EX VIVO EXPANSION AND INDUCED TRANSDIFFERENTIATION OF RABBIT BONE MARROW MESENCHYMAL STEM CELLS TO CORNEAL LINEAGE BY SIMULATING CORNEAL STEM CELL NICHE

A DISSERTATION SUBMITTED

BY

SAUMI MATHEWS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM – 695 011

DECLARATION

I, **Saumi Mathews**, hereby declare that I had personally carried out the work depicted in the dissertation entitled **"*Ex vivo* expansion and induced transdifferentiation of rabbit bone marrow mesenchymal stem cells to corneal lineage by simulating corneal stem cell niche"** under the direct supervision of **Dr.T.V.Kumary**, Scientist G, Tissue Culture Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "***Ex vivo* expansion and induced transdifferentiation of rabbit bone marrow mesenchymal stem cells to corneal lineage by simulating corneal stem cell niche**" submitted by **Saumi Mathews** in partial fulfillment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by **her** under my supervision and guidance at **Tissue culture laboratory**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram 695012.

Thiruvananthapuram

Date 28.08.10

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The Dissertation Entitled

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Submitted

By

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Dedicated To My Family & Friends

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Saumi Mathews

ABBREVIATIONS

^{3}H- Tymidine	Tritiated thymidine
ABC	ATP-Binding Cassette
ABCG2	ATP-Binding Cassette sub family G member 2
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ATP	Adenosine Tri Phosphate
BM MSC	Bone Marrow Mesenchymal Stem Cells
BMP	Bone Morphogenetic Protein
BRCP-1	Breast Cancer Resistance Protein -1
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFU-F	Colony Forming Unit - Fibroblast
CK-12	Cytokeratin-12
CK-3	Cytokeratin-3
CM	Conditioned Medium
CO_2	Carbon dioxide
COL	Collagen
CS	Culture System
Cx 43	Connexin 43
DMEM	Dulbecco's Modified Eagle's Medium
DMEM LG	Dulbecco's Modified Eagle's Medium Low Glucose
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
dT	Deoxy Thymine
DTT	Dithiothreitol
ECM	Extra Cellular Matrix
EDTA	Ethylene diamine tetra acetic acid
EEO	Electroendosmosis

EtBr	Ethidium Bromide
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
hESC	Human Embryonic Stem Cell
HLA	Human Leukocyte Antigen
hMSC	Human MSC
HSC	Hematopoietic Stem Cells
IAEC	Institutional Animal Ethics Committee
ICAM-1	Intracellular Adhesion Molecule -1
IgG	Immunoglobulin G
K	Keratin
LESC	Limbal epithelial stem cell
LFA-1	Leukocyte Function –Associated Antigen -1
LSC	Limbal Stem Cell
LSCD	Limbal Stem Cell Deficiency
MACS	Magnetic Activated Cell Sorting
MHC	Major Histocompatibility Complex
M-MuLV RT PCR	Moloney Murine Lenti Virus RT PCR
MSC	Mesenchymal Stem Cell
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P1	Passage 1
P5	Passage 5
PAX 6	Paxillin 6
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PECAM-1	Platelet/ Endothelial Cell Adhesion Molecule-1
PFA	Paraformaldehyde
PI	Propidium Iodide
Rb MSC	Rabbit MSC

RCF	Relative centrifugal force
RPM	Revolutions Per Minute
RT- PCR	Reverse Transcription Polymerase Chain Reaction
SFA	Fibroblast Surface Antigen
SPARC	Secreted Protein Acidic and Rich in Cysteine
TAC	Transient Amplifying Cells
TAE	Tris Acetic Acid EDTA
TCPS	Tissue Culture Poly Styrene
TE	Tissue Engineering
TGF-β	Transforming Growth Factor- β
UV	Ultra Violet
VCAM-1	Vascular Cell Adhesion Molecule-1
VIM	Vimentin
WHO	World Health Organization
α-MEM	Minimum Essential Medium Alpha

NOTATIONS

%	Percentage
&	And
μ	Micron
μg	micro gram
μl	Micro Litre
bp	Base Pair
cm^2	Square Centimeter
h	Hour
kDa	Kilo Dalton
M	Molar
mg	Milligram
min	Minute
ml	Milli Litre
mM	milli Molar
mm^2	Millimeter Square
ng	nano gram
nm	nanometer
°	Degree
° C	Degree Celsius
s	second
α	Alpha
β	Beta

CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

Blindness is one of the most feared disabilities which can happen due to a variety of ocular diseases or trauma. Around 6.8 million people in India are affected by corneal blindness where majority is attributed to damage or dysfunction of the limbal stem cell population due to different inherited or acquired conditions. The prognosis and treatment of such corneal damages in many cases depends on the extent of deficiency of Limbal Stem Cells (LSCs). Due to the disadvantages in treatment methodologies involved, present research activities focus on the evaluation of alternate autologous cell source for LSCs. The present study aims in transdifferentiating the Mesenchymal Stem Cells (MSCs) to corneal epithelial lineage to act as an alternate autologous source of limbal stem cells for ocular surface reconstruction.

The thesis consists of four chapters namely Introduction & Review of Literature, Materials & Methods, Results & Discussion and Summary & Conclusion.

Chapter I introduces the topic addressed in the thesis and reviews the literature related to the study. This also gives background for the present study and objectives. The cornea on the front surface of the eye is our window to the world; hence maintenance of corneal tissue transparency is essential for vision. The integrity and functionality of the outermost corneal layer, the epithelium, plays a key role in refraction of light on to the retina at the back of the eye. Corneal integrity and function is dependent upon the self-renewing of putative stem cells located in the basal region of the limbus. Any damage or dysfunction of the limbal stem cell population is known as limbal stem cell deficiency (LSCD). The prognosis and treatment of such corneal damage depend on the extent of deficiency. Long term restoration of visual function requires renewal of the corneal epithelium, viz replacement of the stem cell population which has traditionally been achieved by grafting limbal auto or allograft. In the case of bilateral deficiency, allogenic tissue transplantation is the only alternative. These procedures carry risks such as availability of donor tissue, damage to healthy eye by removal of autologous tissue or side effects from long-term immunosuppression with allogenic tissues. Hence present research activities focus on the evaluation of alternate autologous cells for LSCs in

treatment involving bilateral deficiency. Recently it has been reported that other stem cell populations including human embryonic stem cells, buccal mucosal cells and hair follicle stem cells can be transdifferentiated towards a corneal epithelial-like phenotype. However reports are scanty and the results of long term follow up study in such cases are less satisfactory.

Recent reports on the presence of marrow specific cells in cornea, expression of specific markers such as ABCG2 (common to limbal epithelial stem cells), availability and the potential to differentiate into a multiple cell lineages, including those of epithelial lineage put forward MSCs to be an ideal candidate for ocular surface regeneration in bilateral limbal stem cell deficiency. The present study focuses on transdifferentiation of MSCs to corneal epithelial lineage *in vitro*, as an alternate source of limbal stem cells for ocular surface reconstruction. In order to achieve this aim the study objectives were

1. Isolation and maintenance of mesenchymal stem cells.
2. Optimization of culture conditions for mesenchymal stem cell culture and characterization.
3. Isolation, maintenance and characterization of limbal fibroblast cells
4. Simulation of niche factors for transdifferentiation of mesenchymal stem cells to corneo-epithelial lineage using collagen as substrate and limbal fibroblast conditioned medium.
5. Monitoring of differentiation status

Chapter II describes the experimental approach adopted which includes isolation and maintenance of bone marrow mesenchymal stem cells using direct plating method. Optimization of culture conditions for *in vitro* expansion and maintenance of mesenchymal stem cells were done by selection of media. Different commercially available media were used to culture cells and its effect in modulating cell adhesion and proliferation was assessed by phase contrast microscopy and MTT assay. The cells were analyzed for maintenance of stemness by immunostaining and flow cytometry using markers specific to mesenchymal stem cells such as vimentin, ABCG2, CD105, CD29 and CD90.

The limbal fibroblast cells were isolated by enzymatic digestion, cultured and characterized using antibodies against SPARC and vimentin. Conditioned medium from limbal fibroblast culture was stored and used to provide necessary factors for differentiation of MSCs. Effect of microenvironmental factors such as collagen and limbal fibroblast conditioned media were assessed for differentiating MSCs to corneal epithelial cells. Three different culture systems (Culture system-1 collagen + basal medium, Culture system 2- collagen + conditioned medium, Culture system 3- conditioned medium) were analyzed and monitored for its effect in inducing differentiation to corneo epithelial lineage. The differentiation was assessed by immunostaining, RT-PCR and flow cytometry using mesenchymal stem cells markers (ABCG2 & CD29) and corneal epithelial specific markers (CK3/12).

Chapter III includes results of all experiments, data analysis and discussion. Isolation of MSC resulted in a heterogeneous population of cells with fibroblast morphology. α–MEM, selected as optimal medium for culturing MSCs based on high rate of cell adhesion and proliferation, was used for further studies. Immunostaining and flow cytometry analysis of cells cultured in α-MEM showed maintenance of stem cell characteristics such as expression of ABCG2, vimentin, CD105, CD29 and negative expression of CD90. For studying the effect of microenvironment in cell differentiation, cells were cultured in 3 different culture systems. Results of culture system 1 indicated that collagen alone was not able to induce differentiation as the expression of cytokeratins was negative and stem cell markers were only positive. Differentiation of MSCs towards corneal lineage in culture systems 2 (collagen + conditioned media) & 3 (conditioned media alone) indicated an increased expression of cytokeratin and decreased expression of stem cell markers ABCG2 and CD29 by flow cytometry.

Chapter IV summarizes the study and provides drawn out conclusions. Mesenchymal stem cells from rabbit bone marrow were successfully isolated by direct plating method, cultured, maintained and transdifferentiated to corneal lineage by simulating corneo epithelial niche *in vitro*. The present findings provided evidence to the hypothesis that conditioned medium simulating autocrine and paracrine factors secreted by limbal microenvironment can induce transdifferentiation of MSCs into corneal epithelial-like phenotype.

Introduction

CHAPTER I

INTRODUCTION

1.1 Background

Blindness is one of the most feared disabilities which may occur due to different inherited or acquired conditions. According to the World Health Organization (WHO), in 2002, an estimate of 161 million people worldwide were visually impaired, of which 124 million people suffer from low vision and 37 million from total blindness. Approximately 90% of world's blind people live in developing countries. It is estimated that 23.5% of the world's total blind population lives in India (Thomas *et al.,* 2005). According to a report, 6.8 million people are affected by corneal blindness (Sinha *et al.,* 2005). Even though blindness often affects older people; 1.4 million children are affected by blindness worldwide. Over 10 million people worldwide experience bilateral corneal blindness where majority of the cases are due to limbal stem cell deficiency. Damage or dysfunction of the limbal stem cell population can occur due to different inherited or acquired conditions such as Stevens Johnson syndrome, chemical and thermal injuries, ocular cicatricial pemphigoids, corneal dystrophies, recurrent surgeries etc. This can compromise ocular surface integrity resulting in scarring, opacification and even blindness. One of the successfully used therapeutic strategies for treating such damages is the transplantation of limbus taken from related donors or cadavers. Such transplantation methodologies has very low success rate due to graft rejection and recurrent infections and also necessitates long term immunosuppression. The availability of donor tissue also limits such methods of treatment. Alternate approach to overcome such problems is the use of tissue engineering methodologies like *ex vivo* expansion and transplantation of autologous cell sheets engineered from limbal epithelial cells taken from contra lateral healthy eye. But there is a greater risk of surgically induced LSCD in contra lateral healthy eye and is not feasible in the case of bilateral deficiency, which necessitates the use of an alternate cell source other than LSCs for ocular surface reconstruction. However a very few reports are available on the use of various cell sources for corneal surface reconstruction. More over long term follow up studies done after transplantation did not yield satisfactory results. Such problems

underscore the importance of seeking an autologous cell source other than LSCs for cell therapy of corneal disorders especially in case of bilateral deficiency.

1.2 Review of literature

1.2.1 Eye

Eye is an important sensory organ, the origin of which can be traced from the Cambrian era of evolution. Nature has well protected the eye ball by covering it with fatty layer and placed in a bony cavity in skull. It consists of multiple layers and can be broadly divided into two segments – anterior (specialized in light perception) and posterior (specialized in light sensing). Inner portions between the anterior and posterior regions are filled with transparent jelly like fluid named aqueous and vitreous humor respectively (Figure 1).

Anatomically eye can be divided into three layers: outer consisting of sclera and cornea, middle layers of choroids, ciliary body and iris, and the inner layer consisting of retina. Functionally the eye focus and sense light rays and convert them to impulses which are interpreted by brain and give us a visual idea of our habitat. Similar to artificial equipments like camera, eye consists of an aperture system (pupil), lens system (lens) and film system (retina) (Guyton & Hall, 2000).

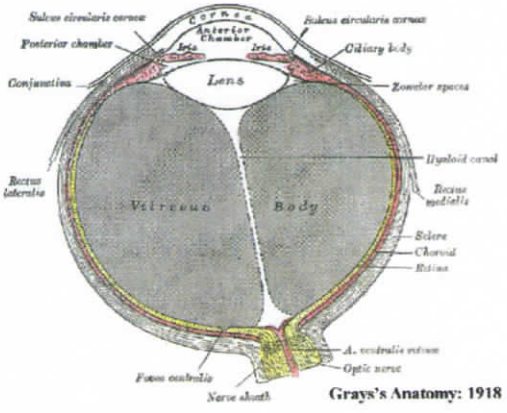

Figure 1 Structure of Eye depicting different parts

1.2.2 The Cornea

Cornea is the transparent anterior window of eye covering iris and pupil and has a major role in vision. It provides a clear transparent path for light and contributes to 70% of refractive power of eye (Karring *et al.*, 2004). It protects eye from external environment and also filter out harmful UV rays. For providing a clear transparent

path for light, cornea is avascular in nature and is not exposed to circulatory immune cells. Even though limbal regions (junction of cornea and sclera) are highly vascularized, the growth of blood vessels is terminated before reaching cornea. The cornea is made up of five major layers – the outermost corneal epithelium followed by Bowman's membrane, stroma, Descemet's membrane and endothelium (Figure 2) (Gipson *et al.,* 1994). All these layers play an important role in maintaining corneal transparency for vision.

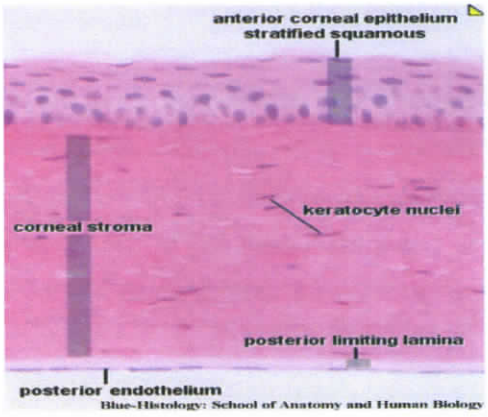

Figure 2 Transverse section of cornea depicting different layers showing stratified squamous epithelium, stroma and the endothelium

1.2.3 Layers of cornea

1.2.3.1 Corneal epithelium

Corneal epithelium (constituting 10% of total corneal surface) provides a smooth transparent surface for accurate refraction of light and also acts as a physical barrier preventing the entry of external agents. It consists of 4 - 6 layers of non keratinized stratified cells which include superficial squamous cells, central supra basal cells and inner columnar cells.

The superficial squamous cells are post mitotic terminally differentiated cells with strong junctional complexes having a few organelles (Klyce, 1972). The junctional complexes between adjacent lateral cells have high electric resistance and low permeability and acts as a protective barrier (Boulton & Albon, 2004). They have microvilli like structures and a buffy coat on their surface. The microvilli help to increase the surface area and buffy coat is responsible for the adherence of tear film to the corneal surface. The corneal epithelial turn over and renewal takes place

in every seven days, by the stem cells residing in limbus known as limbal stem cells (LSCs).

Suprabasal cells or wing cells are polyhedral cells, representing a transitional stage in between differentiation of basal cells to squamous epithelial cells. They divide and migrate superficially to differentiate into squamous epithelial cells. These cells express structural components like tonofilaments which helps in maintaining their polyhedral shape.

Basal columnar cells are progenitor cells that give rise to new suprabasal cells and also secrete matrix factors important for basement membrane and stromal function. These cells play a key role in meditating cell migration during epithelial injury, by regulating organization of hemidesmosomes and local complexes that help in the attachment of cell to underlying basement membrane (Pajoohesh & Stepp, 2005).

The corneal epithelium exists in a state of dynamic equilibrium, with constant renewal of epithelium by limbal stem cells. This heterogeneous population of cells exhibit characteristic molecular markers which could be used as clues in characterization. The peripheral and superficial layers contain more differentiated cells and are less proliferative in nature. Characteristic corneal epithelial cell markers are listed below.

Cytokeratins (CK) are subfamily of intermediate filament proteins that are expressed in epithelial tissues. There are at least 20 different types of cytokeratins. Based on the isoelectric pH, cytokeratins can be divided into two subgroups - type I and type II. Type I/ acidic cytokeratins comprises of keratins (K) 9 to 20, while type II comprises of basic to neutral cytokeratins from keratins 1 to 8. Different types of keratins are expressed in combination in different types of epithelial cells and are used to characterize the type of epithelium. Different types of cytokeratins expressed in cornea are given in Table 1.

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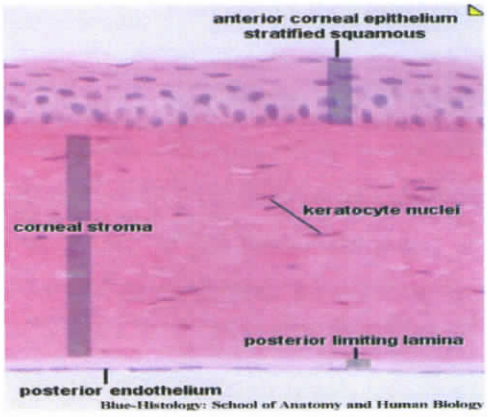

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Table 1 Cytokeratin expression in the cornea (Barnard, 2005)

Keratins	MolecularWeight (KDa)	Acidic/ Basic	Pair	Association
Keratin 3	64	Basic	K 12	Corneal Epithelium
Keratin 5	58	Basic	K 14	Basal Corneal Population
Keratin 6	56	Basic	K 16	Hyperproliferative Population
Keratin 12	55	Acidic	K 3	Corneal Epithelium
Keratin 14	50	Acidic	K 5	Basal Corneal Population
Keratin 16	48	Acidic	K 6	Hyperproliferative Population
Keratin 19	40	Acidic	None	Basal Limbal Epithelium

Connexins (Cx) are gap junctional proteins found in specialized cell surface membrane structures that directly connect the cytoplasm's of adjacent cells and provide direct pathway for sharing ions, nutrients and small metabolites between adjacent cells to maintain homeostasis in tissue. They are building blocks of gap junctions. Currently more than 20 related connexin isoforms are identified in human and mice (Chen *et al.,* 2006). Cx43 has been found to be expressed in a number of epithelial tissues like follicular epidermis, interfollicular epidermis and in corneal epithelium of mice and human and serves as a negative marker for stem cell containing population of limbal epithelial cells implicating that its expression is acquired during the process of TAC differentiation.

1.2.3.2 Bowman's membrane (anterior limiting membrane)

Bowman's membrane is an acellular region constituting mainly of collagen and proteoglycans which acts as a separating layer between epithelium and underlying stroma. It protects stromal layers from injury by preventing the penetration of external agents.

