millipore
21	IgG2a	1:25	Millipore
22	IgG2b	1:25	Millipore

3.4.9.3 Microarray Experiment:

The material and methods of the current microarray experiments are discussed in detail in the coming fifth chapter.

3.4.9.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

The bone marrow stromal cells of the second passage were expanded and induced in a T75 culture flask and used for isolating whole RNA for RT-PCR experiments. Briefly, the neural induced cells were washed with 1X PBS and lysed in TRIZOL (Invitrogen). Whole RNA extraction was done according to the manufacturer's protocol. The first strand was synthesized using M-MuLV RT purchased from MBI Fermentas and subsequent amplification was performed with Taq DNA polymerase purchased from Bangalore Genei. 1μg of whole RNA isolated by trizol method was used for PCR. The Housekeeping gene, β- actin was used to assess the quality of the first strand. Primers for neural markers like Neuron specific Enolase, Tau, Neurofilament, GAP43 and Synaptophysin were designed using FastPCR primer designing software. The PCR was carried out with cycling conditions (Table 3.3). The PCR product was later analyzed by agarose gel electrophoresis and documented.

Table 3.3: Primer sequences for RT-PCR

Name	Accession No	Primer Sequence	Product size
Beta Tubulin 3 / Tuj1	NM_006086.2	F- 5' tcaagcgcatctccgagcag3' R- 5' accgtaaaacgtcaggcctggag 3'	444 bp
Synaptophysin	NM_003179.2	F- 5' tgacctcagcatcgaggtcgag 3' R- 5' acgaaccacaggttgccgac 3'	477 bp

GAP43/ Growth cones	NM_002045.2	F- 5' tccaaggctgaagatgccccag 3' R- 5' acagactcacagacgtgagcagg 3'	377 bp
Neurofilament	NM_006158.1	F- 5'tcagcgctatgcaggacacga R-5'ggtggacatcagataggagctgg	278 bp
NSE	NM_016835.2	F- 5'catcgacaaggctggctacacg 3′ R- 5'gacagttgcaggccttttcttc 3	329 bp
Tau	NM_016835	F-5′gtaaaagcaaagacgggactgg-3′, R-5′- atgatggatgttgcctaatgag-3′;	512 bp
GAPDH3	M33197	F- 5' gccaaggtcatccatgacaac 3' R-5' gtccaccaccctgttgctgta 3'	497bp

3.5 Results:

3.5.1 Isolation and Culturing of MSC-BM: To select a preparation of MSC-BM for detailed examination, bone marrow aspirates were obtained from 15 human volunteers (Table 3.4). There was no apparent correlation between yield of mononuclear cells and volume of marrow obtained with an aspirate. Mononuclear cells were stained with giemsa for morphological characterization (Figure 3.1). After discarding the nonadherent cells by changing the medium, they were washed with PBS three times, 48hrs after primary culture. MSCs were seen to attach to culture dishes sparsely and the majority of cells displayed a spindle like shape (Figure 3.1). These cells began to proliferate at about day 4, and gradually grew to form small colonies (Figure 3.1). By day 7, the number of cellular colonies with different size had obviously increased. In large colonies cells were more densely distributed and showed a spindle shape (Figure 3.1). As the cells

continued to grow, colonies gradually expanded in size and reached confluency by day 10. Passaged MSC-BM behaved similarly to those in primary cultures.

Table 3.4: List of human bone marrow samples used and yield of mononuclear cells.

S.No	Patient ID	Age/Sex (yrs/M/F)	Volume of Sample (ml)	Yield of Mononuclear Cells (millions)	Yield of MNC's per ml of bone marrow
1	P127637	53/M	2	11.0	5.5
2	P300209	35/F	2	34.0	17.0
3	P262674	64/M	2	19.3	9.65
4	P298264	45/F	2	9.7	4.85
5	N110313	3/M	1.5	8.9	5.93
6	N115300	1/1/2/M	1	11.2	11.2
7	N115025	1/M	1.5	5.6	3.73
8	P279517	61/M	1.5	4.5	3.0
9	P085151	21/2/M	1	9.6	9.6
10	P282907	5/F	1	8.5	8.5
11	P279647	64/M	1.5	11	7.3
12	P085157	48/M	2	12	6.0
13	N108718	2/M	1	8.0	8.0
14	P279913	21/2/F	1	5.0	5,0
15	P228289	64/M	1.5	4.8	3.2

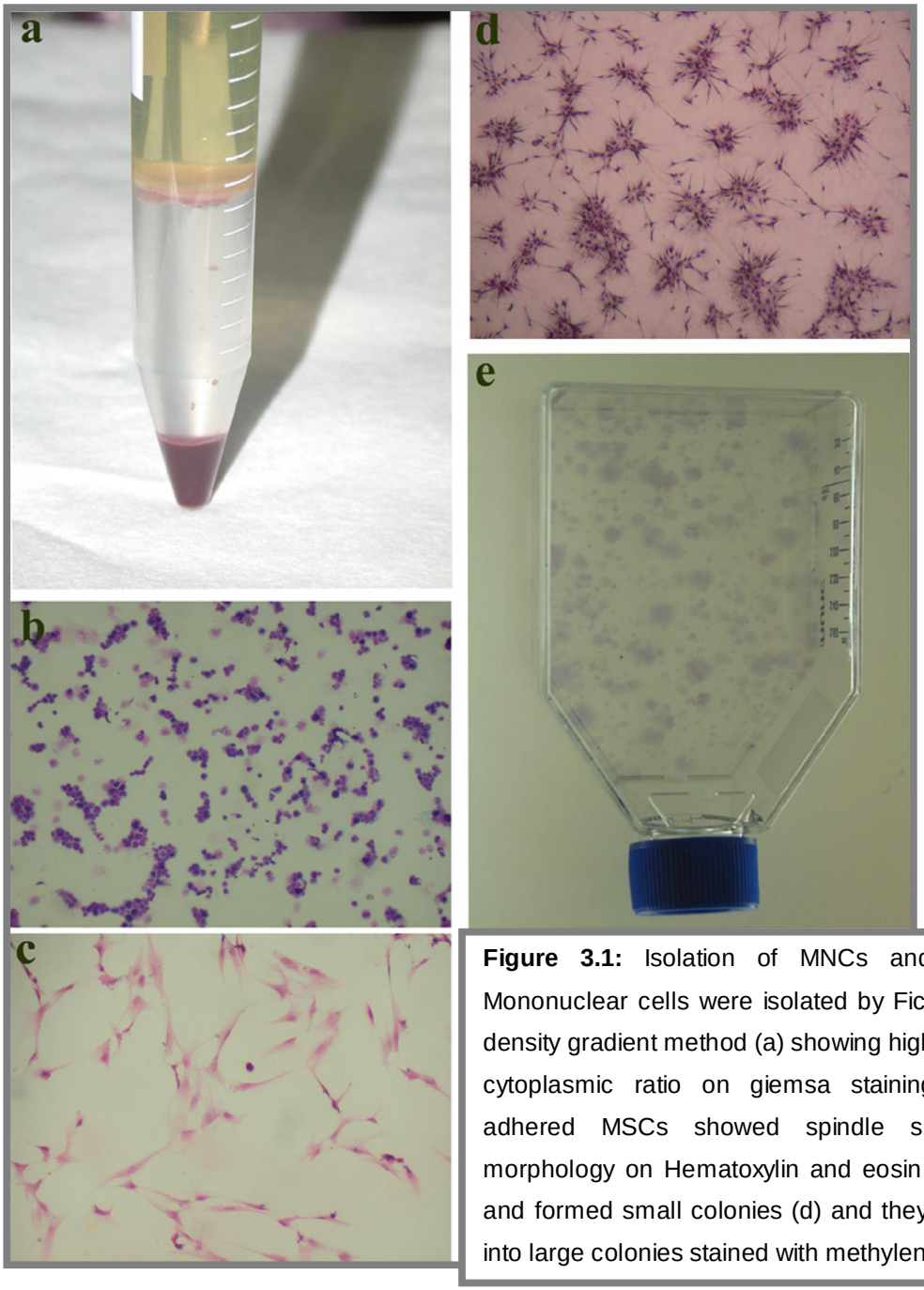

Figure 3.1: Isolation of MNCs and MSCs – Mononuclear cells were isolated by Ficoll-hypaque density gradient method (a) showing high nucleus to cytoplasmic ratio on giemsa staining (b). The adhered MSCs showed spindle shaped like morphology on Hematoxylin and eosin staining (c) and formed small colonies (d) and they grew them into large colonies stained with methylene blue (e)

3.5.2 Characterization of MSC's

3.5.2.1 Flow Cytometry: The adherent MSCs expressed mesenchymal markers (CD90, CD105, CD29, CD71), endothelial makers (VCAM), and MHC Class I, but were negative for MHC class II and hematopoietic markers (CD34, CD45,

CD11a, CD11c, CD38, CD138, CD68, CD25, CD4, CD8, CD11b and CD14 (Table 3.5). The expression profile of MSC showed a decrease in expression of VCAM/CD106 and CD105 after passage 4 (Figure 3.2) and CD71 and CD29 at passage 5.

Table 3.5: Surface antigen profile of MSCs - The table summarizes the results of immunophenotyping of MSCs by FACs analysis from passage 0 to passage 5. Percentage expressions of markers are given (average values of three such experiments ± standard deviation. Percentage less than 2 is considered as a Negative.

S.N0	Marker	P0	P1	P2	P3	P4	P5
1	CD 34	0.89±0.12%					
2	CD45	1.2±0.45%					
3	CD11a	0.89±0.23%					
4	CD11c	1.5±0.21					
5	CD38	0.00%					
6	CD138	0.23±0.12					
7	CD68	0.87±0.23					
8	CD106	55.76±3.5%	54.76±4.5	47.45±2.5%	48.42±1.2%	10.85±2.45	6.34±2.12
9	CD105	81.33±4.2	83.45±5.6	78.0±6.2	73.34±3.2	44.16±8.9	7.41±2.1
10	CD90	98.45±2.13	98.65±5.2	95.34±2.45	98.87±5.6	92.42±3.6	91.46±5.6
11	CD29	78.42±3.4	80.45±7.6	84.92±4.65	76.78±5.6	82.56±72.5	42.14±3.45
12	CD71	54.34±4.5	52.34±4.6	56.76±2.5	49.56±3.6	51.56±3.4	7.94±2.13
13	HLA-ABC	98.99±1.98					
14	HLA-DR	0.98±0.12					
15	CD31	1.45±0.89					
16	CD4	0.23±0.12					
17	CD8	0.87±0.34					
18	CD11b	0.98±0.12					
19	CD14	1.56±0.34					

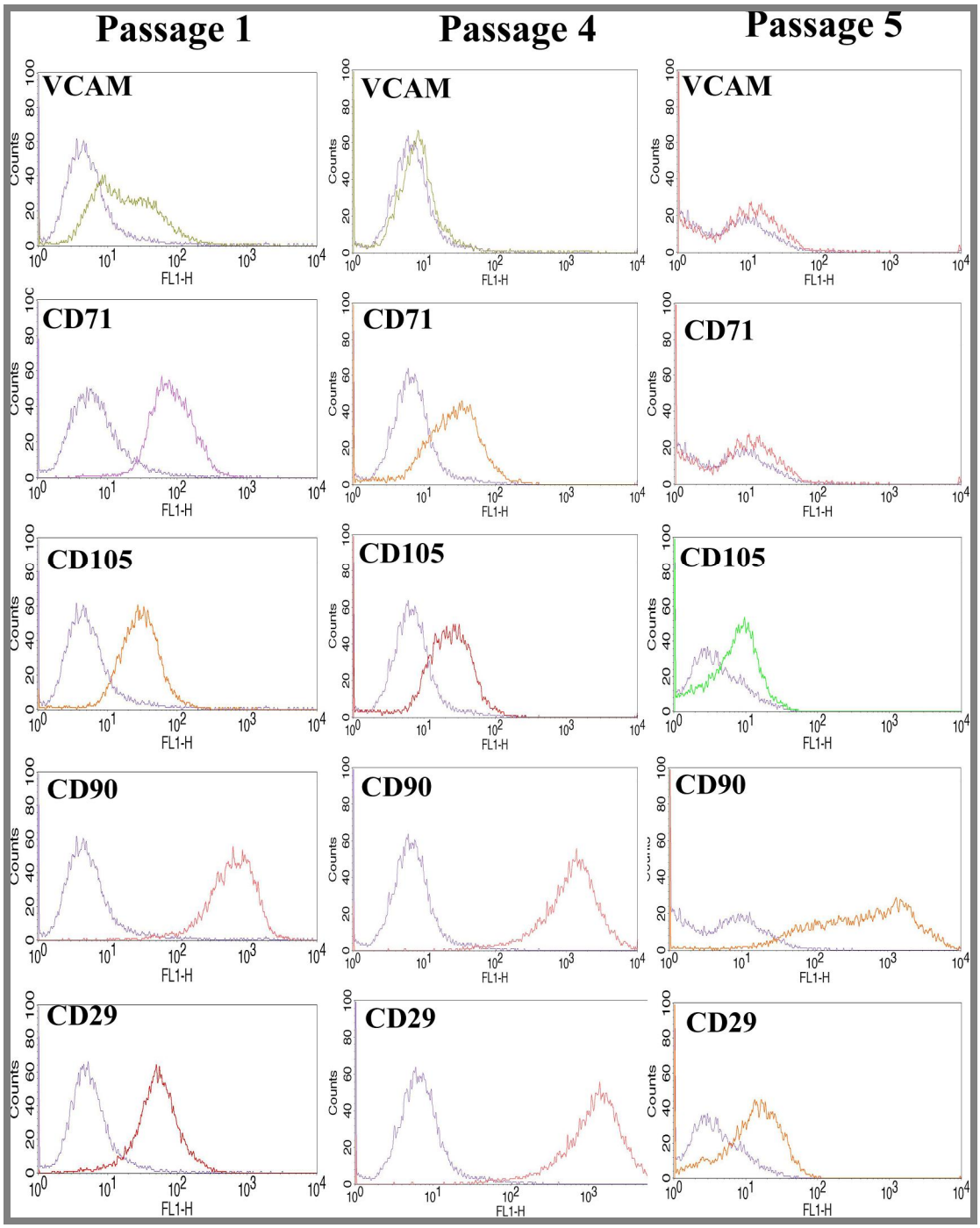

Figure 3.2: Flow cytometry analysis of BMSCs at Passage 1 (P1) passage 4 (P4) and passage 5 (P5). The purple line in the histograms represents the isotype control

3.5.2.2 Immunocytochemistry: Immunocytochemistry examination clearly detected the localization of CD34, CD45 and HLA-ABC on bone marrow mononuclear cells and Vimentin, CD90 and CD29 on MSCs. MSCs were negative for CD45, CD11a, CD34, and CD11c (Figure 3.3).

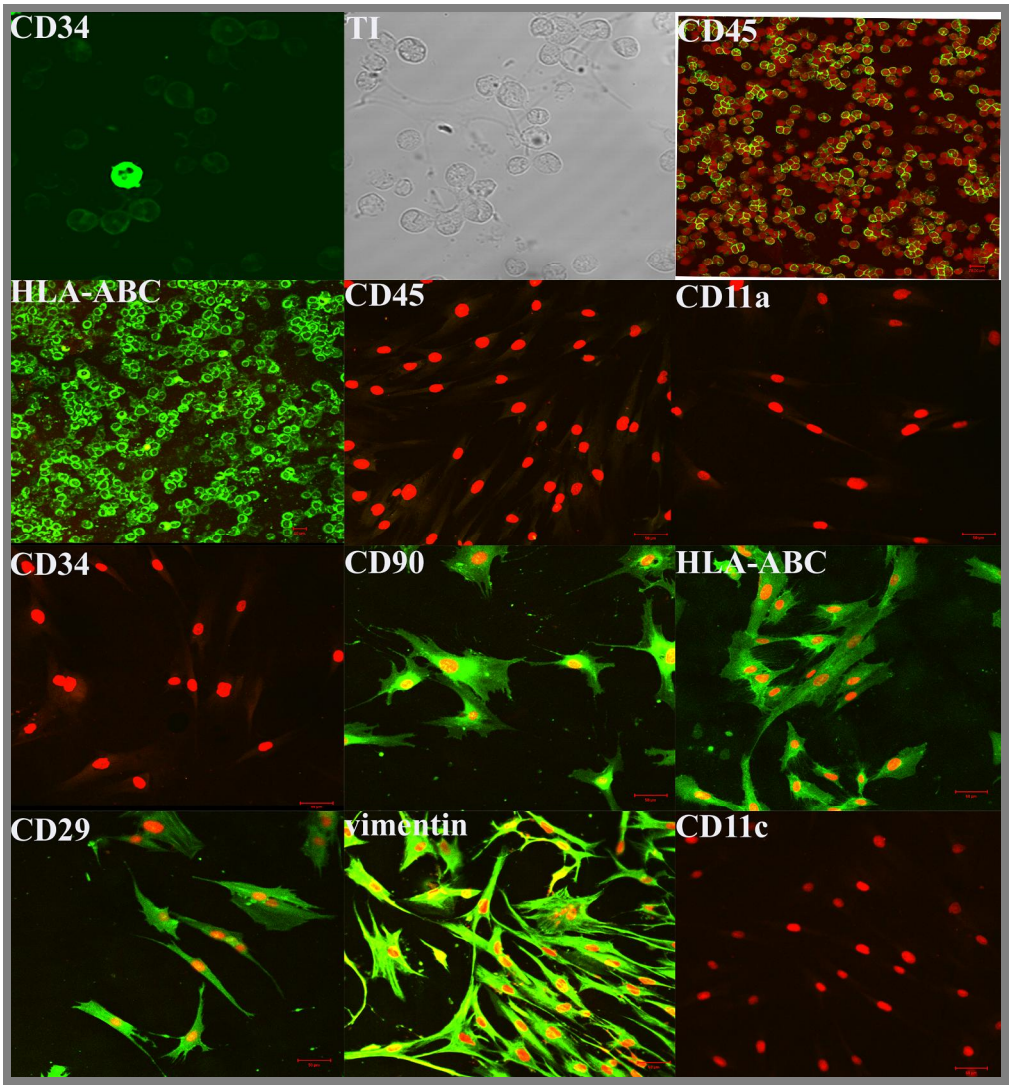

Figure 3.3: Immunophenotypic analysis of bone marrow mononuclear cells showed the positivity for CD34, CD45, HLA-ABC and MSCs-BM showing positive for the CD90, HLA-ABC, CD29, Vimentin and negative for CD45, CD11a, CD34, CD11c. Nucleus was counterstained with propidium iodide (PI). TI – Bright field image of CD34.

3.5.3 Differentiation

3.5.3.1 Adipocytic and Osteocytic Differentiation: MSCs were differentiated *in vitro* using adipogenic and oesteogenic induction media. Following 3 weeks of adipogenic induction, the cells stained Oil red 'O' positive showing lipid laden adipocyte phenotype (Figure 3.4). Similarly, when induced with oesteogenic induction medium for 2-3 weeks, these cells showed osteogensis upon staining with alizarin red for calcium deposits (Figure 3.4).

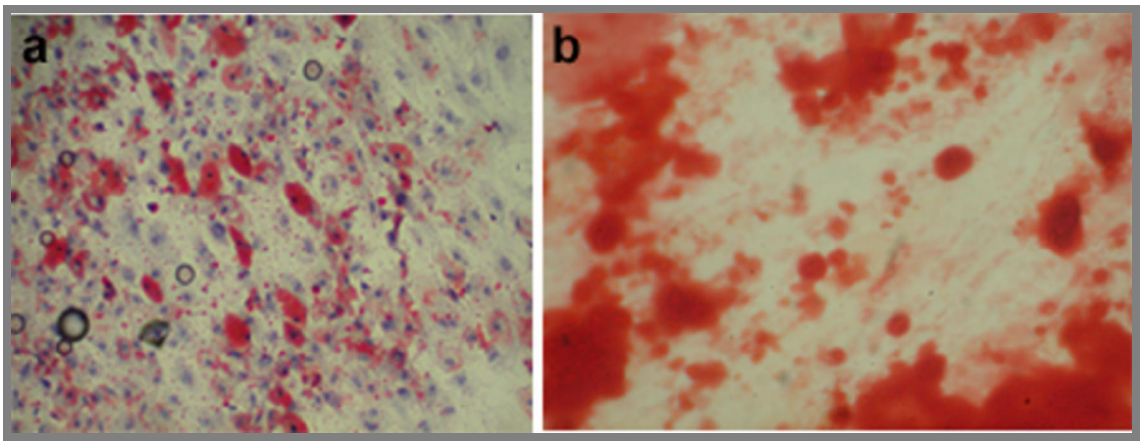

Figure 3.4: Differentiation potential of human bone marrow stromal cells to adipocyte (a) and osteocytes (b).

3.5.3.2 Neural Differentiation*:* MSCs when induced with neural differentiation media for 8 days under serum-free conditions started showing neuron like morphology by day 4 with slender dendritic processes and characteristic aura around soma. Under induced conditions, different types of neuron like cells were observed based on their morphology and axonal polarity like unipolar, bipolar, bipolar pyramidal etc (Figure 3.5). Apart from these, flat glial like cells were also seen extensively. Cultures induced beyond day 10 however gradually lost affinity to polystyrene and floated off the flask surface.

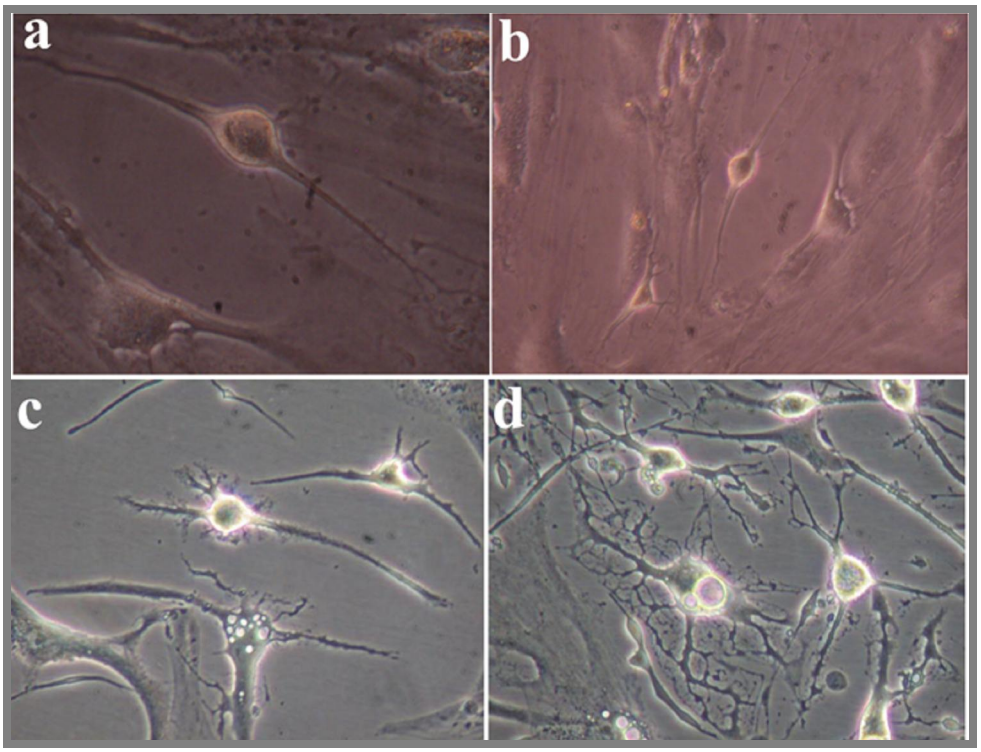

Figure 3.5: Neural induction of MSCs: After the induction by neuronal induction media different types of neuron like cells were observed based on their morphology and axonal polarity like unipolar, bipolar, bipolar pyramidal (a-c). Apart from these, flat glial like cells were also seen extensively (d).

3.5.3.2.1 RT-PCR*:* Neural lineage induced MSCs upon RT-PCR showed very faint expression of neural specific markers like Tau and Synaptophysin while moderate to strong expression of GAP43, neuron specific enolase (NSE) and neurofilament were observed. Synaptophysin, neurofilament and GAP43 showed an up regulatory trend in expression. RNA from undifferentiated sample also showed a varying range of expression of neural markers (Figure 3.6). Expression of the housekeeping gene GAPDH3 was served as an internal control.

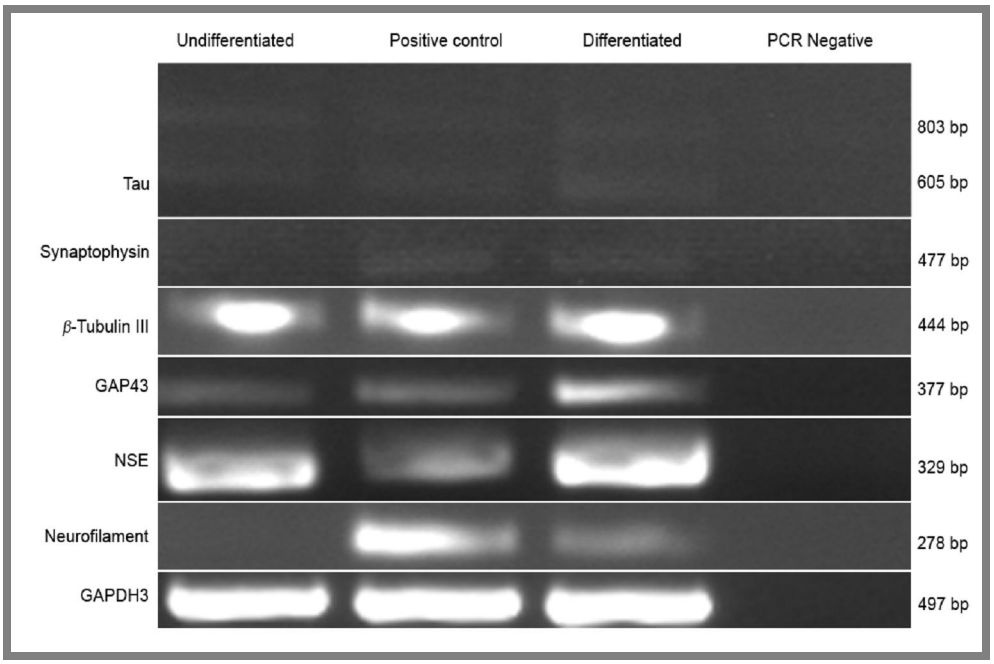

Figure 3.6: Reverse transcription polymerase chain reaction analysis of undifferentiated, differentiated bone marrow stromal cells and the positive control of Neuroblastoma cell line. RT-PCR analysis showed very faint expression of neural specific markers like Tau (as 2 isoforms) and Synaptophysin while moderate to strong expression of Beta-tubulin-III, GAP43, NSE and Neurofilament. Expression of the house-keeping gene GAPDH3 served as an internal control

3.5.3.2.2 Immunocytochemistry*:* On immunocytochemistry the differentiated neural cells stained positive for neural markers like Tau-1, Neurofilament and Growth associated protein- 43 (Figure 3.7).

3.5.3.2.3 Microarray

3.5.3.2.3.1 Quantitity and Quality of RNA

Extracted RNA was pure and of good quality as proven by NanoDrop reading (Tables 3.6, 3.7, 3.8) and electrophoresis on a denaturing agarose gel (Figure 3.8). The two 18S and 28S RNA bands were clearly visible with 28S bands approximately twice in intensity and thickness than the 18S bands. There was no

smearing of the gel in the electrophoresed path (i.e. no degradation of RNA) that was obvious as demonstrated in the below (Figure 3.8)

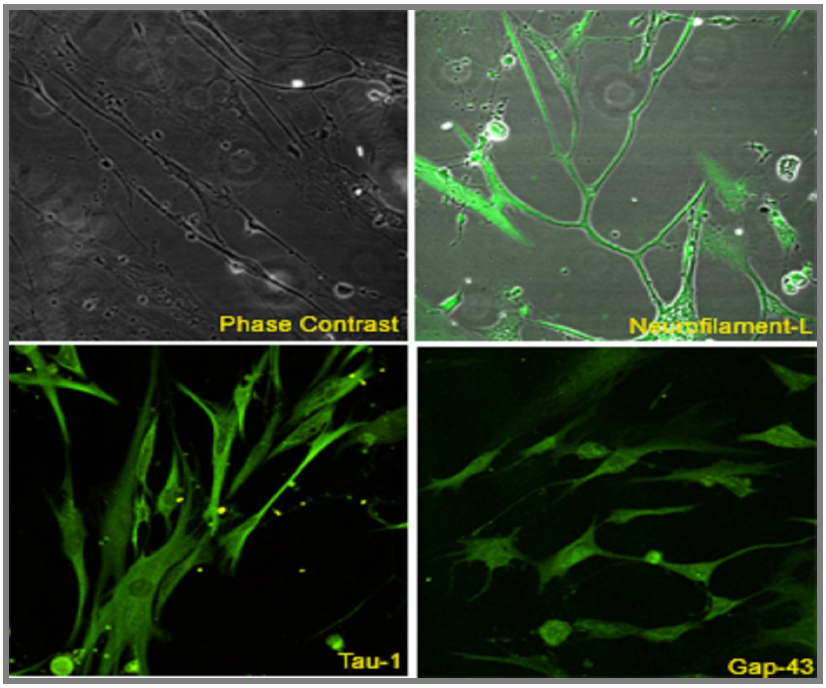

Figure 3.7: Immunocytochemical analysis of differentiated human bone marrow stromal cells expressing the mature neural markers Neurofilament L, Tau-1 and gap 43.

