Characterisation of Cultured Oral Mucosal Epithelial Cells and its Application for Reconstructing the Ocular Surface in Patients with Severe Ocular Surface Diseases

THESIS

Submitted in partial fulfilment

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by

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Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

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BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled "**Characterisation of Cultured Oral Mucosal Epithelial Cells and its Application for Reconstructing the Ocular Surface in Patients with Severe Ocular Surface Diseases**" and submitted by Subhash Gaddipati ID No. 2007PHXF030 for award of Ph.D. degree of the institute, embodies original work done by him under my supervision.

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Abstract

Objectives:

The purpose of this study was to standardise the technique of culturing the human oral mucosal epithelial cells on de-epithelised human amniotic membrane and establish its morphological and phenotypic characters in comparison with corneal and conjunctival epithelial cells. In the second phase, patients with severe bilateral ocular surface disease were treated using autologous cultured oral mucosal epithelial cells and followed up for 2-3 years to establish the long term survival of transplanted oral mucosal epithelial cells.

Methods:

After Institutional Review Board approval and informed consent, Oral mucosal biopsies were harvested from human subjects and grown on de-epithelised human amniotic membrane by explant culture method. Oral mucosal cultures were characterised in comparison with limbal and conjunctival cultures by histological examination, electron microscopy, reverse transcription polymerase chain reaction, microarray and immonohistochemistry. After characterisation of oral epithelial cultures, autologous oral mucosal epithelial cells were transplanted to patient's cornea with limbal stem cell deficiency.

Results:

Sheet of healthy oral epithelial cells was obtained within 3-4 weeks of culture. Electron microscopy demonstrated that the cells formed gap junctions and desmosomes. RT-PCR analysis showed that cultured oral epithelial cells expressed markers of epithelial differentiation such as cytokeratins 3, 4, 13, 15 and connexin 43. The conjunctival cells were positive for *Muc5AC*. The cells also expressed stem cell markers of epithelial cells

such as ΔN isoforms of p63, ABCG2, vimentin as well as p75. The cells did not express cytokeratin 12, a corneal epithelial specific cytokeratin.

A total of 19 eyes of 18 subjects were undergone COMET. The mean follow-up was 22.3 (range: 7 to 48) months. In recipient eyes, recurrence of conjunctivalisation occurred in 2 eyes and persistent epithelial defects occurred in 10 eyes. In 7 (36.8%) eyes the peripheral vascularisation did not progress and the corneal surface was completely epithelialised and stable at 12 months after COMET. Prior to COMET, the BCVA ranged from hand movements to perception of light in all eyes. On the last date of follow-up or before undergoing keratoplasty or keratoprosthesis surgery the BCVA had not improved in 12 (63%) eyes, had improved to counting fingers in 6 (32%) eyes and to 20/125 (5%) in one eye. Three years after PK the BCVA was hand movements. The final BCVA in the four eyes that underwent Boston type 1 keratoprosthesis ranged from 20/20 to 20/30 with a maximum follow-up of 26 months.

The excised corneal buttons showed a five to six cell thickness stratified epithelium with basement membrane. Goblet cells were not observed. Few sub-epithelial vasculatures were seen in close proximity to the basement membrane. Phenotypic characterisation of excised corneal tissue during corneal transplantation showed presence K3, p63 and p75 positive cells in the basal epithelial layers. These findings suggest that the transplanted oral mucosal epithelium maintains its original phenotype without any trans-differentiation to the corneal phenotype.

Conclusions:

Oral epithelial cells can be cultured as explants on de-epithelialised amniotic membrane without using feeder cells. Characterization showed that these cells maintain the phenotypic characteristics of oral epithelial cells and that the culture is a heterogeneous

population of differentiated cells and stem cells. We find the cultured oral epithelial cells usable for ocular surface reconstruction in patients suffering from bilateral ocular surface diseases. Though visual recovery is minimal, COMET is a safe and provides re-epithelisation in 30% of treated patients and symptomatic relief in all patients. Transplanted oral mucosal epithelial cells fail to restore the barrier function of limbus.