1.2.3.3 Stroma (substantia propria)

Stroma lying beneath Bowman's membrane makes major portion of cornea. It consists of stromal fibroblast cells (stromal keratocytes) and collagen fibrils that provide toughness to cornea and help in maintaining ocular pressure. Stromal fibroblasts regulate the synthesis and arrangement of collagen and proteoglycans. Homogeneous arrangement of collagen fibrils is essential for corneal transparency.

The stromal fibroblasts are also transparent and clear due to the presence of corneal crystallins in their cytoplasm (Fini & Stramer, 2005).

The fibroblast cells of limbal stroma are called limbal fibroblasts and are the major cellular components of limbal stroma. Limbal stem cells that are necessary to maintain corneal epithelial homeostasis resides over this limbal stroma. Limbal fibroblasts secrete various cytokines that can promote epithelial wound healing. Stromal niche is believed to modulate the phenotype of overlying epithelium through soluble factors and direct contact.

These cells express proteins like SPARC, vimentin, Fibroblast Surface Antigen (SFA), Serine protease Tissue Inhibitor of Metalloproteinases-2, Human Collagen alpha2(I) chain precursor, 5,10-methylenetetrahydrofolate reductase etc (Shimmura *et al.,* 2006). Vimentin is a type III intermediate filament protein normally expressed in cells of mesodermal origin (Gilles *et al.,* 1999). It participates in cell adhesion, migration, survival and cell signaling in actively proliferating cells and gives mechanostructural integrity for quiescent cells. Secreted Protein Acidic and Rich in Cysteine (SPARC) is 43 kDa protein preferentially secreted by limbal fibroblast cells. It is also known as osteonectin / BM 40 and is expressed by corneal fibroblast cells and are actively involved in the intracellular adhesion of basal limbal epithelial cells (Shimmura *et al.,* 2006), wound healing (Latvala *et al.,* 1996 & Berryhill, 2003), growth and adhesion of vascular endothelial cells (Sweetwyne *et al.,* 2004) and maintaining lens transparency.

1.2.3.4 Descemet's membrane (Posterior limiting membrane)

Descemet's membrane is a thick basement membrane of corneal endothelium. It is a strong sheet of tissue serving as a protective barrier against infections and injuries and is regenerated rapidly. It is mainly composed of proteins like collagen type IV, VIII and proteoglycan (Pajoohesh & Stepp, 2005).

1.2.3.5 Endothelium

Endothelium consists of a single layer of cuboidal cells that are actively involved in regulating fluid balance. In normal healthy eye a homeostatic balance is maintained between the inflow and outflow of fluid (water) with in corneal stroma, by endothelial cells. Without the pumping out action of endothelium, the corneal stroma will swell, become hazy and opaque. Extensive endothelial damage by

disease or trauma usually results in corneal edema and blindness due to its poor regeneration potential. Whole corneal transplantation is the only available therapy in the case of endothelial damage.

1.2.4 Regeneration of cornea

Homeostasis of corneal epithelium is brought about by limbal stem cells residing at the corneo-scleral rim called limbus (Limb & Daniels, 2008). These unipotent stem cells are generally quiescent but when induced, they proliferate by asymmetric division and give rise to stem cells and transient amplifying cells (TACs). These TACs proliferate, undergoes radial migration and differentiation in response to cell loss to repopulate corneal epithelium. According to the reports by Daniel *et al.,* (2001) the first evidence of the presence of stem cells in limbus was given by Davanger and Evanson (1971) where they observed pigmented epithelial migratory lines moving from the limbal area towards the central cornea during wound healing process and suggested that limbal area could be a reservoir of new epithelial stem cells. The second evidence of the presence of stem cells in the limbal epithelium was provided by Cotserilis *et al.,* (1989) who detected label retaining cells at the basal layer of limbal epithelium by labeling with ^{3}H-Thymidine. *In vitro* studies using cells from cornea and limbus showed that the cells from corneal region could be cultured only for a few generations and yielded paraclones whereas the cells from limbal area could proliferate for many generations and often formed large holoclones suggesting the presence of stem cells in the limbal region of the eye. *In vivo* experiments showed that damage to limbal region of eye resulted in insufficient reepithelialization and conjunctival invasion to corneal surface. Clinical studies have shown long term restoration of corneal surface by limbal transplantation, further suggesting the presence of stem cells in the limbus (Daniels *et al.,* 2001). The limbal stem cells reside in the basal layer of limbal epithelium and are interspersed with early TACs. The process of cell differentiation occurs when cells migrate towards the epithelial surface. As a result, from limbus towards corneal epithelium, a gradation of stem cells, TACs and differentiated corneal epithelial cells can be seen (Figure 3). LSC are generally characterized by its small size, high nuclear to cytoplasmic ratio, label retaining potential and slow recycling nature (Arpitha *et al.,* 2008). Many molecules have been suggested to identify LSCs and

underscore the importance of seeking an autologous cell source other than LSCs for cell therapy of corneal disorders especially in case of bilateral deficiency.

1.2 Review of literature

1.2.1 Eye

Eye is an important sensory organ, the origin of which can be traced from the Cambrian era of evolution. Nature has well protected the eye ball by covering it with fatty layer and placed in a bony cavity in skull. It consists of multiple layers and can be broadly divided into two segments – anterior (specialized in light perception) and posterior (specialized in light sensing). Inner portions between the anterior and posterior regions are filled with transparent jelly like fluid named aqueous and vitreous humor respectively (Figure 1).

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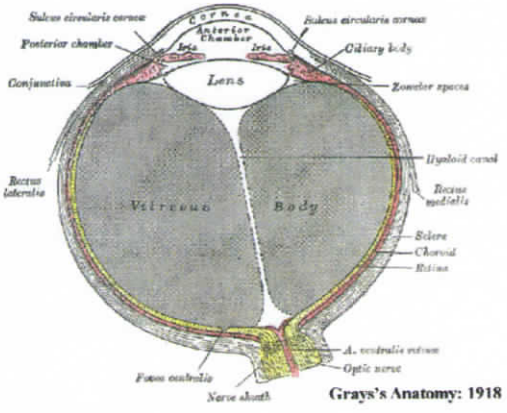

Figure 1 Structure of Eye depicting different parts

1.2.2 The Cornea

Cornea is the transparent anterior window of eye covering iris and pupil and has a major role in vision. It provides a clear transparent path for light and contributes to 70% of refractive power of eye (Karring *et al.*, 2004). It protects eye from external environment and also filter out harmful UV rays. For providing a clear transparent

usually a combination of such markers is used in identifying stem cells in the limbal epithelium. The limbal basal epithelium contains the least differentiated cells and exhibit proliferative potential similar to stem cells. The positive markers for LSCs include vimentin, p63α and Paxillin 6 (PAX-6) and negative markers include CK3, CK12, and Connexin 43 (Cx43) (Kevin *et al.*, 2006).

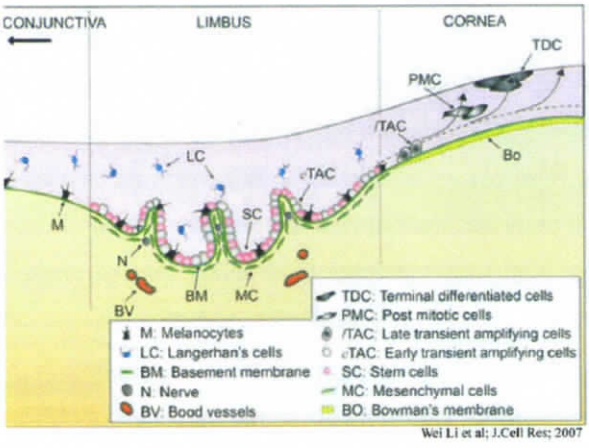

Figure 3 Hypothetical scheme of limbus and its stem cell niche.

p63 is a transcription factor that shares homology with p53, a tumor suppressing transcription factor found mutated often in cancerous tissue (Barnard, 2005). p63 plays a major role in morphogenesis and differentiation of stratified epithelial structures and is required in ectodermal differentiation, proliferation and maintenance of stratified epithelial cells (Pellegrini, 2001). In *in vivo* conditions p63 is found to be expressed in limbal basal epithelium where as in *in vitro* culture it is found to be expressed in limbal epithelial cell derived holoclones with little or no expression in meroclones and paraclones. p63 has been proposed as a putative positive Limbal epithelial stem cell (LESC) marker, however a number of reports have suggested that p63 is not sufficiently specific to act as an LESC marker as it has also been found to be expressed by basal cells of peripheral and central cornea in humans and rats (Chen *et al.*, 2004).

Two isoforms of p63 have been identified, TAp63 and Np63. The roles of these isoforms are not clearly defined, the former is said to be involved in the maintenance of limbal keratinocyte during lesser stages of differentiation and the expression is found to be highest in the basal layers of limbus whereas the latter is

involved in cell cycle control and determines the proliferating activity of limbal keratocytes. Significant increase in the expression of this gene is reported in the peripheral corneal layers (Wang *et al.,* 2005).

ABCG2 or Breast Cancer Resistance Protein 1 (BRCP-1) is a member of ATP binding cassette (ABC) family of cell surface transporter proteins, which include more than 50 members that mediate transfer of diverse array of substrate across the cellular membrane. The principal function of these transporter proteins is to protect the cell from damage by toxins and drugs. Even though ABCG2 expression occurs in variety of normal tissues, its expression is relatively limited to primitive stem cells (Balasubramanian *et al.,* 2008). This protein is immunolocalized to the cell membrane and cytoplasm of some human limbal basal epithelial cells, but not in most limbal suprabasal cells and corneal epithelial cells (Chen *et al.,* 2004). ABCG2-positive cells possess LSC-like characteristics such as higher colony-forming efficiency than ABCG2-negative cells and greater expression in primary cultures of human limbal compared with corneal epithelium (Kevin *et al.,* 2006). De Paiva *et al,* (2005) identified these transporter proteins in clonogenic population of human limbal epithelial cells. This marker is also expressed by bone marrow mesenchymal stem cells.

1.2.5 Limbal stem cell deficiency

The etiology of limbal stem cell deficieny can be related to an insufficient stem cell function or deficiency due to hereditary or acquired reasons. Inherited/ heriditary disorders include aniridia keratitis and keratitis associated with multiple endocrine deficiencies, in which limbal stem cells may be congenitally absent or dysfunctional. Acquired conditions include Stevens Johnson syndrome, chemical injuries, ocular cicatricial pemphigoid, contact lens-induced keratopathy, multiple surgeries, cryotherapies to the limbal region, neurotrophic keratopathy and peripheral ulcerative keratitis. Most commonly the LSCD is often related with external or acquired factors that destroy the limbal stem cells. Majority of cases seen clinically is due to acquired disorders. LSCD is usually manifested by vascularization and chronic inflammation of the cornea, ingrowth of fibrous tissue, and corneal opacification (Fang *et al.,* 2000). Other symptoms include reduced vision, photophobia, recurrent pain, melting and perforation of cornea (Dua *et al.,* 2000).

Based on etiology, LSCD can be broadly classified into two categories – category I or secondary LSCD caused by mechanical or chemical trauma and category II or primary LSCD resulting from gradual loss of stem cell functions over time, probably due to malfunctioning of limbal stroma (Puangsricharern & Tseng, 1995). LSCD may be localized (partial) or diffused (complete). In localized LSCD, some sectors of the limbal and corneal epithelium are normal, and conjunctivalisation is restricted to the regions devoid of healthy epithelium. In total deficiency cases, the entire corneal surface is affected. The diagnosis of the presence of limbal deficiency is crucial in selecting treatment methodologies as these patients are poor candidates for conventional corneal transplantation alone. Moreover conventional corneal transplantation cannot address the problems of inadequate epithelial replacement and subsequent conjunctival in growth, vascularization and inflammation, which ultimately result in graft rejection and failure.

1.2.5.1 Treatment for LSCD

LSCD can arise in two ways - partial LSCD and total LSCD. Treatment is dependent on extent of deficiency.

In the case of partial LSCD if the visual axis gets opaque, then sequential sector conjunctival epithelialectomy is the mode of treatment (Diaz Valle *et al.,* 2007) which involves debridement of conjunctival epithelium to improve vision by enhancing corneal epithelialisation. It is also treated together with amniotic membrane transplantation for increased efficiency. Transplantation of amniotic membrane to superficial keratectomised cornea is another promising approach to prevent recurrent epithelial erosion and visual rehabilitation (Anderson *et al.,* 2001) (Sangwan *et al.,* 2004). An alternative for treating partial LSCD is ipsilateral limbal translocation which involves the translocation of healthy region of limbus to affected area without involving the contra lateral eye. This helps in repopulation of limbal cells and thus enhancing normal healing (Cristina *et al.,* 2001).

Treatment for total LSCD is dependent on whether the LSCD is unilateral or bilateral. In case of unilateral, autograft is preferred and for bilateral, allograft is the only option. Autograft technique involves harvesting sufficient amount of healthy limbal tissue from contralateral eye and transplantation to injured eye (Kenyon &

Tseng, 1989). Use of autograft favours rapid surface healing, increased visual acuity and regression of all symptoms of LSCD (Kenyon, 1989). Autografts can be used in the treatment of secondary LSCD and also for pterygia (Shimazaki, 1996). The uses of autografts are often safe with respect to graft rejection and immunosuppression, but there is a chance for developing surgically induced LSCD in normal contra lateral eye.

During bilateral deficiencies where both eyes are involved, allograft transplantation is the only alternative. Healthy limbal tissue is taken by superficial lamellar dissection after central cornea trephining (Dua & Blanco, 1999) from cadaveric eye or HLA matched donors (Daya & Illari, 2001) and transplanted to the diseased eye. Such transplantation requires long term immunosuppressive therapies and involves a high risk of graft rejection. Efficiency is found to be increased by the use of tissue from HLA matched individuals together with amniotic membrane transplantation (Gomes *et al.,* 2003 & Tseng *et al.,* 1998). However the failure rate is much high compared to use of autografts (Solomon *et al.,* 2002 & Ilari & Daya, 2002). The availability of donor corneas also limits above methods of treatment. Alternate approach to overcome such problems is the use of tissue engineering methodologies to reconstruct damaged cornea to restore vision.

1.2.6 Tissue engineering approach

"Tissue engineering (TE) is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function"(Langer & Vacanti,1993). This field is being broadened by the scientists with the scope of overcoming donor organ shortages. Four major components of tissue engineering identified are cells, matrix, culture conditions and growth factors (Fischer *et al.,* 2007). The application of stem cell and TE in ophthalmology field provide an alternative branch for the ocular surface regeneration.

Various techniques using different cell types with or without scaffold have been evolved for the effective ocular surface reconstruction.

1.2.6.1 Cell choices for ocular surface reconstruction

The limbus of the eye, located at the corneo scleral junction, is the major cell source used for ocular surface reconstruction in patients with LSCD (Polissetty *et*

al., 2008). Both allogenic and autogenic cells are used depending upon the type of deficiency. The limited lifespan and quick differentiation of primary limbal stem cells greatly hinder the progress studies of corneal epithelial cells.

In order to overcome these problems there is a requirement for the search of alternative autologous sources for ocular surface reconstruction. There have been reports of differentiation of embryonic stem cells into corneal epithelial cells *in vitro.* Murine embryonic stem cells, when co-cultured with rabbit limbal corneal epithelial cells in transwell system differentiated into epithelial like cells and express the corneal epithelial cell specific marker cytokeratin 3 /12 (Yu *et al.,* 2001).Culturing of hESC on collagen IV in limbal fibroblast conditioned medium resulted in the loss of pluripotency and differentiation into epithelial-like cells (Ahmad *et al.,* 2007).

Another report suggests that skin stem cells expanded *ex vivo* were effective in restoring a normal corneal surface in the rabbit model of total limbal stem cells deficiency, suggesting their suitability as an alternative for constructing autogenous bioengineered corneas (Huang *et al.,* 2004)

Researchers also suggest that adult hair follicular epithelial stem cells are capable of differentiating into corneal epithelial-like cells *in vitro* when exposed to a limbus- specific microenvironment using extra cellular matrix components and fibroblast conditioned media which supports differentiation. Therefore, the hair follicle may be an easily accessible alternative therapeutic source of autologous adult stem cells for replacement of the corneal epithelium and restoration of visual function in patients with ocular surface disorders (Blazejewska *et al.,* 2009).

Oral mucosal cells has been used as an alternative by Nishida *et al.,*(2004) where a suture less transplantation of carrier-free cell sheets composed of autologous oral mucosal epithelial cells was used to reconstruct corneal surface which restored vision in patients with bilateral severe disorders of the ocular surface.

Mesenchymal stem cells (MSC) from bone marrow were also reported to be used for the reconstruction of corneal surface (Ma *et al.,* 2006) due to their pluripotent nature and expansion potential. Recent reports suggest the use of MSCs to promote wound healing and reconstruction of cornea (Ye *et al.,* 2006). More over the presence

of marrow specific cells in the corneal stroma (Satoru *et al.,* 2006 & Choong *et al.,* 2007), expression of markers such as ABCG2 pertaining to limbal epithelial stem cells (Cintia *et al.,*2005), their potential to differentiate into a variety of cell lineages (Alhadlaq & Mao,2004) including those of epithelial lineage (Sueblinvong *et al.,* 2008) indicates the suitability of using mesenchymal stem cell as an ideal candidate and as an alternate autologous source for ocular surface regeneration in treatment of bilateral limbal stem cell deficiency. Data are rare about the feasibility of MSCs differentiation into corneal epithelial cells, and there is still great controversy regarding the plasticity of MSCs.

1.2.7 Mesenchymal stem cells

Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types. They represent only about 1 in 10,000 nucleated cells in the bone marrow and can expand many folds under *in vitro* conditions. Properties like multipotency, high proliferation rate, immunosuppressive effect and migratory potential to the site of injury make these cells potentially ideal candidates for tissue engineering.

The perception of the presence of non haematopoietic cells with fibroblast morphology in bone marrow was first suggested by the German pathologist Julius Cohnheim (Prockop, 1997). His work raised the possibility that bone marrow may be the source of fibroblast like cells that deposit collagen fibers as a part of normal process of wound repair. Later Friedenstein *et al.,* (1974) reported a rare cell population in bone marrow that developed into colony forming units, exhibiting fibroblast morphology (CFU-F) and plastic adherence.The adherent cells were found to be heterogeneous in nature. Friedenstein's observations were further confirmed by other groups throughout the 1980s (Ashton *et al.,*1980), and it was established that the cells isolated by Friedenstein's method were multipotent and could differentiate into multiple lineages such as those of osteoblasts, chondrocytes, adipocytes, and myoblasts (Castro-Malaspina *et al.,*1986 & Polisetty *et al.,*2008).

Currently these cells are referred to as either mesenchymal stem cells (MSCs), based on their ability to differentiate into mesoderm lineages, or as marrow stromal cells, because they appear to arise from the complex array of supporting structures found in the marrow.

1.2.7.1 Sources and tissue distribution

It has been suggested that cells with mesenchymal stem cell characteristics reside in virtually all postnatal organs and tissues. MSCs have been reported to be isolated and cultured from many species including mice, rats, cats, dogs, rabbits, pigs, and baboons, *albeit* with varying success. The most potential and characterized source of MSC is the bone marrow. In addition to bone marrow, MSC-like cells have been shown to be present in a number of other adult tissues like adipose tissue, trabecular bone, periosteum, vascular wall, synovium and during fetal stage in liver, pancreas, heart, dermis, lung and spleen. The other available sources include umbilical cord blood, placenta and amniotic fluid (Polisetty *et al.,* 2008).