Table 3.6: Quantification of RNA by Nanodrop reading

Sample	Conc., (ng/µl)	260/280	260/230	260	280	230
MSC-BM (2) (Sample 1)	583.9	2.07	0.89	14.59	7.2	16.39
MSC-BM (1) (Sample 1)	809.4	1.92	0.63	20.23	10.5	32.280
MSC-BM (N) (Sample 1)	1118.6	1.97	1.02	27.9	14.20	27.527

MSC-BM (Sample 2)	298.8	2.03	1.09	7.649	3.677	6.840
MSC-BM (N)(Sample 2)	1069.6	2.05	1.29	26.74	13.04	20.671

Table 3.7: Quantification of RNA by Nanodrop reading after column purification

Sample	Conc., (ng/µl)	260/280	260/230	260	280	230
MSC-BM (1)	799.7	2.17	1.53	19.993	9.215	13.026
MSC-BM (N) (1)	1079.4	2.17	1.82	26.985	12.415	14.860
MSC-BM (2)	657.8	2.07	1.16	16.44	7.925	14.213
MSC-BM (N) (2)	970.4	2.11	1.23	24.259	11.517	19.797

Table 3.8: Quantification of RNA by Agarose gel

Sample	Volume loaded (500ng)	By agarose (500ng)
MSC-BM (1)	0.63	0.81
MSC-BM-N (1)	0.46	0.54
MSC-BM (2)	0.76	0.977
MSCBM (N) (2)	0.51	0.655

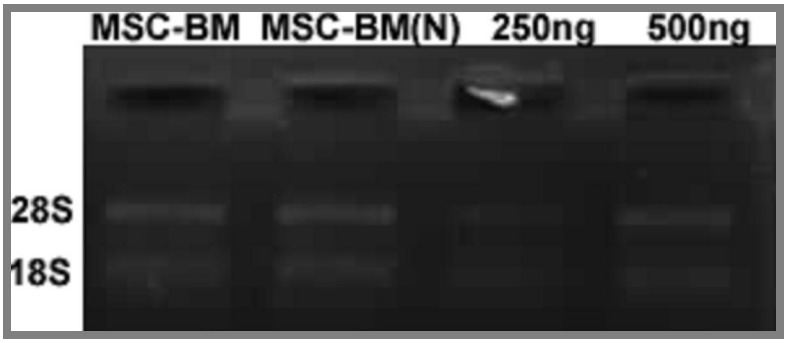

Figure 3.8: Integrity of RNA by agarose gel electrophoresis. Electrophoresed agarose formaldehyde gel revealing good quality RNA from MSC-BM and MSC-BM (N) as indicated by the 28S and 18S bands.

3.5.3.2.3.2: Quantity and Quality of cRNA

Labelled cRNA was of good quality and purity as proven by NanoDrop reading (Table 3.9) and electrophoresis on a agarose gel (Figure 3.9). The specific activity of cRNA was greater than 8.2.

Table 3.9: Quantity of cRNA by Nanodrop reading

Sample	Cy3/cy5 Pmol/µl	Conc., ng/µl	260/280	260	Specific activity
MSC-BM (1) (Cy5)	6.4	487.0	2.38	1.224	13.14
MSC-BM-N (1) (Cy3)	2.4	286.8	2.30	0.724	8.36
MSC-BM (2) (Cy 5)	3.2	317.5	2.26	0.803	10.07
MSC-BM (N) (2) (Cy3)	1.7	203.9	2.17	0.527	8.33

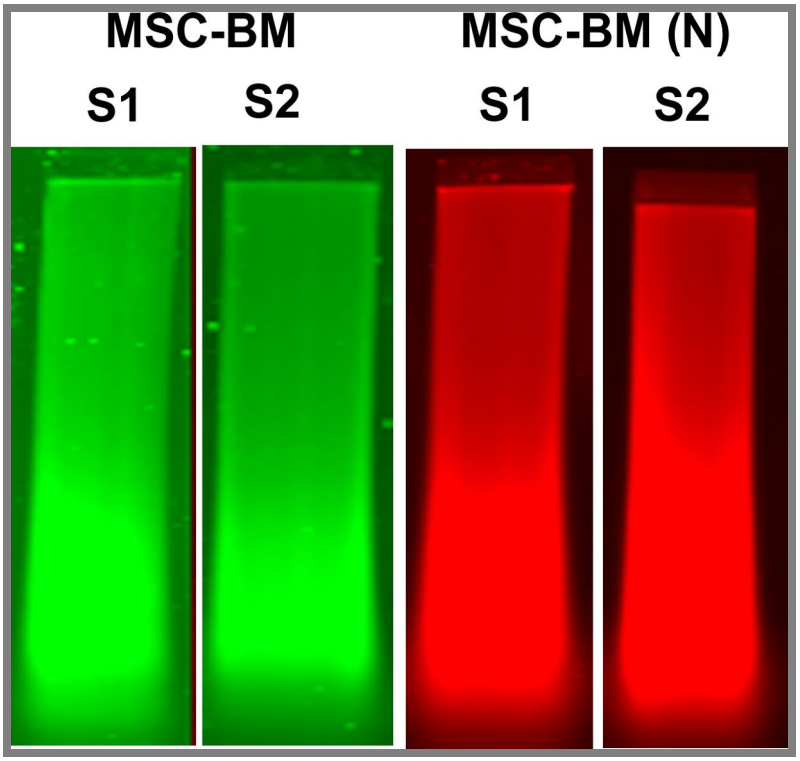

Figure 3.9: cRNA of undifferentiated MSC-BM and differentiated MSC-BM (N). S1 and S2 are the sample 1 and 2

3.5.3.2.3.3 Microarray Analysis

We performed microarray analysis to confirm the neuronal differentiation of MSC-BM and to determine if they can modify their gene expression profile in response to the neurogenic medium. For this study, two different samples of MSC-BM were investigated before and after neurogenic differentiation with Agilent micro array chip. Of probe sets, a total of 780 upregulated genes and 1250 down regulated genes were significantly modulated after differentiation with a fold change of 2.0.

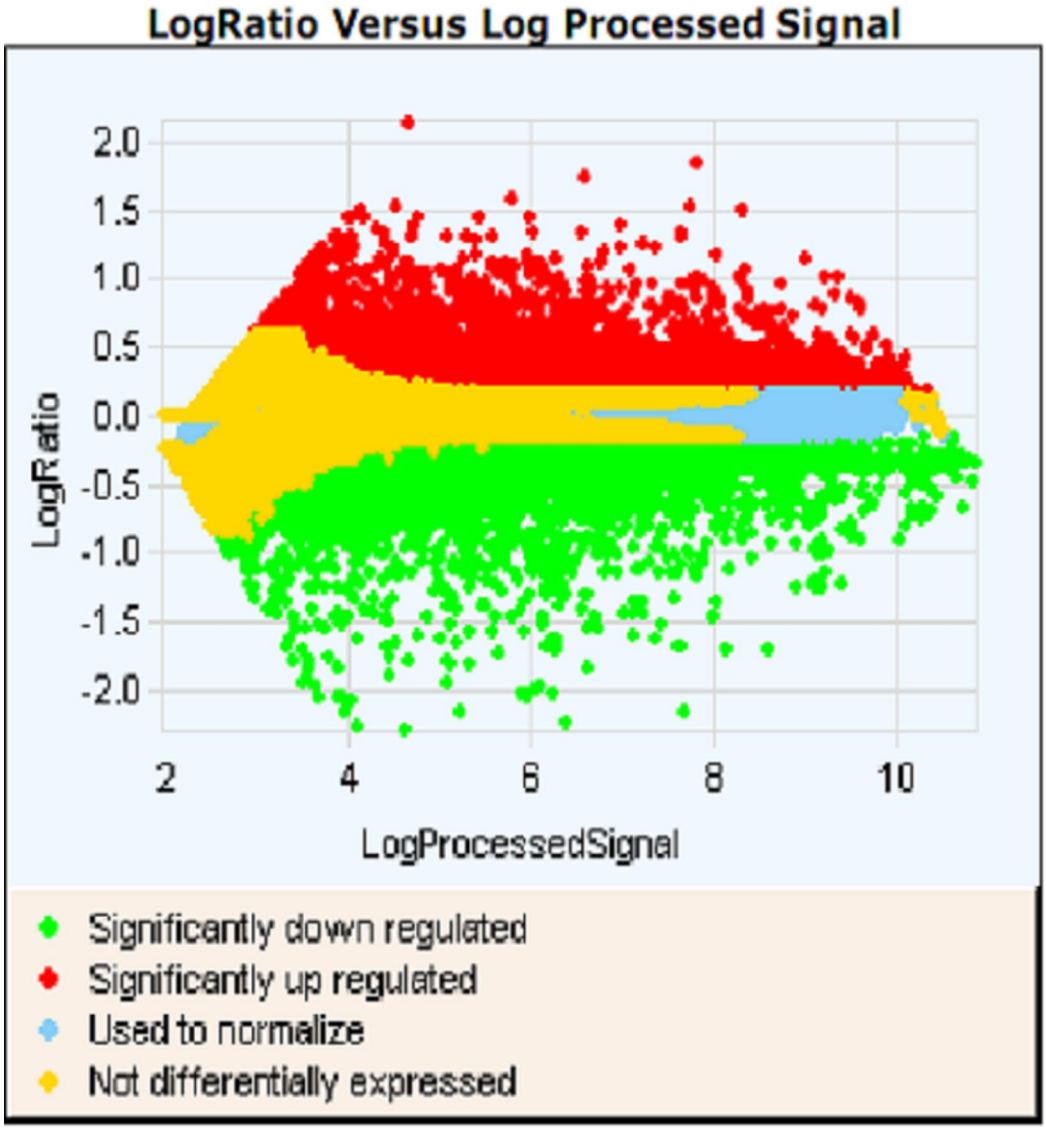

Figure 3.10: Differential gene expression of undifferentiated and differentiated cells

The identified genes were classified into different groups: Neuronal channel/transport, synaptic differentiation/transmission, neuronal development (Fig 3.9), mesodermal differentiation, regarding their function and the pathway, which in they are implicated.

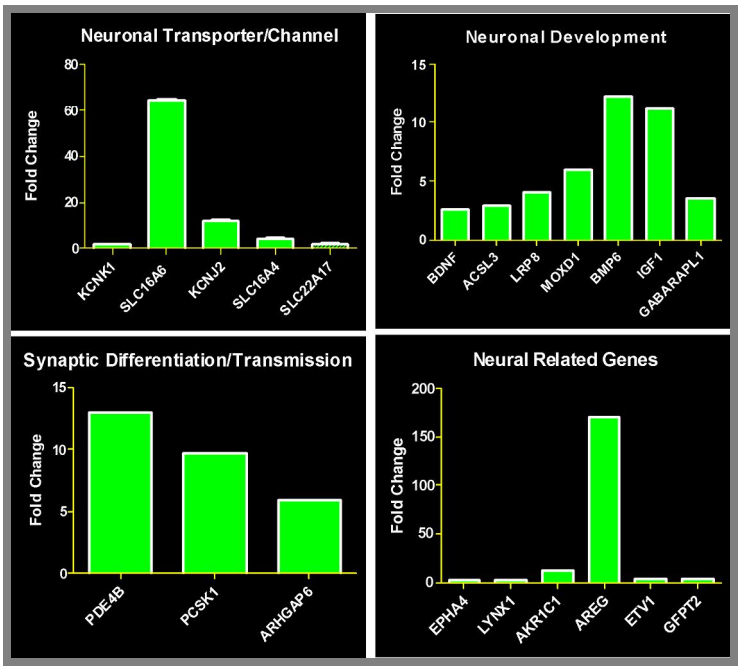

Figure 3.11: Microarray analysis of neural differentiating cells. The upregulated genes were expressed in average fold change of two different samples.

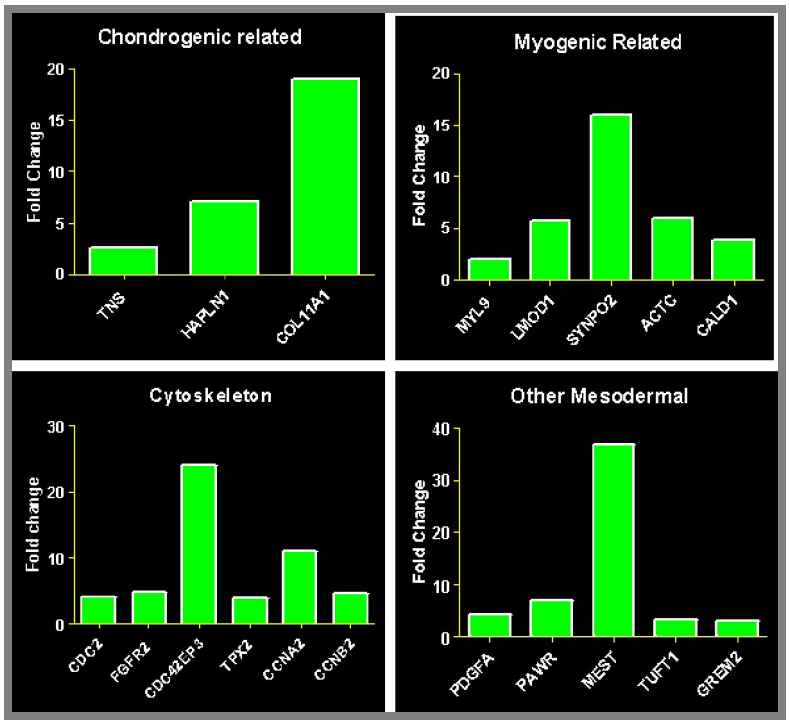

Figure 3.12: Gene expression of Undifferentiated and differentiated cells. Down regulated genes after differentiation were mentioned as average fold change.

3.6. Discussion

Many methods have been used to isolate stromal cells from bone marrow, including plastic adherence (Dexter *et al.,* 1981), gradient density centrifugation (Chen *et al.,* 1991) and immunomagnetic selection by Magnetic Activating Cell Sorter (MACS) (Dezawa *et al*., 2004; Jia *et al.,* 2002) and sorting of bone marrow populations by flow cytometery (FACS). We combined the density gradient centrifugation with plastic adherence and changed the medium three times to obtain a pure isolate of MSC-BM from unstimulated bone marrow samples. The MSCs adhered to the plate and had a fibroblast spindle-shaped morphology, forming colonies when grown in the low plating density. According to the results, this method is relatively simple and can easily be used to obtain pure MSCs.

As there are no specific markers, human MSCs are recognized on the basis of a complex immune phenotype, including the lack of hematopoietic cell markers (such as CD34 and CD45), as well as endothelial markers (such as CD31/PECAM-1), and the expression of a number of surface molecules, including CD105, CD73, CD106, CD44, CD90, CD29 and STRO-1 (Pittenger MF *et al.*, 1999; Tremain N *et al.,* 2001; Le Blanc K *et al.*, 2005; Lee RH *et al.*, 2004; Krampera M *et al*., 2005; Krampera M *et al*., 2006). In this study, we performed phenotypic analysis of these cells. Bone marrow mononuclear cells showed positive results for CD45 and CD34, indicating they are of haematopoietic lineage. MSCs showed negative for CD45, CD11a, CD31, CD18, CD4, CD8, CD64, CD34 and CD28, indicating the absence of any hematopoietic contamination in the culture. Additionally, these cultures were positive for CD90,

CD71, CD105, CD29, CD44, CD106 and vimentin, indicating that they are pure non-hematopoietic stromal cells. Although several groups have used these markers, there is no general consensus on the optimal marker or optimal marker combination for MSCs. Some of this conjecture may be due to variations in sample origin, culture techniques and media composition among laboratories or differences in the age of the donors from which the MSCs were obtained and used for immunophenotyping.

However, as cultures of the cells are expanded under standard conditions, they lose their proliferative capacity and their potential to differentiate into lineages such as adipocytes and chondrocytes (DiGirolamo *et al*., 1999; Sekiya *et al*., 2001). We also observed that as the passage number increases the proliferation capacity of the cells decreases. In this study we observed that the expression pattern of these markers from passages 0 to 6. In passage 0 there is expression of CD90, CD71, CD105, CD29 and CD106, which continued up to passage 3. At passage 4 there is very low-level expression of VCAM and CD105, and at passage 5, CD71 and CD29. The causative factors of these *in vitro* changes are not known. This indicates that culture expanded MSCs-BM might have become a more differentiated cell type, e.g. fibroblasts as described by Marek Honcsarenko and co workers (2005).

As for many other adult stem cells, MSCs are traditionally considered to be capable of differentiating into cell types of their own original lineage, i.e. mesenchymal derivatives. This study supports the findings of many other groups (Muraglia A, 2000) in showing that MSCs are capable of forming osteoblasts,

chondrocytes and adipocytes *in vitro*. The ability of clonally expanded cells to form these three distinct cell types remains the only reliable functional criterion available to identify genuine MSCs and distinguish it from preosteoblast, preadipocyte or prechondreocytic cells which each only give rise to one cell type (Halleux C *et al*., 2001)

The spontaneous expression of neural-specific proteins demonstrated by our MSCs casts doubt on some previously reported protocols that claim neural induction but fail to show the preinduction level of neural-specific proteins. However, rather than calling into question the neural transdifferentiation potential of MSCs, this clarification actually strengthens it by showing the vigorous, spontaneous acquisition of neural properties by induced MSCs. The neural property exhibited by MSCs may be explained by the neural differentiation propensity of stem cells reflected in the development of the nervous system during embryogenesis. It is generally believed that unspecified ectoderm cells differentiate into neural lineage by default unless inhibited by ventralizing factors, such as the bone morphogenic protein-4 (BMP-4) (Wilson PA *et al.*, 1995). So-called neuralizing factors such as noggin, chordin, and follistatin promote neuro-ectoderm specification by inhibiting BMP-4 (Streit A *et al.*, 1999). The embryonic stem cells of murine also show active spontaneous neural differentiation unless inhibited by BMP *in vitro* (Finley MF *et al.*, 1999). Therefore, it is not surprising that MSCs, as multipotent stem cells, may exhibit a neural property in their default state of differentiation *in vitro*, where there are no pro-mesoderm inhibitors such as BMP-4. The expression of some neural markers by preinduced

MSCs is a matter of some controversy in the literature. With the exception of neuron-specific lineage (NSE), Woodbury and coworkers (Woodbury D *et al*., 2000) did not observe any neural-specific protein expression. Sanchez-Ramos *et al*. (2000) reported low levels of Neu N, Nestin, and GFAP expression detectable with Immunocytochemistry, whereas Deng and coworkers (Deng *et al*., 2001) have previously reported expression of vimentin, Map1b, and beta-tubulin but no NFM, GFAP, or S-100-beta. A paper by Tondreau *et al*. (2004), Hermann *et al*. (2006) and Scintu *et al.* (2006) corroborates our findings by reporting significant expression of several neuronal markers, including beta-III tubulin, GAP43, NSE, and Tau by noninduced MSCs. These studies do indicate the presence of pre-termined precursors for neural differentiation within the unstimulated marrow cells, suggesting that they may be infact of multipotent cells (Suon *et al*., 2004).

However, incubation with certain differentiation cocktails, particularly those containing forskolin (Suon *et al*., 2004) produces a rapid and reversible, through transient, transformation of nearly all MSCs into neural like cells bearing elaborate neuritic processes. Moreover, differentiated MSCs express increased quantities of neuronal and glial proteins. We found that the use of our induction protocol, despite causing a vigorous neuron-like morphological change does seem to change the expression of GAP43, NSE, β-tubulin III profile in MSCs evaluated by RT-PCR. . We also found the expression of Neurofilament, Glial fibrillar acidic protein and synaptophysin in differentiated cells but not in undifferentiated cells by RT-PCR and Immunofluorescence. The presence of GFAP in treated MSC may be ascribed not only to differentiation into astrocytes,

but also to neural precursors, that have been demonstrated to express this protein (Scintu *et al*., 2006).

To conclude, the unstimulated bone marrow cells could be a source of robust mesenchymal stem cells, which also show a promise of neural differentiation. The neural differentiation however warrants further studies for functional evaluation.

Mesenchymal Cells from Limbal Stroma of Human Eye

4.1 Introduction:

The limbus of the eye, located at the junction of the cornea and conjunctiva of the ocular surface is now extensively used for ocular surface resurfacing in patients with limbal stem cell deficiency (LSCD) (Pellegrini G *et al*., 1997; Fatima A *et al*., 2006; Sangwan VS *et al*., 2006). It is now established that the progenitor cells that regenerate corneal epithelium reside in the limbus (Schermer A *et al*., 1986; Tseng SC *et al*., 1989). In addition to this regenerative capacity, limbal epithelial cells have also been reported to have features of "plasticity" evident from the neuronal-like differentiation of these cells (Zhao X *et al*., 2002; Seigel GM *et al*., 2003). A report by Dravida and coworkers (Dravida S *et al*., 2005) points to the presence of fibroblast-like cells in the limbal stroma, possessing stem cell-like self-renewal property with plasticity. However, such cells have not been reported from human limbal tissues. We had observed the presence of spindle cell outgrowths in late limbal epithelial cultures, which were non-epithelial in nature. These cultures were serially passaged and characterized for surface markers.

Mesenchymal stem cells or bone marrow stromal cells (MSC-BM) are multipotent stem cells with high self renewing capacity and ability to differentiate into more than two lineages *in vitro* or *in vivo* e.g. into osteoblasts, chondrocytes, myocytes, adipocytes, beta-pancreatic islet cells or neuronal cells. MSC-BM were earlier believed to nurture the hematopoietic stem cells by releasing GSCF, cytokines etc. These differentiated cells do not express hematopoietic and endothelial markers (such as CD45, CD11c and CD31), but express mesenchymal markers CD90, SH2 (endoglin or CD105),

SH3 or SH4 (CD73 and STRO-1) (Simmons PJ *et al*., 1991). MSC-BM have been isolated by means of rapid expansion in serum-containing medium and adherence from several tissues, including bone marrow, amniotic fluid, peripheral blood, adipose tissue, dermis, articular synovium, compact bone, muscle and brain (Friedenstein AJ *et al*., 1974; Javazon EH *et al*., 2004). In response to specific culture conditions, these cells can give rise to multiple mesenchymal-derived cell types, such as osteoblasts (Pittenger MF *et al*., 1999), chondrocytes (Noth U *et al.*, 2002), adipocytes (Zuk PA *et al*., 2002) myloblasts (Labarge *et al*., 2002) and neural cells (Zhao LR *et al*., 2002). In this paper we reproduced the properties of mesenchymal stem cells as rapidly adhering marrow stromal cells with the abilities to form colonies and differentiate into different cell types such as osteocytes and adipocytes. We aimed to investigate if the limbal spindle cells were of mesenchymal origin (MC-L), by comparing them with human MSC-BM both in terms of immunophenotype and plasticity.

4.2. Hypothesis

We hypothesize that the spindle shaped cells derived from extended limbal epithelial cultures are limbal stromal in origin

4.3. Aims

1. To isolate and establish of stromal cell cultures
2. To characterize the limbal stromal cells obtained from explant cultures
3. To differentiate the stromal cells to other mesenchymal lineages

4.4. Material and Methods

The protocol was approved by the Institutional Review Board at L.V.Prasad Eye Institute.

4.4.1. Preparation of Chemicals

All the chemicals and culture media were prepared as described in Appendix I.

4.4.2. Sterility Check of Chemicals & Media:

Following the filter sterilization the media and chemicals were kept for sterility check. A few drops of media/chemicals were inoculated in chocolate agar and thioglycolate broth to screen for both aerobic and anaerobic microorganisms. The inoculated media were then incubated in a bacterial incubator at 37^0C for about 7 days, before the media/chemicals are approved for tissue culture use.

4.4.3. Source of Limbal Tissue:

The technique was initially standardized using corneoscleral rims from rejected eye bank corneas. There after human limbal tissues were harvested from cataract patients, after obtaining informed consent. These tissues were used for standardization of the explant culture technique.

4.4.4. Preparation of HAM

The standard protocol was used to prepare HAM. In brief, the placenta (which has two layers called amnion and chorion) obtained from the caesarian section deliveries was used to obtain the HAM, after screening the donor for HIV, HBs Ag and VDRL the placenta is placed in sterile pan and washed repeatedly (by discarding the water in the sink) with antibiotic containing ringer lactate/Normal saline until clear water is obtained. The placenta was then transferred aseptically to another sterile pan and carried to the laminar

flow hood, which was pre-cleaned, and UV sterilized. HAM was peeled, separating amnion and chorion. The stretched membrane was cleaned using cotton swab and intermittent wetting with ringer lactate/normal saline using wash bottle. Once a clean transparent approximately 2" X 2" area (7.5 x 7.5) was available the nitrocellulose paper was attached on the chorion side keeping the epithelium side up. The HAM was cut around the paper while rolling the edges on the other side of the paper (HAM should be stuck to the nitrocellulose paper perfectly without gaps or air-bubbles). The nitrocellulose paper was then cut into small pieces of membrane as per requirement. The HAM pieces (2.5x2.5, 2.5x5, and 5x5 cm) were then inserted in vials containing DMEM and stored at −70^{o}C. The HAM was thawed at 37^{o}C for 30 min, just prior to use.

4.4.5. Human Corneal Epithelial Medium

We used a modified Human Corneal Epithelial (HCE) medium for culturing limbal tissues. The medium was prepared using 3.98/L MEM with the addition of 16.2g/L Ham's F12 serum, 0.01mg/L epidermal growth factor, 0.25mg/l insulin, 0.1mg/l cholera toxin and hydrocortisone. This was supplemented with 10% FBS/autologous serum at the time of use. MEM, HamsF12, cholera toxin, epidermal growth factor, insulin, fetal bovine serum, trypsin, ethylenediaminetratacetic acid, were obtained from Sigma–Aldrich Chemie (Steinheim, Germany) and Sigma Chemical Co (St.Louis, USA). Filter sterilization was performed using 0.22µ membrane filter procured from Millipore Corporation (Bedford, MA, USA).

4.4.6. Processing of HAM

HAM processed and preserved (as mentioned above) in DMEM at –70^{o}C was obtained from Ramayamma International eye bank. The limbal cells were grown on de-epithealized HAM (2.5 x 5 cm). For de-epithealization, a small piece of glass slide was placed into a 55mm^2 culture plate. The HAM was then transferred onto the slide. With the help of fine and blunt edged forceps, the HAM was peeled from the underlying nitrocellulose membrane and spread onto the glass slide in the culture dish. 1ml trypsin-EDTA was added onto the surface and incubated for 30 minutes at 37^{o}C. The membrane was then scraped using two glass slides. After removal of the cell debris, the membrane was washed thoroughly with PBS (1X) and observed under the phase contrast microscope (Olympus CK 40) to ensure complete denudation, followed by secure tucking around the glass slide piece to obtain a uniform surface.

4.4.7. Explanting of Limbal Epithelial Tissue

The limbal tissue collected in the HCE medium was shredded into tiny bits using sterile surgical blade (no.21). The tissue bits were then picked up with 24-gauge sterile needle and explanted onto the denuded HAM. After 20 minutes of explantation, few drops of HCE medium was added onto the explants and kept in the CO_2 incubator (Binder CB 210) for about 8-10hrs to allow the adherence of the tissue bits to the membrane.

4.4.8. Culture of Limbal Epithelial Cells in HCE Medium

The culture dish with HAM and limbal explants was flooded with 4ml HCE medium with 10% serum autologous/FCS. The medium was changed every alternate day and the growth of the cells was monitored under phase contrast

microscope (Olympus CK 40). As used by other groups in this area, the present study does not make use of feeder cell layers in the culture system. A complete submerged technique of culturing in contrast to airlift technique followed by others has been developed.

4.4.9 Establishment of Stromal Cell Cultures:

The limbal epithelial cultures were established on de-epithealized Human Amniotic Membrane (HAM) as mentioned above. While cultures with a monolayer of epithelial cells growing from the explants in 10–14 days were terminated for transplantation, parallel plates were cultured further for two to three weeks when spindle cell-like outgrowths were seen under a phase contrast microscope (Olympus, Japan). These were then trypsinized and plated on a T25 flask. After two days of plating we observed adherent spindle cells termed limbal mesenchymal cells. Residual epithelial cells were removed by changing the medium. Adherent MC-L was cultured in HCE medium supplemented with 10% fetal bovine serum (FBS) (Sigma, USA). The cultures were maintained at $37^{O}C$ and 5% CO_2 in humidified incubator. When the cells reached 80-90% confluency, cultures were harvested with 0.25% trypsin (Sigma, USA)-1mM EDTA solution (Sigma, USA) from passages P0 through P6.

To confirm the origin of these spindle cells from the limbus, we also grew spindle cells from de-epithealized limbal tissues. Limbal tissues were de-epithealized using dispase (BD, USA) at a concentration of 1.2U/ml, digested with trypsin-EDTA to make a single cell suspension and then plated on the T25 flasks. At confluence, cells were trypsinized and passaged as above.

For comparative analysis MSC-BM were isolated by the ficoll-hypaque (Sigma, USA) density gradient method and cultured on the basis of adherent properties. Briefly, human MSC cultures were established from 5 bone marrow aspirates of healthy donors, after obtaining informed consent. The bone marrow mononuclear cells (BMMNCs) were separated using ficoll-hypaque (Sigma, USA) gradient at 400g for 30 minutes. The mononuclear cells were then plated at a density of $1x10^7$ cells in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% FBS. When cultures reached confluence, cells were passaged using trypsin-EDTA.

4.4.10 Colony-forming Unit (CFU) Assays:

For these assays, cells of both origins (MC-L and MSC-BM) were plated at 2 cells per sq.cm and cultured for 14 days in 75 sq.cm tissue culture flasks. After 14 days the cultures were stained with 0.5% crystal violet in methanol for 5minutes. The colony count was performed excluding colonies that were less than 2mm in diameter or faintly stained.

4.4.11 Population Doublings:

Population-doubling assay was performed on MSC-BM from passage 1 to passage 5 and on MC-L from passages 2 through 6. Passages 1-2 were not included for MC-L, as mesenchymal cells derived from cultured limbal epithelial cells had epithelial cell contamination. $1X10^4$ cells each of MC-L and MSC-BM were seeded at each passage and trypsinized after 10 days and 12 days respectively. The population doubling of cells was calculated as,

Number of Cell Doublings (NCD) = $\log_{10}(y/x)/\log_{10}2$,

Where “y” is the final density of the cells and ‘x’ is the initial seeding density of the cells.

4.4.12 Characterization

4.4.12.1 Flow Cytometry

MC-L and MSC-BM were characterized for a battery of markers (Table 4.1 and Table 3.1) by fluorescent activated cell sorting (FACS). Briefly, a single cell suspension of 0.5 to 1x 10^6 cells each of MC-L and MSC-BM at passage 2 obtained in 100µL of washing buffer solution (See appendix 1) was incubated with saturating concentrations of respective primary antibodies for 40 min. After three washes the cells were centrifuged at 200xg for 5 min, and resuspended in ice cold PBS and then incubated at 4^0C with the FITC-labelled secondary antibody for 30 min in dark. Cell fluorescence was evaluated by flow cytometry in FACS Caliber instrument (Becton Dickinson (BD), Germany and FACS aria (BD, Germany) and data was analyzed by using Cell Quest software (BD, Germany). An isotype control was included in each experiment and specific staining was measured from the cross point of the isotype with a specific antibody graph. A total of 10,000 events were acquired to determine the positivity of different cell surface markers used.