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DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
bp	Base pair
cDNA	Complimentary DNA
cRNA	Complimentary RNA
dNTP	Deoxynucleotide Triphosphate
mg	Milligram
mL	Milliliter
mM	Millimolar
nm	Nanometer
ºC	Degree Centigrade
OD	Right Eye
OS	Left Eye
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rpm	Rotations Per Minute
µg	Microgram
µL	Microliter
µM	Micromolar
min	Minutes
PI	Propidium Iodide
RNase	Ribonuclease
M	Molar
sec	Seconds
hrs	Hours
CLET	Cultured limbal epithelial transplantation
COMET	Cultured oral mucosal epithelial transplantation
5FU	5-Fluorouracil
MMC	Mitomycin-C
LSCD	Limbal stem cell deficiency

KLAL	Kerato limbal allograft
PK	Penetrating keratoplasty
FACS	Florescent activated cell sorter
BCVA	Best corrected visual acuity
HCE	Human corneal epithelium
hAM	Human amniotic membrane
SJS	Stevens–Johnson syndrome
OCP	Ocular Cicatricial Pemphigoid
H and E	Hematoxylin and eosion
PAS	Periodic acid Schiff

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1 Introduction

1.1 Stem Cells

Stem cells are defined as un-differentiated cells having the capacity to both self renew and give rise to differentiated cells in a controlled manner (Till and McCulloch 1980). The term stem cell appears in the scientific literature from 1868 in the works of the German biologist Ernst Haeckel (Haeckel 1868). Haeckel, a major follower of Darwin's theory of evolution, drew phylogenetic trees to represent the evolution of organisms by descent from common ancestors and called these trees "family trees" or "stem trees". In this context, Haeckel used the term "stem cell" to describe the ancestor unicellular organism from which he proposed all multi cellular organisms evolved (Haeckel 1868; Haeckel 1874). In the revised 3rd edition of his book *Anthropogenie* (Haeckel 1877), Haeckel proposed that the fertilized egg also be called stem cell. In earlier studies, the term stem cell referred to the germ line lineage, primordial germ cells and germ line stem cells (Ramalho-Santos and Willenbring 2007). As shown in the figure1.1, a stem cell can divide and give rise to a similar stem cell or can differentiate in to more specialised tissue specific cells.

A lot of definitions have been given by different groups in the context of different tissues for stem cells. The elaborate definition was given by Potten *et al* (Potten and Loeffler 1990). A stem cell can be defined as "a cell which can divide for indefinite periods, often throughout the life span of an organism under right conditions and differentiate to several defined functional cells". These stem cells are a small sub-population of the tissue in a defined location called as niche and have been estimated to make up 0.5% to 10% of the total cell population. Stem cells are thought to share a common set of characteristics including high

proliferative potential and a long cell cycle with the ability to divide indefinitely while maintaining their stem cell identity, to differentiate to form more specialized cells under tightly regulated conditions.