1.2.7.2 Isolation techniques

Regardless of substantial attention to MSCs, there are no universally accepted protocols for their isolation. Initial approaches for the isolation were based on the plastic adherence developed by Friedenstein *et al,* (1974) and still remains as the standard procedure for isolation. This technique involves culturing of bone marrow mononuclear cells *in vitro* on tissue culture plastic surfaces developing a population of fibroblastic cells. However, these cultures exemplify a heterogeneous mixture of cells with varied morphology, cell proliferation rate, histochemical properties and differentiation potential.

In order to obtain a homogenous population of cells, a ficoll or percoll density gradient centrifugation method was suggested (Kolf *et al.,* 2007). The majority of modern culture techniques still take a CFU-F (Colony forming unit fibroblast) approach, where raw unpurified bone marrow or ficoll-purified bone marrow monocytes are plated directly into cell culture plates or culture flasks.

Other isolation procedures include cell isolation based on membrane sensitivity of MSCs and hematopoietic cells to adenosine triphosphate (ATP) ions, size dependent sieving through a porous membrane, sorting of cells by magnetic properties (MACS), flow cytometry, or surface marker expression (FACS) such as STRO-1 (Alhadlaq & Mao, 2004) .

1.2.7.3 *In vitro* growth and expansion of MSC

Under *in vitro* culture conditions, MSCs are known to form a monolayer of adherent cells with fibroblast - like morphology with an initial lag phase of about 3 - 4 days followed by a rapid exponential growth phase and ends with deceleration. Population doubling time depends up on the donor and the initial plating density (Payushina *et al.,* 2006).

1.2.7.4 Characteristics

Phenotypically, MSCs express a number of markers, none of which are specific to MSCs. In humans, MSCs are positive to CD105, CD73, CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM-1]), CD166 (activated leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1 and CD29 and are negative for hematopoietic markers CD45, CD34, CD14 or CD11 as well as co stimulatory molecules CD80, CD86, CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function-associated antigen-1 [LFA-1]), or CD56 (neuronal cell adhesion molecule-1).

MSCs from other species do not express all the same molecules as those on human cells. Although human and rat MSCs have been shown to be CD34 negative, some papers report variable expression of CD34 on murine MSCs (Peister *et al.,* 2004). It is generally accepted that all MSCs are devoid of the hematopoietic marker CD45 and the endothelial cell marker CD31. The differences in cell surface expression of many markers may be influenced by factors secreted by accessory cells in the initial passage. The *in vitro* expression of some markers by MSCs does not always correlate with their expression patterns *in vivo*. There is also variable expression of many of the markers mentioned due to variation in tissue source, the method of isolation and culture, and species differences. Table 2 represents a comparison of cell surface epitopes of MSCs between rabbit MSCs and rodent / human MSCs.

Table 2 Cell surface epitopes of mesenchymal stem cells (Lapi *et al.*, 2008)

Cell surface epitope	Rabbit MSC	Rodent/human MSC
CD29	+	+
CD44	+	+
CD45	-	-
CD90	-	+/-
CD105	+	+
MHC I	-	+/-
MHC II	-	-
Vimentin	+	+
Cytokeratin	-	-

1.2.7.5 Therapeutic Applications

MSCs are of intense therapeutic interest because they represent a population of cells with the potential to treat a wide range of acute and degenerative diseases. MSCs are advantageous over other stem cell types as they are readily available, relative ease of expansion in culture, do not involve any ethical issues as that in embryonic stem cell research, are immuno-privileged, can be differentiated into multiple cell lineages and therefore suitable for allogenic transplantation, reducing the risks of rejection and complications of transplantation. Recently, there have also been significant advances in the use of autologous mesenchymal stem cells to regenerate human tissues, including those of cartilage, meniscus, tendons etc (Gao *et al.*, 2001).

However, compared with *in vitro* characterization, there is less information on the *in vivo* behavior of MSCs. Some reports showed that MSCs when transplanted into fetal or neonatal animals or engrafted underwent site-specific differentiation to contribute to many different tissues such as chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma without provoking an immune response (Liechty *et al.*, 2000). Many studies have also investigated the use of MSCs for gene therapy, including transplantation of MSCs transfected with vascular endothelial growth factor for the improvement of heart function after myocardial infarction in rats (Barbash *et al.*, 2003 & Orlic, 2001), interferon-β delivery into tumors in mice, MSCs expressing BMPs to promote bone formation (Horwitz *et al.*, 1999). There is much evidence to support the theory that MSCs can home to tissues, particularly when injured or inflamed, involving migration across endothelial cell layer (Gao *et al.*, 2001). The mechanism by which MSCs home to

tissues and migrate across endothelium is not yet fully understood, but it is likely that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury, as is the case with recruitment of leukocytes to sites of inflammation. Chemokine receptors and their chemokine ligands are essential components involved in the migration of leukocytes into sites of inflammation, and it has recently been shown that MSCs also express some of these molecules.

1.2.7.6 Differentiation

MSCs have a large capacity for self renewal while maintaining their multipotency. The standard test to confirm multipotency is differentiation of the cells into osteoblasts, adipocytes and chondrocytes as well as myocytes and possibly neuron-like cells. However, the degree to which the culture will differentiate varies among individuals and how differentiation is induced *in vitro*. MSCs can be directed toward extra-mesenchymal lineages also.

1.2.7.6.1 Directed Differentiation of MSCs towards intra-mesenchymal lineages

Ever since scientists Ernest McCulloch and James Till first revealed the clonal nature of marrow-derived mesenchymal cells in the 1960s, it has been understood that MSCs are characterized by plasticity and their fate can be determined by environmental cues. They discovered that culturing marrow stromal cells in the presence of osteogenic stimuli such as ascorbic acid, inorganic phosphate, and dexamethasone could promote their differentiation into osteoblasts. Alternatively, the addition of transforming growth factor-beta (TGF-β) could induce chondrogenic markers. Myocyte and adipocyte differentiation can be similarly induced. More recently, it has also been demonstrated that MSCs have the potential to differentiate into beta-pancreatic islets cells.

1.2.7.6.2 Directed Differentiation of MSCs towards extra-mesenchymal lineages

Directed differentiation of autologous MSCs towards extra-mesenchymal lineages is also an interesting area of stem cell biology, with the potential to repair tissues where resident stem cells are not accessible, such as the brain. Transdifferentiation - the conversion of adult stem cells from one lineage to another - is a hotly contested concept, as it challenges the dogma that cells are progressively restricted in their lineage during development. It has been suggested that MSCs may

have the capacity to transdifferentiate into neuronal cells. However, some members of the research community believe that experimentation in this area is still inconclusive. Much basic research is needed in this area, as these mechanisms are not well understood.

An *in vivo* study suggested that systemically transplanted MSCs can engraft to an injured cornea and promote wound healing by differentiation, proliferation, and synergizing with hematopoietic stem cells (Ye *et al.,* 2006). Transplantation of human mesenchymal stem cells (MSCs) could reconstruct the corneal damage and grafted MSCs differentiate into corneal epithelial cells provides the first line of evidence that MSCs can be used for reconstruction of damaged corneas, presenting a new source for autotransplantation in the treatment of corneal disorders (Ma *et al.,* 2006). Another *in vivo* report suggested that transplantation of human mesenchymal stem cells (hMSCs) could reconstruct the damaged cornea. The therapeutic effect of the MSC transplantation may be associated with the inhibition of inflammation and angiogenesis (Wu *et al.,* 2007). Recent reports suggest that MSC might be able to maintain their stem cell-like character or to transdifferentiate to putative epithelial progenitor cells with regard to the results of ABCG2, β1-integrin and connexin 43. The findings of this *in vivo* study were first step towards the utilization of MSC as an autologous source for corneal epithelial regeneration without immunogenic risk (Reinshagen *et al.,* 2009).

Data are scarce about *in vitro* studies regarding the transdifferentiation of MSCs. Gu *et al.,* (2009) has showed that rabbit MSCs can differentiate into corneal epithelial cells both *in vivo* and *ex vivo*. A very recent study gives evidence that MSCs induced by corneal stromal cells can transdifferentiate into corneal epithelial cells *in vitro*. The data provides evidence that induced MSCs on an amniotic membrane have remarkable effects on the treatment of corneal alkali burn and the reconstruction of the corneal surface of rats (Jiang *et al.,* 2010).

Differentiated MSCs to corneal lineage will provide an alternate autologous cell source against LSCs and will be a major solution for patients suffering from bilateral deficiency.

1.3 Hypothesis

Presence of marrow specific cells in cornea, expression of specific markers such as ABCG2, potential to differentiate into a multiple cell lineages, including those of epithelial lineage provides the foundation for the present study, presuming that Mesenchymal stem cells can be transdifferentiated into corneal epithelial like cells by simulating corneal epithelial niche factors *in vitro*.

1.3.1 Objectives of the study

1. Isolation and maintenance of mesenchymal stem cells.
2. Optimization of culture conditions for mesenchymal stem cell culture and characterization.
3. Isolation, maintenance and characterization of limbal fibroblast cells
4. Simulation of niche factors for transdifferentiation of mesenchymal stem cells to corneo-epithelial lineage using collagen as substrate and limbal fibroblast conditioned medium.
5. Monitoring of differentiation status.

Materials & Methods

1X antibiotic, and incubated at 37 °C, 5% CO_2, 95% humidity in a CO_2 incubator (Sanyo, MCO18AIC) in 25 cm^2 culture flasks for 21 days. Medium change was performed on every 3rd day there after and cells were maintained till it reached 80% confluency and then sub cultured.

2.1.2.2 Selection of medium for mesenchymal stem cell culture

To analyze the effect of medium on cell growth and proliferation three different media were used. Approximately 2 x 10^3 cells/ cm^2 were seeded and cultured in different commercially available mediums; α-MEM, DMEM and DMEM-LG in 12 well plates under controlled conditions in a CO_2 incubator. The cell adhesion and proliferation were assessed by phase contrast microscopy and MTT assay respectively at specific time periods.

2.1.2.2.1 Cell adhesion

The cell adhesion of cultured MSCs was monitored using inverted phase contrast microscope (Leica, Germany) at the end of 24h and 72h of culture. The images were acquired using DC180 camera attached to Leica inverted phase contrast microscope.

2.1.2.2.2 Cell proliferation

The cell proliferation of cultured MSCs was monitored by evaluating the cellular activity by MTT assay at the end of 24 h and 72 h.

MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase of the cells to an insoluble, coloured (dark purple) formazan product which are then solubilised with isopropanol and measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the cell proliferation.

After removing the culture medium from 12 well plate, cells were incubated with serum free medium containing 0.5 mg/ml MTT reagent for a minimum of 4 h at 37°C. MTT reagent was removed, and the cells were rinsed with PBS and incubated with equal amounts of isopropanol for 20 min with continuous shaking. The cell lysate were transferred to a 96 well plate and absorbance was measured in a multiwell plate reader (Biotek PowerWave XS, USA) at 570 nm. The results were expressed as mean ± standard deviation (n=21) and statistical statistical significance was tested using

students t test with graph pad software. For all analyses, differences were considered significant when $p<0.05$.

2.1.2.3 Characterization of Mesenchymal Stem Cells

The medium found optimum for cell proliferation was used for further cell culture purpose. The cells grown under optimized conditions were characterized and analyzed for the maintenance of stemness by immunocytochemistry and flow cytometry.

2.1.2.3.1 Immunocytochemistry

Immunocytochemistry is a technique which uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. For immunostaining purpose the cells were grown on a processed cover slip, stained and examined by confocal microscope. Cells were analyzed for the expression of markers specific to mesenchymal lineage like vimentin, CD105, ABCG2 and negative marker CD90.

The cells were seeded at a density of 1 x 104 cells / cm_2 to coverslips and cultured for 48 h. After removing the culture medium, the cells were washed thrice with PBS and fixed in 3.7% paraformaldehyde for 30 min to 1h at room temperature or overnight at 4 °C. The fixed cells were washed thrice with PBS for 5 min and permeabilized with 0.1% Triton X-100 for 5 min. Permeabilized cells were then washed thrice with PBS and incubated with 1% BSA for 10 min to block nonspecific binding. 10µl of Primary antibody (1:100) as depicted in Table 3, was transferred to a parafilm and the cover slips with cells were kept cell side down and incubated for 1h in a humidified chamber. After incubation the cells were rinsed thrice with PBS and incubated with 10µl of anti mouse FITC (1:100) secondary antibody as applicable for 1h in a humidified chamber. The cells were rinsed again with PBS and counter stained with Propidium Iodide (1: 100) (0.05µg/ml) for 2 min and washed thrice with PBS for 5 min. The cells on cover slips were mounted using fluorescent mounting medium and were observed under Laser Scanning Confocal Microscope (LSM510 META, Carl Zeiss, Germany) using argon laser for getting an excitation wavelength of 488 nm for FITC and HeNe 543 for getting an excitation wavelength of 536 nm for PI. Fluorescence emission was detected using a band pass filter of width 530 - 545 nm and long pass filter of width 610 nm respectively.

Table 3 Antibodies used for immnunostaining/ FACS for mesenchymal stem cell characterization

Primary antibody	**Characteristic protein**
Monoclonal mouse anti cow vimentin clone (Vim 3B4)	Intermediate filaments, vimentin
MsX Hu integrin β1 Flour	Integrin
FITC mouse anti human CD 90	Thymine 1
ABCG2 (BRCP1) purified mouse anti human monoclonal antibody	Transmembrane protein
Endoglin rat monoclonal IgG_{2a} CD 105	TGFβ receptor

2.1.2.3.2 Flow cytometric analysis

Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells within heterogeneous populations. Flow cytometers are used in a range of applications from immunophenotyping to cell counting.

For quantitative immunophenotyping of rabbit MSCs, flow cytometry was performed. Cells cultured on 25 cm^2 culture flask were harvested by adding 1X - trypsin-EDTA. The cells were gently pipetted until all the cells were detached from the surface and made as single cell suspension by passing through cell strainer of pore size 70 μm. Cells were then fixed with 3.7% paraformaldehyde for 30 min and pelletted by centrifuging at 600g for 10 min and then washed with PBS. The cells were then permeabilized with 0.1% triton X 100 in PBS for 5 min and pelleted. Nonspecific binding was prevented by incubating the cells in 1% BSA for 10 min and centrifuged at 600 g for 10 min. Cells were then incubated with the primary antibody (1:100) (1ng/μl) (Table 3) for 45 min at room temperature. After washing twice with PBS, the cells were incubated with appropriate secondary antibody as applicable (1:100) for 45 min. The cells were washed and the pellet was resuspended to a final volume of 300μl with PBS. FACS analysis was performed with flow cytometer (FACS Aria, BD USA) and analyzed by BD FACS Diva software.

2.2 Isolation maintenance and characterization of limbal fibroblast cells

2.2.1 Materials

Minimum Essential Medium,Alpha (α-MEM), (Invitrogen, USA), Fetal Bovine Serum (FBS), (Lonza, USA), Penicillin/Streptomycin 100X, Trypsin- EDTA 10X (PAA laboratories, Germany), Collagenase type I, Bovine serum albumin, Triton-X-100, MTT reagent, Propidium iodide, Anti mouse IgG (whole molecule) – FITC antibody (Sigma Aldrich, Germany), Monoclonal mouse anti cow vimentin clone Vim 3B4, Fluorescent Mounting Medium (Dako cytomation, USA), Osteonectin / SPARC (QED bioscience, USA) Paraformaldehyde (PFA), Chloroform, Ethanol (99.9%), IsoPropanol (MERCK, USA), TRIsoln reagent, M-MuLV RT-PCR kit, GeNei Red Dye PCR kit, Agarose low EEO, 50X TAE buffer, Ethidium Bromide, (Bangalore Genei, India), 25 cm^2 culture flasks, 60 x 15 mm TCPS, 12 well plate, 96 well plate, (Cell Star), Cell strainer (BD Bioscience)

2.2.2 Methods

2.2.2.1 Isolation and culture of limbal fibroblast cells

For isolation and culture of limbal fibroblast cells rabbit eye balls were used. The samples were collected from animals euthanized for other experimental purpose after obtaining prior permission from Institute Animal Ethics Committee.

Briefly the corneas were enucleated and collected in 0.1M PBS containing 1X antibiotic (Penicilln: streptomycin) and incubated for 10 min at room temperature. Excess tissues were dissected out and a 360° excision of limbus was performed. The dissected out limbal ring was cut into 1mm size explants and incubated for 2 h in collagenase- type I (1 mg/ml), at 37 °C in a CO_2 incubator and The explants were removed and digest was aspirated and transferred to 15ml tubes containing serum free α-MEM and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet was then resuspended in 1-2ml of complete α-MEM containing 10% FBS and transferred to 60 x 15 mm cell culture dishes and cultured till confluence under controlled conditions. The cell growth was monitored using phase contrast microscope and was characterized by immunostaining, flow cytometry and RT-PCR.

2.2.2.2 Preparation of conditioned medium

After reaching 50% confluency, the culture medium of limbal fibroblast cells was collected and processed. The harvested media designated as conditioned medium (CM) was centrifuged at 3000rpm for 15 min. The supernatant collected was filtered using 0.22 micron syringe filter, aliquoted and stored at -80 ^{o}C till use. For all differentiation experiments, CM was used.

2.2.2.3 Characterization of limbal fibroblast cells

The cultured limbal fibroblast cells were characterized by flow cytometry, immunostaining and RT – PCR for the presence of markers specific to fibroblast cells such as SPARC, CD 90 and vimentin.

2.2.2.3.1 Immunostaining

For immunostaining, cells were seeded at a density of 2.83 x 10^3 cells / cm^2 on to cover slips and incubated in α-MEM supplemented with 10% FBS for 48 h. At the end of 48h the cells were washed twice with 0.1 M PBS and analysed by immunostaining as per the protocol mentioned in section 2.1.2.3.1 for the expression of markers specific to fibroblast cells.

2.2.2.3.2 Flow cytometry

Limbal fibroblast cells were cultured till confluence under controlled conditions. Cells were trypsinized and made into single cell suspension and stained using markers specific for fibroblast cells like vimentin, SPARC and CD 90 as per the procedure mentioned in section 2.1.2.3.2.

2.2.2.3.3 Reverse transcription - Polymerase chain reaction (RT - PCR)

Reverse transcription polymerase chain reaction (RT - PCR) is a variant of polymerase chain reaction (PCR) commonly used to generate multiple copies of a DNA sequence. In RT - PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase and the resulting cDNA is amplified using traditional PCR using specific primers. The RT - PCR was done in 3 steps (a) RNA isolation (b) cDNA synthesis and (c) cDNA amplification using specific primers.

a) RNA Isolation

Total RNA was isolated from the cells maintained in culture for seven days. For RNA isolation, TRIsoln was used and isolation was done as per the instructions in

the product data sheet as follows. After removing the culture medium approximately 1 ml TRIsoln was added to lyse the cells. The lysate was collected in a sterile 2ml eppendorf tube and 200µl chloroform was added, mixed well and centrifuged at 11,000 rpm for 10 min at 4 °C. The aqueous phase was collected, transferred to another sterile eppendorf tube and equal volume of isopropanol was added and centrifuged at 11,000 rpm for 10 min. The pellet was then rinsed with 75% ethanol and centrifuged again at 7,500 rpm for 5 min. The RNA pellet was air dried, reconstituted in nuclease free water and quantified spectrophotometrically (BioPhotometer, Eppendorf).

b) cDNA Synthesis

cDNA was synthesized using M - MuLV RT - PCR kit as per the kit specifications in an Eppendorf thermalcycler (Eppendorf, USA).