Table 4.1: List of antibodies used for flowcytometry

S.No.	Marker	Dilution	Company
1	K3	1:50 (1mg/ml)	Millipore
2	K14	1:50	Millipore
3	ICAM/CD54	1:50	Millipore
4	CD166	1:50	Millipore
5	SSEA1	1:50	Millipore
6	TRA-1-61	1:50	Millipore
7	TRA-1-81	1:50	Millipore
8	VE-Cadherin	1:50	Millipore

9	Flk1	1:50	Millipore
10	Flt1	1:50	Millipore
11	CD25	1:50	Millipore

4.4.12.2 Immunocytochemistry

Expression of selected markers (Table 4.2) was further confirmed by immunocytochemistry. Cells were seeded into 24 well plates and cultured upto confluency. Cells were then fixed with ice cold methanol or 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2) for 20 minutes and processed for immunocytochemistry. Non-specific reactions were blocked with 5% FCS for 30min at room temperature. Fixed cells were then incubated for one hour with primary antibodies and detected using FITC conjugated secondary antibody, counter stained with propidium iodide (PI). The stained preparations were screened with a laser scanning confocal microscope (LSM510; Carl Zeiss) using a fluorescent light source (excitation wavelength 480 and 540 nm).

Table 4.2: List of antibodies used for immunocytochemistry

S.No.	Antibody	Dilutions	Company
1	CD90	1:200	Millipore
2	CD45	1:200	Millipore
3	CD14	1:200	Millipore
4	CD29	1:200	Millipore
5	CD34	1:200	Millipore
6	HLA-ABC	1:200	Millipore
7	FITC- Conjugated 2^0 Antibody	1:250	Millipore
8	Vimentin	1:250	Dako Cyomation
9	HLA-ABC	1:100	Millipore

10	HLA-DR	1:100	Millipore
11	Nestin	1:250	Millipre
12	GAP43	1:200	Millipore
13	TUBB3	1:200	Millipore
14	NF-L	1:150	Millipore
15	NSE	1:250	Millipore
16	Alpha-SMA	-	DAKO cytomation
17	CK14	1:300	Chemicon
18	CK3/CK12	1:400	Chemicon
19	CK19	-	BiogeneX
20	PAX-6	1:100	abcam
21	CDH1	1:200	Millipore
22	ABCG2	1:100	ebiosciences

4.4.12.3 RT-PCR Analysis:

Selective epithelial and corneal cell type related marker expression in MC-L and MSC-BM was studied by reverse transcription polymerase chain reaction (RT-PCR). Both the stromal cell types were evaluated for epithelial stem cell related marker p63 α and integrin α9, pax-6 selectively expressed by cells of neuroectodermal origin and during ocular development, corneal epithelium related cytokeratin pair K3/K12, epidermal growth factor receptor (EGFR) and low affinity nerve growth factor (NGF) receptor p75, gap junction protein connexin 43 and glycolytic enzyme α-enolase against limbal epithelial cells. Expression of pax-6 was studied to confirm the origin/resemblance of MC-L to mesenchymal or epithelial cells.

Total RNA was extracted from 2-3-week-old limbal epithelial cultures and MC-L and MSC-BM at passage 2 using Trizol™ as per the manufacturers

protocol. The RNA so obtained was quantified by reading the absorbance at 260nm and its purity evaluated from the 260/280 ratio of absorbance in spectrophotometer (Model UV-1601, Shimatzu, Japan). 2µg of this RNA was used for cDNA preparation using Murine Leukemia Virus Reverse transcriptase (Fermentas, #EP0451) and was subjected to a semi-quantitative PCR with primers (Table 4.3), at initial denaturation of 94^{O}C for 3 minutes, denaturation at 94^{O}C for 1 min, annealing temperature given in the table for each marker for 40sec, extension of 72^{O}C for 1 minute a final extension of 10 min at 72^{O}C for a total of 35 cycles. The PCR products were analysed on a 2% agarose gel and scanned using an UV gel doc (Uvtec Ltd, Cambridge, UK). The expression of various markers was normalized using GAPDH as an internal control.

Table 4.3: Primers used in this study

Gene	PRIMER SEQUENCE	**Fragment Length**	**Annealing Temperature**
Vimentin	F: CAGGAACAGCATGTCCAAATCG R: TGTACCATTCTTCTGCCTCCTGC	127	59.5
Collagen type 1 alpha 1	F: TCCCCAGCCACAAAGAGTCTA R: TTTCCACACGTCTCGGTCA	201	59.5
S100A2	F: GATCCATGATGTGCAGTTCTCT R: GTTCTGCTTCAGGGTCGGT	310	59.5
Nestin	F: CACCTGTGCCAGCCTTTCTTAA R: CCACCGGATTCTCCATCCTTA	361	59.5
TGFB1	F: GACTACTACGCCAAGGAGGTC R: TCAACCACTGCCGCACAACTC	332	59.5
RPL35A	F: GAACCAAAGGGAGCACACAG R: CAATGGCCTTAGCAGGAAGA	236	59.5

OTX1	F: CTCCACCCAGCTGTTAGCAT R: CGCATGAAGATGTCAGGGTA	221	59.5
PAX-6	F: GAATCAGAGAAGACAGGCCA R: GGTAGGTATCATAACTCCG	302	59.5
S100A4	F: GATGAGCAACTTGGACAGCAA R: CTGGGCTGCTTATCTGGGAAG	123	59.5
VEGFA	F: ATGCGGATCAAACCTCACC R: ATCTGGTTCCCGAAACCCTG	358 304 269 172	60.0
TUBB3	F: TCAAGCGCATCTCCGAGCAG R: ACCGTAAAACGTCAGGCCTGGAG	444	59.5
CDH11	F: GTGCCTGAGAGGTCCAATGT R: GGGTAGGGCTGTTCTGATGA	165	59.5

4.4.13. *In Situ* Localization by IHC

We attempted to study the localization of these cells in native limbal tissue by immunohistochemistry. Human corneal scleral rim sections (5 µm thick) were thawed, dehydrated and fixed in cold methanol (for cytoplasmic staining) or 4% paraformaldehyde (for all membrane protein staining) at 4^0C for 10 min. Sections were blocked with 5% bovine serum albumin (BSA) in PBS for 1 hr to decrease non- specific antibody interactions. Sections were then incubated for two hours with primary antibodies at room temperature. Secondary antibodies, i.e., anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) or FITC conjugated were then applied in a dark chamber for 1 hr followed by counterstaining for 10 sec with PI. After washing with PBS, a coverslip was applied. The stained preparations were screened with a laser

scanning confocal microscope using a fluorescent light source (excitation wavelength 480 and 540 nm).

4.4.14. Differentiation

4.4.14.1 Adipogenic Differentiation

Passage 2 cells of MC-L were seeded on cover slips in 6 well plates and cultured in complete medium upto confluency. At confluency, the cells were switched to an adipogenic medium (Appendix 1) and further cultured upto 21 days with the medium being changed every alternate day. After 21 days, the adipogenic cultures were fixed in 4% paraformaldehyde for atleast 1hr and stained with fresh 0.3% oil Red-O solution for 2hr. After staining, the cultures were washed three times and counter stained with haematoxylin.

4.4.14.2 Osteogenic Differentiation

Passage 2 cells MC-L were seeded on cover slips in 6 well plates and cultured in complete medium upto confluency. The medium was then replaced with a calcification medium (Appendix 1) and incubated for 21 days. These cover slips were stained with fresh 0.5% alizarin red solution.

4.5. Results:

4.5.1 Establishment of Cell Cultures

Spindle cell cultures were established from both extended limbal epithelial cultures and de-epithealized limbal cultures. The cells appeared fibroblastic, elongated and spindle shaped with a single nucleus under a phase contrast microscope (Figure 4.1a). These cells showed the ability to form colonies, with occasional cell sphere formation giving an impression of embryoid bodies (Figure 4.1b). The fibroblastic morphology was confirmed by giemsa staining (Figure 4.1c).

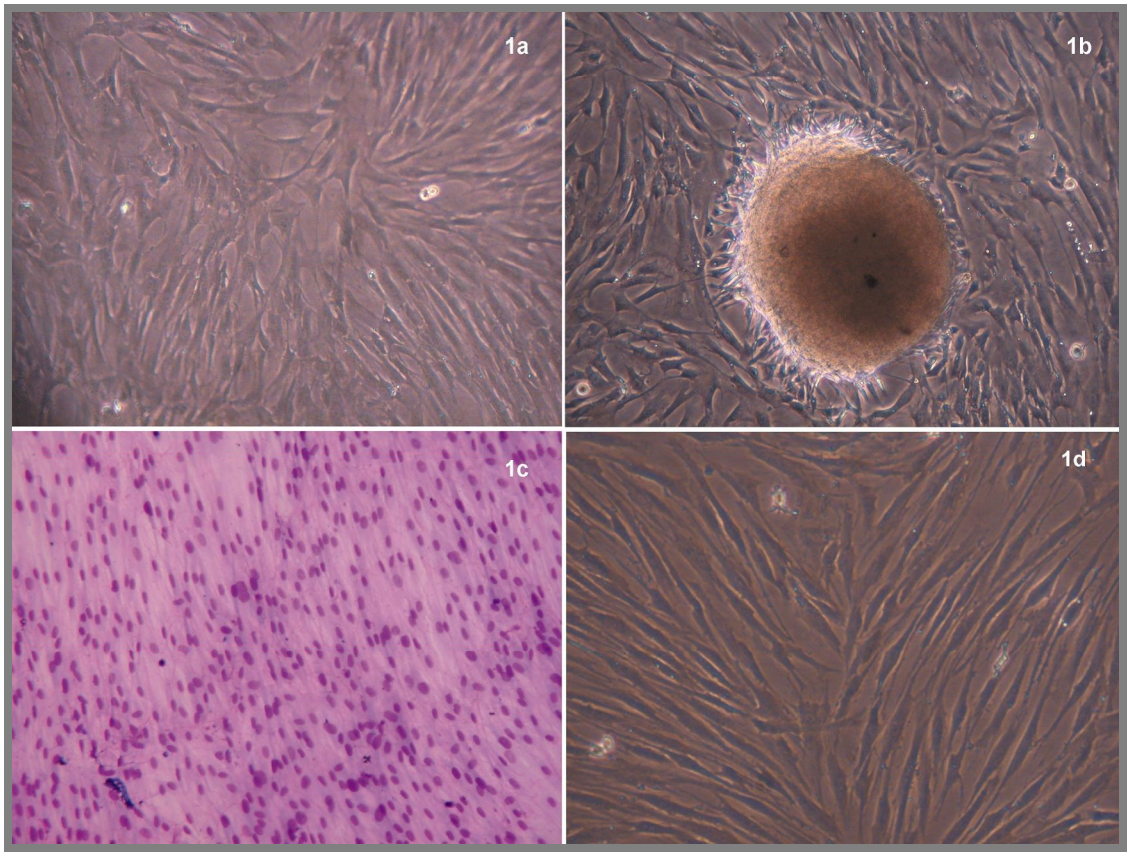

Figure 4.1: Phase contrast microscope picture of MC-L showing spindle morphology (x200) (1a), cell sphere formation in the MC-L cultures giving impression of embryoid body formation (x200) (1b), spindle shaped morphology of MC-L confirmed by giemsa stain (Light microscope, x200) (1c), culture of MSC-BM (x200) (1d).

Spindle shaped MSC-BM were established from unstimulated bone marrow specimen from patients. The cells appeared spindle shaped with a single nucleus under phase-contrast microscope (Figure 4.1d).

4.5.2 Colony Forming Unit Assay:

When plated at 2 cells/cm^2, MC-L in culture showed a colony forming efficiency between 30-40% at passage 2 (Figure 4.2a), 10-15% at P3 and 8% at P4 while MSC-BM showed a CFU of 20% at passage 2 (Figure 4.2b), 8-12% at passage 3 and 2-4% at passage 4. At P5 the cells showed no colony forming ability showing a decrease in the colony forming ability of the cells with increasing passages.

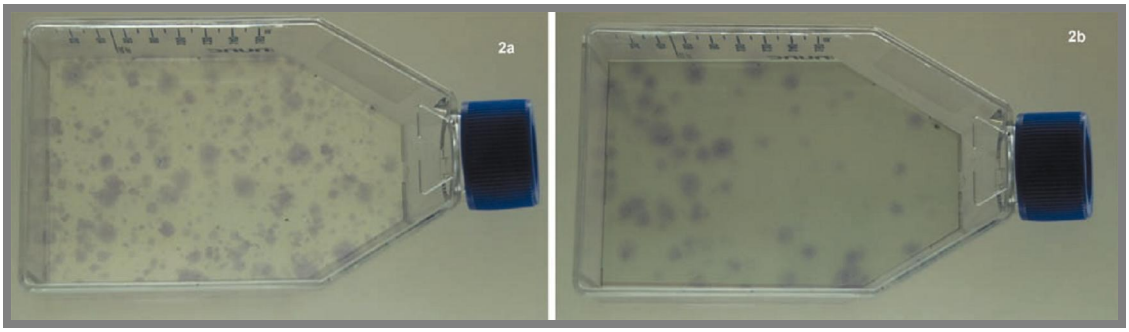

Figure 4.2: CFU assay – T75 flasks showing crystal violet stained colonies of MC-L 2a, and MSC-BM 2b

4.5.3 Population Doubling Assay:

While MC-L showed 22.95 population doublings, MSC-BM showed 30.98 cell doublings. The results of this assay are summarized in tables 4.4 and 4.5, which show the population doublings from passage 0-6.

Table 4.4: Population Doublings of cultured MC-L from P0 through P6

Passage number	Initial cell density	Incubation time (Days)	Mean final cell number (x millions	Number of Cell Doublings (NCD)	Accumulative NCD	Population Doubling Time (Hrs)
P0		23 days				
P1		3-4 days	1.8			
P2	$1X10^4$	10 days	3.0	8.2295	8.2295	29.1633
P3	$1x10^4$	10 days	1.3	7.0230	15.2525	34.1734
P4	$1x10^4$	10 days	0.13	3.7008	18.9533	64.8508
P5	$1x10^4$	10 days	0.08	3.0002	21.9535	79.9946
P6	$1x10^4$	10 days	0.02	1.000	22.9535	240 hrs

Table 4.5: Population Doublings of cultured MSC-BM from P0 through P5

Passage number	Initial cell number	Incubation time (Days)	Mean final cell number (X millions)	Number of Cell Doublings (NCD)	Accumulative NCD	Population Doubling Time (Hrs)
P0	$1x10^7$	13days	1.2			
P1	$1x10^4$	13 days	1.5	7.2295	7.2295	33.1973
P2	$1x10^4$	13 days	1.4	7.1299	14.3594	33.6610
P3	$1x10^4$	13 days	1.4	7.1299	21.4893	33.6610
P4	$1x10^4$	13 days	0.72	6.1705	27.6598	38.8947
P5	$1x10^4$	13 days	0.1	3.322	30.9820	72.2412

4.5.4 Characterization

4.5.4.1 Flow Cytometry

The FACS analysis revealed similarities in surface marker expression of MC-L with MSC-BM (Figure 4.3a). Table 4 summarizes the surface marker expression profile of cultured MC-L and MSC-BM. The cells have shown no expression of embryonic markers (Figure 4.3b) and other endothelial markers (Figure 4.3c).

Table 4.6: Surface antigen profile of MC-L vs. MSC-BM. – indicates negative expression for a marker while + indicates positive expression of a marker. Average values of three such experiments ± standard deviation for a marker are given within the brackets.

S. No.	Marker	MC-L	MSC-BM
1	CD 34	- (1.03±0.4)	- (0.19%±0.02)
2	CD45	- (0.95%±0.43)	- (0.89%±0.2)
3	CD11a	- (0.28%±0.1)	- (0.87%±0.2)
4	CD11c	- (0.0%)	- (0.0%)
5	CD138	- (0.98%±0.2)	- (1.35%±0.5)

6	CD106/VCAM	+ (50.0 % ±5.57)	+ (54.67 % ± 5.86)
7	CD105	+ (21.42 % ± 4.133)	+ (71.33 % ± 6.66)
8	CD90	+ (95.63 % ± 2.11)	+ (94.57 % ± 2.00)
9	CD29	+ (86.33 % ± 3.06)	+ (84.0 % ± 2.65)
10	CD71	+ (66.07 % ± 2.57)	+ (45.27 % ± 4.15)
11	HLA-ABC	+ (93.44 % ± 4.32)	+ (91.33 % ± 2.75)
12	HLA-DR	- (0.67%±0.1)	- (0.87%±0.14)
13	CD4	- (0.99%±0.13)	- (0.15%±0.09)
14	CD8	- (0.78%±0.15)	- (0.66%±0.12)
15	K3	- (0.67%±0.27)	
16	K14	- (0.95%±0.2)	
17	CD 68	- (0.84%±0.43)	- (0.76%±0.025)
18	ICAM/CD54	+ (28.13 % ± 4.01)	+ (24.0 % ± 4.58)
19	CD166	+ (81.67 % ± 3.51)	+ (83.67 % ± 2.08)
20	CD31	- (0.45± 0.23)	- (0.65% ±0.37)
21	CD14	+ (1.2%±0.43)	+ (1.4%±0.5)
22	SSEA1	- (0.00%)	
23	TRA-1-61	- (0.00%)	
24	TRA-1-81	- (0.00%)	
25	VE-Cadherin	- (0.00%)	
26	Flk1	- (0.00%)	
27	Flt1	- (0.00%)	
28	CD25	- (0.64±0.24)	- (0.45±0.12)

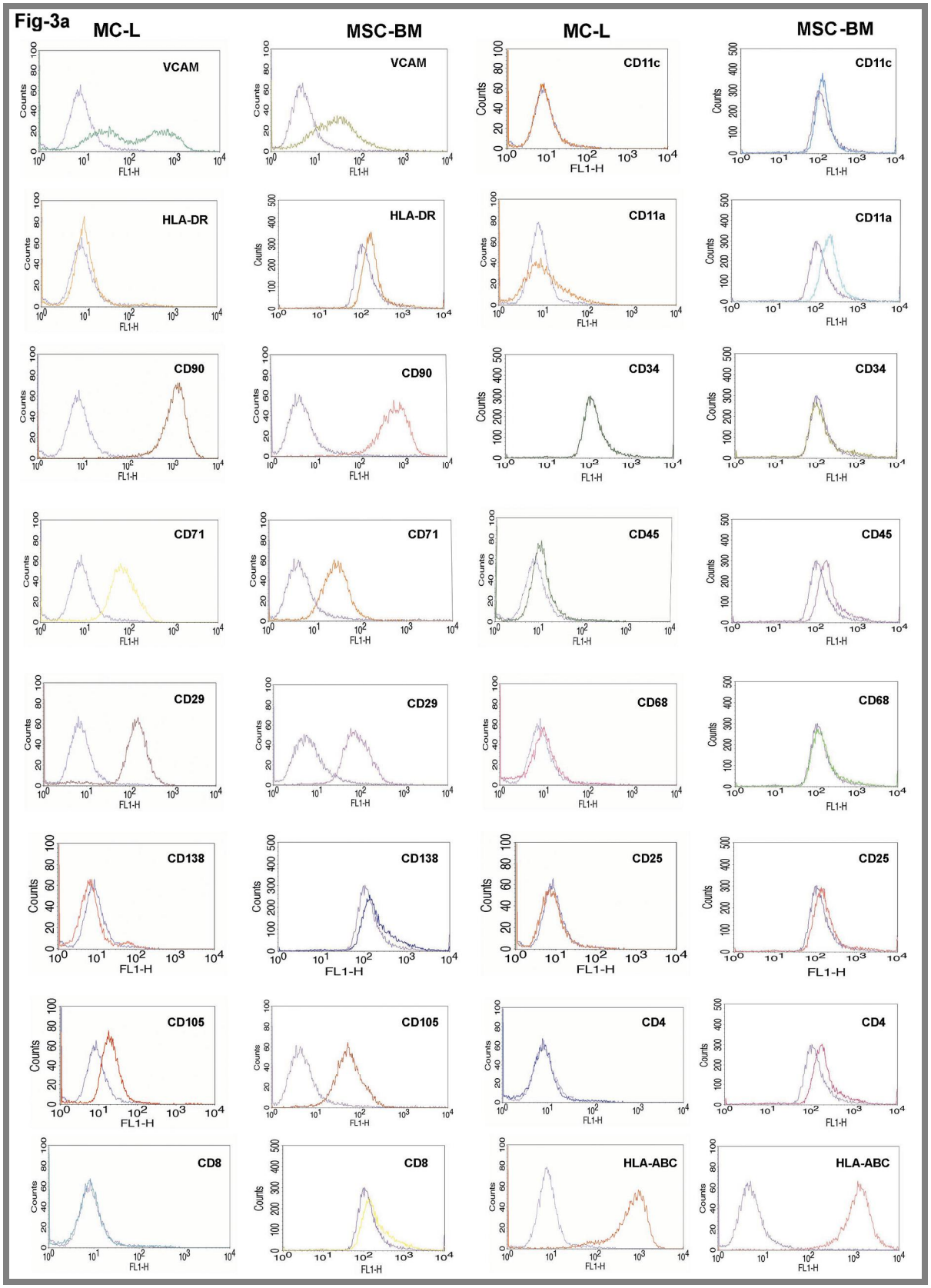
Fig-3a
MC-L
MSC-BM
MC-L
MSC-BM
VCAM
VCAM
CD11c
CD11c
HLA-DR
HLA-DR
CD11a
CD11a
CD90
CD90
CD34
CD34
CD71
CD71
CD45
CD45
CD29
CD29
CD68
CD68
CD138
CD138
CD25
CD25
CD105
CD105
CD4
CD4
CD8
CD8
HLA-ABC
HLA-ABC
Counts
FL1-H

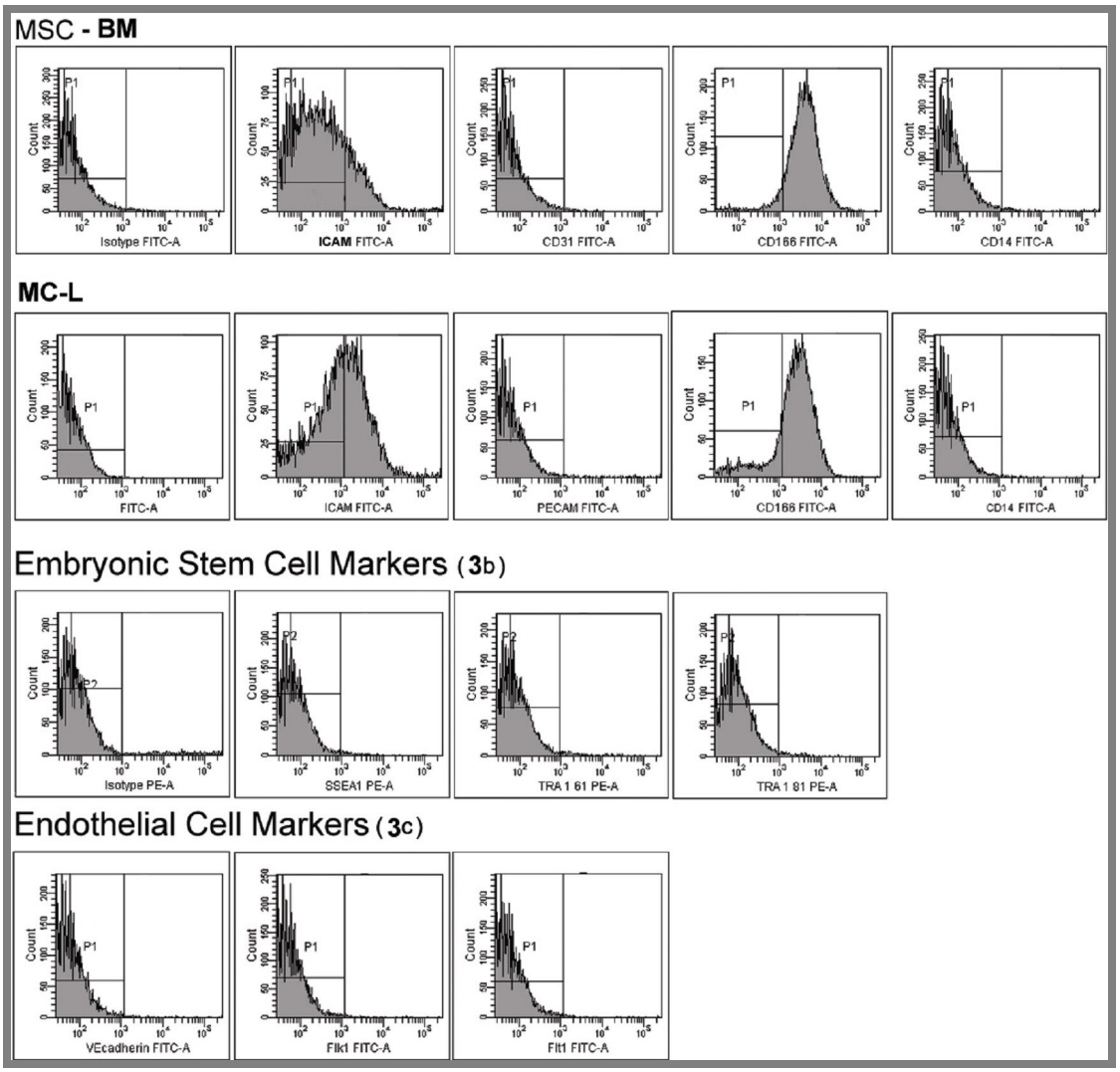

Figure 4.3: Flowcytometry analysis of MC-L cells in comparison to MSC-BM [The purple line in the histograms represents the isotype control] (3a). FACS analysis of MC-L for embryonic stem cell marker expression (3b) and endothelial markers (3c) is also shown to be negative

4.5.4.2 Immunocytochemistry

On immunostaining LEC expressed the markers of ABCG2, CK14, CK19, CK3/CK12, CDH1, vimentin and PAX-6 (Figure 4.4). MC-L were positive for mesenchymal CD90, CD29, vimentin and negative for hematopoietic markers. (Figure 4.5 & 4.6). The MC-L also showed negative staining for epithelial markers K3 and K14 (Figure 4.6).

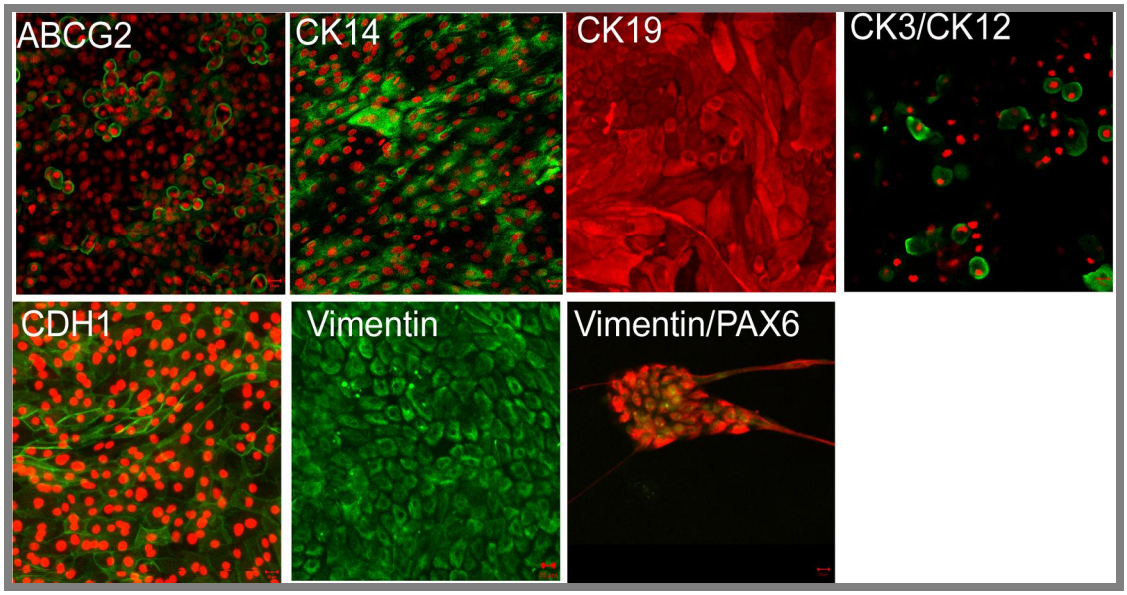

Figure 4.4: Immunostaining of limbal epithelial cells: Limbal explant culture derived epithelial cells on denuded human amniotic membrane expressed ABCG2 (green), cytokeratin 3/12, Cytokeratin19 (red), Cykokeratin 14, E-cadherin, vimentin. Nucleus was conterstained with porpidium iodide (ABCG2, CK14, CK3/CK12, CDH1). Double immunostaining on limbal epithelial cells expressed vimentin (red) and pax-6 (green).

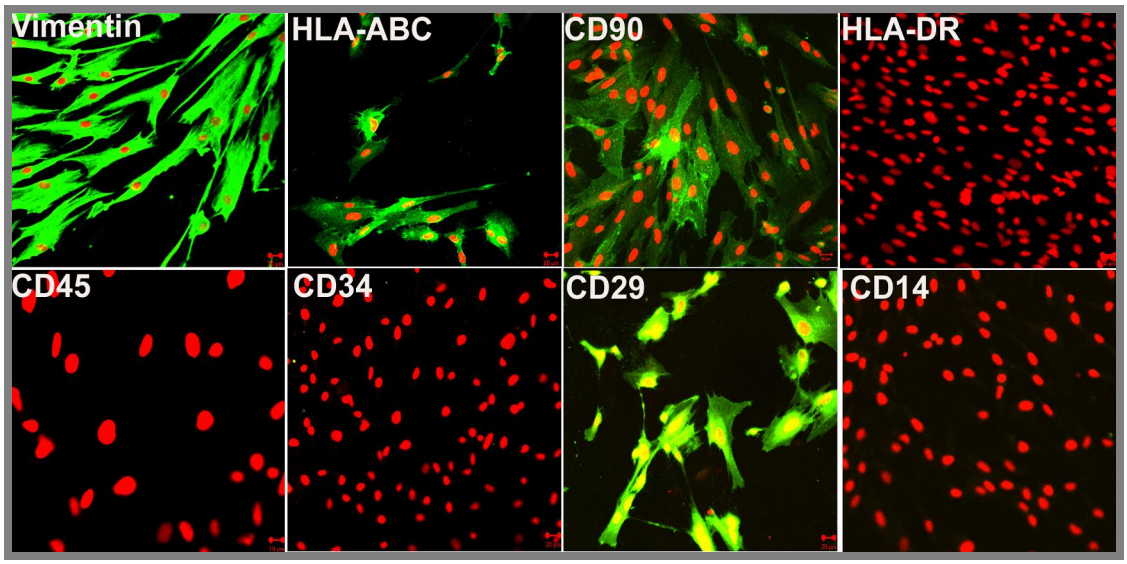

Figure 4.5: Immunostaining of limbal explant culture derived mesenchymal like cells: MC-L derived from limbal explant cultures expressed vimentin, CD90, CD29 (green) cell surface antigens and were negative for CD45, CD34, CD14 or HLA-DR. Nucleus was counterstained with propidium iodide (red).