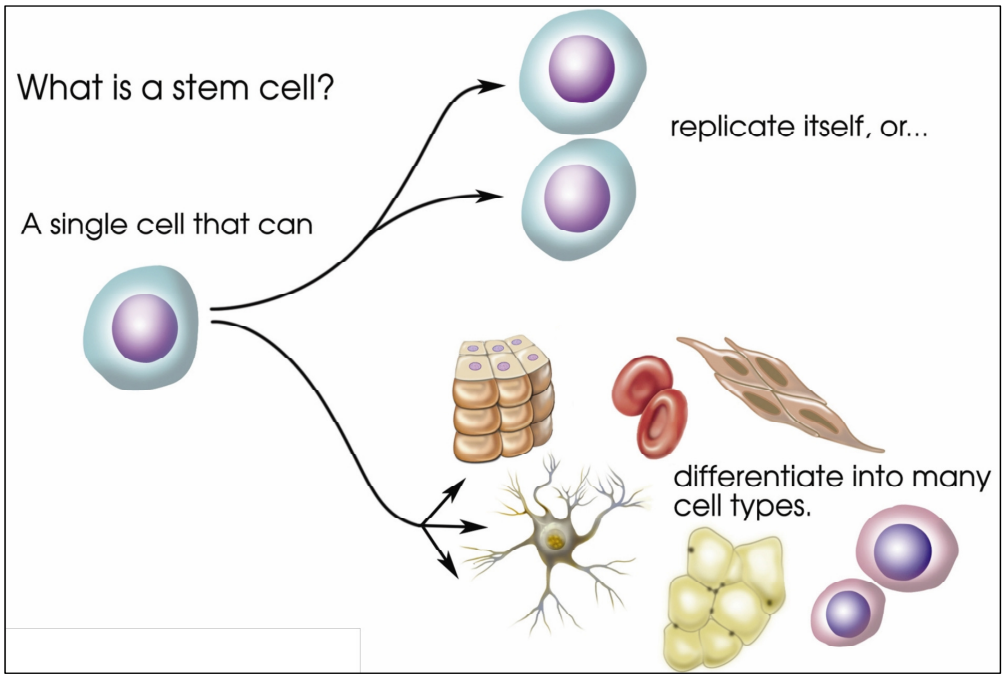


Figure 1.1: Self renewal and plasticity of a stem cell (http://www.nationalacademies.org/stemcells)

The four main characteristics of stem cells are

Undifferentiated cells-Stem cells are undifferentiated at the genotypic and phenotypic level which has the ability to derive a group of specialised cells such as a heart, liver or lung cell.

Self renewability-This is the ability of a cell to divide asymmetrically and give rise to one progenitor cell (stem cell) and one specialised cell (transient amplifying cell).

Differentiation capacity-Stem cells have the potential to form specialised cells of various tissues under defined conditions. This is the ability of a stem cell to cross the germline barrier called as 'plasticity'.

Regeneration- Stem cells have the ability to regenerate in case of any damage to the tissue.

1.2 Stem Cell Niche

Asymmetric division of a stem cell is important for the maintenance of stem cell population and homeostasis of tissues like skin, blood and intestine. The kinetics of stem cell division and differentiation is regulated by different intrinsic factors and micro environment created by extrinsic molecules produced from the surrounding cells, such region is called as 'niche'. The stem cell niche can be defined as "a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self renewing".(Morrison *et al.* 1997; Spradling *et al.* 2001; Ohlstein *et al.* 2004) Different types of niche are shown in the figure 1.2.

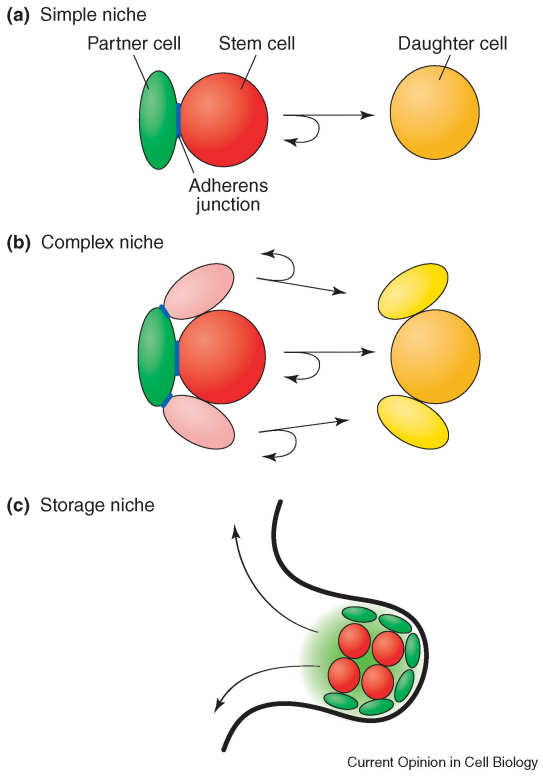


Figure 1.2: Schematic representation of different types of niche.(Ohlstein *et al.* 2004)

In simple niche model (a) stem cell is associated with the partner cell and divide asymmetrically to give a stem cell and specialised cell. In complex niche model (b) stem cells are surrounded by one or more partner cells. Storage niche (c) maintains the stem cells in quiescent state until activated by external signals to divide and migrate.