For cDNA synthesis approximately 1000ng RNA was used. 1µl of oligo (dT) 18 primer was added and the reaction volume was made up to 10µl using nuclease free water. The reaction mixture was then incubated at 65° C for 10 minutes and 27° C for 2 min to remove secondary structures. After the initial incubation step 10µl of PCR reaction mix (Table 4) was added and the total reaction volume was made upto 20µl. The contents were then incubated at 37° C for 1h and 95° C for 2 min. The cDNA synthesized was stored at 4° C till use.

Table 4 M-MuLV PCR reaction mixture

Reagent	**Volume**
Rnase inhibitor	1µl
0.1 M DTT	1µl
RT Buffer(5X)	4 µl
30mM dNTP mix	2 µl
M-MuLV reverse transcriptase	0.5 µl
Nuclease free water	1µl

c) cDNA Amplification

The cDNA was amplified by PCR using GeNei Red Dye PCR kit according to kit instructions. The reaction mixture was prepared as depicted in Table 4 and PCR

amplification was performed using specific primer pairs designed from reported gene sequences for different markers given in Table 5 as per the cycling conditions given below

An initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 30s, specific annealing temperature as given in Table 6 for 30s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR reaction was set for 40 cycles. The PCR products were subjected to electrophoresis using 2% agarose gel containing 0.4 µg/ml ethidium bromide and imaged using a Fluorescent image analyzing system. (Fujifilm FLA 5100, Japan) and analyzed using Multi Guage version 3.0 software.

Table 5 PCR reaction mixture for cDNA amplification

Red Dye PCR Master Mix	8 µl
Sterile Water	9 µl
cDNA	2 µl
Forward Primers (100ng/µl)	0.5 µl
Reverse Primers (100ng/µl)	0.5 µl

Table 6 Primer sequence and annealing temperatures of genes analyzed

Gene	**Primer sequence**	**Annealing temperature (°C)**	**Product length (bp)**
Vimentin	FP-ATGCTTCTTTGGCACGTCTTGACCT RP-ACTGCACCTGTCTCCGGTATTCGTT	65	338
βActin	FP- ATCGTGATGGACTCCGGCGA RP-AGGAAGGAGGGCTGGAACAG	52	350

2.3 Simulation of corneal niche factors for differentiating mesenchymal stem cells to corneal lineage45

2.3.1 Materials

Limbal fibroblast conditioned media, Penicillin/Streptomycin 100X, Trypsin-EDTA 10X (PAA laboratories, Germany), Pure Col™ (Nutacon, Netherlands), Bovine serum albumin, Triton-X-100, MTT reagent, Propidium iodide, Anti mouse IgG (whole molecule) – FITC antibody (Sigma Aldrich, Germany), Minimum

Essential Medium Alpha (α-MEM) (Invitrogen, USA), Fetal Bovine Serum (FBS), (Lonza, USA) monoclonal mouse anti human cytokeratin clone AE1/AE3, Fluorescent Mounting Medium (Dako cytomation, USA), ABCG2 (BRCP1) purified mouse anti human monoclonal antibody (BD pharmingen, USA), MsX Hu integrin β1 Flour (Millipore, USA) Paraformaldehyde (PFA), Chloroform, Ethanol 99.9% purity, IsoPropanol (MERCK,USA), TRIsoln reagent, M-MuLV RT-PCR kit, GeNei Red Dye PCR kit, Agarose low EEO,50X TAE buffer, Ethidium bromide (Bangalore Genei, India), 25 cm^2 culture flasks, 60 x 15 mm TCPS, 12 well plate, 96 well plate, (Cell Star), Cell strainer (BD Bioscience).

2.3.2 Methods

Mesenchymal stem cells were isolated, characterized and maintained in complete ⇨-MEM containing 10% FBS in a CO_2 incubator, expanded *in vitro* and used for further experiments.

2.3.2.1 Effect of collagen and medium in cell adhesion and cell proliferation

The native extra cellular matrix of cornea predominantly contains collagen-IV and hence was used as a substrate for culturing cells. The effect of collagen on cell growth, proliferation and maintenance were analyzed in three different media. Approximately 2.5 x 10^3 cells /cm^2 were seeded and cultured in different media on collagen coated substrate. Cell adhesion and proliferation was assessed by phase contrast microscopy and MTT assay respectively.

2.3.2.1.1 Coating Extra Cellular Matrix components

Glass cover slips, TCPS dishes and culture flasks were coated with ECM type IV collagen (1.5mg/ml). 1ml of collagen solution was added, spread uniformly over the surface and excess collagen solution was aspirated. The coated surface was then incubated for 1h in a CO_2 incubator at 37 °C. The culture surfaces were gently washed with PBS under a laminar flow hood and dried at 37° C before seeding the cells.

2.3.2.1.2 Cell adhesion.

The adhesion and growth of cells seeded on processed cover slips were monitored using an inverted phase contrast microscope (Leica, Germany) at the end of 24h and 72h. The images were acquired using DC180 camera attached to Leica DMIL inverted phase contrast microscope.

2.3.2.1.3 Cell proliferation

The cell proliferation of cultured MSCs was monitored by evaluating the cellular activity by MTT assay at the end of 24h and 72h respectively as described in section 2.1.2.2.2. The cell lysate was transferred to 96 well plate and absorbance was measured in a multiwell plate reader (Biotek Power Wave XS, USA) at 570 nm. The results were expressed as mean ± standard deviation (n=3) and statistical significance was analyzed using students t test with graph pad software. For all analyses, differences were considered significant when $P< 0.05$

2.3.2.2 Culture and differentiation of mesenchymal stem cells

For differentiation studies three different culture systems were used as given in the Table 7.

Table 7 Constituents of 3 different culture systems used for mesenchymal stem cell differentiation

	Culture system
1	Collagen coated surface with basal media
2	Collagen coated surface with conditioned media
3	Uncoated surface with conditioned media

MSCs were trypsinized and seeded at a density of 3 x 10^4 cells/ cm^2on collagen coated and uncoated surface and incubated under controlled conditions in complete α-MEM supplemented with 10% FBS, in a CO_2 incubator till the cells attained sub confluence. After attaining sub confluence, cells were maintained in 3 different culture systems as depicted in Table 5. Approximately 1 ml of CM medium was added twice daily for a period of 7 days in culture system 2 and 3, while culture system 1 was supplemented with α-MEM. The cultures were viewed regularly under an inverted phase contrast microscope (Leica, Germany) and photographs were acquired at specific periods using DC180 camera attached to Leica inverted phase contrast microscope.

2.3.2.3 Monitoring of mesenchymal stem cells for differentiation

The differentiation of MSC to corneal lineage was assessed using markers specific to corneo epithelial lineage. The differentiation was analyzed at the end of 3^{rd} and 7^{th} day by immunostaining, flow cytometry and RT – PCR.

2.3.2.3.1 Immunostaining

The cells cultured in different culture systems were analyzed for differentiation at the end of 3rd and 7th day by immunostaining. The cells were analyzed for the expression of markers specific to corneal epithelial lineage CK3/12 as well as for mesenchymal stem cell lineage markers ABCG2 and CD29. Immunostaining was done as per the procedure listed in section 2.1.2.3.1.

2.3.2.3.2 Flow Cytometry

To determine the extent of differentiation of MSCs, flow cytometry was performed as depicted in section 2.1.2.3.2. The cell suspensions obtained by trypsinization were analyzed for the expression of corneal epithelial markers CK3/12 as well as markers for mesenchynmal stem cell lineage.

2.3.2.3.3 RT-PCR

To confirm differentiation of MSCs to corneal epithelial lineage, RT-PCR analysis was done as per the procedure depicted in section 2.2.2.3.3 using specific primer sequences as given in Table 8.

Table 8 Primer sequence and annealing temperatures for gene profiling.

Gene	Primer sequence	Annealing temperature (°C)	Product length (bp)
βActin	FP- ATCGTGATGGACTCCGGCGA RP-AGGAAGGAGGGCTGGAACAG	52	350
CK 3	FP-GGCAGAGATCGAGGGTCTC RP-GTCATCCTTCGCCTGCTGTAG	64	145
CK 12	FP-ACATGAAGAAGAACCACGAGGATG RP-TCTGCTCAGCGATGGTTTCA	63	150

Results & Discussion

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation, maintenance and characterization of mesenchymal stem cells

3.1.1 Isolation and culture of mesenchymal stem cells

Bone marrow contains hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs produce all blood cells and MSCs are known to differentiate into cells of mesenchymal lineages (Castro-Malaspina *et al.,* 1986 & Polisetty *et al.,* 2008). Preliminary reports by Friedenstein *et al.,* (1974) suggested that MSCs can be isolated from heterogenous population of cells by direct plating method where unpurified bone marrow are plated directly onto cell culture plates or culture flasks, and the selection of cells is based on plastic adherence. Other isolation procedures such as usage of density gradient fractionation have been previously reported (Kolf *et al.,* 2007). However cells isolated by such methods yielded a heterogeneous mixture of cells with varying proliferation and differentiation potentials. Thus most of culture techniques still take a traditional method suggested by Friedenstein *et al.,* (1974) for isolation and culture of MSCs.

In the present study MSCs were isolated from raw unpurified bone marrow of rabbits by direct plating method where the cells were isolated based on its property of plastic adhesion. Most of the non-adherent cells were removed during the first media change. On the 3rd day after seeding, elongated adherent cells were observed; these cells exhibited extensive proliferation between days 5 and 9, with colony formation around day 10 to 14. The cell population reached confluence within 3 weeks in culture (Figure 4) (Friedenstein *et al.,* 1974 & Ashton *et al.,* 1980) which were further expanded *in vitro* and maintained for 6 generations (Figure 5). Morphological variation was observed after sixth passage with reduced adhesion and proliferation similar to reports by Conget & Minguell (1999). In later passages (>P5), the spindle-shaped cells began to display a broadened, flat morphology with a decrease in proliferative potential and thus further experiments were performed only on cells from P1 to P5.

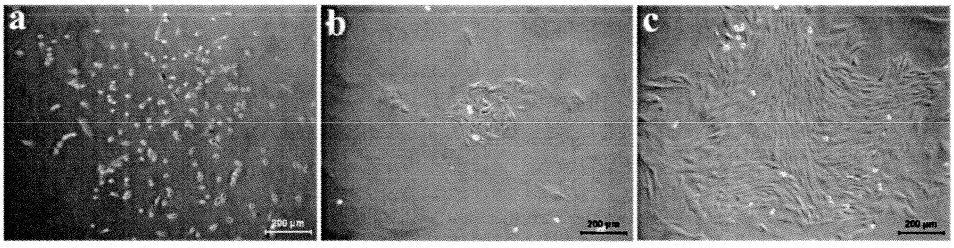

Figure 4 Isolation and culture of MSCs: cell adhesion on 3rd day showing elongated adherent cells (a) 7th day exhibiting colony formation (b) and 14th day showing sub confluence with elongated fibroblast morphology (c).

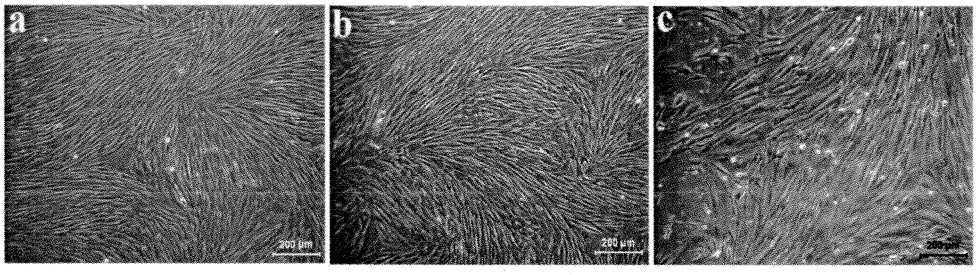

Figure 5 Phase contrast micrographs of MSCs at passage-2 (a) & passage-4 (b) exhibiting normal fibroblast morphology and passage-6 (c) showing changes in morphology

3.1.2 Selection of media for mesenchymal stem cell culture

A critical factor in mesenchymal stem cell biology is to propagate cells with suitable stem cell characteristics, multipotency and high proliferative potential for its use in various biomedical applications such as tissue engineering and cell-based therapies. Under *in vitro* conditions, medium plays a major role in growth and multiplication of cells. Thus selection of optimal medium for culture and expansion of stem cells is crucial. In the present study, for optimization of culture conditions, MSCs after first passage was cultured at a cell density of 2 x 10^3 cells /cm^2 in different commercially available media and analyzed for adhesion and proliferation. The experiment was done at two different time points to check the cell proliferation. An increased cell adhesion was noticed in α-MEM on comparison with DMEM and DMEM LG (Figure 6).

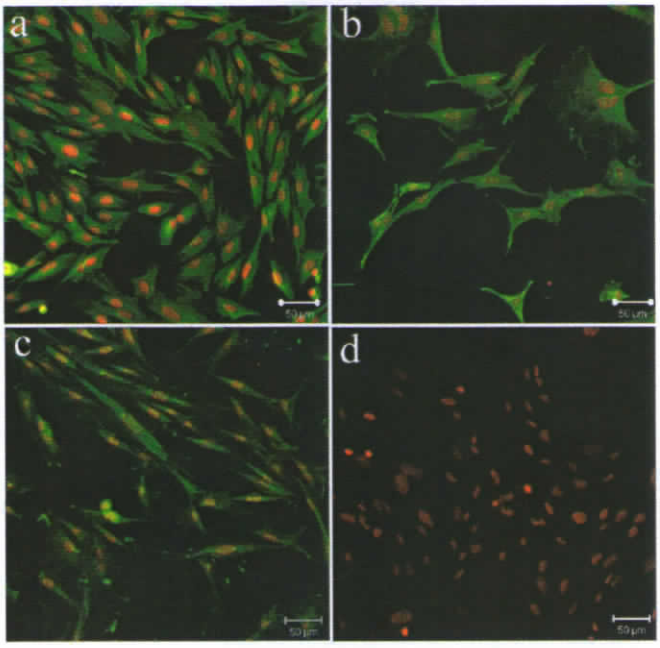

Figure 8 Laser scanning confocal micrographs of MSC showing cytoskeletal filamentous expression pattern of vimentin (a), cytoplasmic localization of ABCG2 (b), membrane bound expression of CD105 (c) and negative expression of CD90 (d). Cells were stained using FITC tagged antibodies and counter stained with PI.

Quantitative analysis of expression of various markers by flow cytometry (Figure 9) showed that more than 90% of cells were positive for expression of monoclonal antibodies directed against cell surface epitopes such as vimentin , CD 105, ABCG2 as described to be present on rabbit MSC but showed a reduced expression of CD29 and negative expression of CD90 (Table 10). Flow cytometry analysis of different surface antigens on hMSCs, like CD105 (71.33 ± 6.66%), CD29 (84.0% ± 2.65) and CD90 (94.57 ± 2.00%) were reported to be positive by Polisetty *et al.,* (2001). In this culture a reduced expression of CD29 was noted with negative expression of CD90. However an elevated expression of CD105 was observed. It is presumed that the variability of the markers on MSCs may be due to different stage of cell commitment and culturing conditions (Lapi *et al.,* 2008). The data is displayed in the form of single color histograms, evidence the presence of mesenchymal stem cells further substantiating the data obtained from immunostaining.

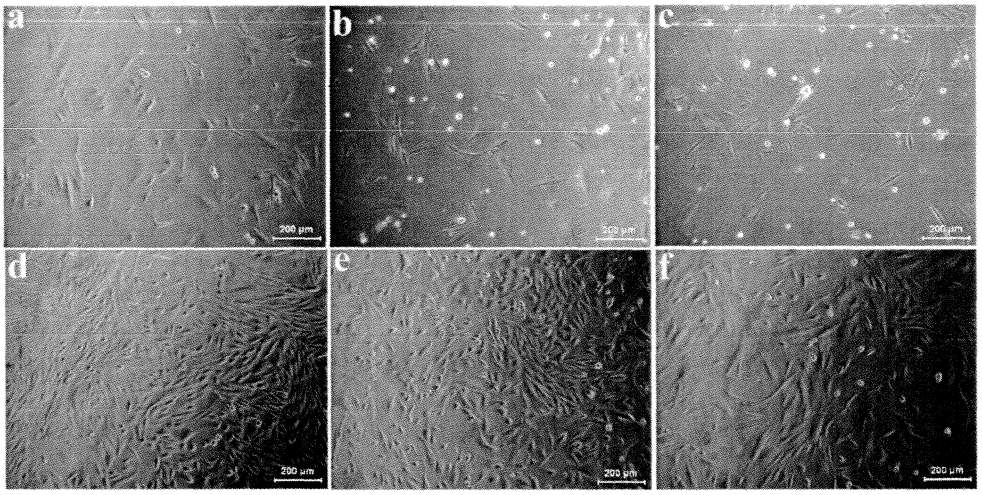

Figure 6 Cell adhesion and growth of MSCs after 24h and 72h using α-MEM (a & d), DMEM (b & e) and DMEM LG (c & f). Cell adhesion was found to be higher in α-MEM

Cell proliferation was analyzed by MTT assay and the graphs were plotted with mean and standard deviation (Table 9) (n=21). As seen from the graphs significantly high cell proliferation was seen on cells cultured in α-MEM compared with cells cultured on DMEM and DMEM-LG (Figure 7).

Table 9 Mean ± standard deviation of cells grown in different media

	α-MEM	DMEM	DMEM-LG
24h	0.067 ± 0.0017	0.039 ± 0.0005	0.055 ± 0.005
72h	0.13125 ± 0.0009	0.1215 ± 0.001	0.07625 ± 0.001

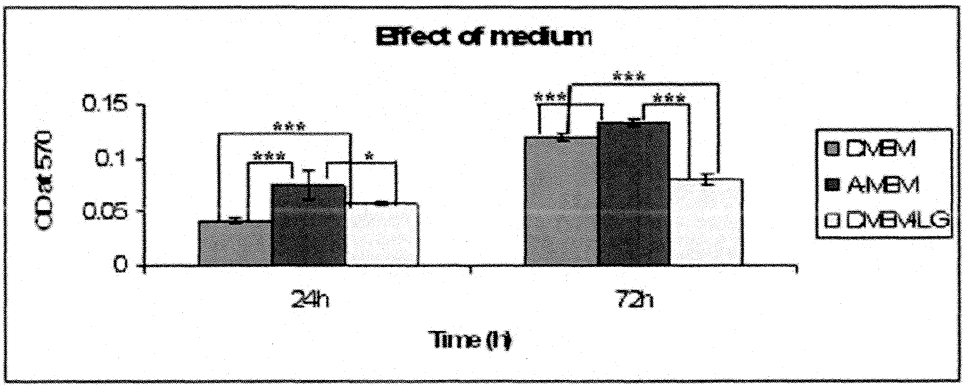

Figure 7 MTT profile of MSCs seeded in different media after 24h and 72h. Significant difference in cell proliferation was noted in cells cultured in α-MEM (*p value –0.05- 0.001; *** p value < 0.001)

α-MEM together with 10%FBS was found to be ideal for rabbit MSC culture. Earlier reports suggested the use of growth factors & higher concentration of FBS (Lapi *et al.,* 2008). The medium parameters in our study was set at 10% FBS avoiding the use of growth factors for *in vitro* expansion of cells. Based on these results, α-MEM was selected as a cost effective optimum basal medium for further studies.

3.1.3 Characterization of mesenchymal stem cells

Although no unique marker is known for MSCs, their cell-surface antigen profile has been well explored. As per Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy guidelines (Dominici *et al.,* 2006), human mesenchymal stem cells were reported to express CD105, CD73, and CD90 and negative for CD45, CD34, CD14 CD79a and HLA-DR. A few reports are available for the characterization of rabbit MSCs. Recently reports on flow cytometric study of rabbit MSCs by Lapi *et al.,* (2008) showed that these cells are positive for the expression of CD105, CD29, Vimentin and ABCG2 but was negative for the expression of CD90.