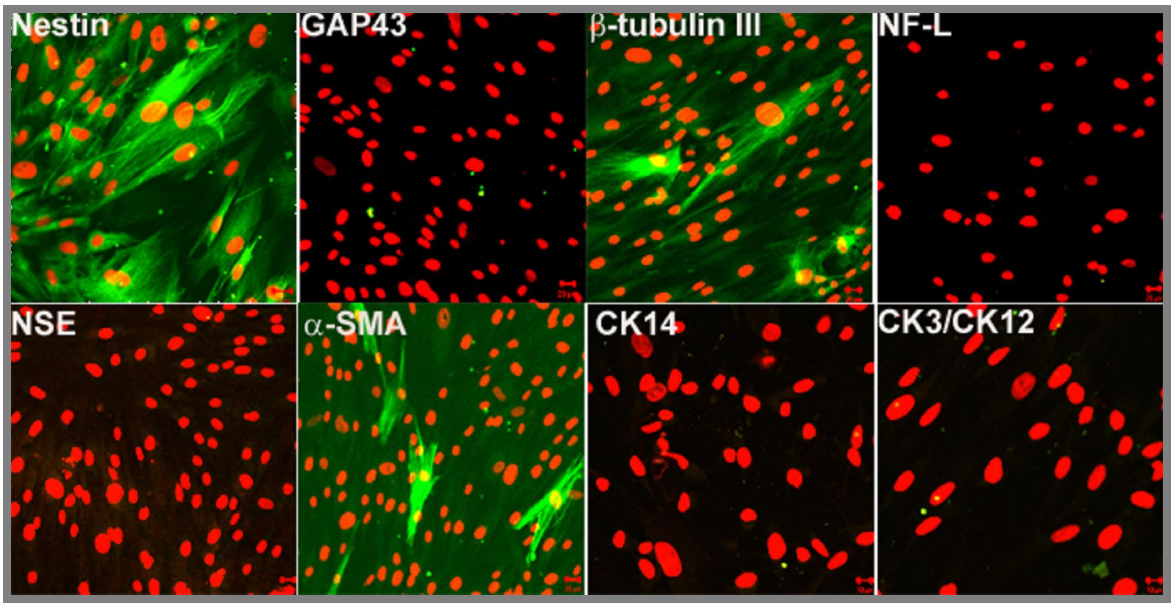

Figure 4.6: Immunophenotyping of limbal stromal cells. The stromal cells show immunoreactivity for nestin, few cells expressed β-tubulin III, α-SMA and all cells were negative for GAP43, NF-L, NSE, CK14, and CK3/CK12. Nucleus was counterstained with propidium iodide (red).

4.5.4.3 RT-PCR Analysis

RT-PCR analysis showed the expression of S100A2 and transcription factors paired homeobox-6 (PAX-6), orthodenticle homeobox 1 (OTX1) only in LEC. Vimentin and vascular endothelial growth factor A isoforms (121, 148, 165) were expressed in LEC, MLC-L and MSC-BM. Collagen type 1 alpha 1 expressed in MLC-L and MSC-BM. S100A4 showed expression in both LEC and MLC-L (Figure 4.7).

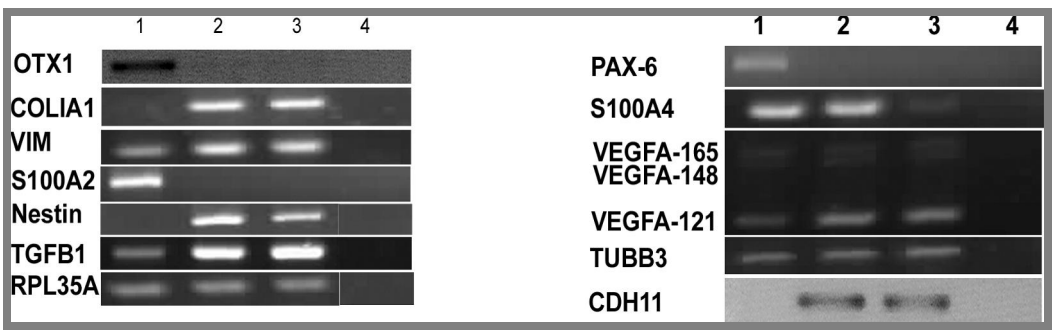

Figure 4.7: RT-PCR analysis of limbal epithelial and mesenchymal cells of limbus and bone marrow. 1- LEC, 2-MC-L, 3-MSC-BM, 4- -RT control

4.5.5 *In Situ L*ocalization by IHC

In situ localization was performed on frozen sections of corneo-scleral rims. On immunocytochemistry Cytokeratin (CK) AE1/AE3 and CK3/12 expression was noted in the cytoplasm of the suprabasal cells of limbal epithelium and corneal epithelium, but not the basal layer of limbus. No staining was observed in corneal and limbal stroma for AE1/AE3 and CK3/12 (Figure 4.8).

On immunocytochemistry Vimentin stained the basal layer of limbus but was absent in the suprabasal and corneal epithelial cells. Vimentin protein was also detected in stromal cells of both the limbus and cornea. CD29 stained the basal layer epithelial cells of limbus and cornea, walls of blood vessels and stroma of limbus occasionally in corneal stroma. Vascular adhesion cells molecule and Nestin expression was observed in limbal suprabasal and corneal epithelial cells and also observed in limbal stroma but not in corneal stroma. CD44 expression (red) was noted in limbal basal epithelia, corneal basal epithelia and limbal stroma but not in corneal stroma (Figure 4.9).

In situ localization showed CD90 and CD71 in the suprabasal limbal stroma though very few cells in the corneal stroma expressed the CD90. Alpha SMA was detected in the limbal stroma where it stained the vessels, but its expression was not seen in the corneal stroma, as the cornea is avascular (Figure 4.10).

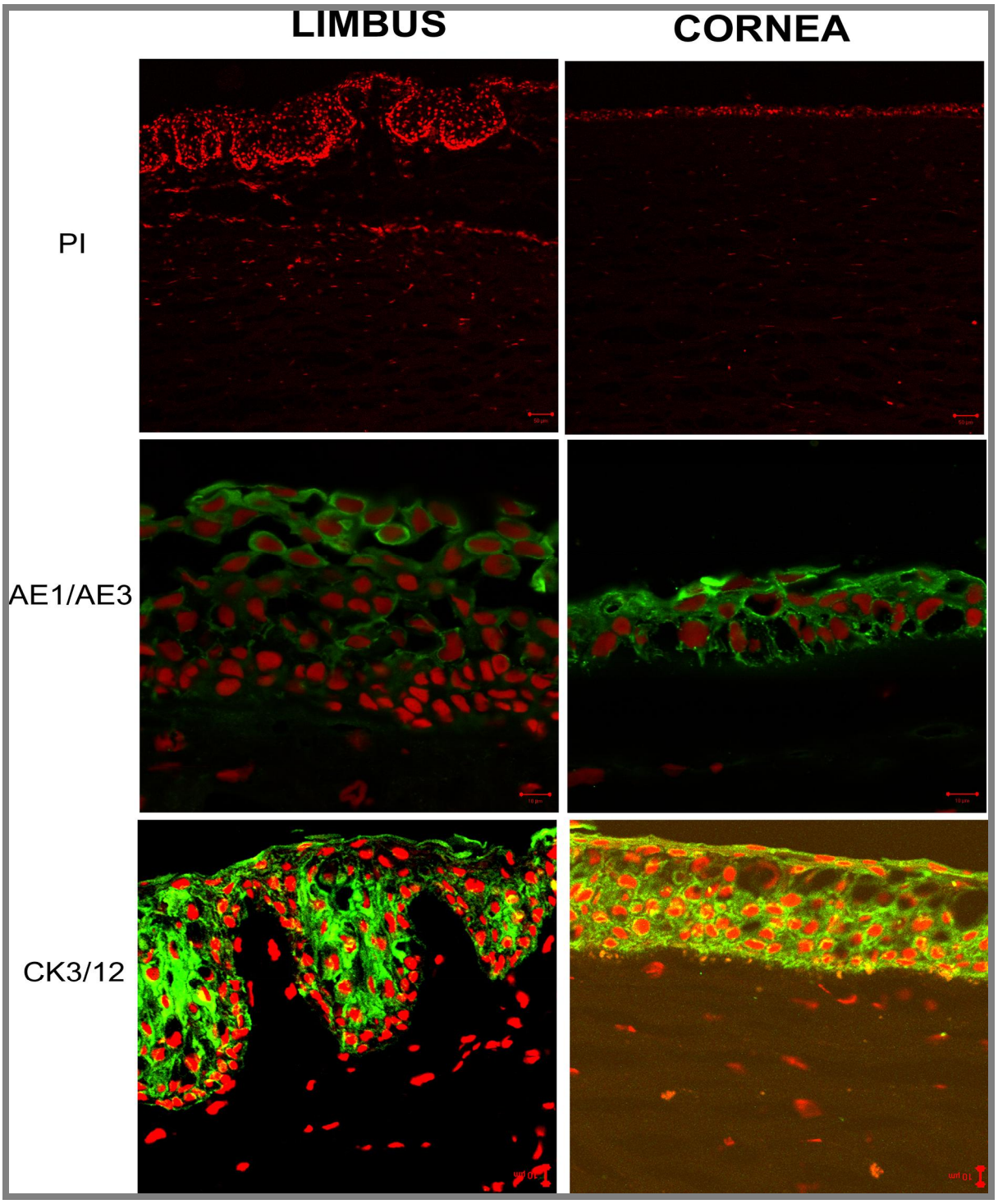

Figure 4.8: Immunohistochemical analysis of corneo-limbal sections shows expression (green) of cytokeratin AE1/AE3 and CK3/12 suprabasal limbal epithelia and entire corneal epithelial cells. Note the absence of staining in the stroma of cornea and limbus.

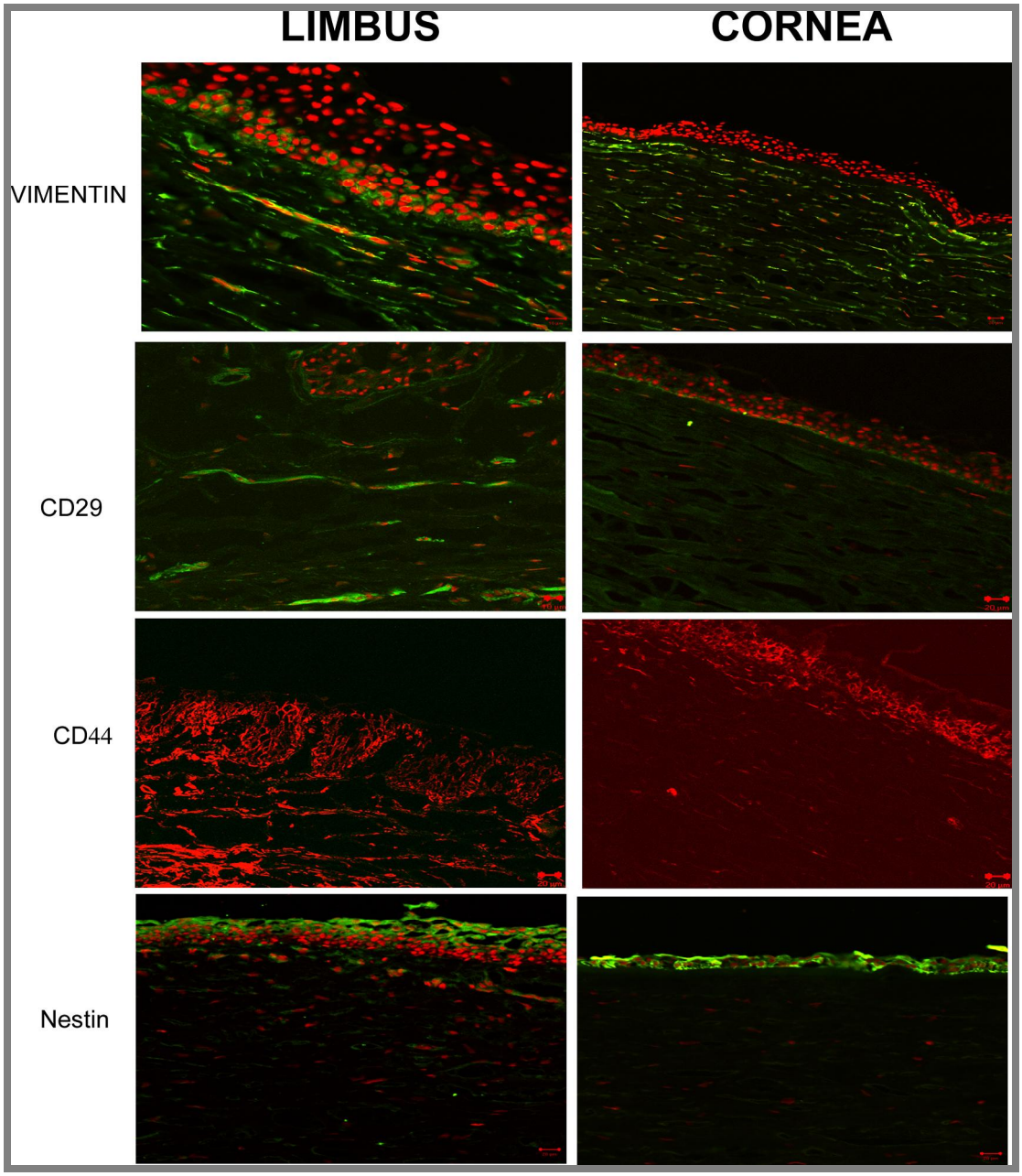

Figure 4.9: The sections from the corneo-limbal region show vimentin positivity within the basal layer of limbus and stroma of limbus and cornea. CD29 stained the epithelial cells of limbus and cornea, blood vessels and stroma of limbus occasionally in corneal stroma. VCAM and nestin expression was seen in suprabasal cells of limbus and corneal epithelial cells. CD44 expression (red) was noted in limbal basal epithelia, corneal basal epithelia and limbal stroma. Few cells in the corneal stroma also showed expression of CD44.

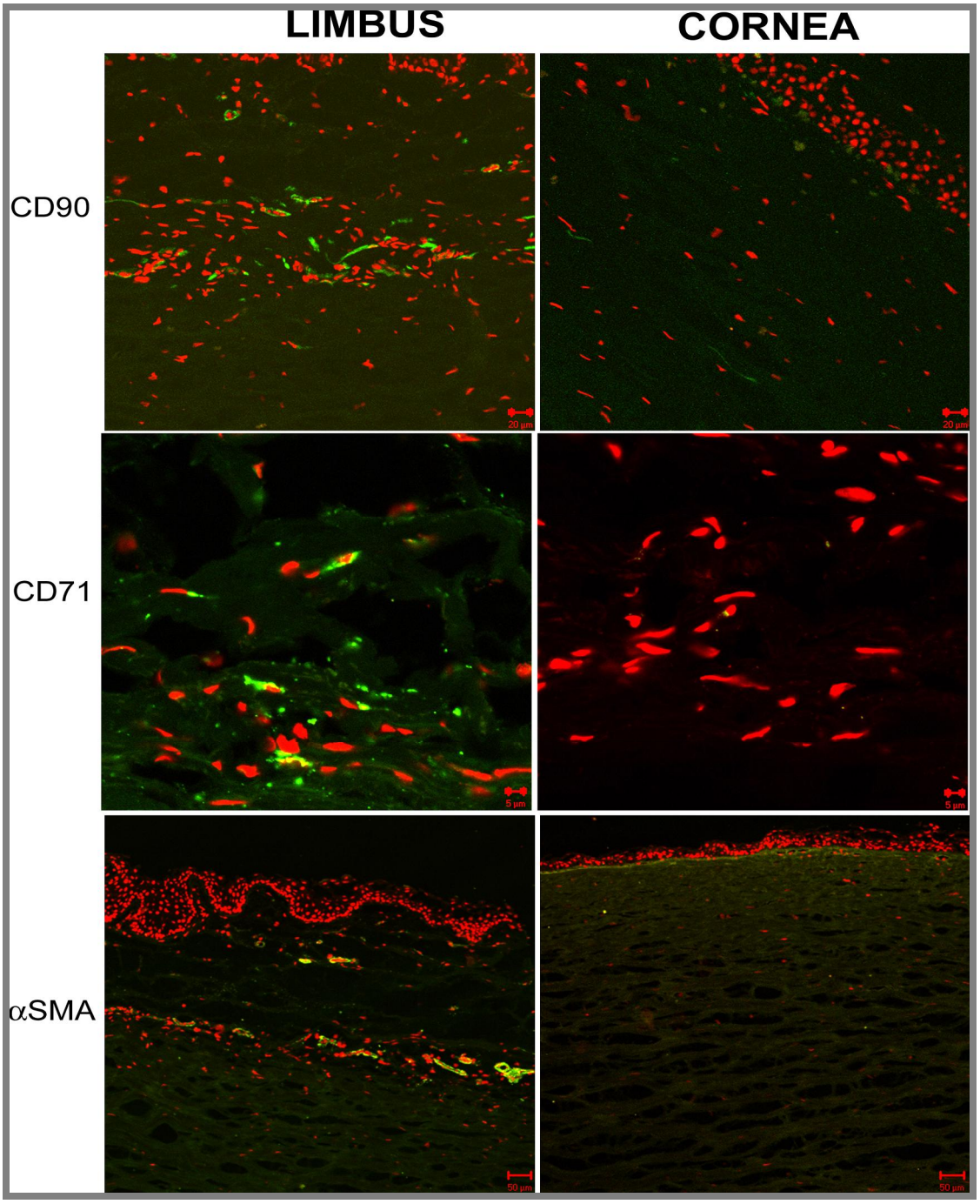

Figure 4.10: *In situ* localizaton of corneo scleral rims. CD90 stained the blood vessels and stroma of limbus whereas CD71 stained the limbal stroma. Alpha-SMA positive cells were seen in the blood vessels of limbal stroma. Nuclei were counterstained with propidium iodide (PI) except in CD44 staining.

4.5.6 Differentiation

MC-L were differentiated *in vitro* using adipogenic and oesteogenic induction medium. Following 3 weeks of adipogenic induction, the cells

stained Oil red 'O' positive showing lipid laden adipocyte phenotype. (Figure 4.11 a & b). Similarly, these cells induced with oesteogenic induction for 2-3 weeks showed calcification when stained with alizarin red for calcium deposits (Figure 4.11c & d).

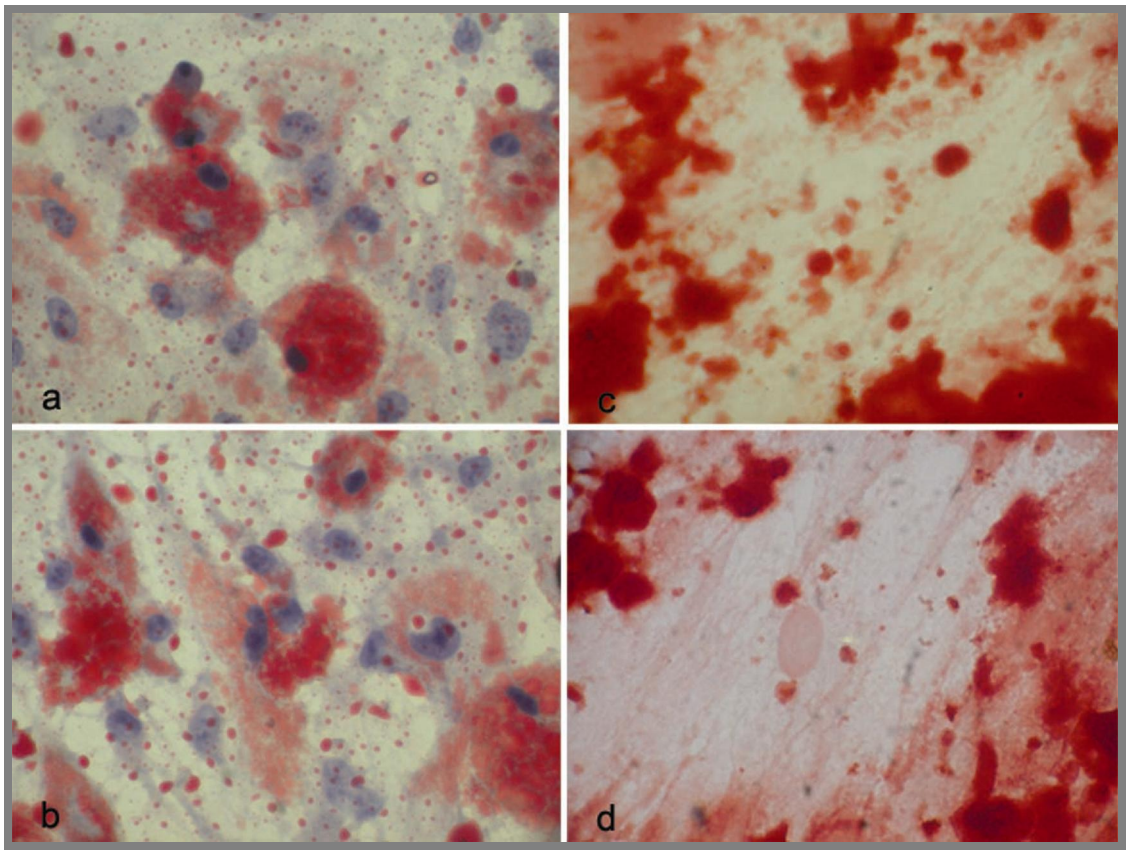

Figure 4.11: Differentiation of MC-L and MSC-BM into adipocytes and osteocytes showing the presence of oil-red positive lipid laden cells in MSC-BM (a) and MC-L (b) and presence of alizarin stained calcium deposits in MSC-BM (c) and MC-L (d) showing calcification after three weeks of culture in respective differentiation media at 20X magnification

4.6 Discussion:

Limbal epithelial cells are cultivated *in vitro* and used for clinical application in patients with LSCD in many centers worldwide including ours (Pellegrini *et al.,* 1999, Fatima *et al.,* 2006, Sangwan *et al.,* 2006). We made an interesting observation that when in culture for a longer time, these epithelial cells give rise to fibroblast-like cells. We speculated that these cells

were of mesenchymal origin, as they had a longer initial lag phase in comparison to limbal epithelial cells, in culture. The presence of similar cells in de-epithealized tissue further pointed towards their mesenchymal origin. This is similar to the observations made by Dravida and coworkers (Dravida *et al.,* 2005) who showed the existence of fibroblast-like cells in the limbal stroma, with embryonic stem (ES) cell-like features. Another study by Funderburgh and coworkers (Du *et al.,* 2005), evaluated the stromal cells isolated from corneal stroma with features of stem cells as proved by ABCG2 and pax-6 expression and side population studies. They also demonstrated that the location of such cells was more towards the limbus than the central cornea.

Cultured human corneal epithelial stem cells from the limbus have been successfully used for corneal reconstruction. The phenotypic characterization of these stem cells has been well documented. This population of MC-L is different from the above-mentioned limbal epithelial stem cells (LESCs) in their origin. Limbal epithelial stem cells are cultured from the corneo-limbal epithelium (H.S. Dua *et al.,* 2000), while isolated and cultured LSC (from extended limbal epithelial cultures) were adherent to the plastic surface. Morphologically, LESCs were relatively small, cuboidal with 10.1µm diameter, structurally and biochemically primitive with low cellular granularity and pigmentation. MC-L on the other hand was characteristically elongated and spindle shaped. LESCs are shown to express the vimentin, cytokeratin 3/12, cytokeratin 14, Cytokeratin 19, E-cadherin and PAX-6, whereas MC-L do not express epithelial markers.

There is sufficient work done on the human MSC-BM, from their characterization and differentiation to clinical application. Human MSC-BM are

currently being tested in a number of animal models for human diseases (Schwarz *et al*., 1999 and 2001), and several clinical trials making use of these cells have been initiated (Koc *et al.,* 2000, Horwitz *et al.,* 1999). For most of these experiments and trials MSC-BM are prepared with a standard protocol in which nucleated cells are isolated from a bone marrow aspirate with a density gradient and then both enriched and expanded in the presence of FBS by their tight adherence to plastic tissue culture dishes. The MC-L in the present study were cultured in a similar manner based on their adherent property to plastic dishes. Morphologically, these cells looked similar to MSC-BM under the phase contrast microscope. The observation of decrease in proliferative capacity of cells with increasing passages (rigorous growth at passages 2 and 3, proliferation rate decrease at passage 4 and 5 with slight change in morphology and no further proliferation at passage 6) and minor morphological changes in culture have made us restrict our study till passage 6. These cells showed colony forming efficiency of 30-40% at passage 2, which decreased to 10-15%, and further with increasing passages, which is similar to MSC. The MC-L showed 22.9 population doublings, which was in close approximation to MSC-BM, which showed 30.9 population doubling. This resembles the reported in vitro life span of human MSC-BM (22-23 doublings beginning at primary culture (Banfi *et al.,* 2000) and 15 at passage 1 (Digirolamo *et al.,*1999). Moreover the cultures undergo subtle changes as they expand, with a marked decrease in the rate of proliferation and plasticity (Digirolamo *et al.,* 1999; Zohar *et al.,* 1997] as observed both in MC-L and MSC-BM.

The immunophenotyping of MC-L cells showed a remarkable similarity with the surface antigen profile of MSC-BM, as evident from the results of both immunofluorescence and flow cytometry. Like the MSC-BM, the MC-L showed similar expression patterns for CD106 (VCAM), CD54 (ICAM), CD166 (ALCAM), CD90 (Thy-1), CD29 (integrin β1), and CD71 (transferrin receptor) markers and negative for hematopoietic markers. The data shows a difference in expression patterns of CD105 between MC-L (21.42% ± 4.133) and MSC-BM (71.33 % ± 6.66). While the expression levels in MSC-BM are similar to those reported by previous studies (Oswald *et al*., 2004) and the present data on the lower expression levels in mesenchymal cells of limbal origin is not sufficient to derive any further conclusions. Since the endothelial markers (Flt1, Flk1, VE-Cadherin, CD31 as shown in table 4) were negative in limbal derived mesenchymal cells, we speculate that they are probably neither endothelial derived nor does it point towards endothelial differentiation. We also observed a negative expression profile of MC-L for embryonic stem cell (ESC) markers such as SSEA1, Tra-61 and Tra81, which is in contrast to the observations made by Dravida and co-workers. This difference could be attributed to the difference in the source of cells i.e, sorted and unsorted cells.

Differentiated epithelial markers K3/K12 and AE1/AE3 (Chen Z *et al*., 2004) were expressed only in limbal epithelial (suprabasal) cells and as expected were absent in MC-L and MSC-BM. Vimentin (Joseph A et al., 2004) is an intermediate filament that is found in mesenchymal cells other than muscle (Kivela T *et al*., 1998). Lauweryns *et al* identified a subpopulation of “transitional cells” in normal limbal tissue that co-expressed CK19 and vimentin and speculate that these might be stem cells (Lauweryns *et al*., 1993

and 1993). We found that the basal layer of the limbal epithelial cells and stroma of cornea and limbus expressed vimentin.

Predominant *in-situ* localization of some markers (CD44, CD90, CD29 and CD71) in limbal stroma as compared to central and peripheral corneal stroma further strengthens our postulation that these cells are possibly derived from limbal stroma, the anatomic niche of limbal epithelial cells. It would be logical to speculate that the reported mesenchymal cells from different sources (fat, bone marrow, skin *etc*) are possibly part of a common microenvironment or niche. However this would require further studies for confirmation.

In this study we also demonstrated the multilineage differentiation of MC-L into adipocytes and osteocytes, similar to the plasticity of MSC-BM. Also, the low level and absence of MHC-II molecules in MC-L is similar to their levels in MSC-BM (data not reported here). Though the evidence points towards their limbal stromal location, the *in vivo* role of these cells is not known and extrapolation is beyond the scope of this study. Literature review however points towards the presence mesenchymal stromal cells in corneal stroma similar to our hypothesis. Choong and coworkers, in their study on keratocytes that were isolated from adult human cornea tissues found them to be CD13, CD29, CD44, CD56, CD73, CD90, CD105, $CD133^{+}$/ HLA-DR, CD34, CD117, $CD45^{-}$ markers which is similar to that of MSC-BM. These cells were also able to differentiate into adipocytes and osteocytes. Yamagami and coworkers also evaluated for the presence of bone marrow-derived cells in normal human corneal stroma and showed that the CD45-positive cells in anterior stroma of the central and paracentral cornea and stromal layers of the

peripheral cornea also uniformly expressed CD11b, CD11c, CD14 and HLA-DR antigen but not CD3, CD19, CD56 or CD166, indicative of bone marrow-derived monocyte lineage cells. They concluded that these cells could play a role in immune responses in the human cornea [Yamagami S *et al*., 2006]. An independent study by McCallum et al phenotypically compared epithelial and nonepithelial components of human corneal and conjunctival microenvironments using a panel of monoclonal antibodies for epithelial cell maturation, mesodermal-derived fibrous tissue and vessels, specific keratins, and MHC Class I and II antigens. The study suggested that cornea and conjunctiva had similar antigenically defined pathways of maturation [McCallum RM *et al*., 1993].

However we know that MSC-BM comprises a multifunctional tissue consisting of heterogeneous cell populations that provide a specialized microenvironment for controlling the process of hematopoiesis [Dexter TM *et al*., 1989]. Since the MC-L form part of the niche for limbal stem cells (cells derived from the limbal stroma underlying the limbal epithelium) and did show similarities in phenotypic profile of MSC-BM viz the adherent nature, similar surface antigen expression, low immunogenicity and colony forming capability, self-renewal capacity and plasticity (unpublished data), we speculate that the niche stromal cells are special cells which might play a role in providing specialized microenvironment in limbal stem cell maintenance. Its role in diseased and normal states cannot be extrapolated in the current study.