1.2.1 Factors Involved in the Stem Cell Self Renewal

Stem cell self renewal by asymmetric division may be governed by microenvironment and the orientation of plane of cell division. Previous studies by different groups on mechanism involved in stem cell division and control observed that both intrinsic and extrinsic factors play a major role in self renewal.(Mori *et al.* 1990; Irvin *et al.* 2003; Ho 2005; Akala and Clarke 2006; Darr and Benvenisty 2006)

a) Intrinsic factors

Intrinsic factors includes transcription factors (NF-Y, Wnt downstream proteins-lymphoid enhanced factor (Lef), T cell factor (Tcf) and c-myc) (Cadigan and Nusse 1997; Bungartz *et al.* 2011), cell cycle regulators (Cul1, p27), anti senescent factors (Telomerase, poly ADP ribose polymerases and TATA box binding protein related factor 2)(Klapper *et al.* 2001; Beneke and Burkle 2007) and Notch signalling(Bjornson *et al.* 2011) play a major role in self renewal and maintenance of quiescence.

b) Extrinsic factors

Extrinsic factors helps for the asymmetric division of stem cells. In the niche model of different animal tissues, proteins secreted from the niche cells like bone morphogenic proteins (BMP) (Chen and McKearin 2003; Song *et al.* 2003; Zheng *et al.* 2011), Janus kinase and signal transducer and activator of transcription (JAK-STAT) (Kiger *et al.* 2001), angiopoietin-

like (ANGPTL) molecules play a major role in asymmetric stem cell division (Broxmeyer *et al.* 2011).

Mode of stem cells division depends on the conditions as shown in the figure 1.3. When epithelial stem cells are dividing in perpendicular (a) of basement membrane (gray line) generates two similar daughter cells that are stem cells. If the division is parallel (b) to the plane of the basement membrane generates one stem cell and one differentiated cell. This type of asymmetric division is predominant during stratification and normal homeostasis of the tissue. Stem cell pool expansion during embryonic development occurs in symmetric fashion (c). In normal homeostasis cells will divide asymmetrically and maintain constant number of stem cells (d). In case of wound stem cell will divide symmetrically to increase the stem cell pool and start covering the wound by differentiating in asymmetric cell divisions (e). Defective regulation of the cell division (f) lead to tumorigenesis or poor wound healing response.