In order to ascertain that the culture-expanded cells were MSCs, the cells were examined by immunostaining and quantitatively assessed by flow cytometry. Immunostaining showed positive expression of CD105, ABCG2, and vimentin and negative expression of CD90. The characteristic cytoskeletal expression of vimentin illustrated the filamentous nature (Figure 8a) and confirmed the significant role of vimentin in supporting and anchoring, the expression of ABCG2 was found to be localized in the cell membrane as well as in cytoplasm (Figure 8b) similar to the reports by Balasubramanian *et al.,* (2008). CD 105 was found to be localized in the membrane (Figure 8c) as reported earlier by Arufe *et al.,* (2009). It has been reported that great variation exists in the expression of CD90 which are reported to be expressed mostly by mesenchymal stromal cells. The expression of CD90 (Figure 8d) was found to be negative.

It is presumed that the variability of the markers on MSCs may be due to different stage of cell commitment and culturing conditions. The immunoreactivity obtained against ABCG2, CD 105 and vimentin as well as the negative reaction for CD90 concord with previously reported findings by Lapi *et al.,* (2008).

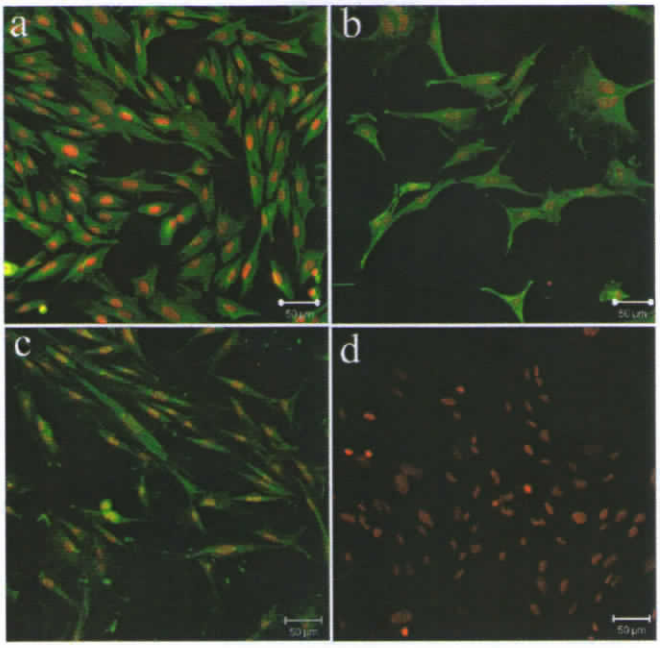

Figure 8 Laser scanning confocal micrographs of MSC showing cytoskeletal filamentous expression pattern of vimentin (a), cytoplasmic localization of ABCG2 (b), membrane bound expression of CD105 (c) and negative expression of CD90 (d). Cells were stained using FITC tagged antibodies and counter stained with PI.

Quantitative analysis of expression of various markers by flow cytometry (Figure 9) showed that more than 90% of cells were positive for expression of monoclonal antibodies directed against cell surface epitopes such as vimentin , CD 105, ABCG2 as described to be present on rabbit MSC but showed a reduced expression of CD29 and negative expression of CD90 (Table 10). Flow cytometry analysis of different surface antigens on hMSCs, like CD105 (71.33 ± 6.66%), CD29 (84.0% ± 2.65) and CD90 (94.57 ± 2.00%) were reported to be positive by Polisetty *et al.,* (2001). In this culture a reduced expression of CD29 was noted with negative expression of CD90. However an elevated expression of CD105 was observed. It is presumed that the variability of the markers on MSCs may be due to different stage of cell commitment and culturing conditions (Lapi *et al.,* 2008). The data is displayed in the form of single color histograms, evidence the presence of mesenchymal stem cells further substantiating the data obtained from immunostaining.

Table 10 FACS analysis of mesenchymal stem cells

Markers analyzed	Vimentin	ABCG2	CD 105	CD 29	CD 90
Expression (%)	99±0.44	99 ±0.63	99 ±0.54	75 ±5.63	1 ±0.11

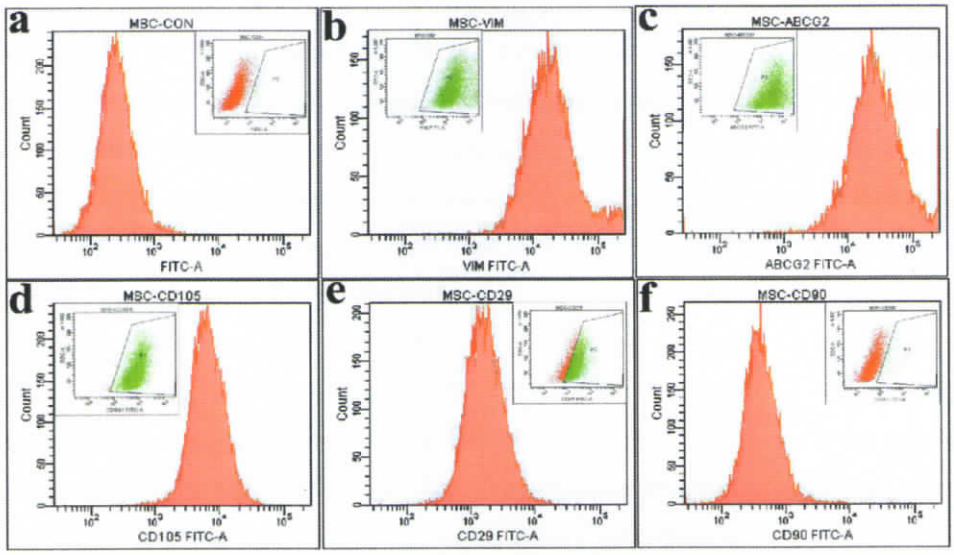

Figure 9 Fluorescence-activated cell sorting (FACS) analysis of MSCs showing positive expression of mesenchymal stem cell markers Vimentin (99±0.44%) (b), ABCG2 (99±0.63%) (c), CD105 (99±0.54%) (d), CD29 (75±5.63%) (e) and negative expression of CD90 (1±0.11%) (f) as indicated by histograms. Unstained cells were used as control (a)

In this study, rabbit bone marrow, MSCs were successfully isolated with a characteristic spindle shape. Flow cytometry analysis and immunocytochemistry revealed that most cells expressed the mesenchymal markers like vimentin, ABCG2, CD105, CD29 and did not express CD90. Morphology, surface antigen profile characteristics, and differentiation characteristics provided evidence that the cells isolated were MSCs.

3.2 Isolation, maintenance and characterization of limbal fibroblasts

3.2.1 Isolation and culture of limbal fibroblast cells

Stem cell maintenance and function are regulated by various intrinsic and extrinsic factors provided by a unique local microenvironment or niche. Common components of such niches are signaling molecules and growth factors from

neighboring cells as well as extra cellular matrices. The limbal stem cells and their progeny reside within small clusters in the basal epithelium in close spatial relationship with specific basement membrane and matrix components as well as with stromal fibroblasts which provides increased levels of growth and survival factors Limbal fibroblasts conditioned media are known to influence the differentiation of stem cells to corneal epithelial lineage under *in vitro* conditions (Ahmad *et al.,* 2007 & Blazejewska *et al.,* 2009). In order to obtain conditioned media, limbal fibroblasts were isolated from deepithelialized limbal explants by enzymatic digestion method and cultured. Under phase contrast microscope the cells appeared fibroblastic, elongated, and spindle shaped (Figure 10a) similar to the reports by Ahmad *et al.,* (2007). The cells attained confluence after 10 days of culture (Figure 10b) in DMEM-10% FBS. Cells were first passaged after 10 days and thereafter at every 5 days (Figure 10c). The culture conditions for isolation and expansion of rabbit limbal fibroblast cells were standardized and fibroblast conditioned medium was collected for further differentiation studies. To date, only isolation procedures for human limbal fibroblasts have been reported (Ahmad *et al.,* 2007 & Blazejewska *et al.,* 2009).

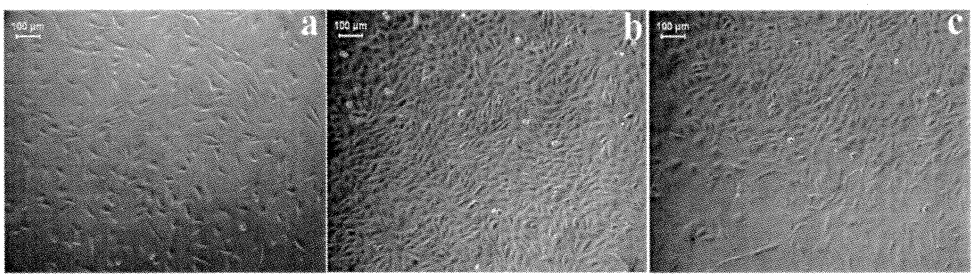

Figure 10 Phase Contrast Micrograph of limbal fibroblast cells in primary culture showing elongated fibroblast morphology on 4th day (a), confluency on 10th day (b) and cells on 5th day after first passage (c) maintaining the same morphology

3.2.2 Characterization of limbal fibroblasts

A recent report by Dravida *et al.,* (2005) suggested the presence of fibroblast - like cells in the limbal stroma, possessing stem cell-like self-renewal property and multipotency similar to mesenchymal stem cells. In order to determine whether the isolated cells were genuine limbal fibroblast cells, immunocytochemistry, flow cytometry and RT - PCR was performed for markers specific to fibroblast lineage. Limbal fibroblast cells were previously reported to express cell surface markers;

Vimentin, SPARC (Shimmura *et al.,* 2001) and CD90 (Ahmad *et al.,* 2007). SPARC is one among the few proteins detectable by proteomic analysis in the limbal fibroblast supernatant, suggesting a functional role of this protein in maintenance of the homeostasis of the limbal structure (Shimmura *et al.,* 2001). A school of thought suggests that soluble factors expressed by limbal fibroblasts may be involved in maintaining the undifferentiated phenotype of limbal stem cells. Relatively higher expression of SPARC is reported in limbal fibroblasts cells and hence is regarded as a potential marker for selection and identification of limbal fibroblast cells.

Immunocytochemistry of cultured cells showed that cells were positive for the expression of the markers like vimentin (Figure11a) and SPARC (Figure 11b). The expression of vimentin and SPARC was found localized within the cytoplasm with FITC appearing as green in colour, the nucleus counter stained with propidium iodide appearing red. Similar staining pattern was reported previously in the works of Yan & Sage (1999). Contrary to the reports by Ahmad *et al.,* (2007) rabbit fibroblast cells were found to be negative for the expression of CD90 (Figure 11c).

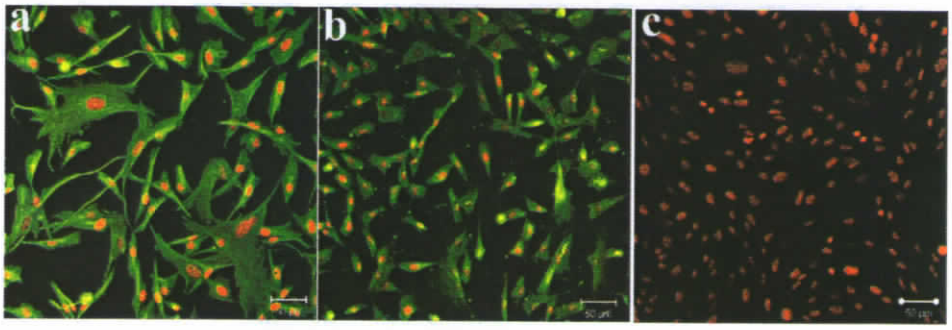

Figure 11 Laser scanning confocal micrographs of limbal fibroblasts showing cytoskeletal filamentous expression pattern of vimentin (a), cytoplasmic expression of SPARC (b) and negative expression of CD 90 (c). Cells were stained using FITC tagged antibodies and counter stained with PI.

Flow cytometry analysis of confluent fibroblast cultures was consistent with the results obtained from immunocytochemisry. Flow cytometric analysis indicated that the cells were strongly positive for the expression of vimentin (98%) and SPARC (99%) but was negative for the expression of CD 90 (0.3%) (Figure 12) suggesting that the cells isolated were limbal fibroblast cells.

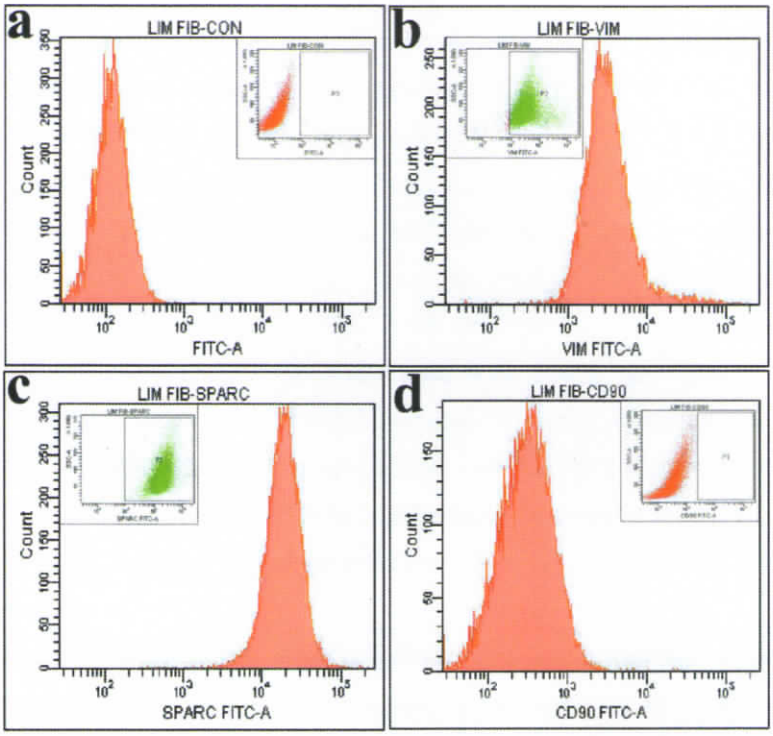

Figure 12 Fluorescence-activated cell sorting (FACS) analysis of limbal fibroblasts: FACS histograms confirming the positive expression of vimentin (98%) (b), SPARC (99%) (c) and negative expression of CD 90 (0.3%) (d). Unstained cells were used as control (a).

RT - PCR analysis of cells for genes specific to vimentin and β-actin as internal control showed the expression of vimentin further confirming fibroblast lineage (Figure 13).

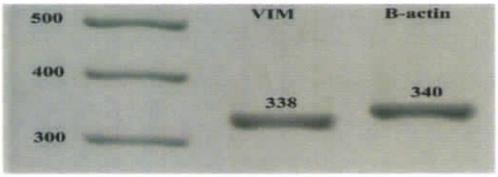

Figure 13 Gene expression profiles of fibroblast cells showing positive expression of marker vimentin (338bp) in limbal fibroblasts. β actin served as internal control (340bp).

3.3 Simulation of niche factors for differentiating mesenchymal stem cells to corneal lineage.

It is 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. Some of the possible factors for niche regulation are proximity to vasculature, the basement membrane composition with respect to specific isoforms

of collagen IV, laminin and fibronectin and the presence of limbal fibroblasts in the underlying stroma, which produce various cytokines. Previous studies using different stem cells into the desired epidermal and corneal epithelial lineages have analyzed the inductive properties of these niches. Those studies demonstrated that corneal epithelial-like cells can be obtained if provided with limbus-specific differentiation signals viz *in vivo* delivery of stem cells (Ma *et al.,* 2006 & Ye *et al.,* 2006) and by replicating micro environmental factors, such as type IV collagen and limbal fibroblast CM (Ahmad *et al.,* 2007 & Blazejewska *et al.,* 2009). In order to prove the hypothesis that MSCs could be transdifferentiated to corneo epithelial lineage by simulating specific niche factors, the MSCs were cultured for a period of 7 days on different culture systems. In this study two major niche parameters were analyzed for its efficiency for inducing transdifferentiation – collagen and limbal fibroblast conditioned media

3.3.1 Effect of collagen and medium in cell adhesion and cell proliferation

Since collagen is considered as an additional parameter for differentiation, the effect of collagen was analyzed using cell adhesion and proliferation. MSCs were seeded on collagen coated surface and maintained in different commercially available media. Phase contrast micrographs (Figure 14) showed increased cell adhesion and proliferation of cells cultured in α-MEM. Cell proliferation was analyzed by MTT assay and the graphs were plotted with mean and standard deviation (Table 11). Results showed significantly high cell adhesion and proliferation of MSCs on collagen coated surface in α-MEM on 24 h and 72 h compared to that of cells in DMEM and DMEM LG (Figure 15). $p < 0.05$ was considered to be statistically significant (n=21). There was no statistically significant difference in cell adhesion and proliferation between DMEM and DMEM LG after 24 h. The α-MEM was previously shown to enhance cell adhesion and growth under normal culture conditions (Figure 6) which further confirmed α-MEM to be the optimal medium for culture and *in vitro* expansion of mesenchymal stem cells.

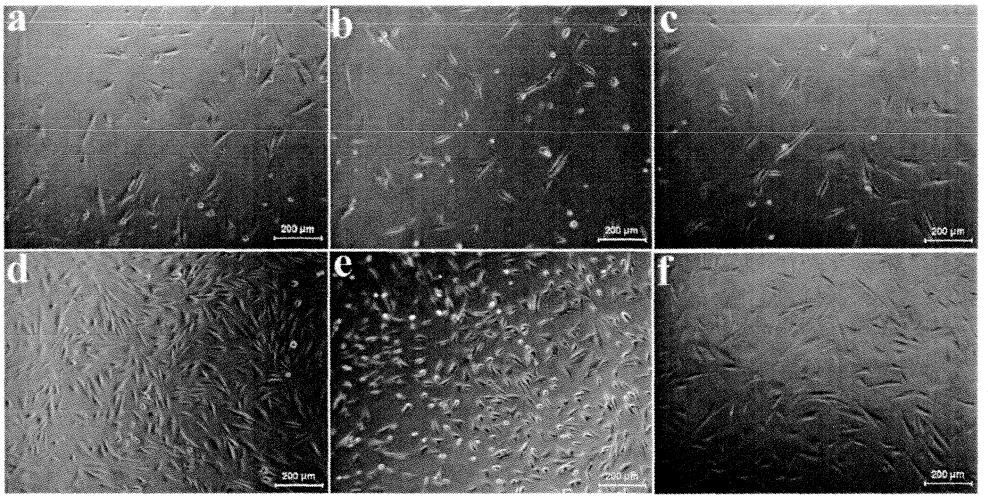

Figure 14 Cell adhesion and growth of MSCs after 24h and 72h on collagen coated surface in α-MEM (a & d), DMEM (b & e) and DMEM LG (c & f). Cell adhesion and proliferation was found to be higher in α-MEM.