In conclusion, the stromal cell cultures from limbal explants are of stromal origin and fibroblastic in nature and share properties with MSC-BM.

Thus our study shows that the limbal stroma supporting the limbal epithelium possesses a unique population of cells, similar to MSC-BM in their culture characteristics, phenotypic marker expression profile, colony forming efficiency, population doubling capacity and low immunogenicity. However, the role of these cells *in vivo* and potential application *in vitro* needs to be further explored.

GENE EXPRESSION PROFILE OF LIMBAL EXPLANT CULTURE DERIVED CELLS

5.1 Introduction

One of the most important advances made in translational research is in the field of ocular surface reconstruction using cell therapy (Sangwan VS *et al*., 2004, 2006). This technology owes its success not only to the surgical advances but also to the increasing amount of knowledge pertaining to the location, characteristics and functioning of LSC (Cotsarelis G *et al* 1989, Wolosin JM *et al*., 2000, Kinoshita S *et al*., 2001). In the normal uninjured state, LSC are mitotically quiescent and maintained in a specialized limbal stromal microenvironment or "niche". However, on corneal epithelial wounding, stem cells located in the limbus proliferate to generate more stem cells and transient amplifying cells so as to replace the damaged epithelium. Though a specific signature of LSC is not known, it is generally agreed that they are characterized by special location in the limbus, clonality, cytokeratin profile, p63 delta isomers and ABCG2 expression (Figueira EC *et al.,* 2007, De Paiva CS *et al*., 2006). Recent report by Majo and coworkers (Majo F *et al*., 2008) suggests the presence of stem cells not only in the limbus but also in the central cornea. This report now shifts the focus from the epithelial stem cells to the role of niche in maintaining the "stemness" *in-vivo*. It is also well established that the niche plays an important role in the maintenance of stem cell properties in several tissues and this is expected to be true in the case of the LSC niche as well (Scholtzer-Schrehardt U *et al*., 2005, Itskovits-Eldor J *et al*., 2000, Schofield R *et al*., 1983, Li L *et al.,* 2006). Some of the implied factors for niche regulation include proximity to vasculature, (Gipson *et al*., 1989); the basement membrane composition with respect to specific isoforms

of collagen IV, laminin and fibronectin (Ljubimov *et al*., 1995); and the presence of limbal fibroblasts in the underlying stroma, which produce various cytokines (Li and Tseng, 1995).

There have been some reports in literature, which suggest that limbal fibroblast-like cells from adult corneo-limbal tissue may have stem cell like properties (Figueira *et al*., 2007), and also their conditioned media has the ability to convert human embryonic stem cells to corneal epithelial-like cells (Ahmad *et al*., 2007). Our earlier work documented the growth of spindle shaped MSC-L cells in extended limbal explant cultures, which bear a striking resemblance to the MSC-BM (Polisetty *et al*., 2008).

Gene expression profiling is an emerging technique of identifying stem cells which have contributed to the understanding of several cellular pathways and intrinsic factors that characterize LSC in normal human corneas (Adachi *et al*., 2006, Diehn *et al*., 2005, Jun *et al*., 2001). In these studies, the entire cornea was used as the starting material, whereas Zhou and coworkers have shown the gene expression profiles of stem cell-enriched limbal basal cell population in mice (Zhou *et al*., 2006). In the present study, we evaluated the transcriptome of the limbal explant culture derived epithelial and mesenchymal like cells by microarray and identified expression of unique genes and biological pathways that characterize both these cell types. To evaluate our hypothesis that the MSC-L possibly act as one of the “niche” derived intrinsic feeder cells, we compared the profile of these cells to that of the MSC-BM, which form the supporting niche for the hematopoietic system.

5.2. Hypothesis:

We hypothesized that limbal explant derived mesenchymal cells have similar gene expression profile with that of Bone marrow mesenchymal stem cells.

5.3. Aims

- Ø To perform gene expression profile of limbal explant culture derived cells in comparison to bone marrow derived mesenchymal stem cells

5.4. Material and Methods

The protocol was approved by the Institutional Review Board at L.V.Prasad Eye Institute.

5.4.1. Preparation of Chemicals

All the chemicals and culture media were prepared as described in the Appendix I.

5.4.2. Sterility Check for Chemicals & Media:

Following the filter sterilization the media and chemicals were kept for sterility check. A few drops of media/chemicals were inoculated on chocolate agar and in thioglycolate broth to screen for both aerobic and anaerobic microorganisms. The inoculated media was then incubated in a bacterial incubator at 37^0C for about 7 days, before the media/chemicals were approved for tissue culture use.

5.4.3 Establishment of Cell Cultures

In an ongoing clinical trial, which is approved by the Institutional Review Board, limbal tissues were obtained as mentioned in an earlier chapter (Chapter 4).

5.4.4 Microarray:

Microarrays serve as a powerful tool to screen different biological specimens or regions within the same tissue specimens for primary differences in the expression of mRNAs that accompany, and may regulate physiological and pathological change.

5.4.4.1 Isolation of Total Cellular RNA (Trizol-Method):

The Isolation of RNA from cultured cells involves the following steps:

(I) Homogenization, (II) Phase Separation, (III) RNA precipitation, (IV) RNA wash (V) Resuspension of the RNA pellet.

(I) Homogenization

Cells were dislodged from the culture flask using 1 ml TRIzol and passed several times through a pipette for homogenization.

(II) Phase Separation

Homogenized samples were incubated for 5 mins at 15-30^{o}C. 0.2 ml of chloroform was added for every 1 ml TRIzol reagent added. Tubes were shaken vigorously for 15-20 seconds, incubated at 15-30^{o}C for 2-3mins and centrifuged at 12,000g for 15 mins at 2-8^{o}C. After centrifugation the lower red phenol-chloroform phase, an interphase, and an upper aqueous phase is formed.

III] RNA Precipitation

The aqueous phase containing (organic phase - for DNA, Protein isolation) the RNA was transferred to a fresh tube. RNA was then precipitated by isopropyl alcohol (0.5 ml/1ml TRIzol) and incubated at 15-30^{o}C for 10 mins. The sample was then centrifuged at 12,000g for 10mins at 2-8^{o}C. The RNA precipitate, often invisible forms a gel like pellet on bottom/side of the tube.

IV] RNA Wash:

The supernatant was decanted and RNA pellet was washed in 75% ethanol (1ml/1ml of TRIzol). The sample was mixed by vortexing and centrifuged at 7,500x*g* for 5 mins at 2-8^{o}C.

V] Resuspension of the RNA

RNA pellet was then dried (air dried) and dissolved in RNase-free water and stored at -70^{o}C.

5.4.4.2 Quality and Quantification of RNA

Nanodrop 1000 Spectrophotometer: Molecular biology techniques and other scientific methods continue to rapidly evolve using progressively smaller amounts of samples. NanoDrop technology has successfully addressed the growing demand for micro-volume quantitation and analysis by being the first to solve the sample conservation issue using fiber optic technology and surface tension properties.

Good quality RNA will have an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater. This is because nucleic acid is detected at 260nm, whereas protein, salt and solvents are detected at 280 and 230 nm respectively. A high OD of 260/280 and OD of 260/230 ratios therefore indicate that you have extracted RNA devoid of any these contaminants.

The isolated RNA was quantified by nanodrop spectrophotometer by adding 1 µl of sample to the NanoDrop 1000 spectrophotometer and measuring the OD of 260/280 and 230/260 ratio.

5.4.4.3 Purification of RNA:

RNeasy spin column kit

A maximum of 100 µg of RNA can be cleaned up in this protocol. This amount corresponds to the RNA binding capacity of the RNeasy column.

Procedure:

1) The sample was made up to 100 µl with RNase free water to which 350 µl of buffer RLT was added.
2) 250µl Alcohol (96-100%) was added to the diluted RNA and mixed well by pipetting.
3) 700 µl of the sample was transferred to an RNeasy mini spin column, which was placed in a 2ml collection tube. The lid was closed gently and centrifuged for 15s at 8,000x*g*. After centrifugation, the flow through was discarded.
4) 500 µl of buffer RPE was added to the RNeasy spin column. This was centrifuged for 15s at 8,000x*g*.
5) 500 µl of RPE was added again to the RNeasy spin column and centrifuged for 2 min at 8,000x*g*.
6) After centrifugation the RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 min.
7) Again the RNeasy spin column was placed in a new 1.5 ml collection tube to which 30 µl of RNase free water was directly added. The lid was gently closed and centrifuged for 1 min at 8,000x*g*.

5.4.4.4 Quality and Quantity of RNA after Column Purification

After column purification of RNA, the quality and quantity was checked by Nanodrop 1000 spectrophotometer and also by electrophoresing on a denatured agarose gel (0.8%).

Agarose gel electrophoresis:

Gel Preparation:

1. Required amount (0.8%) of agarose was added to RNAase fee water.

2. Agarose was boiled in microwave and the flask was swirled to ensure even mixing.
3. Melted agarose was cooled to a tolerable temperature (~55^{o}C).
4. Eight ml of 10X MOPS buffer and 14.4 ml of formaldehyde was added to it.
5. The melted gel was poured in the casting apparatus with an inserted comb.
6. The gel was allowed to stand till it solidified.
7. The comb was removed gently from the gel plate after solidification.

Sample preparation:

1. RNA (500ng) products were mixed with 2 µl of RNA loading Dye (Bromophenol blue), 10 µl of formamide, 4 µl of formaldehyde and 2 µl of 10X MOPS.
2. It was incubated at 70^{0} C for 15 min.

Gel loading and running

1. The gel plate was placed in the electrophoresis tank
2. 1X MOPS buffer was poured to cover the wells
3. The samples were loaded the samples along with standards - 250ng and 500ng. The gel was run in 1x MOPS buffer at 100V.
4. The gel was run for approximately half an hour at a voltage supply of 10V/cm till bromophenol migrated to atleast half the distance of the gel
5. The gel was removed from the tank and was placed on UV transilluminator (UV tec) and the amplification was documented in a gel doc system
6. After running, the amount of RNA was quantitated by comparing with known concentrations of 250ng and 500ng.

5.4.4.5 cDNA and cRNA Preparation

1. 500 ng of total poly A RNA was added to 1.5 ml microcentrifuge tube.
2. 1.2 µl of T7 promoter primer was then added to it.

Table 5.1: Ingredients for cDNA preparation

Sample	T7 promoter primer (µl)	RNA (500ng) (in µl)	Nuclease free water	Total volume
LEC	0.6l	2.23	2.92	5.75
MSC-L	0.6	1.1	4.05	5.75
MSC-BM	1.2	1.62	8.68	11.5
MSC-BM (N)	0.6	0.54	4.61	5.75

3. Nuclease free water was used to bring total reaction volume to 11.5 µl.
4. The primer and the template were denatured by incubating the reaction at 65^0 C in a circulating water bath for 10 min.
5. Thereafter the samples were placed on ice for 5 min.
6. Prior to use the components were mixed gently and added in the order mentioned below and placed on ice (Table 5.2).

Table 5.2: cDNA master mix

Component	Volume per reaction (in µl)
5X First Strand Buffer	4
0.1M DTT	2
10mM dNTP mix	1
MMLV-RT	1

RNase Out (RNA Inhibitor)	0.5
Total volume	8.5

7. Each sample was spun briefly to bring down the contents from the walls of the tube and lid.
8. 8.5 µl of MSC-BM and 4.5 µl LEC, MSC-L and MSC-BM (N) of cDNA master mix was added to the each sample and mixed by pipetting up and down
9. Samples were incubated at 40^0C in a circulating water bath for 2 hrs.
10. Samples were then placed at 65^0C circulating water bath and incubated for 15 min
11. Samples were then placed on ice for 5 min and spun to bring down the contents along the wall and lid.
12. Just prior to use, the components were mixed gently and added in the following order (Table 5.3).

Table 5.3: Transcription Master Mix

Component	Volume per reaction (in µl)
Nuclease free water	15.3
4X transcription buffer	20
0.1 M DTT	6
NTP mix	8
50%PEG	6.4
RNase out	0.5
Inorganic pyrophosphatase	0.6

T7 RNA polymerase	0.8
Cyanine-3-CTP or cyanine 5-CTP	2.4
Total volume	60

13. 60 µl of MSC-BM and 30 µl of LEC, MSC-L, MSC-BM of transcription master mix was added to each sample tube and gently mixed by pipetting.
14. Samples were incubated in a circulating water bath at 40^0 C for 2 hrs.

5.4.4.6 Purification of the labelled/amplified RNA

1. Nuclease free water was added to the cRNA sample to make up the total volume to 100µl.
2. 350 µl of Buffer RLT was added and mixed well by pipetting
3. 250 µl of ethanol (96% to 100% purity) was added and mixed well by pipetting
4. 700 µl of the cRNA sample was transferred to an RNeasy mini column in a 2 ml collection tube. The samples were then centrifuged at 13,000rpm at 4^0 C for 30 seconds. After centrifugation the flow-through was discarded.
5. The RNeasy column was transferred to a new collection tube and 500µl of buffer RPE (containing ethanol) was added to the column. The samples were then centrifuged at 13000 rpm, 4^0C for 30 sec.
6. 500 µl buffer RPE was added to the column. The samples were centrifuged at 13,000 rpm, 4^0 C for 60 sec.
7. The cleaned cRNA sample was eluted by transferring the RNeasy column to a new 1.5 ml collection tube. 30 µl of RNase free water was

directly added on to the membrane and centrifuged at 4^0C for 30 seconds at 13,000 rpm

8. The flow-through containing cRNA was placed on ice.

5.4.4.7 Quality and Quantification of cRNA

Labelled cRNA was quantified by using Nandodrop 1000 UV-VIS spectrophotometer. The specfic activity of cRNA was calculated as follows

Specific activity = Conc. Of Cy3 or Cy5 / Concentration of cRNA *1000 = pmol cy3 per µg of cRNA.

Quality of cRNA was checked by running the sample (200ng each) on 0.6% agarose gel.

5.4.4.8 Hybridization

(I) Preparation of 10X blocking agent

1. 500 µl of nuclease free water was added to a vial containing lyophilised 10X blocking agent supplied with agilent gene expression hybridization kit. Mixed by vortexing.
2. Any material adhering to the walls and cap of the tube was brought down by centrifuging for 5 to 10 sec

(II) Preparation of hybridization samples

1. Water bath was set at 60^0 C
2. For each microarray, the components were added as indicated in the table 5.4

Table 5.4: Ingredients for hybridization

Sample	Cy3 825ng	Cy5 825ng	Blocking agent	Nuclease free water	25X fragmentation buffer	Total
LEC 1 vs MSC-BM 1	2.586	1.69	11	37.514	2.2	55
LEC 1 VS MSC-L 1	2.586	5.16	11	34.054	2.2`	55
MSC-L 1 vs MSC-BM 1	5.63	1.69	11	34.48	2.2	55
MSC-BM (N) 1 vs MSC-BM 1	2.87	1.69	11	37.24	2.2	55
LEC 2 vs MSCBM2	4.014	2.598	11	35.188	2.2	55
LEC 2 vs MCL 2 (CY5)	4.014	8.104	11	29.682	2.2	55
MCL (Cy3) (2) vs MSCBM (2)	9.55	2.598	11	29.652	2.2	55
MSCBM (N) (2) vs MSCBM (2)	4.016	2.598	11	35.156	2.2	55

3. Samples were incubate at 60^0 C for exactly 30 min to fragment RNA
4. 55 µl of 2x GEx hybridization buffer HI-RPM was added to makeup total volume to 110 µl.
5. Samples were mixed by centrifugation and used immediately.

(III) Preparation of hybridization assembly

1. A clean gasket slide was loaded into the agilent surehyb chamber base with the label facing up
2. 100 µl of hybridisation sample was slowly dispersed on to the gasket well in a drag and dispense manner, being sure not to touch the gasket walls.
3. The array "active side' down onto the surehyb gasket slide, so that the "Agilent"-labelled barcode is facing down and the numeric barcode is facing up.
4. The SureHyb chamber cover was placed onto the sandwitched slides and slide the clamp assembly onto both pieces.
5. The clamp was hang-tightened on the chamber
6. The assembled slide chamber was placed in rotisserie in a hybridization oven set to 65^0 C at 10rpm for 17hrs.

5.4.4.9 Microarray Wash

1. The slide was placed onto a dish containing gene expression wash buffer 1 and rotated for 1 min.
2. The slide rack was transferred to a slide containing gene expression wash buffer 2, which is prewarmed at 37^0 C overnight.
3. The slide rack was removed slowly as to minimize droplets on the slides.
4. The slides were scanned to minimize the impact of environmental oxidants on signal intensities.

5.4.4.10 Microarray Image and Data Analysis

Microarray image analysis was done using Feature extraction version 9.5.3.1 (Agilent Technologies) and data analysis was done using Gene Spring version 10 (Agilent Technologies). The background corrected intensity values

were used for analysis. Normalization was done using LOWESS algorithm. Similar expressed genes were filtered on the basis of standard deviation between two biological replicates with the cut off of less than one. Fold changes were calculated and genes more than two fold were selected.

5.4.5 Validation of Microarray using Semi quantitative RT-PCR and Real-time-PCR

To confirm the gene expression profile determined by microarray, a number of selected genes (Table 5.5) were subjected to RT-PCR analysis, using total RNAs derived from the two independent samples of LEC, MSC-L and MSC-BM that were used for the microarray experiments, as well as an additional pair of LEC, MSC-L and MSC-BM samples. RPL35a, a ribosomal protein served as an internal control.

A 2 µg quantity of RNA was reverse transcribed using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA, http://www.appliedbiosystems.com), and 1/100th of the reaction was used per 20µl PCR reaction. PCR reactions were performed with DyNAZYME master mix (Finnzymes Oy, Espoo, Finland, http://www.finnzymes.com). The PCR products were resolved on a 2% agarose gel containing ethidium bromide. Real-Time PCR quantitation was performed in an ABI prism 7900 HT sequence detection system and analysed with SDS 2.1 software (Applied Biosystems). The reactions were identical to those described above, except that DyNAMO^{TM}SYBERgreen 2X mix (Finnzymes) was used in place of DyNAZYME MIX. The sequences of primers are shown in table 5.6. Amplification of RPL35a was performed for each cDNA (in triplicate) for normalization of RNA content. Threshold cycle number (Ct) of amplification in

each sample was determined by ABI Prism Sequence Detection System software (Applied Biosystems). Relative mRNA abundance was calculated as the average for Ct for amplification of a gene-specific cDNA minus the average Ct for RPL35a and fold change over control has been calculated as follows

$\Delta ct = ct_{gene} - ct_{RPL}$

$\Delta\Delta ct = \Delta ct$ (one cell type) $- \Delta ct$ (another cell type)

Fold Change = $2^{-\Delta\Delta ct}$

Three individual gene-specific values thus calculated were averaged to mean ± standard deviation and fold change was expressed as log 2 ratios.

Table 5.5: Primers used in this study

S.N0	Gene name	FORWARD PRIMER (5'->3')	Reverse primer (5'->3')	Length in base pairs
1	Fibroblast Growth Factor 2 (FGF2)	GGAGAAGAGCGACCCTCAC	GTGCCACATACCAA CTGGTG	221
2	(Angiopoietin 1 (Ang-1)	CCCAGAAACTTCAACATCTGG	GGACTGTGTCCATC AGCTC	537
3	RPL35A	GAACCAAAGGGAGCACACAG	CAATGGCCTTAGCA GGAAGA	236
4	E-cadherin	AAGGTGACAGAGCCTCTGGAT AGA	TCTGATCGGTTACCG TGATCAA	124
14	CD24	AACTAATGCCACCACCAAGG	cctgtttttccttgccacat	188
15	PBX-1	ACCCTTCGCCATGTTATCAG	attgctgggagatcagttgg	189
16	OTX-1	CTCCACCCAGCTGTTAGCAT	cgcatgaagatgtcagggta	221
17	FOXA1	AGGGCTGGATGGTTGTATTG	Aggcctgagttcatgttgct	150
18	SHC3	GACATCTACAGCACGCCAGA	CAAGGGCTGGTTCT TGAGAG	186
19	FOXF1	TTGGCAATATTTGCCGTGTA	ctgcactctagcagccaaaa	209
20	CDH6	TCGAGAAAACAGGGAGCAGT	cggtggagaagattcaggag	175
21	CDH11	GTGCCTGAGAGGTCCAATGT	gggtagggctgttctgatga	165
22	Collagen VI alpha 1	ACAGTGACGAGGTGGAGATCA	GATAGCGCAGTCGG TGTAGG	122
23	Collagen IV alpha 2	TTGGCGGGTGTGAAGAAGTTT	CCTTGTCTCCTTTAC GTCCCTG	178
25	Fibronectin 1	GCAGTAACCACTATTCCTGCA C	TCCTGATACAACCAC GGATGAG	192
26	T-cell differentiation protein 2 (MAL2)	TTGCCTCCTCCAATGTTCCTC	CAGTTAGCATCAATT TGAGCCAC	133

27	CTGF	CAGCATGGACGTTCGTCT	CCAACCACGGTTTG GTCCTT	117
28	SPARC (osteonectin)	CGAGACCTGTGACCTGGACAA TG	TCCGGTACTGTGGA AGGAGTGG	127
29	SFlt-F	TGAGCACTGCAACAAAAAGG	TCCTCCGAGCCTGA AAGTTA	172
30	FLT-F	GGCTCTGTGGAAAGTTCAGC	GCTCACACTGCTCAT CCAAA	223
31	FGFR1			
32	Brain derived nerve growth factor	GATGCTCAGTAGTCAAGTGCC	GCCGTTACCCACTC ACTAATAC	168
33	Chemokine (C-C motif) ligand 2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACC CACTTCT	190
34	Chitanase 3 like 1	GAAGAGGCCCTGTCTAGGTA	AGATGATGTGGGTA CAGAGG	250
35	Matrix Metallo proteinase 2 (MMP2)	CCGTCGCCCATCATCAAGTT	CTGTCTGGGGCAGT CCAAAG	169
36	Interleukin 1 aplha (IL-1A)	TGTGACTGCCCAAGATGAAG	CGCCTGGTTTTCCA GTATCT	238
37	*Decorin*	AGTTGGAACGACTTTATCTGTC C	GTGCCCAGTTCTATG ACAATCA	160
38	*Neurotrophin tyrosine kinase receptor 2*	GATAAGCTGGACTCGGCACG	GGACGACATCCCTA GCAGCC	152
39	*Connexin 43*	TGTCCTTAAGTCCCTGCTAA	GTAGCTGAGGAATG ATGAAAAAG	245

5.5 Results

5.5.1 Quantity and Quality of RNA

A good quality intact RNA was successfully isolated from LEC, MCL and MSCBM using TRIzol reagent. After electophoresis of total RNA samples in the presence of ethidium bromide, the 28S and 18S human rRNA was clearly visible under UV illumination. The intensity of the 28S RNA was twice the intensity of the 18S RNA (Figure 5.1).

Table 5.6: Quality and quantity of RNA by Nanodrop 1000 Spectrophotometer

Sample	Conc. (ng/µl)	260/280	260/230	260	280	230
MSC-BM (1.2)	583.9	2.07	0.89	14.59	7.2	16.39
MSC-BM (1.1)	809.4	1.92	0.63	20.23	10.5	32.280
LEC 1	397.8	1.96	0.73	9.945	5.082	13.705
MSC-L 1	1613.5	1.89	0.55	40.338	21.3	73.978
LEC2	853	2.08	1.05	21.73	10.27	20.33
LEC3	280	2.04	1.35	6.72	3.56	7.7
MCL-2	288	2.05	0.68	6.8	3.3	9.9
MCL-2.1	223.8	2.05	1.62	5.59	2.73	3.4
MCL-3	1003	2.05	1.35	25.09	12.23	18.5
MSC-BM 2.1	360.2	2.04	1.10	9.004	4.4	8.18
MSC-BM 2.2	455.9	2.02	1.25	11.398	5.631	9.155
MSCBM 3	298.8	2.03	1.09	7.46	3.677	6.8

Table 5.7: Quality and quantity of RNA by Nanodrop 1000 spectrophotometer after column purification

Sample	Conc.(ng/µl)	260/280	260/230	260	280	230
LEC	261.8	2.17	2.02	6.546	3.022	3.236
MSC-L	648.7	2.21	1.76	16.217	7.341	9.198
MSC-BM	799.7	2.17	1.53	19.993	9.215	13.026
LEC2	534	2.09	0.89	13.35	6.39	15.72
LEC3	328.5	2.10	1.96	8.212	3.919	4.186
MCL 2	679.4	2.08	1.58	16.985	8.169	10.735

MCL 3	711.9	2.07	1.73	17.797	8.58	10.30
MSC-BM 2 (Combined 2.1 and 2.2)	657.8	2.07	1.16	16.44	7.925	14.213
MSC-BM 3	162.9	2.13	0.43	4.072	1.194	9.488

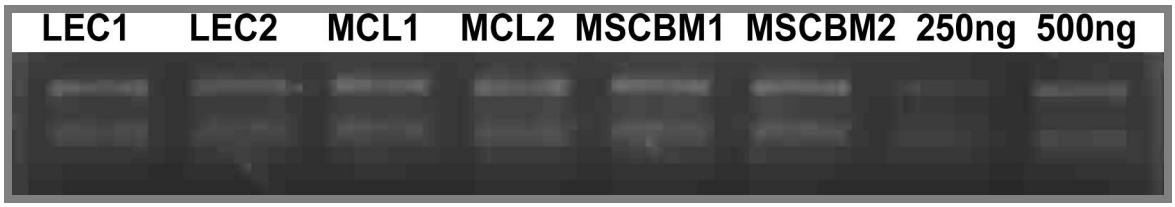

Figure 5.1: Integrity of RNA by agarose gel electrophoresis. Electrophoresed agarose formaldehyde gel revealing good quality RNA from LEC, MCL and MSC-BM as indicated by the 28S and 18S bands.

Table 5.8: Quantity of RNA by agarose gel quantification

Sample	Volume loaded for 500ng	Conc. of RNA by agarose for 500ng
LEC 1	1.9	2.23
MSC-L 1	0.77	1.09
MSC-BM 1	0.63	0.81
LEC 2	0.94	1.05
LEC3	1.52	1.95
MCL 2	0.73	0.821
MCL 3	0.7	0.78
MSCBM 2	0.76	0.977
MSCBM3	3.06	3.44

5.5.2 Quality and Quantity of cRNA

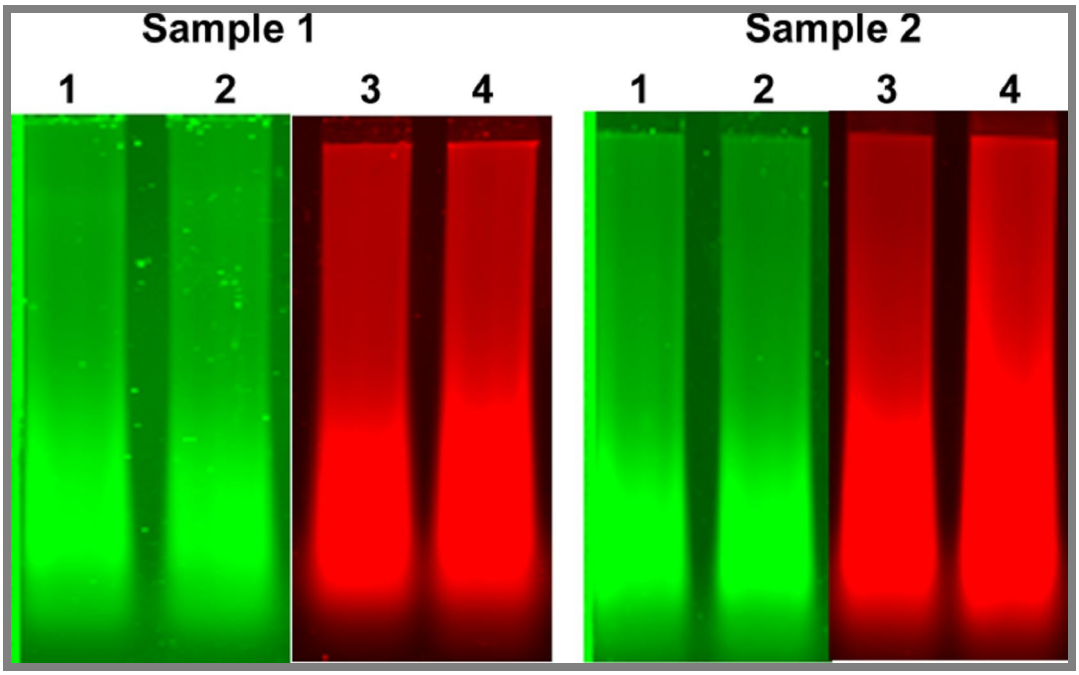

Figure 5.2: Quality of cRNA by agarose gel electrophoresis. 1- LEC, 2-MCL (Cy3), 3-MSC-L (Cy5), 4-MSC-BM

Table 5.9: Quantity of cRNA by nanodrop 1000 spectrophotometer

Sample	Cy3/cy5 Pmol/µl	Conc., ng/µl	260/280	260	Specific activity
LEC (Cy3)	3.7	319	2.27	0.802	11.59
MSC-L (Cy3)	1.8	146.4	2.35	0.366	12.29
MSC-L (Cy5)	2.2	159.7	2.30	0.406	13.77
MSC-BM (Cy5)	6.4	487.0	2.38	1.224	13.14
LEC 2	1.9	205.5	2.16	0.526	9.2
MSCL 2 (Cy3)	1.0	86.3	2.18	0.216	11
MSCL 2 (Cy 5)	1.2	101.8	2.16	0.257	11.78
MSCBM 2 (Cy 5)	3.2	317.5	2.26	0.803	10.07

5.5.3 Microarray Data Analysis

Microarray experiments were performed to assess the differential expression of genes between LEC, MSC-L and MSC-BM. In this study two different samples of LEC, MSC-L and MSC-BM were labelled using (Cy3 and Cy5) dyes and competitive hybridization was performed as described in the methods section. The data has been deposited in NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with GEO series accession number (GSE16763). Analysis of the data considering a three fold difference, suggested differential expression of 3484 genes between LEC and MSC-L; 1579 genes between MSC-L and MSC-BM and 4837 between LEC and MSC-BM. KEGG pathway analysis was used to identify gene ontologies that preferentially expressed in the limbus and bone marrow. The differentially/highly expressed genes in LEC, MSC-L and MSC-BM were shown in table 5.10. The groups were segregated (Table 5.10) based on the fold expression towards one lineage as compared to others, i.e., genes that are highly representative of

a) LEC > 25 fold higher expression as compared to MSC-L and MSC-BM

b) MSC-L > 15 fold higher expression as compared to LEC and MSC-BM

c) MSC-BM > 20 fold higher expression as compared to LEC and MSC-L

d) MSC-L and MSC-BM > 20 fold higher expression in mesenchymal cells compared to LEC

e) Limbal explant culture derived cells – It shows the genes highly expressed in LEC and MSC-L compared to MSC-BM over the fold change of 10.