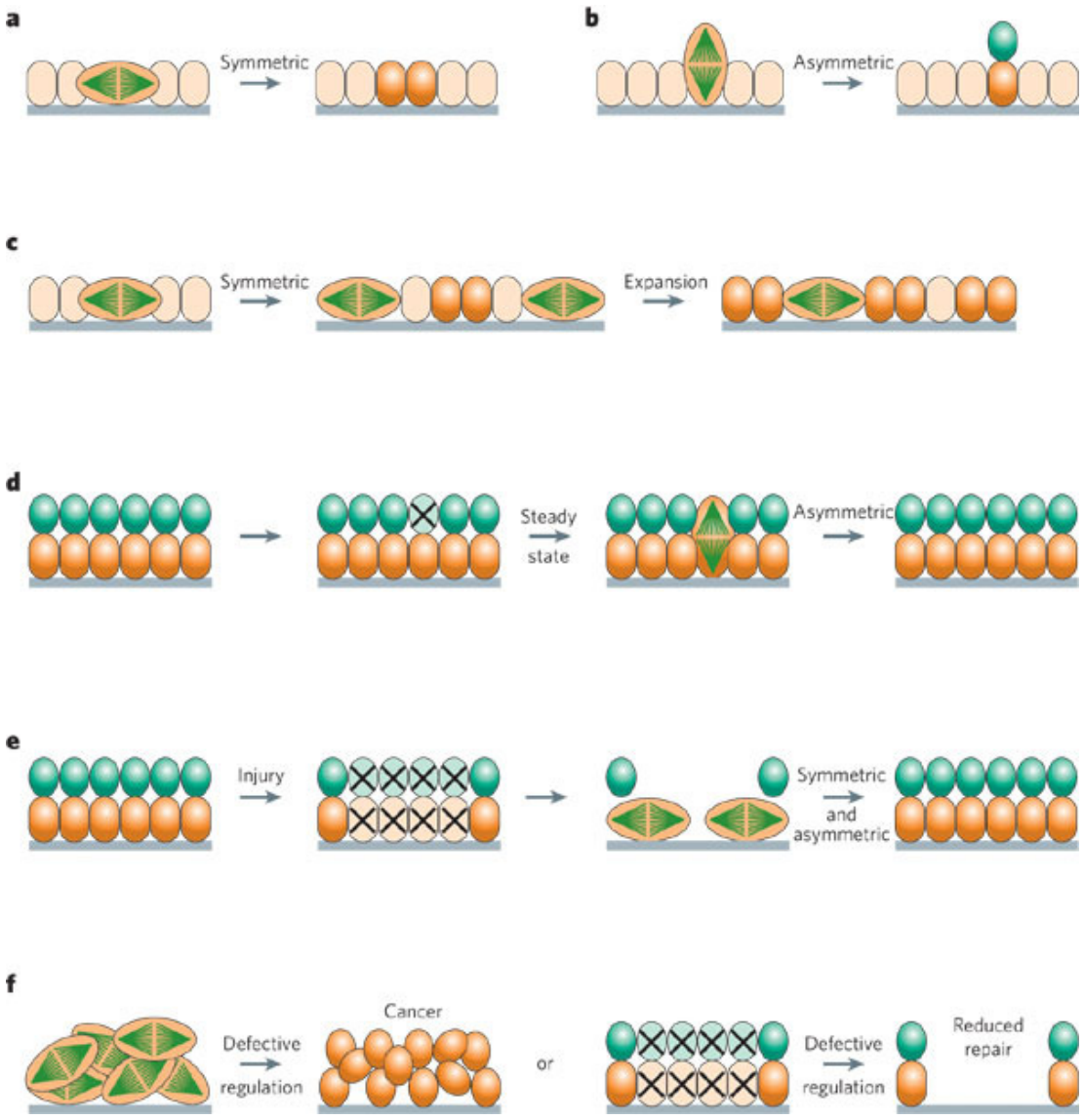


Figure 1.3: Symmetric and asymmetric division of stem cells in different conditions (Morrison and Kimble 2006).

1.3 Classification of Stem Cells

1.3.1 Classification of Stem Cells Based on the Origin of the Cells

Stem cells are classified depending on the origin into embryonic stem cells (ESC), fetal stem cells and adult stem cells (ASC). Embryonic stem cells are from the inner cell mass (ICM), which is part of the early stage (5 days old) embryo called the blastocyst. Once harvested, the

cells of the inner cell mass can be cultured *in vitro* and are capable of unlimited number of symmetrical divisions without undergoing differentiation. In presence of appropriate conditions, they can differentiate in to all cell types of the body.

Fetal stem cells are primitive cell types found in the organs of fetus. Neural stem cells, hematopoietic stem cells and pancreatic islet progenitors have been isolated from aborted fetus (Beattie *et al.* 1997). Fetal neural stem cells found in the fetal brain were shown to differentiate into both neurons and glial cells (Brustle *et al.* 1998; Villa *et al.* 2000). Fetal blood, placenta, amniotic membrane and umbilical cord are also rich sources of fetal hematopoietic stem cells (Seshareddy *et al.* 2008; Chularojmontri and Wattanapitayakul 2009; Mihu *et al.* 2009).

ASC are isolated from the tissues of mature adults. These cells are either unipotent, bipotent or multipotent stem cells, as the number of cell types which they can differentiate into is limited. ASC serve as the reserve stem cell source for generating differentiated cells during tissue regeneration process in living multi cellular organisms. The advantage of ASC is that they offer the opportunity to utilize small samples of patient's own cells for their expansion *in vitro* and subsequent autologous transplantation. This avoids all the problems that accompany allogenic transplantation like immune suppression, graft rejection and also does not pose any ethical concerns. ASC identification or isolation from different adult mammalian tissues were summarised in the below table 1.1 and figure 1.4.

Table 1.1: Evidence for the existence of adult stem cells in different tissues of mammals