Table 11 Mean ± standard deviation of cells grown in different media on collagen coated surface

	α-MEM	DMEM	DMEM-LG
24h	0.06 ± 0.001	0.038 ±0.002	0.036 ±0.002
72h	0.1075 ± 0.002	0.0675 ± 0.008	0.06525 ± 0.001

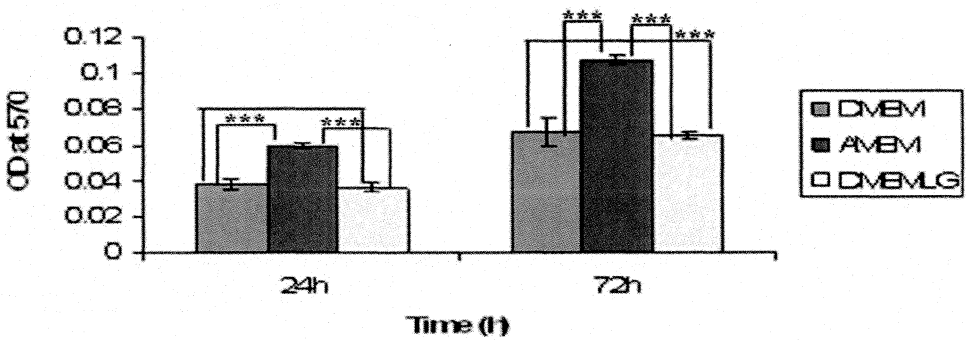

Figure 15 MTT profile of MSCs seeded on collagen coated surface in different media after 24h and 72h. Significant difference in cell proliferation was noted in cells cultured in α-MEM ***p value <0.0001. There was no significant difference in cell proliferation on DMEM and DMEMLG after 24h.

3.3.2 Monitoring of mesenchymal stem cells for differentiation

Cytokeratins are cytoskeletal proteins which play an important structural and protective role in maintaining the integrity of the epithelium. Different types of

keratins are expressed in combination in different types of epithelial cells and are used to characterize the type of epithelium. The corneal epithelium is known to express CK3/12.

To induce differentiation of MSCs to corneal epithelial lineage, MSCs were seeded on 3 different culture systems (Culture system 1 - collagen with basal media, Culture system 2 - collagen with conditioned media and Culture system 3 - conditioned media respectively) and expanded *in vitro*. The differentiation was monitored at regular intervals on day 3, day 7 and day 10. Phase contrast microscopy of MSC cultured in all the three culture systems did not show any change in cell morphology till 7th day of culture (Figure 16, 17, 18) compared with undifferentiated MSCs. By day 10 the cells started showing the signs of senescence (Figure 19) hence the monitoring of differentiation was restricted to day 7.

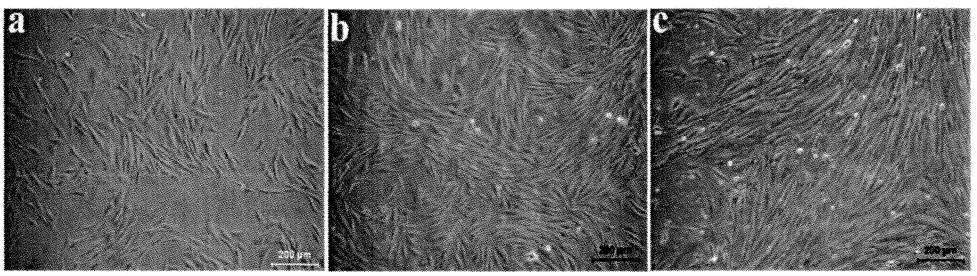

Figure 16 Phase contrast micrographs of MSCs on culture system- 1 showing elongated spindle morphology by day 1 (a), day 3 (b) and day 7 (c).

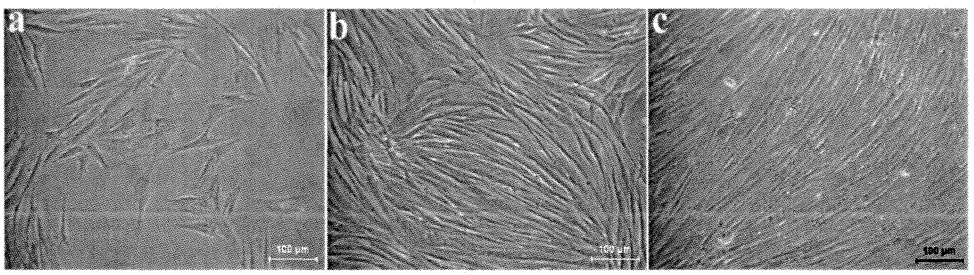

Figure 17 Phase contrast micrographs of MSCs on culture system- 2 showing elongated spindle morphology by day 1 (a), day 3 (b) and day 7 (c).

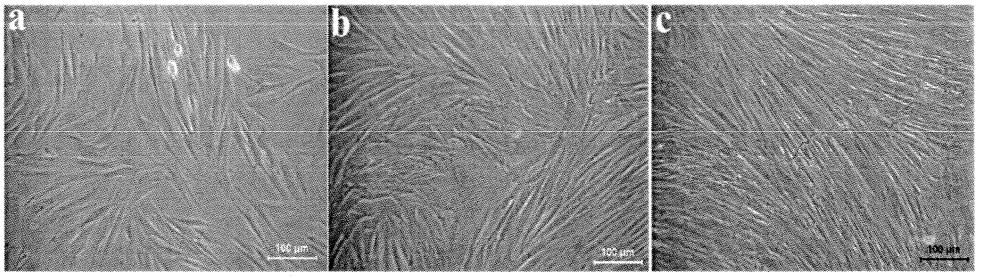

Figure 18 Phase contrast micrographs of MSCs on culture system- 3 showing elongated spindle morphology by day 1 (a), day 3 (b) and day 7 (c).

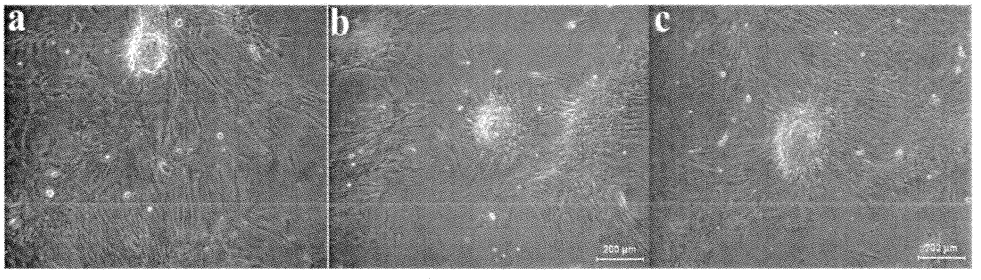

Figure 19 Phase contrast micrographs of MSCs on 10^{th} day showing morphological variation and the signs of senescence in culture system 1 (a), culture system 2 (b) and culture system 3 (c).

In order to determine whether the collagen coating alone has any effect in transdifferentitaing MSCs to corneal epithelial lineage, MSCs seeded on culture system 1 was analyzed for the expression of stem cell specific markers CD 29, ABCG2 and corneal epithelial specific marker, cytokeratin (CK) by immunostaining, flow cytometry and RT PCR. Immunocytochemistry demonstrated that MSCs cultured in α-MEM were positive for mesenchymal stem cell marker ABCG2 (Figure-20 a & b) but was negative for the expression CK (Figure 20 c & d) on 3rd and 7th day. Cytokeratins are known to be immunolocalized to cytoplasmic filaments (Harkin *et al.,* 2004). Unlikely to these reports such staining pattern was not obtained in culture system 1 indicating the negative expression of cytokeratins.

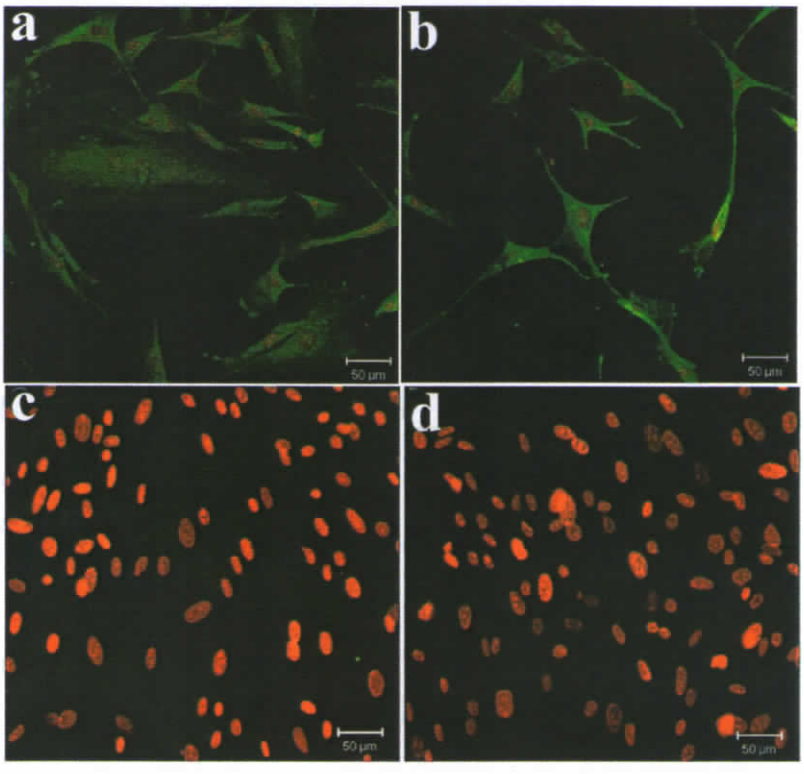

Figure 20 Laser scanning confocal micrographs of mesenchymal stem cells in culture system 1 on 3rd & 7th day showing cytoplasmic expression of ABCG2 (a & b), and negative expression of cytokeratin (c & d). Cells were stained using FITC tagged antibodies and counter stained with PI.

RT-PCR analysis with β-actin as internal control showed the expression of vimentin and β-actin but was found to be negative for expression of cytokeratin 3 and 12, pertaining to corneal lineage. (Figure 21)

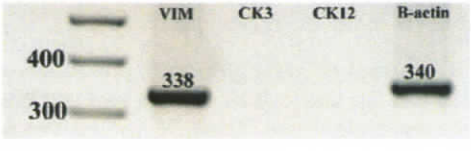

Figure 21 Gene expression profiles of selected markers vimentin (338bp), CK3 (145bp) and CK12 (150bp) in MSC cultured in culture system 1 showing positive expression for vimentin and negative expression of CK3 and CK12.β actin served as internal control.

Quantitative data by flow cytometry (Figure 22) indicated that cells were positive for the markers ABCG2, CD 29 with no expression of cytokeratin confirming immunocytochemistry analysis. These results indicated that collagen alone did not induce transdifferentiation and there is requirement of simulating other niche factors.

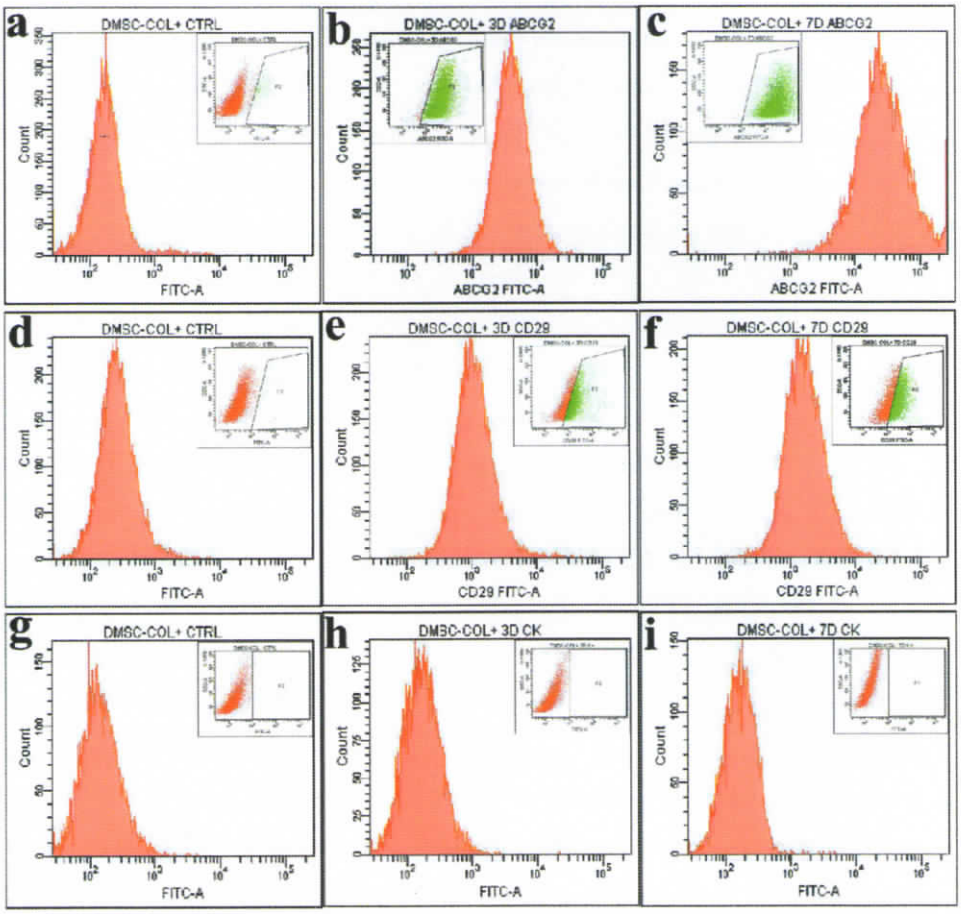

Figure 22 Fluorescence-activated cell sorting (FACS) analysis of MSCs showing positive expression of mesenchymal markers ABCG2 (b & c) and CD 29 (e & f) and absence of CK (h & i) expression in culture system-1 on 3rd and 7th day. Unstained cells were used as control (a, d & g).

One major criticism of studies describing plasticity of bone marrow stem cells is the heterogeneity of the cell population. Although we cannot rule out the possible existence of heterogenous subpopulations of committed cells, the chances are rare as cells do not proliferate during the differentiation in presence of conditioned media and collagen. Usage of single clone of cells for differentiation purpose can also prove the

plasticity of MSCs eventually. However, transdifferentiation capability of adult MSCs was considered controversial because of possible progenitor cell contamination or fusion of MSCs with primitive stem cells in the target tissue. Furthermore, it is unclear if precommitted progenitor cells and committed cells possess multidifferentiation potential to a similar extent as stem cells. Our results in culture system-1 ruled out the possibility of committed cells to be the reason for transdifferentiation as the cells does not show any signs of differentiation indicated by negative staining for keratins and positive expression of stem cell markers.

Hence to induce differentiation, MSCs were cultured on two different culture systems 2 and 3 and its efficacy to transdifferentiate MSCs into epithelial-like cells was analyzed by PCR, flow cytometry, and immunocytochemistry. Immunostaining using antibodies specific for ABCG2 showed cytoplasmic/membrane bound expression and cytokeratin showed cytoskeletal organization on 3rd and 7th day in culture systems 2 and 3 (Figure 23 & 25) where as cells cultured in culture system- 1 exhibited negative staining pattern for cytokeratin (Figure 20). Together, these observations indicate important parallels regarding stem cell markers and differences regarding differentiation markers between the MSCs and corneal epithelial cells. From these results, it can be inferred that cells in culture system 2 and 3 may have differentiated into epithelial cells. The current observations substantiate the importance of the limbal stem cell niche, which normally regulates the behavior and differentiation of progenitor limbal stem cells, for inducing transdifferentiation into a corneal epithelial-like phenotype. This data suggest that replication of niche factors in vitro increases the efficiency and lineage determination of stem cell based cultivation methods.

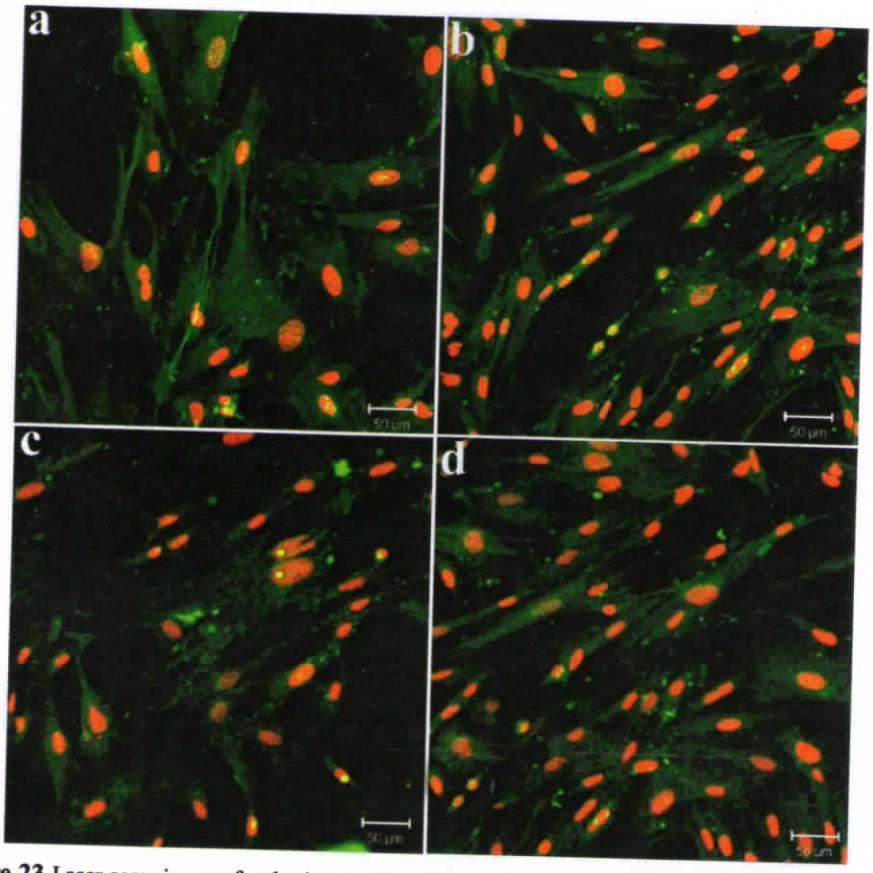

Figure 23 Laser scanning confocal micrographs of mesenchymal stem cells in culture system 2 on 3rd & 7th day : showing cytoplasmic expression of ABCG2 (a & b) and cytoskeletal expression of cytokeratin (c & d). Cells were stained using FITC tagged antibodies and counter stained with PI.