Table 5.10: The differential gene expression between LEC, MSC-L and MSC-BM, as measured by the fold change difference of the corresponding genes (Ribosomal genes excluded)

a. Genes differentially expressed in LEC (25 fold over expression in LEC compared MSC-L and MSC-BM)

Keratin 12, T-cell differentiation protein 2 (MAL2), Nebulette, v-myc myelocytomatosis viral related oncogene, ets homologous factor, calbindin 1, Kringle containing transmembrane protein 2, glucosaaminyl (N-acetyl) transferase 2, carcinoembryonic antigen-related cell adhesion molecule 6, Apolipoprotein B mRNA editing enzyme catalytic polypeptide –like 3a, tumor associated calcium signal transducer 1, sciellin, serine peptidase inhibitor kazal type 5, carcinoembryonic antigen-related cell adhesion molecule 1, interleukin 1 alpha, interleukin 18, dual adaptor of phosphotyrosine and 3-phosphoinositides (DAPP1), Transmembrane channel-like 5, claudin 1, defensin beta 1, WAP four-disulfide core domain 5, chemokine (C-X-C motif) ligand 1, dystonin, desmocollin 2, cadherin 1, transforming growth factor alpha, S100 calcium binding protein A8, Serpin peptidase inhibitor clade B member 5, visinin-like 1, interleukin 1 beta, desmoglein 3, matrix metallopeptidase 10, tumor protein p73-like (p63), homeoboxdomain-only protein (HOP), amphiregulin.

b. Genes differentially expressed in MSC-L (15 folds over expression in MSC-L compared to LEC and MSC-BM)

Vascular endothelial growth factor receptor 1, cadherin 6, forkhead box F1, glutamate receptor ionotrophic, collectin sub-family member 12,

SHC (Src homology 2 domain containing) transforming protein 3 (SHC3), AF052115, BC073929.

c. Genes differentially expressed in MSC-BM (20 folds over expression in MSC-BM compared to LEC and MSC-L)

growth differentiation factor 6 (GDF6), Urea transporter, erythrocyte (SLC14A1), neurotrophic tyrosine kinase, receptor, type 2 (NTRK2), early growth response 2 (Krox-20 homolog, Drosophila) (EGR2), secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1) (SPP1), myogenic factor 6 (herculin) (MYF6), collagen, type XI, alpha 1 (COL11A1), olfactomedin 4 (OLFM4), hepatitis A virus cellular receptor 2 (HAVCR2), homeo box A11, antisense (HOXA11S) on chromosome 7, homeobox C9 (HOXC9), HELAD1S mRNA for helicase, phosphodiesterase 1C, calmodulin-dependent 70kDa, opioid binding protein/cell adhesion molecule-like (OPCML), transcript variant 2, zinc finger, matrin type 4 (ZMAT4),

d. Genes differentially expressed in limbal explant culture derived cells (LEC and MSC-L) over bone marrow (10 fold difference)

semaphorin 3D (SEMA3D), matrix metallopeptidase 1 (interstitial collagenase) (MMP1), vitrin (VIT), Lysophosphatidic acid receptor Edg-7 (LPA receptor 3) (LPA-3), keratin 18 (KRT18), transcript variant 1, insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3), myelin basic protein (MBP), contactin 3 (plasmacytoma associated) (CNTN3),

e. Genes differentially expressed in MSC-L, MSC-BM over epithelial cells (over 20 fold difference)

nucleosome assembly protein 1-like 3 (NAP1L3), thymocyte selection-

associated high mobility group box (TOX), axin 2 (conductin, axil) (AXIN2), phosphodiesterase 11A (PDE11A), potassium voltage-gated channel, Isk-related family, member 4 (KCNE4), dermatan sulfate epimerase-like (DSEL), chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), G protein-coupled receptor 124 (GPR124), protocadherin 18 (PCDH18), hyaluronan and proteoglycan link protein 1 (HAPLN1), collagen, type V, alpha 2 (COL5A2), alpha-2-macroglobulin (A2M), decorin (DCN), cerebellar degeneration-related protein 1, 34kDa (CDR1), ependymin related protein 1 (zebrafish) (EPDR1), formin 2 (FMN2), Platelete derived growth factor receptor alpha, frizzled homolog 7, dapper, antagonist of beta-catenin, homolog 3, microfibrillar associated protein 5, lysyl oxidase, integrin, alpha 8, junctional adhesion molecule 2, protein kinase C, alpha, platelet-derived growth factor receptor, beta polypeptide.

The other highly expressed genes in LEC include the CD24 (48 and 155 folds over expression compared to MSC-BM and MSC-L respectively), FOXA1 (27 and 631 folds over expression compared to MSC-BM and MSC-L respectively), and KRT13 (11 and 15 folds), LAMA3 (7.4 and 7.3), ITGA6 (22 and 10) and CDH3 (6.0 and 7.4). To explore the interdependence of LEC and MSC-L, we looked at the growth factor and cytokine profile of these cells (Table 5.11). LEC showed high expression of growth factors like transforming growth factor alpha (TGF), Amphiregulin (AREG), epiregulin (EREG), hepatocyte binding epidermal growth factor (HB-EGF), growth factor receptor-

bound protein 14 (GRB14), (fibroblast growth factor 11 (FGF11) and cytokine like chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2. The MSC-L showed higher expression of growth factors like FGF7, FGF2 and cytokine CXCL12.

Table 5.11: Differential gene expression between LEC and MSC-L in growth factors and cytokine related genes

Gene	LEC vs MCL	LEC vs MSCBM	MCL vs MSCBM
amphiregulin (schwannoma-derived growth factor)	167.6 (Down)	131.25 (down)	3.74 (down)
transforming growth factor, alpha (TGFA)	57.21 (down)	28.49 (down)	
fibroblast growth factor binding protein 1 (FGFBP1)	38.75 (down)	23.011 (down)	
fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) (FLT1)	31.0 (up)		96.42 (down)
ALPHA PLATELET-DERIVED GROWTH FACTOR RECEPTOR	26.10	33.93 (up)	
platelet-derived growth factor receptor, beta polypeptide (PDGFRB)	20.52 (up)	21.90 (up)	
insulin-like growth factor binding protein 5 (IGFBP5)	17.65 (up)	14.81 (up)	

growth factor receptor-bound protein 14 (GRB14)	17.23 (down)	5.82 (down)	2.95 (down)
fibroblast growth factor 2 (basic) (FGF2)	13.78 (up)		
fibroblast growth factor 11 (FGF11)	12.83 (down)		
keratinocyte growth factor-like protein 1 (KGFLP1)	10.9 (up)		9.85 (up)
fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome) (FGFR1)	10.50 (up)	8.96 (up)	
insulin-like growth factor 2 (somatomedin A) (IGF2)	9.9 (up)	6.2 (up)	
connective tissue growth factor (CTGF)	9.77 (up)	15.45 (up)	2.3 (up)
pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1) (PTN)	8.54 (up)	9.9 (up)	
insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1)	8.10 (up)		
fibroblast growth factor 1 (acidic) (FGF1)	8.03 (up)	5.41 (up)	7.41 (down)
Insulin-like growth factor binding	7.65	14.2 (up)	2.96 (up)

protein 4 (IGFBP4)			
fibroblast growth factor 7 (keratinocyte growth factor) (FGF7)	7.51 (up)	15 (up)	4.51 (up)
platelet derived growth factor D (PDGFD)	5.97 (up)	12.7 (up)	
fibroblast growth factor binding protein 3 (FGFBP3)	5.68 (up)	4.4 (up)	
insulin-like growth factor binding protein 5 (IGFBP5)	5.61 (up)	5.4 (up)	2.95 (down)
fibroblast growth factor receptor 2 (fgfr2)	4.50 (down)		4.89 (up)
Hepatocyte growth factor precursor (Scatter factor) (SF) (Hepatopoeitin-A)	4.05	10.06	
transforming growth factor beta 1 induced transcript 1 (TGFB1I1), transcript variant 2	3.94 (up)	2.97 (up)	
nerve growth factor, beta polypeptide (NGFB)	3.83 (up)		2.6 (down)
chemokine (C-X-C motif) ligand 2 (CXCL2)	79.06 (down)	4.5 (down)	
chemokine (C-X-C motif) ligand 3	26.25 (down)	16.0 (down)	3.88 (up)

(CXCL3)			
chemokine (C-X-C motif) ligand 1 (CXCL1)	65.87 (down)	64.9 (down)	
chemokine (C-X-C motif) ligand 11 (CXCL11)	58.344 (down)	14.42 (down)	
chemokine (C-X-C motif) ligand 10 (CXCL10)	7.71 (down)	6.97 (down)	
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) (CXCL12)	34.26	82.68 (up)	
chemokine (C-C motif) ligand 26 (CCL26)	11.4 (up)	2.49 (up)	
chemokine (C-C motif) ligand 2 (CCL2)	23.43 (up)		3.48 (down)
chemokine (C-C motif) ligand 13 (CCL13)	12.42 (up)		2.34 (up)
interleukin 1, alpha (IL1A)	78.72 (down)	63.61 (down)	
interleukin 1, beta (IL1B)	48.57 (down)	28.89 (down)	
interleukin 1 receptor, type II (IL1R2)	12.17 (down)	21.03 (down)	3.92 (up)
interleukin 1 receptor antagonist (IL1RN)	20.06 (down)	15.10 (down)	
interleukin 20 receptor, alpha	210.69		

(IL20RA)	(down)		
interleukin 18 (interferon-gamma-inducing factor) (IL18)	78.26	56.72 (down)	
interleukin 23, alpha subunit p19 (IL23A)	28.40 (down)	29.29 (down)	
interleukin 11 receptor, alpha (IL11RA)	15.58 (up)		
neurotrophin 5 (NTF5)	14.96 (down)	11.64 (up)	
neurotrophin 3 (NTF3)	16.40 (up)	7.50 (up)	7.48 (down)
nerve growth factor, beta polypeptide (NGFB)	3.8 (up)		
Glial cell line-derived neurotrophic factor precursor (Astrocyte-derived trophic factor 1)	4.24 (up)	6.56 (up)	
GDNF family receptor alpha 1 (GFRA1)	6.5 (up)	6.28 (up)	
brain-derived neurotrophic factor (BDNF)	9.15 (up)	3.69 (up)	8.68 (down)

The analysis showed the differential gene expression between MSC-L and MSC-BM. Various gene ontology terms were picked up and analysed from the microarray data. The gene ontology terms were classified into groups like osteogenic, chondrogenic, myoblast, adipogenic, MHC-class II related, Homeobox genes, extracellular and other genes (Table 5.12).

Table 5.12: Comparisons of genes expressed in MSC-L and in MSC-BM cells for selected terms of gene ontology.

Gene Name	LEC vs MSC-L	LEC vs MSC-BM	MSC-L-MSC-BM
Osteogenesis			
Osteonectin	5.8	3.89	
Collagen, type I, alpha 2	10.7	10.15	
Connective tissue growth factor	9.7	15.45	
Collagen, type V alpha 2	16.7	22.2	
Osteopontin		3.8	29.4
Runt related transcription factor 2		5.9	5.5
PDZ and LIM domain 7, transcript variant 4	5.3	3.29	
Gremlin 2	16.1	14.29	
Myogenesis			
Transgelin	4.2	5.31	
Meltrin alpha	10	13.78	
Myosin light chain 9, transcript variant 2	4.7	4.87	
Synocoilin 1	8.2	5.7	
Tropomyosin1 (alpha), transcript variant 3	4.7	6.84	

Tropomyosin 2 (beta), transcript variant 2	3.5	3.75	
Caldesmon 1, transcript variant 1	13.8	17.7	
Desmuslin, transcript variant A	26.4	27.9	
Leiomodin 1	8.5	9.29	
Adipogenesis			
Leptin Receptor	4.94	60.58	26.74
Leptin		6.24	6.89
Serum amyloid A1, Transcript variant 1	6.8 (down)	4.2 (down)	7.7
CEBPA	4.8 (down)		3.23
Chondrogenesis			
Fibromodulin	10.1	13.82	
Decorin, transcript variant A1	27.6	41.45	
Cartilage oligomeric matrix protein		8.4	11.15
Tensin 1	14.9	10.31	
Hyaluronan and proteoglycan link protein	31	47.45	
Collaten, type XI, alpha 1		60.18	46.12
Chitanase 3-like 1		4.72	5.68

Extracellualr Matrix Components			
Microfibrillar associated protein 5	24	28.12	
Syndecan 2	12.1	16.7	
Matrix-remodelling associated 5	11.3	7.2	
Chondroitin sulfate proteoglycan 4	8.3	7.5	
Collagen, type VIII, alpha 1, transcript variant 1	8.6	5.39	
Others			
Procollagen-lysine 1,2-oxoglutarate 5-dioxygenease 1	3.2	3.3	
Low density lipoprotein-related protein 12	3.43	3.3	
Notch homolog 2	3.78	5.2	
Collagen, type VI alpha 1	3.9	2.39	
Cysteine-rich, angiogenic inducer, 61	4	4.8	
Glial cell line-derived neurotrophic factor precursor	4.2	6.5	
Endoglin	4.29	4.17	
Collagen, type 1, alpha 2	4.3`	3.3	
Leukemia inhibitory factor receptor alpha (LIFR)	4.46	5.9	

Neuropilin 1	4.6	4.1	
Colony stimulating factor 1 (Macrophage)	5.6	15.09	
Wingless-type MMTV integration site family, member 5B	5.7	6.06	
Neuronal growth regulator 1	6.13	8.06	
Noggin	6.2	3.7	
Matrix metallopeptidase 2	6.27	6.2	
Collagen, type VI, alpha 3, transcript variant 4	6.27	8.4	
Neuronal PAS domain protein 1	6.38	5.7	
Fibroblast growth factor receptor 1	6.45	8.9	
Neuropilin 1, transcript variant 1	6.7	8.3	
Collagen, type VI, alpha 2	7.4	6.2	
Collagen, type VI, alpha 1	8.0	10.2	
Bone marrow stromal cell antigen	8.2	8.4	
Collagen, type V, alpha 1	8.4	9.3	
Fibronectin 1, transcript variant 7	9.24	10.14	
Fibroblast growth factor receptor 1, transcript variant 1	10.5	7.6	
Collagen, type 1 alpha 2	10.7	10.15	
Angiopoietin 1	14.8	17.1	
Neuronal cadherin	8.4	13.5	

5.5.4 Validation of Microarray:

The gene expression patterns obtained by the Real Time PCR and Semi quantity RT-PCR were in good agreement with that from the microarray analysis, indicating high fidelity in microarray data and analytical methods.

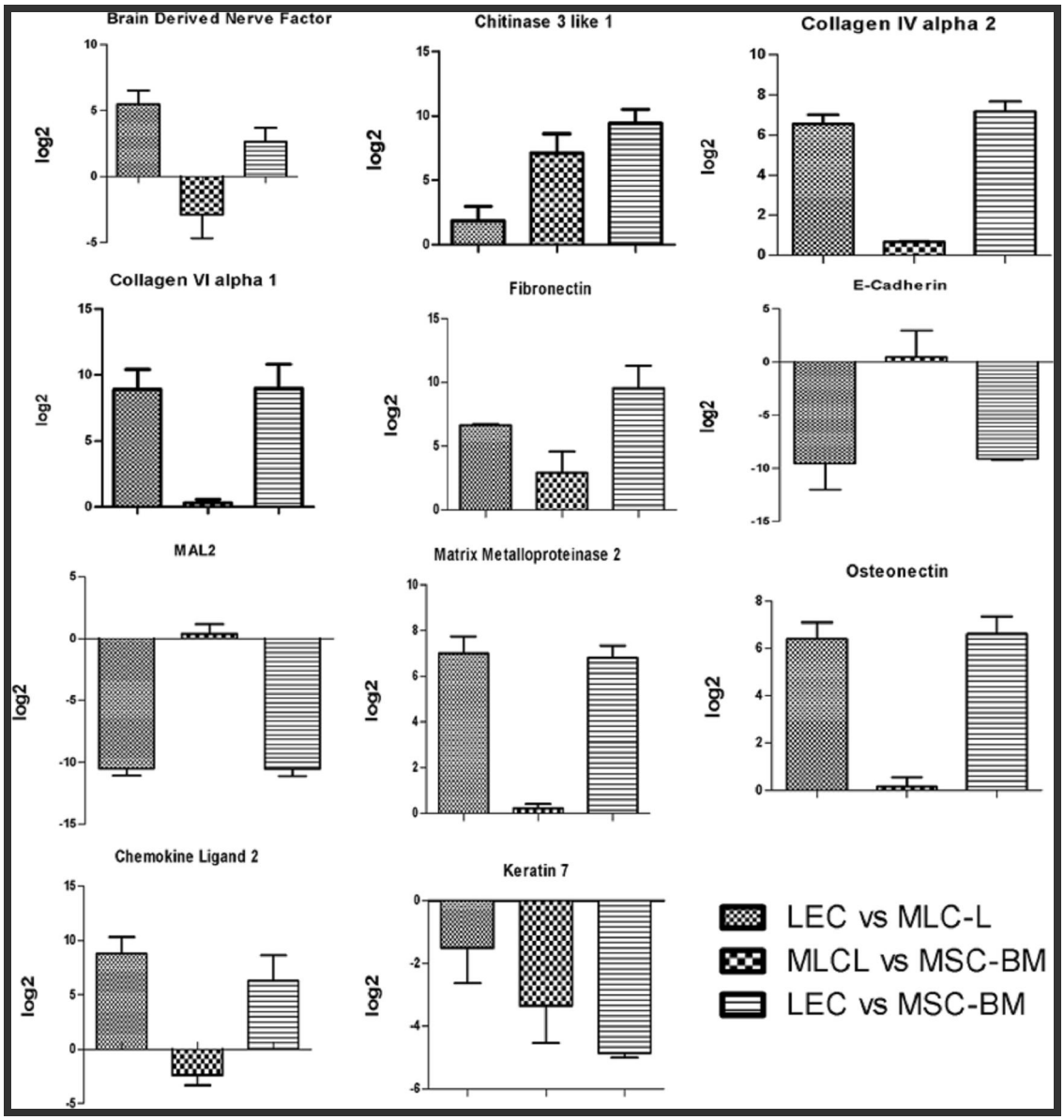

Figure 5.3: Validation of microarray data by Real time RT-PCR. The individual gene-specific values thus calculated were averaged to mean ±standard deviation and fold change was expressed as log 2 ratios (y-axis).

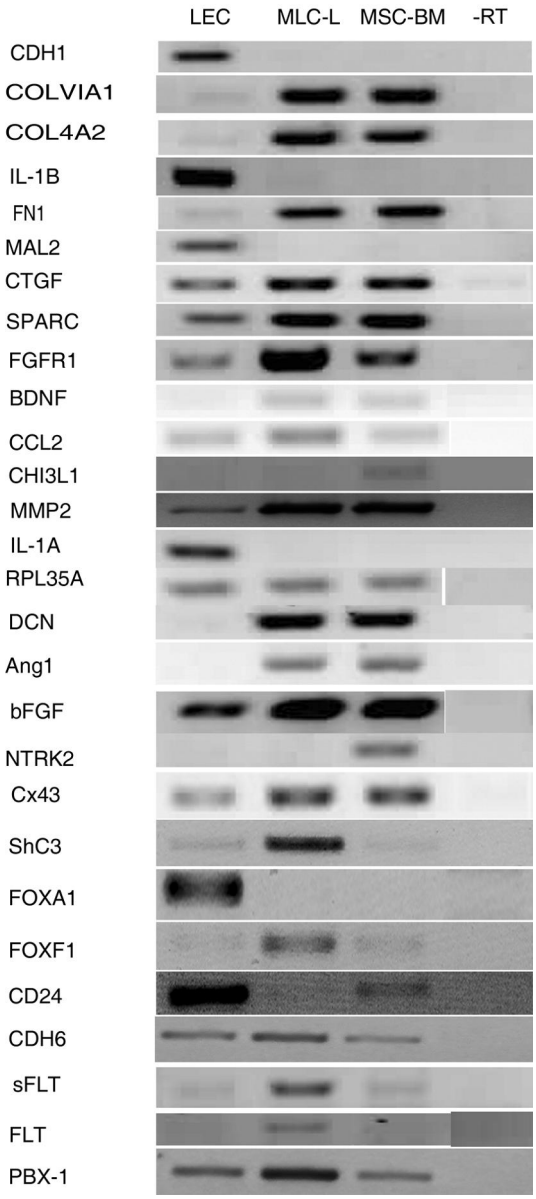

Figure 5.4: Validation of microarray data by semiquantitative RT-PCR. Reverse transcription polymerase chain reaction analysis of the selected differentially expressed genes. Ribosomal protein large 35 (RPL35) was used as an internal control. Abbreviations: LEC – limbal epithelial cells, MSC-L – mesenchymal cells of limbus, MSC-BM – mesenchymal stem cells of bone marrow, -RT – no reverse trasncriptase, CDH1 – cadherin 1 (E-cadherin), COLVIA1 – collagen 6 alpha 1, COL4A2 – collagen 4 alpha 2, IL-1B – interleukin 1 beta, FN1 – fibronectin 1, MAL2 - T-cell differentiated antigen 2, CTGF – Connective tissue growth factor, FGFR1 – fibroblast growth factor receptor 1, BDNF – brain derived nerve growth factor, CCL2 – chemokine ligand 2, CHI3L1 – chitanase 3 like 1, MMP2 – matrix metallo peptidase 2, IL1A – interleukin 1 alpha, KRT7- cytokeratin 7, DCN – decorin, Ang 1 – angiopoientin, bFGF – basic fibroblast growth factor, NTRK2 – neurotrophin tyrosine kinase receptor 2.

5.6 DISCUSSION

Limbal stem cell deficiency has been a challenging clinical problem, the current treatment of which involves replenishing the depleted limbal stem cell pool by either limbal tissue transplantation or use of cultivated limbal epithelial sheets (Pelligrini *et al*., 1997, Tsai *et al*., 2000, Sangwan *et al*., 2004, 2006). As described in the chapter 4, establishment of a feeder cell free method of cultivating the limbal explant tissues on denuded human amniotic membrane. Our results show that limbal explant culture derived MSC-L when expanded exhibit a spindle shaped, fibroblast-like appearance similar to that of MSC-BM (Polisetty *et al*., 2008). Though we had no logical explanation for this in the beginning, the revelation of presence of spindle cells prompts us to postulate that these spindle cells in the explant culture system function like "intrinsic feeder cells". Isolated MSC-L can be distinguished from epithelial cells (lack of expression of KRT3/12, KRT14), fibroblasts (lack of expression of HLA-DR), haematopoietic stem cells (lack of expression of CD34, CD45, CD11b, CD10, CD40, CD40L and CD138), because they are adherent to the surface of tissue culture flasks and express different cell-surface markers (CD90, CD13, CD105 and CD44).

Genes that show differential expression in the LEC when compared to MSC-L and MSC-BM, encode proteins that stabilize epithelial sheets and promote or regulate cell to cell interaction and cell to matrix interaction including keratins (Keratin 13, Keratin 12), laminins (LAMA3, LAMB3), cadherins (CDH3 and CDH1), nebulette, epiregulin, calbindin 1 28 Kda, desmosomal components (DSG3, DSC2), matrix metallo peptidase 10, Serine

peptidase inhibitor clade B5, carcinoembryonic antigen-related cell adhesion molecule 1. In addition, LEC showed high expression of known basal markers (TP73L, ITGA6, epiregulin and HOP) and differentiated epithelial markers (CDH1, KRT12) (Takacs *et al*., 2009). Immunocytochemical analysis showed the expression of limbal epithelial stem cell markers ABCG2, vimentin, KRT14, KRT19 and also expressed the differentiated epithelial markers CDH1, KRT3/12 on cultivated LEC. This further supports the fact that cultivated LEC cells on dhAM in a feeder cell- free culture technique, contain a distinct population of stem cells and differentiated cells which serve to replenish the depleted limbal stem cells when transplanted to the diseased eye (Sangwan *et al*., 2004, 2006, Vemuganti *et al*., 2004). The other highly expressed transcripts in the limbal epithelial cell cultures include the CD24, a surface molecule that has been used to identify different types of human stem cells (Sagrinati *et al*., 2006), OTX1 a transcription factor, is expressed in the presumptive ciliary body and iris that has been shown to be essential for development of these tissues (Zhang *et al*., 2007) and FOXA1 (endodermal stem cell marker) (Conigliaro *et al*., 2008).

Cytokines and the interaction of cells with extracellualr matrix components have been suggested to play an important role in niche regulation. Cytokine and growth factor signalling is an important determinant of the functional state of these cells and of the relationship between LEC and MSC-L (Li *et al*., 1995). The most important growth factors for normal human keratinocyte proliferation are member of EGF family, including TGFA, HB-EGF, ER, and AR and these act in an autocrine manner (Shirakata *et al*., 2000). Our data also reveals the high expression of these four EGF members

in LEC. The fibroblast growth factors, FGF1 and FGF2, are well-characterized growth factors known for their mitogenic effect on a number of cells derived from neuroectodermal or mesodermal origins. FGF1 and FGF2 were mitogenic to corneal and limbal epithelium (Wilson *et al*., 2001) and keratinocyte growth factor (FGF7), epithelium specific growth factor, has been found to be mitogen for several epithelial cells including limbal epithelial cells (Li *et al*., 1995) are highly expressed in MSC-L. Interestingly and as expected their corresponding receptors FGFR1 are expressed in MSC-L, FGFR2 in LEC. The proinflammatory forms of IL-1 (IL1A and IL1B) expression in LEC play significant roles in ocular surface immune and inflammatory responses and wound healing (Wilson *et al*., 2001). The highly expressed chemokine in LEC include CXCL1, 2, 3, 10, 11 and in MSC-L include CXCL12, CCL26, CCL2 and CCL13. The intense expression of chemokine ligand CXCL12 (Stromal cell derived factor 1) in MSC-L is similar to the study by Tristan and coworkers (Bourcier *et al*., 2003). This factor might exert physiological effects on the cornea and could be involved in pathological conditions such as corneal angiogenesis (Bourcier et al., 2003). The neurotrophic factors have been reported to play important roles in maintaining stem cells in the limbus (Qi *et al*., 2007). We also noted a high expression of neurotrophic factors like neurotrophin 3, nerve growth factor and brain derived growth factor in MSC-L while neurotrophin 5 was highly expressed in LEC. Glial derived neurotrophic factor (GDNF) and its receptor GDNF receptor alpha 1 were highly expressed in the MSC-L and MSC-BM similar to the observations made by Qi *et al* in limbal cells (Qi *et al*., 2007). All these features support our hypothesis that

the limbal epithelial cells and stromal cells play a complementary role not only *in vivo* but also *in-vitro* in the explants culture system.

Genes highly expressed in MSC-L include CDH6, vascular endothelial growth factor receptor 1 (VEGFR1 or FLT1) glutamate receptor ionotrophic (GRIA3), collectin subfamily member 12, transcription factor forkhead box F1 (Foxf1), Src homology 2 domoain contataining transforming 3 (SHC3), oxytocin receptor and unknown genes AFO52115 and BCO73929. These genes with such higher expression (>15 fold) can be considered as the markers of mesenchymal like cells of limbus. The FOXF1 is a transcription factor, expressed in mesenchymal cells of the lung, liver, and gall bladder and is shown to be involved in mesenchymal cell migration without changes in cell proliferation and cell survival (Malin *et al*., 2007).

Interesting observation is the high expression of receptors neurophilin 1, platelete derived growth factor receptor alpha, and leprecan-like 2 in MSC-L, which is similar to MSC-BM. (Kim *et al*., 2006). This study supports the characteristics of mesenchymal cells that were previously identified in MSCs, such as vimentin, fibronectin, Collagen Type I and III, collagen type VI, light chain of myosin 9 and matrix metallopeptidase 2 (Nicola et al., 2001, Silva et al., 2003, Klein G et al., 1995, Kim et al., 2006). The genes which show similar gene expression in MSC-L and MSC-BM are those which are related to extracellular components, cell adhesion molecules (microfibrillar associated protein 5, syndecan 2, matrix-remodelling associated 5, chondroitin sulfate proteoglycan 4, collagen 8 alpha 1) and the genes related to osteoblasts (Osteonectin, Collagen type 1, Connective tissue growth factor, OB-Cadherin), chondrocytes (fibromodulin, decorin, tensin 1, hyaluronan and

proteoglycan link protein) and myoblasts (transgelin, sarcoglycan epsilon, caldesmon 1, leimodin, meltrin alpha) (Table 4). The MSC-L also expressed the products showing characteristics of hematopoiesis-supporting stroma, including fibulin-1 and fibulin 2, collagen type VI and stromal cell-derived factor, in the same level as MSC-BM thus supporting our hypothesis that these cells possibly act as intrinsic feeder cells in explant culture system. Nevertheless, some differences were observed between expression profiles of MSC-L and MSC-BM. Among the genes that were expressed at higher levels by MSC-BM are growth differentiation factor 6, neurotrophic tyrosine kinase receptor 2, urea transporter erythrocyte, myogenic factor 6. Other genes highly expressed at higher levels in MSC-BM include chondrogenesis related genes (COMP, COL11A1, CHI3L1), osteogenic related genes (RUNT2, SPP1) and adipogenic related genes (CEBPA, LEP, SAA1). The MSC-BM are more committed to the osteoblastic, chondrogenic and adipocytic lineages. This suggests that in addition to some common signatures of niche supporting cells, mesenchymal cells from different sources possibly carry tissue specific signatures, which reflect their tissue of origin.

In summary, this study highlights the gene expression profile of limbal epithelial stem cells, mesenchymal like cells from limbal stroma in the ex-vivo expanded feeder cell free limbal explant tissue culture system. Their lineage specific signatures, evidence of interdependent pathways with limbal epithelial cells, striking resemblance to the signatures of bone marrow derived mesenchymal cells support our hypothesis that the limbal stromal cells are similar to bone marrow derived mesenchymal cells and could possibly be an important component of limbal niche.