S.No	Adult tissue or organ	Location of adult stem cells
1	Skin	Bulge cells, Basal cells of epithelium (Lavker and Sun 1982; Barrandon and Green 1987)
2	Cornea	Limbus (Schermer *et al.* 1986; Pellegrini *et al.* 1999)
3	RPE	Scattered in RPE layer (Salero *et al.* 2012)
4	Gastro intestinal track	Crypt bases (Merritt *et al.* 1995)
5	Lung	Bronchio-alveolar duct cells (Boers *et al.* 1998)
6	Dental	Dental pulp (Gronthos *et al.* 2000)
7	Oral mucosa	Basal cells (Nakamura *et al.* 2007)
8	Pituitary	Anterior portion (Gleiberman *et al.* 2008)
9	Thyroid, parathyroid	Lobes (Thomas *et al.* 2006)
10	Pancreas	Pancreatic ducts (Bernard-Kargar and Ktorza 2001)
11	Liver	Biliary epithelial cells (Alison *et al.* 1996; Alison *et al.* 1998)
12	Brain	Subventricular zone, Hippocampus (Eriksson *et al.* 1998)
13	Retina	Pigmented ciliary epithelial cells (Coles *et al.* 2004; Cicero *et al.* 2009)
14	Skeletal muscle	Satellite-cell (Schalbruch and Hellhammer 1977), Myoblasts (Rando and Blau 1994)
15	Heart	Myocytes of heart (Beltrami *et al.* 2001)
16	Endothelial cells	Blood (Asahara *et al.* 1997)
17	Bone marrow	Marrow stromal cells (Fridenshtein *et al.* 1973) Hematopoietic stem cells (Till and Mc 1961)
18	Testis	Testicular parenchyma (Withers *et al.* 1974)
19	Ovary	Ovarian surface epithelium (Liu *et al.* 2007)
20	Adipose tissue	Adipose stromal cells (Zhu *et al.* 2009)
21	Kidney	Bowman's capsule (Sagrinati *et al.* 2006)

Bone marrow, skin and corneal epithelial stem cells have been the most extensively studied and used for the regenerative therapy in humans till date (Gallico *et al.* 1984; Appelbaum 2007).

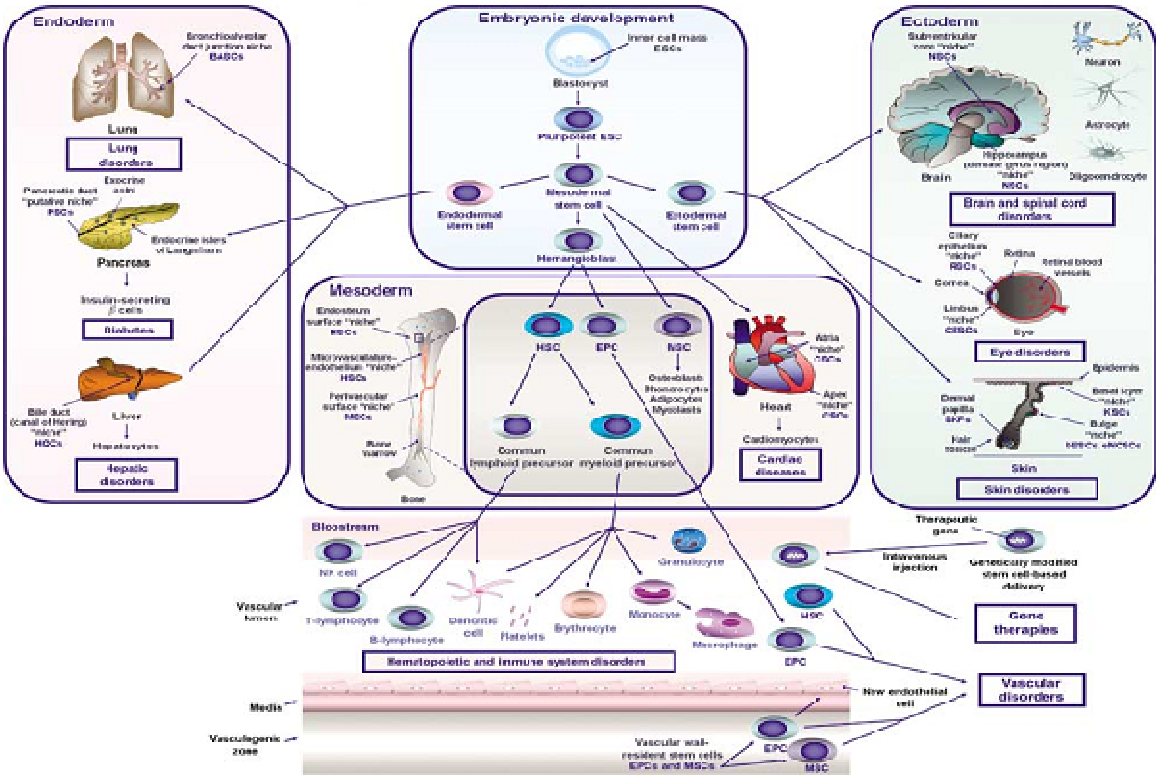


Figure 1.4: Scheme showing the potential therapeutic applications of embryonic and tissue-specific adult stem cells in cellular and gene therapies (Mimeault *et al.* 2007).