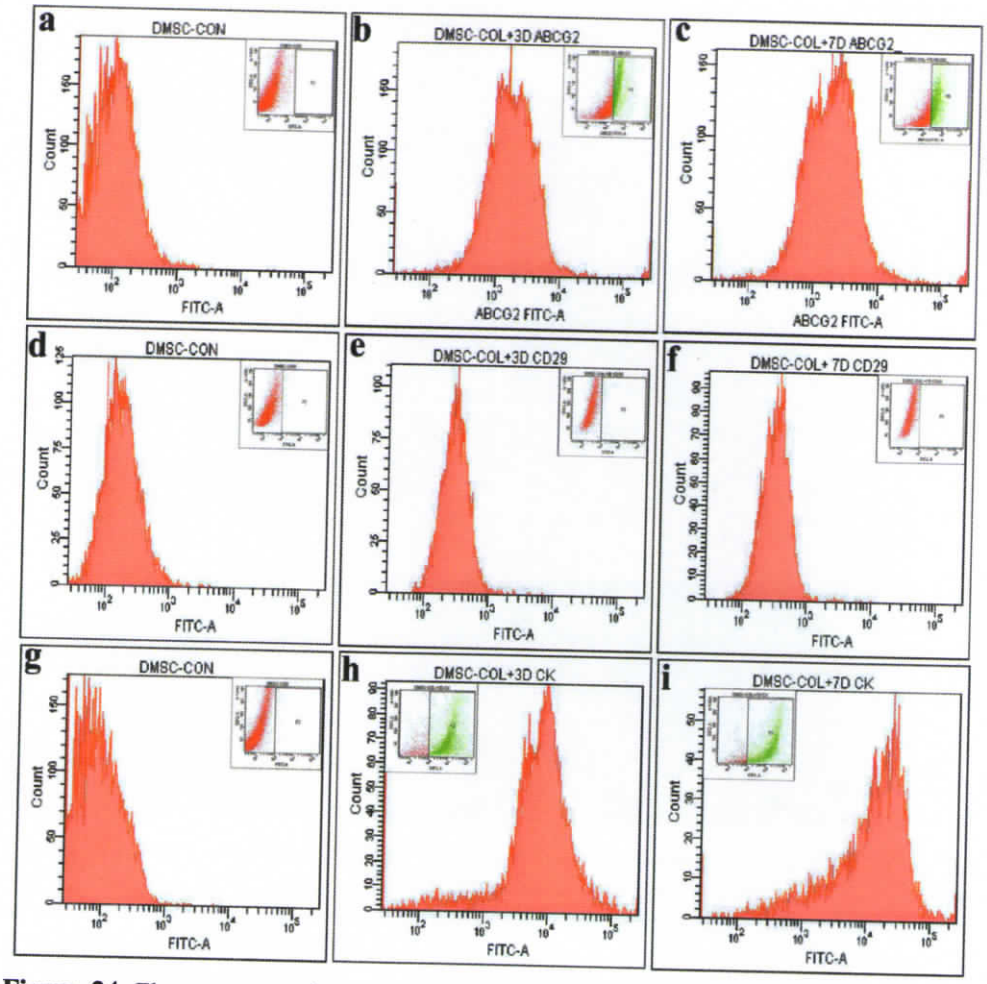

Figure 24 Fluorescence-activated cell sorting (FACS) analysis of MSCs. showing positive expression of mesenchymal markers ABCG2 (b & c), CK (h & i) and absence of CD 29 expression (e & f) in culture system 2 on 3rd and 7th day as evidenced by histogram. Unstained cells were used as control (a, d & g).

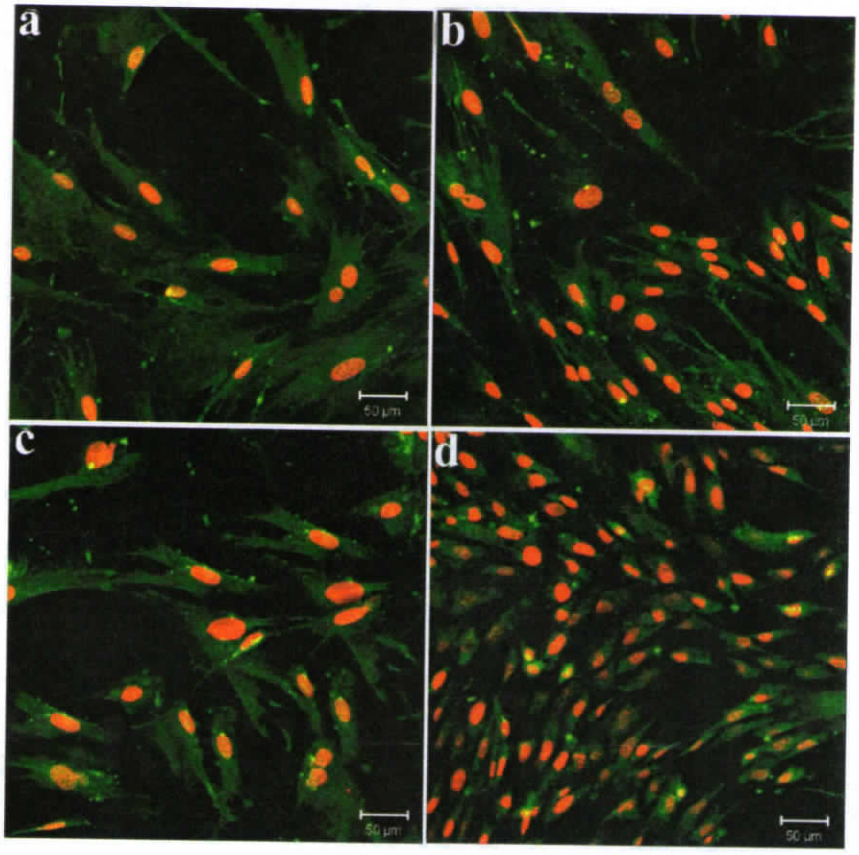

Figure 25 Laser scanning confocal micrographs of mesenchymal stem cells in culture system 3 on 3rd & 7th day : showing cytoplasmic expression of ABCG2 (a & b) and cytoskeletal expression of cytokeratin (c & d). Cells were stained using FITC tagged antibodies and counter stained with PI.

For further confirming the transdifferentiation, MSCs were analyzed quantitatively using FACS. FACS analysis of MSCs grown in culture system 2 and 3 showed an increase in the expression of cytokeratins (Figure 24, 26 (h & i)) which was found to be negative in culture system 1. The cells showed a gradual decrease in expression of stem cell marker ABCG2 (Figure 24, 26 (b & c)) in both culture system 2 and 3 but were found to be negative for the expression of CD 29 (Figure 24, 26 (e & f)). These stem cell markers were found to be highly expressed in culture system 1 under normal culture condition in the presence of ECM component, collagen. The quantitative values of different markers as analyzed by flow cytometry in different culture systems are given in Table 12.

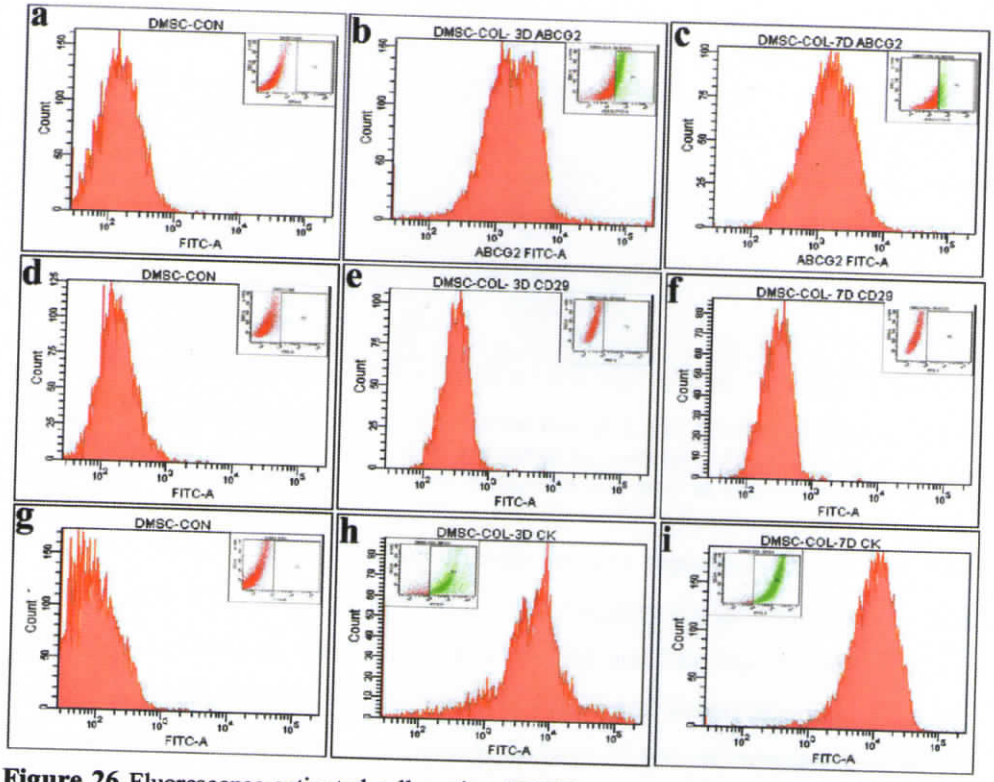

Figure 26 Fluorescence-activated cell sorting (FACS) analysis of MSCs. showing positive expression of mesenchymal markers ABCG2 (b & c), CK (h & i) and absence of CD 29 expression (e & f) in culture system 3 on 3rd and 7th day as evidenced by histogram. Unstained cells were used as control (a, d & g).

Table 12 Expression level of various antigens in different culture system

Culture system	Cytokeratin		ABCG2		CD 29	
	3rd day	7th day	3rd day	7th day	3rd day	7th day
1	0.5±0.01%	0.2±0.11%	97±1.65%	98±1.22%	53±2.33%	52±3.22%
2	86±2.31%	86±3.44%	34±6.33%	39±2.66%	1±0.87%	1±0.44%
3	84±3.21%	88±4.11%	36±2.33%	21±3.22%	0.3±0.011%	0.4±0.22%

There was reduction in the expression of ABCG2 in culture system 2 and 3 as shown by FACS analysis. Since generalized antibody for keratins was used for FACS and immunostaining, further confirmation of the expression of keratin specific to corneal lineage was analyzed by PCR (Figure 27).

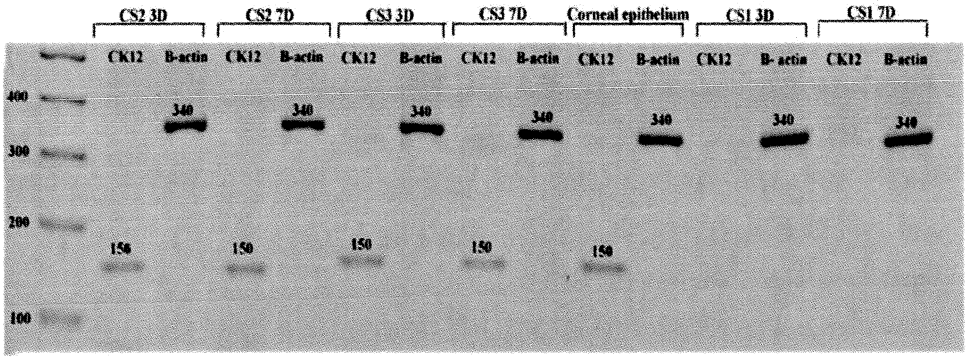

Figure 27 Gene expression profiles of selected marker CK12 (150bp) in different culture systems (CS) 1, 2 & 3: showing positive expression by systems 2 & 3 on 3rd & 7th day and negative expression by system 1 .Corneal epithelium was used as positive control . β actin served as internal control.

The PCR analysis of MSCs showed positive expression of CK12 on both culture system 2 and 3 by 3rd and 7th day, but negative for the expression of CK3. The MSCs cultured on culture system 1 for 3rd and 7th day did not show any expression of CK12 reflecting the results of FACS and immunostaining. The data obtained concords well with the previous findings of transdifferentiating other cell types to corneal epithelial lineage by replication of niche factors. Collectively, these data indicate that the transdifferentiation of mesenchymal stem cells can be influenced by specific microenvironment and that factors derived from limbal fibroblasts appear to induce expression of the corneal epithelial marker CK12.

Reconstruction of the ocular surface epithelium in patients with bilateral limbal stem cell deficiency is one of the most exigent problems. The use of autologous cells is highly favoured for the replenishment of stem cells. This is likely to avoid the the risk of allogenic immune rejection and the requisite for immunosuppression. A key technique employed for resurfacing corneal epithelium includes the use of autologous cells, which can be used to reconstruct the ocular surface. Another technique relies on differentiation potential of cells such as embryonic stem cells and hair follicle cells (Ahmad *et al.*, 2007 & Blazejewska *et al.*, 2009) for reconstruction of ocular surface in patients with limbal stem cell deficiency However, insufficient enduring outcome justify a continued search for new autologous stem cell source.

The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for an autologous or allogeneic cell therapy. Clinical trials for the treatment of various diseases have proven the therapeutic relevance of transplanted MSCs. Consistently, it has been demonstrated that mesenchymal - like cells are found in the stroma of limbus (Polisetti *et al.*,2010) and in lens epithelium that can differentiate into myofibroblasts (Walker *et al.*, 2010). These remarks provided the foundation for the present study, presuming that mesenchymal stem cells might in turn be able to transdifferentiate into a corneal epithelial phenotype in response to corneal or limbus specific microenvironmental conditions. Transdifferentiation implies that adult stem cells maintain the multidifferentiation potentials even after being exposed to certain inductive factors.

It is time-honored that the niche plays an important role in the maintenance of stem cell properties in several tissues. Some of the promising factors for niche regulation are the basement membrane components and cytokines and growth factors produced by limbal fibroblasts in the underlying stroma (Jiang *et al.*, 2010). Various studies have made use of inductive properties of niches to direct the differentiation of stem cells into the corneal epithelial lineages which were accomplished by means of techniques like co culture, use of conditioned media, (Ahmad *et al.*,2007), and enrichment via injection in vivo (Ma *et al.*, 2006 & Ye *et al.*, 2006). Those studies demonstrated that corneal epithelial-like cells can be obtained on providing limbus-specific microenvironment (Ahmad *et al.*, 2007 & Blazejewska *et al.*, 2009). Expression levels of CK3/CK12 were used as a signal of differentiation into a corneal epithelial lineage.

In this study these microenvironmental conditions have been partially simulated in vitro using characteristic extracellular matrix components and fibroblast conditioned media containing specific soluble factors and signaling molecules. Results from our study showed that rabbit bone marrow MSCs is capable of transdifferentiating into corneal epithelial cells in vitro, which make them attractive candidates for the development of autografts. The findings in our study also provide evidence to the hypothesis that conditioned medium can induce transdifferentiation of MSCs into corneal epithelial-like phenotype as shown by the concomitant induction of CK12 by limbal CM. This study also gives evidence that *in vitro* differentiation

potential of MSCs is not restricted to mesodermal lineages but also into other lineages like corneal epithelial cells could be realized.

The current observations substantiate the importance of the limbal stem cell niche, which normally regulates the behavior and differentiation of limbal stem and progenitor cells, for inducing transdifferentiation into a corneal epithelial-like phenotype. These findings need further authentication using *in vivo* studies in animal models, to prove the biofunctionality of these cells in ocular surface reconstruction. Furthermore the exact molecular mechanisms that trigger this transdifferentiation process needs to be evaluated.

Our findings may support the development of autologous transdifferentiated tissue - engineered constructs which could represent an attractive source and a promising therapeutic tool for ocular surface reconstruction and restoration of visual function in patients with ocular surface disorders. Clinical studies will have to prove whether the systemic application of predifferentiated corneal epithelium may have positive effects in patients with ocular surface disorders. Transdifferentiated MSCs could also be beneficial in the bioengineering of corneal epithelium which is an essential requirement for the successful ocular surface reconstruction.

Summary & Conclusion

CHAPTER IV

SUMMARY AND CONCLUSION

The ocular epithelium is composed of stratified epithelial cells whose maintenance is achieved by limbal stem cells. The damage or loss of limbal stem cells due to variety of factors can compromise LSC population which in turn can lead to severe ocular defects and blindness. At present there are no ideal therapies available for treating such disorders, especially in the case of bilateral deficiency. Normally allografts are used for transplanatation in such cases. The major shortcomings of allograft transplantation include availability of tissues, significant immune rejection rates and the possibility of infections. Developments in the areas of tissue engineering and regenerative medicine have effectively transformed the handling of ocular cell surface disorders. Corneal LSC transplantation is a well known procedure in the treatment of the ocular surface disorders, and outstanding result has been reported upon using autologous limbal epithelial tissue from the unaffected eye. But there is a greater risk of developing limbal stem cell deficiency in the healthy eye.

Thus there is a definite need to develop alternate cell sources like buccal epithelial cells, embryonic stem cells etc. These cells serve as a potential treatment regimen for congenital and/or acquired ocular surface disease. In this context the present study was aimed to analyze the possibility of transdifferentitaing MSCs to corneal epithelial lineage by simulating micro environmental factors. The study was conducted in three phases.

First phase included isolation, maintenance and characterization of mesenchymal stem cells. Isolation of MSCs resulted in a heterogeneous population of cells with fibroblast morphology Optimization of culture conditions for *in vitro* expansion and maintenance of mesenchymal stem cells by selection of appropriate media was done by assessing cell adhesion and proliferation in different commercially available media. α-MEM together with 10% FBS was found to be ideal for rabbit MSC culture . The medium parameters in our study was set at 10% FBS avoiding the use of growth factors for *in vitro* expansion of cells. Earlier reports required use of growth factors and higher concentration of FBS which was not cost effective. In this study high rate of cell adhesion and proliferation was observed in α-MEM and used

for further studies. Immunostaining and flow cytometry analysis of cells cultured in α-MEM showed maintenance of stem cell characteristics with positive expression of ABCG2, vimentin, CD 105,CD 29 and negative expression of CD 90.

In the second phase, limbal fibroblast cells were isolated, cultured and characterized using antibodies against SPARC and vimentin. The culture conditions for isolation and expansion of rabbit limbal fibroblast cells was standardized. As far as the author's knowledge is concerned this is the first report on isolation of limbal fibroblast cells from rabbit. Positive expression of SPARC and vimentin and negative expression of CD 90 was noted. Conditioned medium from limbal fibroblast culture was used to provide paracrine and autocrine factors of niche for inducing differentiation of MSCs.

In the third phase effect of microenvironmental factors were assessed for inducing transdifferentiation of MSCs to corneal epithelial cells using collagen and limbal fibroblast conditioned media. Three different culture systems (culture system-1 collagen + basal medium, culture system 2- collagen + conditioned medium and culture system 3- conditioned medium) were employed and monitored for differentiation by immunostaining, RT - PCR and flow cytometry of both mesenchymal stem cells markers (ABCG2 and CD 29) and corneal epithelial specific markers (CK3/12). Differentiation of MSCs towards corneal lineage was confirmed by increase in the expression of cytokeratin 12 and decrease in expression of stem cell markers ABCG2 and CD 29 in culture systems 2 & 3. Results of culture system 1 indicated that collagen alone was not enough to give necessary stimuli for differentiation. The present findings provide evidence to the hypothesis that limbal fibroblast conditioned medium can induce transdifferentiation of MSCs into corneal epithelial-like phenotype contrary to previous reports where both collagen and conditioned medium were required to induce differentiation of MSCs. Only single previous study reported the maintenance of differentiated cells for 3 days. In this study it was possible to maintain the culture for longer duration (7 days).

In conclusion an optimized, cost effective cell culture condition for isolation and maintenance of rabbit MSCs was standardized and rabbit limbal fibroblast cells culture system was developed for the first time. The MSCs were possible to be differentiated using limbal fibroblast conditioned medium alone avoiding the use of

collagen and could be maintained for longer time periods than previous reports further substantiating an optimized environment for long term maintenance of differentiated cells.

The success of the present study of transdifferentiating MSCs to corneal epithelial lineage will provide an alternate autologous cell source against LSCs and will be a major solution for patients suffering from bilateral deficiency as this could avoid other set backs due to use of xenogenic or allogenic sources. These findings need further authentication using *in vivo* functional studies in animal models, to use autologous mesenchymal stem cells for replacement of the corneal epithelium in therapeutic applications. Moreover, MSCs and corneal epithelial cells do not belong to the same germ layer. Thus the exact mechanism behind reprogramming remains to be determined. Future studies will be needed to elucidate the role of key factor and the transcription signals that regulate this process.

Our findings may support the development of tissue engineered ocular surface reconstructs based on autologous MSCs. Transdifferentiated MSCs could also be beneficial in the engineering of corneal epithelium which is an essential requirement for the successful ocular surface reconstruction. Due to their multipotency, easy accessibility, and high proliferation rate *ex vivo*, these represent an attractive source of autologous adult stem cells and a promising therapeutic tool for ocular surface reconstruction and restoration of visual function. Clinical studies will have to prove whether the systemic application of transdifferentiated corneal epithelial MSCs may have positive effects in patients with ocular surface disorders.