SUMMARY

The field of stem cell biology is gaining a lot of importance in therapeutics and the role of these cells in regenerative medicine is being explored in a number of clinical trails worldwide. Various source of cells that are being evaluated in clinical trails include embryonic, fetal, umbilical cord and adult stem cells. With the belief that adult autologous cells have a better acceptance in clinical trials, we explored the potential of bone marrow derived stromal cells (MSC-BM) to transdifferentiate into neuronal lineage. Our initial aim was to establish and characterize the MSC-BM of rat and human origin and explore their stemness and plasticity. Around the same time, our lab has reported a new observation of finding stromal cells in limbal cultures, which showed features similar to MSC-BM. So I pursued the objective of comparing the phenotype of these mesenchymal cells by various techniques was added.

In this thesis we have addressed different aspects of mesenchymal stem cells from bone marrow and limbus, which include:

1. **Rat Bone Marrow Stromal Cells** - Isolation, characterization and differentiation potential
2. **Human Bone Marrow Stromal Cells** – Isolation, characterization and differentiation potential
3. **Limbal Stromal Cells** – Isolation, characterization, *in-situ* localization and differentiation potential
4. **Gene Expression Profile** – Gene expression profile of limbal explant culture derived cells in comparison to MSC-BM.

1. Rat Bone Marrow Stromal Cells

Bone marrow is a complex tissue containing stem cells with hematopoietic properties. The hematopoietic stem cells, which are the primary source of blood cells in the adult body, are regulated within a microenvironment of stromal cells in the bone marrow (Colter DC *et al*., 2001). The stromal cells exert their effects on the hematopoietic cells through direct cell-cell interactions as well as by the release of soluble factors (Yanai N *et al*., 1994). Stromal cells isolated from bone marrow are heterogeneous and fibroblastic in appearance (Prockop *et al*., 1997) and were initially named plastic-adherent cells or colony-forming-unit fibroblasts and subsequently referred to as either marrow stromal cells or mesenchymal stem cells (MSC-BM), due to their potency to differentiate into various connective tissue lineages including adipocytes, osteoblasts, chondrocytes or myoblast (Pittenger MF *et al*., 1999; Jiang *et al*., 2002). MSC-BM have been isolated from a variety of species, including mouse (Peister A *et al*., 2004), rat (Javazon EH *et al*., 2001), rabbit (Johnstone B *et al*., 1998) and human subjects (Colter DC *et al*., 2001). Although MSC-BM from different species have similar characteristics in part, some data suggest that variations occur among species. MSC-BM from human bone marrow are relatively easy to harvest and to expand in culture (Sekiya *et al*., 2002a), whereas rodent MSC-BM have proven more difficult (Friedestein AJ *et al*., 1974; Simmons DJ *et al*., 1991), although this is not without controversy (Javazon EH *et al*., 2001). The technical difficulties in preparing MSC-BM from rodent bone marrow have limited the number of experiments, because animal transplantation models are required for preclinical

studies. The selection of suitable cell populations is apparently crucial for the outcome of *in vivo* experiments with MSC-BM. Although there are many methods to isolate MSC-BM, no optimal method is available.

In our study we have isolated and established bone marrow stromal cell MSC-BM by the simple and reliable method of combining density gradient centrifugation with plastic adherence. The stromal cells were characterized and differentiated into adipocytes, osteocytes and neuronal like cells.

Salient findings:

- Ø The rat bone marrow mononuclear cells isolated by ficoll density gradient centrifugation expressed the hematopoietic markers CD45, CD11a, CD18, and CD31.
- Ø Pure populations of MSC-BM were obtained by combining method of density gradient and plastic adherence compared to solo density gradient centrifugation and plastic adherence.
- Ø The adherent MSC-BM showed spindle shaped morphology and formed colonies. On immunostaining the MSC-BM expressed CD90, fibronectin and vimentin and were negative for hematopoietic (CD45, CD11a, CD18) and endothelial markers (CD31).
- Ø The RT-PCR results show the expression of vimentin and collagen type 1 alpha 1 transcripts in isolated MSC-BM. Thus proving that the isolated cells are MSC-BM with little or no contamination from hematopoietic cells.
- Ø When induced with adipogenic induction medium, these cells showed lipid globules on staining with Oil red ‘O’. On osteogenic induction for 2-3

weeks, these cells showed calcium deposits when stained with alizarin red. Similarly, when induced with neuronal induction medium, the cells appeared bi and multipolar cells resembling neurons as observed under phase contrast microscope. These cells were positive for nestin, synaptophysin, and neurofilament-L.

2. Human Bone Marrow Stromal cells

Among the adult stem cells, MSC-BM are being explored extensively in the hope that they will lead the way to autologous stem cell-based replacement therapies as well as in treating graft versus host disease (Dezawa M *et al.*, 2004). For this reason, the MSC is one of the most extensively studied types of adult stem cell with respect to transdifferentiation potential (Pittenger MF *et al.*, 1999). Of all the lineages, the particular interest is neural differentiation as it holds promise for developing therapeutics for neurodegenerative diseases (Sugaya K, 2003). However, due to the lack of universally defined cell surface markers to characterize the MSC-BM (Baksh D *et al.*, 2004), it remains enigmatic with regard to both its identity and qualification as a true stem cell (Javazon EH *et al.*, 2004).

A number of different approaches have been reported to trigger this apparent transdifferentiation *in vitro*. Some groups have used chemical treatments such as DMSO, (Suzuki H *et al*., 2004) whilst others have opted for the use of growth factors (Hermann A *et al.*, 2004). However, it appears that early positive results obtained using DMSO-based protocols are unreliable, as the neural-like morphology and gene expression displayed by MSCs after

treatment were in fact due to toxicity (Lu P *et al*., 2004). Regardless of the ongoing debate about the nature of this differentiation and the possibility of artifacts, MSC transdifferentiation has been widely used and reported (Wislet-Gendebien S *et al*., 2005, Hermann A *et al*., 2004).

In this study, we attempted to establish cultures of human MSC-BM and evaluate their phenotype using surface markers over time. We evaluated the potential of these cells to differentiate to mesenchymal and non-mesenchymal cell lineages, i.e., their potential for neural differentiation.

Salient findings:

- Ø The human bone marrow mononuclear cells showed high nucleus to cytoplasmic ratio and showed expression of CD34, CD45 and HLA-BC.
- Ø The MSC-BM showed spindle shaped morphology and the colony forming efficiency was 30%.
- Ø The established MSC-BM expressed CD90, CD29, CD105, CD106, CD71 and vimentin and negative for CD31, CD11a, CD11c, and CD45.
- Ø With increasing passages, the MSC-BM tend to lost the expression of CD106 and CD71 and differentiate into more fibroblastic phenotype.
- Ø When induced by adipogenic induction medium, these cells showed fat globules in the cytoplasm on staining with Oil red 'O'. On osteogenic induction for 2-3 weeks, these cells showed calcium deposits when stained with alizarin red. Similarly, when induced by neuronal induction medium, the cells expressed gap-43, tau-1 and neurofilament-L.

- Ø Microarray experiments show that after neural differentiation, the genes related to synaptic differentiation, neuronal channel/transporter, neuronal development and other neural related genes were upregulated. The down-regulated genes were chondrogenic, myogenic, cytoskeleton and other mesodermal related genes.

3. Limbal Stromal Cells or Mesenchymal Stem Cells of Limbus (MSC-L)

The limbus of the eye, located at the junction of the cornea and conjunctiva of the ocular surface is now extensively used for ocular surface reconstruction in patients with limbal stem cell deficiency (LSCD) (Pellegrini G *et al*., 1997; Fatima A *et al*., 2006; Sangwan VS *et al*., 2006). It is now established that the progenitor cells that regenerate corneal epithelium reside in the limbus (Schermer A *et al*., 1986; Tseng SC *et al*., 1989). Damage to or dysfunction of the limbal stem cell population due to different inherited or acquired conditions results in limbal stem cell deficiency, which has severe consequences for ocular surface integrity and visual function and may lead to functional blindness. One successfully used therapeutic strategy for ocular surface reconstruction is the transplantation of autologous epithelial cell sheets engineered from limbal epithelial cells expanded in vitro using appropriate delivery systems such as amniotic membrane or fibrin gels. L.V. Prasad Eye Institute is the leading institute for transplantation cases for limbal stem cells deficiency by using feeder cell free expanded limbal epithelial cells. Interestingly, while culturing the limbal epithelial cells after 4-5 weeks observed the spindle cell out growth. The next obvious questions raised were regarding the origin of these cells-limbal epithelium or stroma and the role of

these cells. There have been some reports in literature, which suggest that limbal fibroblast-like cells from adult corneo-limbal tissue may have stem cell like properties (Dravida S *et al*., 2005), and also their conditioned media has the ability to convert human embryonic stem cells and hair follicle bulge cells into corneal epithelial-like cells. To understand the role of these cells, they were expanded and characterized for various markers and compared with other mesenchymal cells of bone marrow.

Salient findings:

- Ø From late limbal explant cultures spindle shaped cells were isolated and cultured based on their adhesion property. These cells proliferate and gradually grew to form small colonies.
- Ø The spindle shaped cells were positive for CD90, CD29, CD44, CD105, CD71, CD166, CD54 and vimentin and negative for CD34, CD45, CD31, CD11a, CD11c and epithelial markers K3 and K14.
- Ø MSC-L expressed collagen type 1 alpha 1, vimentin, nestin, ostetoblast cadherin and were negative for PAX-6, S100A2 similar to MSC-BM.
- Ø On adipocytic induction the cells showed fat globules in the cytoplast of cells when stained with Oil red ‘O’. Similarly, these cells when induced with oesteogenic induction medium for 2-3 weeks, showed calcium deposits when stained with alizarin red.

4**) Gene Expression Analysis:**

Gene expression profiling is an emerging technique of identifying stem cells. This technique has contributed to the understanding several cellular pathways and

intrinsic factors that characterize LSC in normal human corneas (Adachi *et al*., 2006, Jun *et al*., 2001). In these studies, the entire cornea was used as the starting material, whereas Zhou and coworkers have shown the gene expression profiles of stem cell-enriched limbal basal cell population in mice (Zhou *et al*., 2006). In the present study, we evaluated the transcriptome of the limbal explant culture derived epithelial (LEC) and MSC-L by microarray and identified expression of unique genes and biological pathways that characterize both these cell types. To evaluate our hypothesis that the MSC-L possibly act as one of the "niche" derived intrinsic feeder cells, we compared the profile of these cells to that of the MSC-BM, which form the supporting niche for the hematopoietic system.

Salient findings

- Ø Gene expression analysis revealed the genes highly representative of LEC, MSC-L and MSC-BM respectively.
- Ø The data also revealed that cytokine networking (growth factor profile) exists between the limbal epithelial and stromal cells.
- Ø It reveals the similar and differential gene expression between MSC-L and MSC-BM.

LIMITATIONS

The work done as a part of this thesis was productive in terms of research outcome and clinical application, but like all other works, it has some limitations, especially owing to the fact of being cell biology related work where objective quantification and characterization of the product can be a problem and therefore has many biological plausibilities. Added to this is a lack on consensus in the markers that characterize adult stem cells. These include,

- The method of isolation used for isolating bMMNCs is not qualitative. In spite of using a simple method of isolating and culturing BMSCs from BMMNCs, the chance of variability in BMMNC yield, percentage of BMSCs, contamination of committed progenitors from sample to sample during marrow processing always exists. This can be overcome by using sorting cells by FACS and MACS to get an enriched population of stem cells.
- Functional evaluation of neural and photoreceptor differentiated BMSCs by electrophysiological studies and biochemical assays *in vitro* and photoreceptor functionality *in vivo* post transplantation of cells by ERG would certainly have added weightage and strengthened our findings.
- While studying the gene expression studies, limbal epithelial cells used in this study are heterogeneous population containing minimal contamination of stromal cells. This can be overcome by using sorting cells by FACS and MACS to get an enriched population of stem cells.

190.

REFERENCES

1. Adachi W, Ulanovsky H, Li Y *et al*. *Invest Ophthalmol Vis Sci*. 2006;47(9):3801-10
2. Ahmad S, Stewart R, Yung S *et al*. *Stem Cells*. 2007;25(5):1145-1155.
3. Akiyama Y, Radtke C, Kocsis JD. *J Neurosci*. 2002;22:6623-6630.
4. Alexanian AR. *Exp Cell Res*. 2005;310(2):383-91.
5. Alonso L, Fuchs E. *Proc Natl Acad Sci U S A*. 2003;100.
6. Ambati BK, Nozaki M, Singh N *et al*. *Nature*. 2006;443(7114):993-7.
7. Ambati BK, Patterson E, Jani P *et al*. *Br J Ophthalmol*. 2007;91(4):505-8.
8. Amel Touhami, Martin Grueterich, Scheffer C. G. Tseng. *Invest Ophthalmol Vis Sci*. 2002;43:987–994
9. Anderson KL, Perkin H, Surh CD *et al. J Immunol*. 2000;164(4):1855-61.
10. Hermann A, Stefan Liebau, Regina Gastl *et al*. *Journal of Neuroscience Research.* 2006;83:1502-1504
11. Angoulvant D, Clerc A, Benchalal S *et al*. *Biorheology.* 2004;41:469-476.
12. Aubin JE. *J Cell Biochem* 1999;72:396-410
13. Awad HA, Butler DL, Boivin GP *et al*. *Tissue Eng*. 1999;5(3):267-77
14. Azizi SA, Stokes D, Augelli BJ *et al*. *Proc Natl Acad Sci USA*. 1998;95:3908.
15. Baddoo M, Hill K, Wilkinson R *et al*. *J Cell Biochem*. 2003;89(6):1235-49.
16. Badiavas EV, Falanga V. *Arch Dermatol*. 2003;139:510-516.
17. Baksh D, Song L, Tuan RS. *J Cell Mol Med.* 2004;8(3):301-16
18. Banfi A, Muraglia A, Dozin B *et al*. *Exp Hematol*. 2000;28(6): 707-15
19. Barbaro V, Testa A, Di Iorio E *et al*. *J Cell Biol*. 2007;177(6):1037-49. Epub 2007.

20. Barrandon Y, Green H. *Proc Natl Acad Sci U S A*. 1987 Apr;84(8):2302-6.
21. Beauchamp JR, Heslop L, Yu DS *et al*. *J Cell Biol*. 2000;151(6):1221-34.
22. Bernardo ME, Avanzini MA, Perotti C *et al*. *J Cell Physiol* 2006.
23. Bickenbach JR, McCutecheon J, Mackenzie IC. *Cell Tissue Kinet*. 1986;19(3):325-33.
24. Blanpain C, Lowry WE, Geoghegan A *et al*. *Cell.* 2004;118(5):635-48.
25. Boiret N, Rapatel C, Veyrat-Masson R *et al*. *Exp Hematol*. 2005;33:219-225.
26. Bonner-Weir S. *Endocrinology*. 2000;141(6):1926-9. Review.
27. Bosnakovski D, Mizuno M, Kim G *et al*. *Cell Tissue Res*. 2005;319(2):243-53. Epub 2004 Nov 20.
28. Bossolasco P, Cova L, Calzarossa C *et al*. *Exp Neurol*. 2005;193(2):312-25
29. Bourcier T, Berbar T, Paquet S *et al*. *Mol Vis*. 2003;9:96-102
30. Brazelton TR, Rossi FM, Keshet GI *et al*. Science. 2000;290(5497):1775-9
31. Brazzell RK, Stern ME, Aquavella JV *et al*. *Invest Ophthalmol Vis Sci*. 1991;32(2):336-40.
32. Bruder SP, Kurth AA, Shea M *et al*. *J Orthop Res*. 1998;16(2):155-62
33. Bruder SP, Ricalton NS, Boynton RE *et al*. *J Bone Miner Res* 1998;13:655-63.
34. Bürkle A, Brabeck C, Diefenbach J *et al*. *Int J Biochem Cell Biol*. 2005;37(5):1043-53.
35. Cadigan KM, Nusse R. *Genes Dev*. 1997;11(24):3286-305. Review.

36. Campagnoli C, Roberts IA, Kumar S *et al*. *Blood*. 2001;98(8):2396-402.

37. Caplan AI, Elyaderani M, Mochizuki Y *et al*. *Clin Orthop Relat Res*. 1997;342:254-569.

38. Chaloin-Dufau C, Sun TT, Dhouailly D. *Cell Differ Dev*. 1990;32(2):97-108.

39. Chen SL, Fang WW, Ye F *et al*. *Am J Cardiol* 2004;94:92-5.

40. Chen Z, de Paiva CS, Luo L *et al*. *Stem Cells*. 2004;22(3):355-66.

41. Chen ZZ, Van Bockstaele DR, Buyssens N *et al*. *Leukemia* 1991;5(9):772-81.

42. Chopp M, Li Y. *Lancet Neurol*. 2002;1:92-100.

43. Chopp M, Zhang XH, Li Y *et al*. NeuroReport. 2000;11:3001-3005.

44. Chunmeng S, Tianmin C. *Med Hypotheses*. 2004;62(5):683-8.

45. Collins AT, Habib FK, Maitland NJ *et al*. *J Cell Sci*. 2001;114(Pt 21):3865-72.

46. Colter DC, Class R, DiGirolamo CM *et al*. *Proc Natl Acad Sci USA* 2000;97:3213-8.

47. Colter DC, Sekiya I, Prockop DJ. *Proc Natl Acad Sci USA* 2001;98:7841-7845

48. Conigliaro A, Colletti M, Cicchini C *et al*. *Cell Death Differ*. 2008;15(1):123-33.

49. Conlon I, Raff M. *Cell*. 1999;96(2):235-44.

50. Cotsarelis G, Cheng SZ, Dong G *et al*. *Cell*. 1989;57(2):201-9.

51. Coulombre JL, Coulombre AJ. *Dev Biol*. 1971;25(3):464-78.

52. Covas DT, Siufi JL, Silva AR *et al*. *Barz J Med Biol Res* 2003; 36:1179-83.

53. d'Adda di Fagagna F, Hande MP, Tong WM *et al*. *Nat Genet*. 1999;23(1):76-80.

54. Daniels JT, Dart JK, Tuft SJ *et al*. *Wound Repair Regen*. 2001;9(6):483-94. Review.

55. Davanger M, Evensen A. *Nature*. 1971;229(5286):560-1.

56. Dazzi F, Ramasamy R, Glennie S *et al*. *Blood Rev*. 2006;20(3):161-71.

57. De Bari C, Dell'Accio F, Vandenabeele F *et al*. *J Cell Biol*. 2003;160:909-918.

58. De Bari C, Dell'Accio F, Tylzanowski P *et al*. *Arthritis Rheum*. 2001;44(8):1928-42.

59. De Paiva CS, Pflugfelder SC, Li DQ. *Stem Cells*. 2006;24:368-375.

60. Deans RJ, Moseley AB. *Exp Hematol*. 2000;28(8):875-884.

61. Delplanque A, Coraux C, Tirouvanziam R *et al*. *J Cell Sci*. 2000;113 (Pt 5):767-78.

62. Deng W, Obrocka M, Fischer I Prockop DJ. *Biochem Biophys Res Commun*. 2001;282:148-152.

63. Dennis JE, Carbillet JP, Caplan AI *et al*. *Cells Tissues Organs*. 2002;170:73-82.

64. Devine SM, Bartholomew AM, Mahmud N *et al*. *Exp Hematol* 2001;29:244-55.

65. Devine SM. *J Cell Biochem Suppl*. 2002;38:73-9

66. Dexter T.N. Testa NG, Allen TD *et al*. *Blood* 1981;58:699-709

67. Dexter TM. *Leukemia*. 1989; 3:469-474.

68. Dezawa M, Kanno H, Hoshino M *et al*. *J Clin Invest*. 2004;113(12):1701-10

69. Dezawa M, Takahashi I, Esaki M *et al*. *Eur J Neurosci* 2001;14:1771–1776

70. Di Iorio E, Barbaro V, Ruzza A *et al*. *Proc Natl Acad Sci U S A*. 2005;102(27):9523-8. Epub 2005 Jun 27.

71. Di Nicola M, Carlo-Stella C, Magni M *et al*. *Blood*. 2002;99:3838-3843.

72. Diehn JJ, Diehn M, Marmor MF *et al*. *Genome Biol*. 2005;6(9):R74.

73. Digirolamo CM, Stokes D, Colter D *et al*. *Br J Haematol*. 1999;107(2):275-81

74. Dittel BN, McCarthy JB, Wayner EA *et al*. *Blood* 1993;81(9):2272-2282.

75. Djouad F, Plence P, Bony C *et al*. *Blood*. 2003;102:3837-3844.

76. Dravida S, Pal R, Khanna A *et al*. *Brain Res Dev Brain Res*. 2005;160(2):239-51.

77. Du Y, Funderburgh ML, Mann MM *et al*. *Stem Cells*. 2005;23(9):1266-75

78. Du Y, Sundarraj N, Funderburgh ML, *et al*. *Invest Ophthalmol Vis Sci*. 2007;48(11):5038-45.

79. Ebato B, Friend J, Thoft RA. *Invest Ophthalmol Vis Sci*. 1987;28(9):1450-6.

80. Erices A, Conget P, Minguell JJ. *Br J Haematol*. 2000;109(1):235-42.

81. Espana EM, Grueterich M, Romano AC *et al*. *Ophthalmology*. 2002;109(11):2004-10.

82. Espana EM, Ti SE, Grueterich M *et al*. *Br J Ophthalmol*. 2003;87(12):1509-14.

83. Fatima A, Sangwan VS, Iftekhar G *et al*. *J Postgrad Med*. 2006; 52(4): 257-61.

84. Fernandez M, Simon V, Herrera G *et al*. *Bone Marrow Transplant*. 1997;20:265-271.

85. Ferrari G, Cusella-De Angelis G, Coletta M *et al*. *Science.* 1998;279(5356):1528-30.

86. Ferraris C, Chevalier G, Favier B *et al*. *Development*. 2000;127(24):5487-95.

87. Fibbe WE, Noort WA. *Ann NY Acad Sci*. 2003;996:235-244.

88. Figueira EC, Di Girolamo N, Coroneo MT *et al*. *Invest Ophthalmol Vis Sci*. 2007 Jan;48(1):144-56

89. Finley MF, Devata S, Huettner JE. *Neurobiol.* 1999;40(3):271-87

90. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. *Cell Tissue Kinet*. 1987;20(3):263-72.

91. Friedenstein AJ, Chailakhyan RK, Latsinik NV *et al*. *Transplantation*. 1974;17(4):331-40

92. Friedenstein AJ, Deriglasova UF, Kulagina NN *et al*. *Exp Hematol*. 1974;2(2):83-92.

93. Friedenstein AJ, Gorskaja JF, Kulagina NN. *Exp Hematol* 1976; 4:267-274

94. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. *J Embryol Exp Morphol*. 1966;16(3):381-90.

95. Fu W, Killen M, Culmsee C *et al*. *J Mol Neurosci*. 2000;14(1-2):3-15.

96. Fuschs S, Satler LF, Kornowski R *et al*. *Am J Cardiol* 2004;94:92-5.

97. Gao J, Dennis JE, Muzic RF *et al*. *Cell Tissues Organs*. 2001;169:10-20.

98. Gardner RL, Beddington RS. *J Cell Sci Suppl*. 1988;10:11-27.

99. Gelmini S, Poggesi M, Distante V *et al*. *Cancer Lett*. 2004;216(1):81-7.

100. Ghen MJ, Roshan R, Roshan RO *et al. Reprod Biomed Online*. 2006;13(4):562-72.
101. Gipson IK, Spurr-Michaud S, Tisdale A *et al*. *Invest Ophthalmol Vis Sci.* 1989;30(3):425-34.
102. Gipson IK. *Eye*. 1989;3(Pt 2):132-140
103. Glennie S, Soeiro I, Dyson PJ *et al*. *Blood*. 2005;105:2821-2827.
104. Goshima J, Goldberg VM, Caplan AI. *Clin Orthop Relat Res*. 1991;(262):298-311.
105. Gronthos S, Graves SE, Ohta S *et al*. *Blood* 1994; 84:4164-73.
106. Gronthos S, Zannettino AC, Hay DJ *et al*. *J Cell Sci* 2003;116:1827-1835.
107. H.S. Dua A. Azuara-Blanco. *Surv. Opthalmol*.2000;44:415-425.
108. Hall PA. *J Pathol*. 1989 Aug;158(4):275-7.
109. Halleux C, Sottile V, Gasser JA *et al*. *J Musculoskelet Neuronal Interact*. 2001;2(1):71-6
110. Hayashi R, Yamato M, Sugiyama H *et al*. *Stem Cells*. 2007;25(2):289-96. Epub 2006 Sep 28.
111. Hermann A, Gastl R, Liebau S *et al*. *J Cell Sci*. 2004;117(Pt 19):4411-22.
112. Hernigou P, Beaujean F. *Clin Orthop Relat Res* 2002;405:14-23.
113. Herrera MB, Bussolati B, Bruno S *et al*. *Int J Mol Med*. 2004;14:1035-1041.
114. Hoffman A, Czichos S, Kaps C *et al*. *J Cell Sci*. 2002;115:769-781.
115. Hoffman RM. *Cell Cycle*. 2006;5(3):232-3.
116. Hofstetter CP, Schwarz EJ, Hess D *et al*. *Proc Natl Acad Sci USA* 2002;99:2199–2204

117. Honczarenko M, Le Y, Swierkowski M *et al*. *Stem Cells*. 2006;24(4):1030-41.

118. Horwitz EM, Gordon PL, Koo WK *et al*. *Proc Natl Acad Sci USA* 2002;99:8932-8937.

119. Horwitz EM, Prockop DJ, Fitzpatrick LA *et al*. *Nat Med* 1999;5:309-13.

120. Huang AJ, Tseng SC. *Invest Ophthalmol Vis Sci*. 1991;32(1):96-105.

121. Hunt P, Robertson D, Weiss D *et al*. *Cell* 1987;48:997–1007.

122. Igura K, Zhang X, Takahashi K *et al*. *Cytotherapy*. 2004;6(6):543-53.

123. Im G, Shin Y, Lee K. *Osteoarthritis Cartilage* 2005;13:845-853.

124. Imitola J, Park KI, Teng YD *et al*. *Philos Trans R Soc Lond B Biol Sci*. 2004;359(1445):823-37.

125. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C *et al*. *Blood*. 2003 15;102(4):1548-9.