1.3.2 Classification of Stem Cells Based on their Potency to Generate Different Cell Types

Based on the differentiation capability of stem cells, they are classified in to the totipotent stem cells, pluripotent stem cells, multipotent stem cells and unipotent stem cells (Table 1.2).

Totipotent Stem Cells: These are the most versatile of the stem cell types. When a sperm cell and egg cell nuclei unite, they form a diploid fertilized egg.This is a totipotent cell, meaning it

has the potential to give rise to all the cell types of the body such as brain, liver, blood, heart cells *etc.* and also the extra-embryonic tissue such as the placenta. The first few cell divisions in embryonic development produce more totipotent cells. The cells of the embryo are totipotent till the morula stage (till 16-cell stage). Later the embryo enters into blastocyst stage forming the inner cell mass and the outer trophoblasts containing more specialised cells.

Pluripotent Stem Cells: These cells, like totipotent stem cells, can give rise to all tissue types of the body. The cells of the inner mass will form all the tissues of the developing human body and are pluripotent. However, they cannot give rise to the extra embryonic structures like the placenta. As these pluripotent stem cells continue to divide, they begin to specialize further. Human embryonic stem cells are derived from the inner cell mass of the blastocyst stage embryo and are pluripotent.

Multipotent Stem Cells: These are less plastic and more differentiated stem cells. They are tissue specific and give rise to a limited range of cells within a tissue type. The offspring of the pluripotent cells become tissue specific progenitors and are multipotent. They can differentiate into multiple cell types of a given organ. For example, a multipotent hematopoietic stem cells can develop into all cell types of blood such as the red blood cells, white blood cells, platelets *etc*.

Unipotent Stem Cells: A unipotent stem cell refers to a cell that can proliferate, self renew and differentiate in to a more mature cell type. Unipotent stem cells are found in many adult tissues. In comparison with other types of stem cells, they have the lowest differentiation potential. The self renew property retained by the ADS helps in the maintenance of reserve

stem cell pool in an adult tissue and plays an important role in tissue regeneration and normal homeostasis.

Table 1.2: Classification of stem cells based on their potency to give rise to various cell types.

Differentiation capability	Potential	Examples
Totipotent	Capable of forming a complete organism including extra embryonic structures like the placenta	Fertilized egg, Embryonal 4 cell stage
Pluripotent	Capable of forming all the 200 different types of tissues of the human body	Cells of ICM harvested from day 5 human blastocyst
Multipotent	Capable of forming many types of specialized cells of a single germ layer	Mesenchymal stem cells, Haematopoietic stem cells of the bone marrow
Oligopotent	Capable of forming a few specialized cells of the same or related tissue/ organ	Hair follicle and bulge epithelial stem cells, Gastrointestinal stem cells.
Unipotent	Capable of forming a single type of specialized cells	Satellite cells of muscles, Limbal stem cells of corneal epithelium

1.4 Strategies of Stem Cell Identification

1. Identification based on *in-situ* labelling with molecular markers which includes stem cell specific markers and cell cycle related markers (Table 1.3)
2. Identification based on *ex-vivo* expansion, labelling, transplantation, assessing repopulated cells

3. Identification based on isolation, *in vitro* expansion in presence of different growth factors, identification of the differentiated or trans differentiated cells.
4. Identification based on clonal expansion**1.4.1 Putative Stem Cell Markers**

Stem cells can be identified and isolated from a heterogeneous population by using markers like ABCG2, PAX6 *etc.* Up to now there is no universal marker to identify stem cells from different tissue sources. Battery of markers is useful for the identification of a stem cell population. Different putative stem cell markers used for identification and isolation were mentioned in the table 1.3

Table 1.3: Putative stem cell markers used for the isolation and identification of stem cells from different tissues in mammals.