Bibliography

BIBLIOGRAPHY

Ahmad S, Stewart R, Yung S, Kolli A, Armstrong L, Stojkovic M, Figueiredo F, Lako M. Differentiation of human embryonic stem cells into corneal epithelial-like cells by *in vitro* replication of the corneal epithelial stem cell niche. *Stem Cells*.2007; 25:1145–1155.

Alhadlaq A and.Mao JJ. Mesenchymal stem cells isolation and therapeutics; *Stem Cells and Development* .2004;13: 436-448.

Anderson DF, Ellies P, Renato TF, Scheffer P, Tseng CG. Amniotic membrane transplantation for partial limbal stem cell deficiency.*Br J Ophthalmol*.. 2001;85:567-575.

Arpitha P, Prajna NV, Srinivasan M, and Muthukkaruppan V. A subset of human limbal epithelial cells with greater nucleus-to-cytoplasm ratio expressing high levels of p63 possesses slow-cycling property. *Cornea*. 2008; 27(10):1164-1170.

Arufe MC, De la Fuente A, Fuentes-Boquete,. De Toro FJ, and. Blanco FJ. Differentiation of Synovial CD -105þ Human Mesenchymal Stem Cells Into Chondrocyte-Like Cells Through Spheroid Formation.*Journal of Cellular Biochemistry*. 2009;108:145–155

Ashton BA, Allen TD, Howlett CR. Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. *Clin Orthop Relat Res* 1980; 151:294–307.

Bab I, Ashton BA, Gazit D. Kinetics and differentiation of marrow stromal cells in diffusion chambers *in vivo*. *J Cell Sci* .1986; 84:139–151.

Balasubramanian S, Guruswamy S, Iyer GK and Krishnakumar S. Putative stem cell markers in limbal epithelial cells cultured on intact and denuded human amniotic membrane. *Indian J Med Res*.2008; 128: 149-156.

Barbash IM, Chouraqui P, Baron J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infracted myocardium: Feasibility, cell migration, and body distribution. *Circulation* .2003; 108:863–868.

Barnard Z. Analyses of cytokeratins and p63 isoforms expressed by human limbal epithelial cell .2005.

Berryhill BL, Kane B, Stramer BM, Fini ME, Hassell JR. Increased SPARC accumulation during corneal repair. *Exp Eye Res* 2003; 77:85-92. Erratum in: *Exp Eye Res* 2003; 77: 643.

Blazejewska EA, Schlo¨ tzer-schrehardt U, Zenkel M, Bachmann B, Chankiewitz E, Jacobi C, Kruse FE. Corneal limbal microenvironment can induce transdifferentiationof hair follicle stem cells into corneal epithelial-like cells. *Stem Cells*. 2009;27:642–652.

Boulton M & Albon J. Stem cells in the eye. *Int J Biochem Cell Biol*. 2004; 36 (4): 643-657.

Chen Z, De Paiva CS, Luo L, Kretzer FL. Pflugfelder SC. and Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells*.2004; 22: 355-66.

Chen Z, Evans H, Stephen C, Pflugfelder, Li D. Gap junction protein connexin 43 serves as a negative marker for a stem cell-containing population of human limbal epithelial cells. *Stem Cells*. 2006; 24: 1265–1273.

Choong PF, Mok PL, Cheong SK, Then KY. Mesenchymal stromal cell -like characteristics of corneal keratocytes. *Cytotherapy*. 2007; 9(3): 252- 258.

Cintia S. de Paiva, Zhuo Chen, Rosa M. Corrales, Stephen C. Pflugfelder, De-Quan Li; ABCG2 Transporter Identifies a Population of Clonogenic Human Limbal Epithelial Cells. *Stem Cells* .2005;23:63-73.

Cristina M, Dantas N, Elias P , Dantas C, Ricardo J, Reggi A. Ipsilateral limbal translocation for treatment of partial limbal deficiency secondary to ocular alkali burn . *Br J Ophthalmol* 2001; 85:1031-1033.

Daniels JT, Dart JK, Tuft SJ, Khaw PT. Corneal stem cells in review. *Wound Repair and Regeneration*. 2001; 9(6): 483- 494.

Darwin J. Prockop. Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues. *Science*.1997; 276: 71-4.

Daya SM & Ilari FA.Living related conjunctival limbal allograft for the treatment of stem cell deficiency.*Ophthalmology*. 2001; 108 (1):126-133.

De Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DR. ABCG2 Transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells*.2005; 23: 63-73.

Diaz Valle D, Santoz BE, Benitez Del Castillo JM, Mendez FR, Lopez AC, Martinez JM, Garcia SJ. Sectorial conjuctuival epitheliectomy and amniotic membrane transplantation for partial limbal stem cells deficiency. *Arch Soc Esp Ophthalmol* . 2007; 82(12):769-772

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ and Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8(4)315- 317.

Dua HS & Blanco A. Allolimbal transplantation in patients with limbal stem cell deficiency. *A.Br. J.Ophthalmology*. 1999; 83 (4): 440-490.

Dua HS, Saini JS, Azuara- Blanco A, Gupta P. Limbal stem cell deficiency: concept, etiology, clinical presentation, diagnosis and management. *Indian J Ophthalmol*. 2000; 48 (2): 83-92.

Fang RJ, Tsai MD, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med*. 2000; 343:86-93.

Fini ME, Stramer BM. How the cornea heals: cornea specific repair mechanisms affecting surgical outcomes. *Cornea*.2005; 24(8): 2-11.

Fischer JP, Mikos AG, Bronzino JD.drug delivery in tissue engineering.Fischer JP, Mikos AG, Bronzino JD (eds) Tissue engineering CRC press. 2007 ,13-8-13-15.

Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA.Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol* .1974;2 (2): 83–92.

Gao J, Dennis JE, Muzic RF. The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs*. 2001; 169:12–20.

Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM and Birembaut P Vimentin contributes to human mammary epithelial cell migration. *Journal of Cell Science*. 1999; 112 : 4615-4625.

Gipson IK, Joyce NC and Zieske JD. The anatomy and cell biology of the human cornea, limbus, conjunctiva and adnexa. Foster CS, Azar DT, Dohlman CH (eds) Smolin and Thoft's The cornea: scientific foundations and clinical practice. Lippincott Williams & Wilkins; 2005:1-20.

Gomes JA, Santos MS, Ventura AS, Donato WB, Cunha MC, Hofling- Lima AL. Amniotic membrane with living related corneal limbal /conjuctival allograft for ocular surface reconstruction in Steven –Johnson Syndrome. *Arch Ophthalmol*. 2003; 121 (10):1369-1374.

Gu S, Xing C, Han J, Mark OM, Hong TJ. Differentiation of rabbit bone marrow mesenchymal stem cells into corneal epithelial cells *in vivo* and *ex vivo*. *Molecular Vision*. 2009; 15:99-107.

Guyton AC & Hall JE.Eye.Brenda L.Seagol (ed). Textbook of medical physiology. Philadelphia: Saunders press.; 2000:631-656.

Harkin DG, Barnard Z, Gillies P, Ainscough SL and Apel AJG.Analysis of p63 and Cytokeratin Expression in a Cultivated Limbal Autograft Used in the Treatment of Limbal Stem Cell Deficiency. *Br. J. Ophthalmol*. 2004; 88: 1154–1158

Horwitz EM, Prockop DJ, Fitzpatrick LA. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med*.1999;5:309–313.

Huang B, Wang ZC, Ge J, Chen XG, Liu JB, Fan ZG, Tao J, Liu BQ, Guo FF. A pilot study on transdifferentiation of skin stem cell in reconstructing corneal epithelium. *Natl Med J China*. 2004; 84(10): 838–842.

Ilari L & Daya SM; Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. *Ophthalmology*. 2002; 109(7):1278-84.

Jiang TS, Cai L, Ji WY, Hui YN, Wang YS, Hu D, Zhu J. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. *Molecular Vision*. 2010; 16:1304-1316

JL Walker, N Zhai, L Zhang, BM Bleaken, I Wolff, J Gerhart MGWeinstein, and AS Menko Unique precursors for the mesenchymal cells involved in injury response and fibrosis .*PNAS*; 2010 :107(31)13730–13735.

KarringH, Thøgersen IB, Klintworth GK, Enghild JJ, and Pedersen TM. Proteomic analysis of the soluble fraction from human corneal fibroblasts with reference to ocular transparency. *Molecular & Cellular Proteomics*. 2004; 3:660–674.

Kenyon KR & Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology*. 1989; 96(5):709-22.

Kenyon KR. Limbal autograft transplantation for chemical and thermal burns. *Dev Ophthalmol*. 1989; 18:53-8.

Kevin YH, Kicic CA and Franzco SJW. Limbal stem cells: the search for a marker. *Clinical and Experimental Ophthalmology*. 2006; 34: 64–73.

Klyce SD. Electrical profiles in the corneal epithlelium. *J Physiol*. 1972; 226: 407– 429.

Kolf CM, Cho E and Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Research & Therapy*. 2007; 9:204-14.

Langer R, Vacanti JP. Tissue engineering. *Science*. 1993; 260 (5110): 920-926.

Lapi S, Nocchi F, Lamanna R, Passeri S, Iorio M, Paolicchi A, Urciuoli P, Coli A, Abramo F, Miragliotta V , Giannessi E, Stornelli MR, Vanacore R, Stampacchia G, Pisani G, Borghetti L and Scatena F. Different media and supplements modulate the clonogenic and expansion properties of rabbit bone marrow mesenchymal stem cells. *BMC Research Notes*. 2008; 1(53): 1-7

Latvala T, Puolakkainen P, Vesaluoma M, Tervo T. Distribution of SPARC protein (osteonectin) in normal and wounded feline cornea. *Exp Eye Res*. 1996; 63: 579-84.

Liechty KW, MacKenzie TC, Shaaban AF. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat Med* .2000; 6:1282–1286.

Limb GA & Daniels JT. Ocular regeneration by stem cells: present status and future prospects. *British Medical Bulletin* .2008; 85: 47–61.

Ma Y, Xu Y, Xiao Z, Yang W, Zhang C, Song E, Du Y, Li L. Reconstruction of chemically burned rat corneal surface by bone marrow–derived human mesenchymal stem cells. *Stem Cells*. 2006; 24:315–321.

Nishida K, Yamato M. Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, Okano T, and Tano Y. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium; *N Engl J Med* .2004; 351: 1187-96.

Orlic D, Kajstura J, Chimenti S. Mobilized bone marrow cells repair the infracted heart, improving function and survival. *Proc Natl Acad Sci USA* .2001;98:10344–10349.

Pajoohesh GA & Stepp MA. In search of markers for the corneal epithelium. *Biol Cell* 2005;97: 265–276.

Payushina OV, Domaratskaya EI, and. Starostin VI. Mesenchymal Stem Cells: Sources, Phenotype, and Differentiation Potential. *Biology Bulletin*. 2006;33:12–18.

Peister A, Mellad JA, Larson BL. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood*. 2004; 103:1662–1668.

Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin, D, McKeon F and De Luca M. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 2001; 98: 3156- 3164.

Polisetti N, Agarwal P, Khan I, Kondaiah P, Sangwan VS, . Vemuganti GK. Gene expression profile of epithelial cells and mesenchymal cells derived from limbal explant culture.*Molecular Vision*. 2010; 16:1227-1240

Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK. Mesenchymal cells from limbal stroma of human eye. *Molecular Vision*. 2008; 14: 431-442.

Puangsricharern V, Tseng SC. Cytolgic evidences of corneal diseases with limbal stem cell deficiency. *Ophthalmology* .1995; 102 (10):1476-1485.

Reinshagen H, Auw-Haedrich C,Sorg RV, Boehringer D, Eberwein P, Schwartzkopff J, Sundmacher R and Reinhard T. Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits; *Acta Ophthalmologica*. 2009;21:1-8.

Sangwan VS, Matalia HP, Vemuganti GK, Rao GN. Amniotic membrane transplantation for reconstruction of corneal epithelial surface in cases of partial limbal stem cell deficiency. *Indian J Ophthalmol* .2004;52:281.

Satoru Y, Nobuyuki E, Tomohiko U, Seiichi O, Shiro A. Bone Marrow derived cells in normal human corneal stroma. *Archives of Ophthalmology*. 2006; 124:62-69.

Shimazaki J, Yang HY, Tsubota K. Limbal autograft transplantation for recurrent and advanced pterygia. *Ophthalmic Surg Lasers*. 1996; 27(11): 917-2.

Shimmura S, Miyashita H, Higa K, Yoshida S, Shimazaki J, Tsubota Km. Proteomic analysis of soluble factors secreted by limbal fibroblasts. *Molecular Vision*. 2006; 12: 478-84.

Sinha R, Sharma N, Vajpayee RB. Corneal blindness – present status. *Cataract and refractive surgery t*oday. 2005; (Oct):59-61.

Solomon A, Ellies P, Anderson DF, Touhami A, Grueterich M, Espana EM, Ti SE, Goto E, Feuer WJ, Tseng SC. Long-term outcome of keratolimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. *Ophthalmology*.2002; 109(6):159-66.

Sueblinvong V, Loi R, Eisenhauer PL,. Bernstein IM, Suratt BT, Spees JL and Weiss DJ. Derivation of Lung Epithelium from Human Cord Blood–derived Mesenchymal Stem Cells. *American Journal of Respiratory and Critical Care Medicine.* 2008; 177: 701-711.

Sweetwyne MT, Brekken RA, Workman G, Bradshaw AD, Carbon J, Siadak AW, Murri C, Sage EH. Functional analysis of the matricellular protein SPARC with novel monoclonal antibodies. *J Histochem Cytochem.* 2004; 52:723-33.

Thomas R, Paul P, Rao GN, Muliyil JP, Mathai A. Present status of eye care in India. *Surv Ophthalmol.* 2005; 50 (1): 85-101.

Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol.* 1998; 116(4): 431-441.

Wang DY, Cheng CC, Kao MH, Hsueh YJ, David HK, Ma and JK Chen. Regulation of limbal keratinocyte proliferation and differentiation by TAp63 and Np63 transcription factors. *IOVS.* 2005; 46(9):3102-3108.

Wu Y, Chen L, Scott PG. Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells.* 2007;25:2648–2659.

Yan Q and Sage EH. SPARC, a matricellular glycoprotein with important biological functions. *The Journal of Histochemistry & Cytochemistry.* 1999;47(12): 1495–1505

Ye J,Yao K Kim JC. Mesenchymal stem cell transplantation in rabbit corneal alkali burn model;engraftment and involvement in wound healing.*Eye*.2006;20(4):482-490.

Yu L, Ge J, Wang ZC, Huang B, Yu KM, Long CD , Chen XG. The preliminary experimental study of induced differentiation of embryonic stem cells into corneal epithelial cells. *Eye Science.* 2001; 17: 138–143.

Appendix

APPENDIX

α MEM (MINIMUM ESSENTIAL MEDIUM ALPHA) STOCK

10.2 g of α MEM powder was suspended in 970.7 ml sterile milliQ water. pH was adjusted to 4.0 and the medium was sterilized by membrane filtration. It was then stored at 2-8°C and in dark till use

α MEM (MINIMUM ESSENTIAL MEDIUM ALPHA) SERUM FREE

α MEM stock solution	-	96.07 ml
7.5% Sodium bicarbonate	-	2.93 ml
Antibiotics (penicillin/streptomycin)	-	1 ml

Adjusted the pH to 7.4 and sterilized by filtration

α MEM (MINIMUM ESSENTIAL MEDIUM ALPHA) WITH 10% FBS(100ml)

α MEM serum free	-	90 ml
FBS (Fetal Bovine Serum)	-	10 ml

Adjusted the pH to 7.4 and sterilized by filtration

DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) STOCK

9.6 g of DMEM powder was suspended in 930 ml sterile milliQ water. pH was adjusted to 4.0 and the medium was sterilized by autoclaving at 121°C at 15psi for 15 min. It was then stored at 2-8°C and in dark till use

DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) SERUM FREE (100ml)

DMEM stock solution	-	92.07 ml
200mM Glutamine	-	2.0 ml
7.5% Sodium bicarbonate	-	4.93 ml
Antibiotics (penicillin/streptomycin)	-	1 ml

Adjusted the pH to 7.4 and sterilized by filtration

DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) WITH 10% FBS (100ml)

DMEM serum free	-	90	ml
FBS (Fetal Bovine Serum)	-	10	ml

Adjusted the pH to 7.4 and sterilized by filtration

DMEM LG (DULBECCO'S MODIFIED EAGLE'S MEDIUM- LOW GLUCOSE)

9.95 g of DMEM LG powder was suspended in 940.7 ml sterile milliQ water.

pH was adjusted to 4.0 and the medium was sterilized by membrane filtration.

It was then stored at 2-8°C and in dark till use.

DMEM LG (DULBECCO'S MODIFIED EAGLE'S MEDIUM- LOW GLUCOSE) SERUM FREE (100ml)

DMEM stock solution	-	94.07	ml
7.5% Sodium bicarbonate	-	4.93	ml
Antibiotics (penicillin/streptomycin)	-	1	ml

Adjusted the pH to 7.4 and sterilized by filtration.

DMEM LG (DULBECCO'S MODIFIED EAGLE'S MEDIUM- LOW GLUCOSE) WITH 10% FBS (100ml)

DMEM LG serum free	-	90	ml
FBS (Fetal Bovine Serum)	-	10	ml

Adjusted the pH to 7.4 and sterilized by filtration.

PARAFORMALDEHYDE (3.7%)

3.7gm of paraformaldehyde was added in 100ml PBS and stirred until it dissolves in a magnetic stirrer. 1ml aliquot was made in eppendorff tube and stored at -20°C. It was thawed whenever required.

0.1 M PBS (PHOSPHATE BUFFERED SALINE) 10 X

NaCl	-	8.0	g
KCl	-	0.2	g
Na_2HPO_4	-	1.15	g
$KH_2 PO_4$	-	0.2	g

Dissolved the contents in 100 ml Milli Q water.

Adjusted the pH to 7 and sterilized.

0.1 M PBS (PHOSPHATE BUFFERED SALINE) 1 X

NaCl	-	8.0	g
KCl	-	0.2	g
Na_2HPO_4	-	1.15	g
$KH_2 PO_4$	-	0.2	g

Dissolved the contents in 1000 ml Milli Q water.

Adjusted the pH to 7 and sterilized.

COLLAGENASE (1mg/ml)

1mg of collagenase was dissolved in 1 ml DMEM serum free media and sterilized by filtration.

PENCILLIN-STREPTOMYCIN (1X)-WORKING

1ml of 100X stock pencillin.-streptomycin stock was diluted to 100ml in 0.1 M PBS (1X).

TRYPSIN –EDTA (1X)- WORKING

1ml of 10X stock TRYPSIN-EDTA was diluted to 10ml with 0.1M PBS (1X).

Never freeze and store after thawing.

TRITON X 100 - 0.1%

0.01 ml of triton X - 100 was added to 9 .99 ml of 0.1M PBS (1X).

BSA (1%) - 10 mg /ml

10 mg BSA was dissolved in 1 ml of 0.1M PBS (1X).

0.1 M NaOH

400 mg NaOH was dissolved in 100 ml distilled deionized water and sterilized by membrane filtration.

7.5% SODIUM BICARBONATE

7.5g of sodium bicarbonate was dissolved in 100 ml distilled deionized water and sterilized by membrane filtration.

COLLAGEN – WORKING SOLUTION

Pure Col ^{TM} (3mg/ml)	- 8 ml
1X PBS	- 6 ml
10 X PBS	- 1 ml
0.1 M NaOH	- 1 ml

Sterilized by membrane filtration and stored at 4 °C.

200mM GLUTAMINE

2.93g of glutamine was dissolved in 100 ml distilled water and sterilized by membrane filtration.