126. Int Anker PS, Noort WA, Scherjon SA *et al*. *Haematologica* 2003;88:845-52

127. Itskovitz-Eldor J, Schuldiner M, Karsenti D *et al*. *Mol Med*. 2000;6:88-95.

128. Jan YN, Jan LY. *Nature*. 1998;23;392(6678):775-8.

129. Javazon EH, Beggs KJ, Flake AW. *Exp Hematol.* 2004;32(5):414-25

130. Javazon EH, Colter DC, Schwarz EJ *et al*. *Stem Cells* 2001;19:219-225

131. Jia L, Young MF, Powell J *et al*. *Genomics* 2002; 79(1):7-17.

132. Jiang XX, Zhang Y, Liu B *et al*. *Blood* 2005;105:4120-4126.

133. Jiang Y, Jahagirdar BN, Reinhardt RL *et al*. *Nature* 2002;418(6893):41-49

134. Jiang Y, Vaessen B, Lenvik T *et al*. *Exp Hematol*. 2002;30(8):896-904.

135. Johnstone B, Hering TM, Caplan AI *et al*. *Exp Cell Res* 1998;238:265-272

136. Joseph A, Powell-Richards AO, Shanmuganathan VA *et al*. *Br J Ophthalmol*. 2004;88(3):393-398.

137. Jun AS, Liu SH, Koo EH *et al*. *Arch Ophthalmol*. 2001;119(11):1629-34

138. Jung J, Moon N, Ahn JY *et al*. *Stem Cells Dev*. 2009;18(4):559-71.

139. Kadiyala S, Young RG, Thiede MA *et al*. *Cell Transplant*. 1997;6(2):125-34.

140. Katz AJ, Tholpady A, Tholpady SS *et al*. *Stem Cells*. 2005;23:412-423.

141. Keilhoff G, Goihl A, Langnase K *et al*. *Eur J Cell Biol* 2006;85:11-24.

142. Kenyon KR, Tseng SC. *Ophthalmology*. 1989;96(5):709-22.

143. Kim CG, Lee JJ, Jung DY *et al*. *Mol Cells*. 2006;30;21(3):343-55.

144. Kinoshita S, Adachi W, Sotozono C *et al*. *Prog Retin Eye Res*. 2001;20(5): 639-673.

145. Kinoshita S, Friend J, Thoft RA. *Invest Ophthalmol Vis Sci*. 1981;21(3):434-41.

146. Kishi T, Takao T, Fujita K *et al*. *Biochem Biophys Res Commun*. 2006;340(2):544-52. Epub 2005 Dec 19.

147. Kivela T, Uusitalo M. *Prog Retin Eye Res*. 1998;17(3):385-428.

148. Klapper W, Parwaresch R, Krupp G. *Mech Ageing Dev*. 2001;122(7):695-712.

149. Klein G, Müller CA, Tillet E *et al*. *Blood*. 1995;86(5):1740-8.

150. Koc ON, Gerson SL, Cooper BW et *al*. *J Clin Oncol*. 2000;18:307-316.

151. Kolf CM, Cho E, Tuan RS. *Arthritis Res Ther*. 2007;9(1):204.

152. Kopen GC, Prockop DJ, Phinney DG. *Proc Natl Acad Sci USA* 1999;69:10711-10716.

153. Korinek V, Barker N, Moerer P *et al*. *Nat Genet*. 1998;19(4):379-83.

154. Korkko J, Ibberson D, Kopen GC *et al*. *Stem Cells*. 2001;19(5):408-18.

155. Kotton DN, Ma BY, Cardoso WV *et al*. *Development*. 2001;128(24):5181-

156. Krampera M, Cosmi L, Angeli R *et al*. *Stem Cells*. 2006;24(2):386-98.

157. Krampera M, Glennie S, Dyson J *et al*. *Blood*. 2003;101:3722-3729.

158. Krampera M, Pasini A, Rigo A *et al. Blood.* 2005;106(1):59-66. Epub 2005 Mar 8

159. Kruse FE, Tseng SC. *Ophthalmologe.* 1993;90(6):669-78.

160. Kruse FE, Tseng SC. *Invest Ophthalmol Vis Sci*. 1993;34(6):1963-76.

161. Kruse FE, Völcker HE. *Curr Opin Ophthalmol*. 1997;8(4):46-54.

162. LaBarge MA, Blau HM. *Cell*. 2002;111(4):589-601.

163. Lane JM, Yasko AW, Tomin E *et al*. *Clin Orthop Relat Res*. 1999;361:216-227.

164. Lauweryns B, JJ van den Oord, L Missotten. *Invest Ophthalmol Vis Sci*. 1993;34(6):1991-1999.

165. Lauweryns B, vanden Oord JJ, De Vos R *et al*. *Invest Ophthalmol Vis Sci*. 1993;34(6):1983-1990.

166. Lavker RM, Dong G, Cheng SZ *et al*. *Invest Ophthalmol Vis Sci*. 1991;32(6):1864-75.

167. Lavker RM, Sun TT. *Eye (Lond).* 2003;17(8):937-42.

168. Lavker RM, Tseng SC, Sun TT. *Exp Eye Res*. 2004;78(3):433-46. Review.

169. Lavker RM, Wei ZG, Sun TT. *Invest Ophthalmol Vis Sci*. 1998;39(2):301-7.

170. Lazarus HM, Koc ON, Devine SM *et al*. *Biol Blood Marrow Transplant* 2005;11:389-90.

171. Le Blanc K, Pittenger M. *Cytotherapy*. 2005;7(1):36-45.

172. Le Blanc K, Rasmusson I, Sundberg B *et al*. *Lancet* 2004;363:1439-41.

173. Le Blanc K, Tammik C, Rosendahl K *et al*. *Exp Hematol* 2003;31:890-6.

174. Lee JH, Kosinski PA, Kemp DM. *Exp Cell Res* 2005;307:174-82.

175. Lee RH, Hsu SC, Munoz J *et al*. *Blood.* 2006;107:2153-61.

176. Lee RH, Kim B, Choi I *et al*. *Cell Physiol Biochem.* 2004;14(4-6):311-24

177. Li DQ, Tseng SC. *J Cell Physiol*. 1997;172(3):361-72.

178. Li DQ, Tseng SC. *J Cell Physiol*. 1995;163(1):61-79.

179. Li L, Neaves WB. *Cancer Res*. 2006;66:4553-4557.

180. Li L, Xie T. *Annu Rev Cell Dev Biol*. 2005;21:605-31.

181. Li M, Price D, Smith A. *Symp Soc Exp Biol*. 2001;(53):29-42.

182. Li W, Hayashida Y, Chen YT *et al*. *Cell Res*. 2007;17(1):26-36. Review.

183. Liechty KW, MacKenzie TC, Shaaban AF *et al*. *Nat Med 2000*;6:1282-6.

184. Lindberg K, Brown ME, Chaves HV *et al*. *Invest Ophthalmol Vis Sci*. 1993;34(9):2672-9.

185. Ljubimov AV, Burgeson RE, Butkowski RJ *et al*. *Lab Invest*. 1995;72(4):461-73.

186. Loeffler M, Grossmann B. *J Theor Biol*. 1991;150(2):175-91.

187. Lou S, Gu P, Chen F *et al*. *Brain Res*. 2003;968:114-121.

188. Lu B, Jan LY, Jan YN. *Curr Opin Genet Dev*. 1998;8(4):392-9.

189. Lu P, Blesch A, Tuszynski MH. *J Neurosci Res*. 2004;77(2):174-91

190. Luria EA, Panasyuk AF, Friedenstein AY. *Transfusion*. 1971;11:345-9.

191. Ma L, Feng XY, Cui BL *et al. Chin Med J* (Engl). 2005;118(23):1987-93.

192. Magaki T, Kurisu K, Okazaki T. *Neurosci Lett*. 2005;384(3):282-7

193. Majo F, Rochat A, Nicolas M *et al*. *Nature*. 2008;456(7219):250-4

194. Makino S, Fukuda K, Miyoshi S *et al*. *J Clin Invest*. 1999;103:697-705.

195. Malin D, Kim IM, Boetticher E *et al*. *Mol Cell Biol*. 2007;27(7):2486-98.

196. Mann I. A study of epithelial regeneration in the living eye. *Br J Ophthalmol*. 1944;28(1):26-40.

197. Mareschi K, Ferrero I, Rustichelli D *et al*. *J Cell Biochem* 2006;97:744-54.

198. Martin GR. *Proc Natl Acad Sci U S A*. 1981;78(12):7634-8.

199. Matic M, Petrov IN, Rosenfeld T *et al. Invest Ophthalmol Vis Sci.* 1997;38(3):600-9.

200. Mauney JR, Nguyen T, Gillen K *et al*. *iomaterials*. 2007;28(35):5280-90. Epub 2007 Aug 31.

201. McCallum RM, Cobo LM, Haynes BF. *Invest Ophthalmol Vis Sci*. 1993;34(5):1793-803.

202. Meachem S, Von Schönfeldt V, Schlatt S. *Reproduction*. 2001 Jun;121(6):825-34. Review.

203. Melnikova L, Biessmann H, Georgiev P. *Genetics*. 2005;170(1):221-35.

204. Mezey E, Chandross KJ, Harta G *et al. Science*.2000;290(5497): 1779-82

205. Mitchell JB, Mclntosh K, Zvonic S *et al*. *Stem Cells* 2006;24:376-85.

206. Miura M, Gronthos S, Zhao M *et al*. *Proc Natl Acad Sci U S A*. 2003;100(10):5807-12.

207. Modlin IM, Kidd M, Lye KD *et al*. *Keio J Med*. 2003;52(2):134-7.

208. Moscoso I, Centeno A, Lopez E *et al*. *Tranplant Proc* 2005;37:481-2.

209. Muguruma Y, Yahata T, Miyatake H *et al*. *Blood*. 2006;107:1878-87.

210. Muraglia A, Cancedda R, Quarto R. *J Cell Sci*. 2000;113 (Pt 7):1161-6

211. Nagaya N, Fujii T, Iwase T *et al*. *Am J Physiol Heart Circ Physiol* 2004;287:H2670-6.

212. Nakamura T, Kinoshita S. *Cornea*. 2003;22(7 Suppl):S75-80.

213. Neuhuber B, Gallo G, Howard L et al. *J Neurosci Res*. 2004;77(2):192-204

214. Neuhuber B, Himes BT, Shumsky JS *et al*. *Brian Res*. 2005;1035:73-85.

215. Nicola T, Jarmo K, David I *et al*. *Stem Cells*. 2001;19(5):408-418.

216. Noth U, Osyczka AM, Tuli R *et al*. *J Orthop Res*. 2002;20: 1060–1069

217. Oliver JA, Maarouf O, Cheema FH *et al*. *J Clin Invest*. 2004;114(6):795-804.

218. Oritz LA, Gambelli F, McBride C *et al*. *Proc Natl Acad Sci USA* 2003;100:8407-8411.

219. Oswald J, Boxberger S, Jørgensen B *et al*. *Stem Cells*. 2004;22(3):377-84.

220. Ozerdem U, Alitalo K, Salven P *et al*. *Invest Ophthalmol Vis Sci*. 2005;46(10):3502-6.

221. Pajoohesh-Ganji A, Ghosh SP, Stepp MA. *Dev Dyn*. 2004;230(3):518-28.

222. Parr AM, Tator CH, Keating A. *Bone Marrow Transplant*. 2007 ;40(7):609-19. Epub 2007 Jul 2. Review.

223. Peister A, Mellad JA, Larson BL *et al*. *Blood* 2004;103:1662-1668.

224. Pellegrini G, Dellambra E, Golisano O *et al*. *Proc Natl Acad Sci U S A.* 2001;98(6):3156-61.

225. Pellegrini G, Golisano O, Paterna P *et al*. *J Cell Biol*. 1999;145(4):769-82.

226. Pellegrini G, Traverso CE, Franzi AT *et al*. *Lancet*. 1997; 349(9057): 990-3.

227. Pittenger MF, Mackay AM, Beck SC *et al*. *Science*. 1999;284(5411):143-7

228. Polisetty N, Fatima A, Madhira SL *et al*. *Mol Vis.* 2008;14: 431-442.

229. Prockop DJ. *Science*. 1997;276(5309):71-74.

230. Prota G. *J Invest Dermatol*. 1980;75(1):122-7.

231. Puangsricharern V, Tseng SC. *Ophthalmology*. 1995;102(10):1476-85.

232. Qi H, Chuang EY, Yoon KC *et al. Mol Vis.* 2007;13:1934-41

233. Quarto R, Mastrogiacomo M, Cancedda R *et al*. *N Eng J Med* 2001;344:385-6.

234. Reading L, Still K, Bishop N *et al*. *Bone*. 2000;26, suppl:9S.

235. Rennick D, Yang G, Gemmell L *et al*. *Blood* 1987;69:682–691.

236. Reyes M, Lund T, Lenvik T *et al*. *Blood*. 2001; 98:2615-2625.

237. Rhodes NP, Srivastava JK, Smith RF *et al*. *J Mater Sci Mater Med* 2004;15:397-402.

238. Risbud MV, Shapiro IM, Guttapalli A *et al*. *Spine (Phila Pa 1976).* 2006;31(1):83-9.

239. Romano AC, Espana EM, Yoo SH *et al*. *Invest Ophthalmol Vis Sci*. 2003;44(12):5125-9.

240. Rubio D, Garcia-Castro J, Martin MC *et al*. *Cancer Res* 2005; 65:3035-9.

241. Ryan DH, Nuccie BL, Abboud CN *et al*. *J Clin Invest* 1991;88(3):995-1004.

242. Sagrinati C, Netti GS, Mazzinghi B *et al*. *J Am Soc Nephrol*. 2006 Sep;17(9):2443-56. Epub 2006 Aug 2

243. Sanchez-Ramos J, Song S, Cardozo-Pelaez F *et al*. *Exp Neurol*. 2000;164(2):247-56

244. Sangwan VS, Matalia HP, Vemuganti GK *et al*. *Indian J Ophthalmol.* 2004;52(4): 281-285.

245. Sangwan VS, Matalia HP, Vemuganti GK *et al*. *Indian J Ophthalmol*. 2006; 54(1): 29-34.

246. Santa María L, Rojas CV, Minguell JJ. *Exp Cell Res*. 2004;300(2):418-26

247. Sato N, Meijer L, Skaltsounis L *et al*. *Nat Med*. 2004;10:55-63.

248. Schermer A, Galvin S, Sun TT. *J Cell Biol*. 1986;103(1):49-62.

249. Schmidt D, Mol A, Odermatt B *et al. Tissue Eng*. 2006;12(11):3223-32.

250. Schofield R. *Blood Cells*. 1978;4(1-2):7-25.

251. Schofield R. The stem cell system. *Biomed Pharmacother*. 1983;37(8):375-80. Review.

252. Scholtzer-Schrehardt U, Kruse FE. *Exp Eye Res*. 2005;81:247-264.

253. Schwarz EJ, Alexander GM, Prockop DJ *et al*. *Hum Gene Ther*. 1999;10 (15):2539-49.

254. Schwarz EJ, Reger RL, Alexander GM *et al*. *Gene Ther*. 2001;8(16):1214-23.

255. Scintu F, Reali C, Pillai R *et al*. *BMC Neurosci*. 2006;7:14.

256. Seigel GM, Sun W, Salvi R *et al*. *Mol Vis*. 2003; 9:159-163

257. Sekiya I, Colter DC, Prockop DJ. *Biochem Biophys Res Commun.* 2001;284(2):411-8

258. Sekiya I, Larson BL, Smith JR *et al*. *Stem Cells* 2002a;20:530-541

259. Shake JG, Gruber PJ, Baumgartner WA *et al*. *Ann Thorac Surg* 2002;73:1919-1956.

260. Shi S, Bartold PM, Miura M *et al*. *Orthod Craniofac Res*. 2005;8(3):191-9. Review.

261. Shirakata Y, Komurasaki T, Toyoda H *et al*. *J Biol Chem*. 2000;25275(8):5748-53.

262. Silva WA Jr, Covas DT, Panepucci RA *et al*. *Stem Cells* 2003; 21(6): 661-9.

263. Simmons DJ, Seitz P, Kidder L *et al*. *Calcif Tissue Int* 1991;48:326-334.

264. Simmons PJ, Torok-Storb B. *Blood*. 1991;78(11):2848-53.

265. Sliva GV, Litovsky S, Assad JA *et al*. *Circulation* 2005;111:150-6.

266. Smith RK, Korda M, Blunn GW *et al*. *Equine Vet J*. 2003;35(1):99-102.

267. Sosnová M, Bradl M, Forrester JV. *Stem Cells*. 2005;23(4):507-15.

268. Sottile V, Halleux C, Bassilana F *et al*. *Bone*. 2002;30(5):699-704.

269. Spangrude GJ, Aihara Y, Weissman IL *et al*. *J Immunol*. 1988;141(11):3697-707.

270. Spees JL, Olson SD, Ylosalo J *et al*. *Proc Natl Acad Sci USA* 2003;100:2397-2402.

271. Spradling A, Drummond-Barbosa D, Kai T. *Nature*. 2001;414(6859):98-104.

272. Stamm C, Westphal B, Kleine HD *et al*. *Lancet* 2003;361:45-6.

273. Stingl J, Eirew P, Ricketson I *et al*. *Nature*. 2006;439(7079):993-7.

274. Streit A, Stern CD. *Trends Genet*. 1999;15(1):20-4

275. Sugaya K. *Int Rev Cytol*. 2003;228:1-30

276. Suon S, Jin H, Donaldson AE *et al*. *Stem Cells Dev*. 2004;13(6):625-35

277. Suzuki H, Taguchi T, Tanaka H *et al. Biochem Biophys Res Commun.* 2004;322(3):918-22

278. Takács L, Tóth E, Berta A e*t al. Cytometry A*. 2009;75(1):54-66

279. Thorgeirsson SS, Grisham JW. *Semin Liver Dis*. 2003;23(4):303-12.

280. Till JE, McCulloch EA, Siminovitch L. *Proc Natl Acad Sci U S A*. 1964;51:29-36.

281. Tondreau T, Lagneaux L, Dejeneffe M *et al*. *Differentiation*. 2004;72(7):319-26

282. Torrente Y, Belicchi M, Pisati F *et al*. *Cell Transplant*. 2002;11(1):25-34

283. Touhami A, Grueterich M, Tseng SC. *Invest Ophthalmol Vis Sci*. 2002;43(4):987-94.

284. Tremain N, Korkko J, Ibberson D *et al*. *Stem Cells*. 2001;19(5):408-18

285. Tsai RJ, Li LM, Chen JK. *N Engl J Med*. 2000;343(2):86-93.

286. Tseng SC. *Eye (Lond).* 1989;3 (Pt 2):141-57.

287. Tsujimura A, Koikawa Y, Salm S *et al*. *J Cell Biol*. 2002;157(7):1257-65. Epub 2002 Jun 24.

288. Uccelli A, Moretta L, Pistoia V. *Eur J immunol*. 2006;36:2566-73

289. Van Genderen C, Okamura RM, Fariñas I *et al*. *Genes Dev*. 1994;8(22):2691-703.

290. Vauclair S, Majo F, Durham AD *et al*. *Dev Cell*. 2007 ;13(2):242-53.

291. Vemuganti GK, Kashyap S, Sangwan VS *et al*. *Indian J Ophthalmol*. 2004;52(2):113-20.

292. Vogel W, Grunebach F, Messam CA *et al*. *Haematologica* 2003;88:126-33.

293. Wakitani S, Imoto K, Yamamoto T *et al*. *Osteoarthritis Cartilage* 2002;10:199-206.

294. Wang G, Bunnell BA, Painter RG *et al*. *Proc Natl Acad Sci U S A.* 2005;102(1):186-91. Epub 2004 Dec 22

295. Watanabe K, Nishida K, Yamato M *et al*. *FEBS Lett*. 2004;565(1-3):6-10.

296. Watt FM, Hogan BL. *Science*. 2000;287:1427-1230.

297. Weissman IL. *Cell*. 2000;100(1):157-68

298. Welm BE, Tepera SB, Venezia T *et al*. *Dev Biol*. 2002;245(1):42-56.

299. Wilson PA, Hemmati-Brivanlou A. *Nature.*1995;376(6538):331-3

300. Wilson SE, Mohan RR, Ambrosio R Jr *et al*. *Prog Retin Eye Res* 2001;20:625–637.

301. Wislet-Gendebien S, Wautier F, Leprince P et al. Brain Res Bull. 2005;68(1-2):95-102.

302. Wollert KC, Meyer GP, Lotz J *et al*. *Lancet* 2004;364:141-8.

303. Wolosin JM, Budak MT, Akinci MA. *Int J Dev Biol*. 2004;48(8-9):981-91.

304. Wolosin, JM, Xiong X, Schutte M *et al*. *Prog Retin Eye Res*. 2000;19(2): 223-255

305. Woodbury D, Schwarz EJ, Prockop DJ *et al*. *J Neurosci Res* 2000;61:364–370

306. Xie T, Spradling AC. *Cell*. 1998;94(2):251-60.

307. Xu M, Uemura R, Dai Y *et al*. *J Mol Cell Cardiol* 2007;42(2):441-8. Epub 2006 Dec 20.

308. Yamaguchi Y, Sumikawa Y, Yoshida S *et al*. *Br J Dermatol*. 2005;152:664-772.

309. Yamagami S, Mimura T, Yokoo S *et al*. *Cornea*. 2006;25(10 Suppl 1):S90-2.

310. Yanai N, Sekine C, Yagita H *et al*. *Blood* 1994;83:2844–2850.

311. Ye J, Yao K, Kim JC. *Eye (Lond).* 2006;20(4):482-90.

312. Yoshida S, Shimmura S, Shimazaki J *et al*. *Invest Ophthalmol Vis Sci*. 2005;46(5):1653-8.

313. Young RG, Butler DL, Weber W *et al*. *J Orthop Res*. 1998;16(4):406-13.

314. Zhang Y, Overbeek PA, Govindarajan V. *Mol Vis*. 2007;13:2289-300.

315. Zhao LR, Duan WM, Reyes M *et al*. *Exp Neurol*. 2002;174(1):11-20.

316. Zhao X, Das AV, Thoreson WB *et al*. *Dev Biol*. 2002; 250(2): 317-31.

317. Zhao Y, Glesne D, Huberman E. *Proc Natl Acad Sci U S A*. 2003;100(5):2426-31.

318. Zhou M, Li XM, Lavker RM. *J Biol Chem*. 2006;281(28):19600-9.

319. Zhu W, Zou Y, Aikawa R *et al*. *Circulation*. 1999;100(20):2100-7.

320. Zieske JD, Mason VS, Wasson ME *et al*. *Exp Cell Res*. 1994;214(2):621-33.

321. Zohar R, Sodek J, McCulloch CA*Blood*. 1997;90(9):3471-81.

322. Zuba-Surma EK, Klich I, Greco N *et al*. *Eur J Haematol*. 2009.

323. Zuk PA, Zhu M, Ashjian P *et al*. *Mol Biol Cell*. 2002;13(12):4279-95.

324. Zuk PA, Zhu M, Mizuno H *et al*. *Tissue Eng*. 2001;7(2):211-28.

325. Zvaifler NJ, Marinova-Mutafchieva L, Adams G *et al*. *Arthritis Res*. 2000;2(6):477-88.

APPENDIX 1

(1) Phosphate Buffered Saline

PBS (1X) was used for washing and other purposes. The following chemicals were added to distilled water. After dissolving the chemicals thoroughly the pH was set to 7.2 using a pH meter and the final volume was made to 100ml with distilled water.

Nacl	0.8g
Kcl	0.02g
KH_2PO_4	0.012g
Na_2HPO_4	0.091g

(2) Trypsin Enzyme

Trypsin enzyme was used for dislodging the stromal cells. It was prepared by reconstituting the lyophilized powder of trypsin in PBS, to make 0.25% trypsin solution. To this 0.1mM EDTA solution was added and filter sterilized and stored at 4^oC.

(3) Growth Factors

All the growth factors from Sigma were reconstituted in DMEM medium; aliquots of 50µl were made and stored at $–80^oC$.

(4) Fetal Bovine Serum (FBS)

FBS was obtained from Sigma Aldrich. After filter sterilization aliquots of 50ml were made and stored in $–80^oC$.

(5) Composition of the Human Corneal Epithelium Medium

S.No.	Ingredients	Quantity	Company
1	Minimal Essential Medium	1.99g	Sigma M0643
2	Nutrient Mixture Ham's F-12	3.33g	Sigma N4388
3	Penicillin	75mg	
4	Streptomycin	50mg	
5	Amphotericin	1.25mg	
6	Gentamicin	50µl (4mg/L)	
7	Epidermal Growth Factor	50µl (0.01mg/L)	Sigma E4127
8	Cholera Toxin	50µl (0.1mg/L)	Sigma C8052
9	Insulin	2.5-5 mg	Sigma I1882
10	Sodium bicarbonate	0.690 g	

Method of Preparation: Add the first two ingredients in 100ml of Milli Q water, in a sterile 500ml flask/beaker, add sodium bicarbonate, dissolve and add the remaining ingredients. Ensure that the pH is 7.0. Make up the medium volume to 412 ml. Sterilize the medium with vaccum filter using Millipore filter membrane (0.22µ). After sterilization add a few drops of the medium to chocolate agar plates for sterility check. The medium is then stored at 4^{o}C. The shelf life of the medium is about 15 days after that the pH of the medium increases indicated by change in color (phenol red) towards more pinkish.

Separation of autologous serum: About 10ml blood is drawn from the patient in non-heparin, 15ml graduated falcon tubes. The blood is kept at room temperature undisturbed for a couple of hours to allow the settlement of cellular components. It is then centrifuged at 2000 rpm for 10 minutes. The serum collected is pipetted out in a fresh falcon and filter sterilized using 0.22µ millipore syringe filters, and used in the culture medium in 10% concentration.

(6) Preparation of Dulbecco's Modified Eagle's Medium (DMEM)

S.No.	Ingredients	Quantity	Company
1	DMEM	13.4 g	Sigma - D 7777
2	Gentamycin	100µl (4 mg/L)	
3	Penicillin	150mg	
4	Streptomycin	100mg	
5	Amphotericin	2.50mg	
16	Sodium bicarbonate	3.7 g	

Method of Preparation: Add the first two ingredients in 500ml of Milli Q water, in a sterile 1000ml flask/beaker, add sodium bicarbonate, dissolve and add the remaining ingredients. Make up the final volume to 1000ml. Ensure that the pH is 7.2 – 7.5. Sterilize the medium with vacuum filter using Millipore filter membrane (0.22µ). After sterilization add a few drops of the medium to chocolate agar plates for sterility check. The medium is then stored at 4^{o}C.

(6) Reagents for Flow Cytometry:

Washing buffer solution

The buffer consists of 2% (w/v) fetal calf serum (Sigma, USA) and 0.1% (w/v) sodium azide (Sigma, USA) in PBS. The buffer was filter sterilized and stored at 4^{0} C.

Paraformaldehyde Solution:

For cytoplasmic antigen staining fixation of cells required. For fixation paraformaldehyde soulution is used. Add 2g of paraformaldedehyde powder (Sigma) to 100ml of 1X PBS. Heat to 70^{0}C in a fume hood until the paraformaldehyde goes into solution. Allow the solution to cool to room

temperature. Adjust the pH 7.4 using 0.1M NaOH or HCl. This solution was filter sterilized and stored at 4^{o} C protected from light.

Antibody dilutions:

Stock solutions were diluted to 1:10 with PBS – Sodium azide buffer (working standard) and stored at 4^{o}C. From working standard required dilution of antibodies were prepared accordingly by using PBS – BSA – Sodium azide buffer.

(7) Reagents for Immunocytochemistry

4% paraformaldedehyde solution

Add 4g of paraformaldedehyde powder (Sigma) to 100ml of 1X PBS. Heat to 70^{0}C in a fume hood until the paraformaldehyde goes into solution. Allow the solution to cool to room temperature. Adjust the pH 7.4 using 0.1M NaOH or HCl. This solution was filter sterilized and stored at 4^{o}C protected from light

(8) Adipogenic Induction Media - Adipogenic induction media is prepared by using the components of 0.5 µM dexamethasone (Sigma, USA) 0.5 mM isobutylmethylxanthine (Sigma, USA), and 10µg/ml insulin (Sigma, USA) and were dissolved in DMEM+ 10%FCS. The induction media was filter sterilized and stored at 4^{0}C.

(9) Osteogenic Induction Media - Osteogenic induction media containing DMEM/10% FBS, 100nM dexamethasone, 10mM β-glycerophosphate, and 50µM ascorbic acid (Sigma, USA). The induction media was filter sterilized and stored at 4^{0}C

(10) Growth Factors

Reconstitution of Growth Factors and Inducers for Differentiation

All growth factors during reconstitution were carefully handled at 4^{o}C in ice as they are heat sensitive and maintaining utmost sterility to avoid microbial contamination. The growth factors were reconstituted in sterile DMEM media as per the manufacturer's instructions for the required concentration. The growth factors are mixed gently, but thoroughly so as to make sure the entire lyophilized powder is dissolved in the media. The reconstituted growth factors are then aliquoted into 0.5ml eppendorf tubes to an appropriate volume so as to achieve the final working concentration when mixed to 50ml of induction media. All throughout the procedure the reconstituted growth factors are maintained in ice. Table 1 summarizes the details of reconstitution of all the growth factors.

After Aliquoting, the 0.5ml tubes are arranged in laxbro boxes, labeled appropriately and stored at -80^{o}C until further usage.

Note:

1. The reconstituted growth factor when frozen appears pale to dark yellowish in colour, which is perfectly normal and should not be mistaken for contamination.
2. Reconstituted growth factors should not be syringe filtered to avoid loss of protein as a result of filtration.

Table 1: Growth Factor Reconstitution

S No	Growth Factor	Content per vial	Reconstitution	Final Concentration (per ml)
1	EGF	100µg	1ml of media (50µl/Vial)	100ng/ml
2	NGF	25µg	1ml of media (100µl/Vial)	50ng/ml
3	bFGF	25µg	1ml of media (20µl/Vial)	10ng/ml
4	PDGF -AB PDGF- BB	250ng 10 µg	1Vial for 50ml In 1ml of media and aliquot into 50 µl/Vial	10ng/ml 10ng/ml
5	Forskolin	10mg (Mol Wt 410.5g)	In 10ml of ice cold Ethanol (205µl/Vial)	10µM/ml

LIST OF PUBLICATIONS

1. **Polisetty N**, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK Mesenchymal cells from limbal stroma of human eye. *Mol Vis.* 2008;14:431-42
2. K. Purushotham Reddy, Lakshmi Kiran Chelluri, **Polisetti N**, Geeta K Vemuganti. Histocompatibility testing of cultivated human bone marrow stromal cells – A promising step towards pre-clinical screening for allogenic stem cell therapy. *Cell Immunol*. 2009;259(1):61-5. Epub 2009 Jun 6.
3. Koppula PR, **Polisetti N**, Vemuganti GK. Unstimualted diagnostic marrow tap-a minimally invasive and reliable source of mesenchymal stem cells. *Cell Biol Int*. 2009. Nov 2. (Epub ahead of print)
4. **Polisetti N**, Ganta VJ, Vemuganti GK, Phanithi PB. Characterization and differentiation potential of rat bone marrow stromal cells. *Neurology India* 2009 (Accepted)
5. **Polisetti N**, Prasoon Agarwal, Imran Khan, Paturu Kondaiah, Virender S Sangwan, Vemuganti GK. Gene expression profiles of Limbal explant culture derived epithelial and mesenchymal like cells. (Manuscript submitted to *Invest Ophthalmol Vis Sci*)
6. **Polisetti N**, Koppula PR, Vemuganti GK. Gene expression profiles of bone marrow derved neural like cells (Manuscript Under Preparation)

LIST OF PRESENTATIONS

1. Presented a paper "Mesenchymal stem cells from Limbal stroma: Signature of limbal niche" in **International Symposium on Emerging Trends in Biomedical and Nanobiotechnology:Relevance to Human Health** held during December 19-21, 2009
2. Presented poster entitled "Mesenchymal stem cells from Limbal stroma: Signature of limbal stem cell niche" at the **XXXII All India Cell Biology Conference (AICBC) & International Workshop on Cell Cycle Regulation** held at University of Hyderabad, Hyderabad, India during December 10-13th 2009.
3. Presentated a paper entitled "Mesenchymal stem cells from Limbal stroma of human eye" at the **ASIA-ARVO**, held at the HICC Auditorium, Hyderabad, INDIA during Jan 15-18th 2009
4. Presented a poster entitled " Isolation and characterization of rat bone marrow stromal cells and their plasticity" at the 75th annual meeting of **Society for Biological chemists INDIA (SBCI),** held at the Jawaharlal Nehru University, New Delhi, India, during 7-11th Dec, 2006
5. Presented a Paper entitled Plasticity of Marrow stromal cells at the **Society for Regenerative Medicine**, 2006, held at the Acharya Nagarjuna University, Guntur, India.
6. Presented a paper entitled "Mesenchymal stromal cells from the Limbal stroma of human eye" at the **Indian Eye Research Group (IERG),** 2007, held at L.V. Prasad Eye Insitute, Hyderabad India
7. Presented poster entitled Exploring the plasticity of rat and human bone marrow stromal cells at the 1st annual meeting of **Stem cell research**

forum of India (SCRFI) and international conference on stem cell research, Banglore, India, during 29-1st Feb 2007

AWARDS AND FELLOWSHIPS

1. **Young Scientist Award (Gold Medal)** in International symposium on Emerging Trends in Biomedical and Nanobiotechnology: Relevance to Human Health, held during December 19-21, 2009
2. **Best Presentation Award** in ASIA-ARVO held at HICC auditorium, Hyderabad, India during Jan 15-18th 2009
3. **Young Scientist Award** in meeting of Society for regenerative Medicine, 2007 held at Acharya Nagarjuna University, Guntur, India
4. **Best Poster Award** in 75th annual meeting of society for Biological chemists of India (SBCI), held at the Jawaharlal Nehru University, New Delhi, India, during 8-11th Dec, 2006
5. **Senior Research Fellowship** for the period of 2007-2009 by the council of Scientific and Industrial Research (CSIR), INDIA
6. **Junior Research Fellowship** for the period of 2005-07 by council of Scientific and Industrial Research (CSIR), INDIA
7. Qualified for **GATE-2004** Fellowship