S.No	Cell Source	Putative Stem Cell Markers	References
1	Embryonic stem cells	TRA-1-60, OCT-4, TRA-1-81, SSEA4, NANOG, SOX2	(Thomson *et al.* 1995; Rossant 2001; Henderson *et al.* 2002; Adewumi *et al.* 2007; Atlasi *et al.* 2008)
2	Mesenchymal cells	CD90, CD105, CD29, CD71, CD166	(Jones *et al.* 2002; Fickert *et al.* 2004; Igura *et al.* 2004)
3	Neural cells	Nestin, Vimentin, c-kit	(Kukekov *et al.* 1999)
4	Hematopoietic cells	CD34, CXCR4, CD45, CD11a, CD11c, CD138, CD68, CD25 and Lin	(Krause *et al.* 2001)
5	Intestinal epithelium	LGR5, Mushashi-1, BMI-1	(Potten *et al.* 2003; Barker *et al.* 2007; Sangiorgi and Capecchi 2008)
6	Skin epithelium	β1 integrin, CK 15 and 19	(Jones and Watt 1993; Michel *et al.* 1996; Jih *et al.* 1999)
7	Oral mucosal	p75, OCT4, SOX2,	(Nakamura *et al.* 2007; Marynka-

	epithelium	NANOG	Kalmani *et al.* 2010)
8	Corneal epithelium	ΔNp63α, ABCG2, integrin α9	(Chen *et al.* 2004; Di Iorio *et al.* 2005)
9	Pancreatic tissue	NGN-3, FGFR1-IIIB, PDX-1, Nestin	(Cras-Meneur and Scharfmann 2002; Gu *et al.* 2002; Lin *et al.* 2006)
10	Hepatic tissue	Rex-1, alpha-fetoprotein, CD90, c-kit, CD34	(Duret *et al.* 2007)
11	Lung	Cytokeratin 5, Cytokeratin 14, CCSP, SCA-1	(Hong *et al.* 2001; Hong *et al.* 2004; Kim and Jung 2005)

1.4.1.1 Phenotypic Differentiation Markers Used for Characterisation of Oral mucosal Epithelial Cells Vs Limbal and Conjunctiva Cells

A. Cytokeratins

Cytokeratins are a group of structural proteins that form intermediate filaments in the cytoplasm of epithelial cells. They are expressed in different patterns during differentiation of the stem cell population in to epithelial cells. The cytokeratins comprise of about 20 different proteins, which are expressed as a combination of acidic or basic keratins based on the type of epithelium and state of differentiation (Franke *et al.* 1979).

Using cultured oral mucosal epithelial cells for the ocular surface reconstruction, it is interesting to know the common cykokeratin profile in these cultures. For example, cytokeratin 3 and cytokeratin12 are specifically expressed in differentiated corneal epithelial cells. Epithelial cells in the basal layer of the limbus (corneal stem cell niche) region are devoid of these two cytokeratins (Chaloin-Dufau *et al.* 1990). Cytokeratin 4 is expressed in the limbal epithelium of mouse and rabbit eyes. It helps to maintain the internal epithelial cell integrity (Ness *et al.* 1998). Another intermediate filament vimentin has been found in the

basal cells of human and murine limbal epithelium. Cytokeratin 13 is a specific marker of conjunctival epithelium, expressed in conjunctiva, posterior limbal epithelium and absent in the corneal epithelium (Ramirez-Miranda *et al.* 2011). Cytokeratin 15, also known as a limbal specific marker is located in the basal cells of human limbal epithelium (Yoshida *et al.* 2006).

B. Cell-cell, Cell-matrix interaction molecules

Gap junction proteins are transmembrane proteins that help in communication between the neighbouring cells by allowing diffusion of small molecules. Connexin 43 is abundantly expressed in differentiated corneal epithelial cells and serves as a negative marker for the stem cell population.

Cadherins are a family of calcium ion dependent cell-cell adhesion transmembrane proteins. Epithelial cadherin (E-cadherin) mediates cell to cell contact and results in the contact inhibition and regulates cell proliferation. Cytoplasmic domain of E-cadherin strongly associates with the Wnt signalling protein β catenin when it is inactive. Upon activation of Wnt signalling, β-catenin was localised to the nucleus along with the cleaved product of E-Cadherin and activates the target gene expression. Any de-regulation between these protein interactions leads to cancer (Kanai *et al.* 1994).

Integrins are a large family of heterodimeric transmembrane glycoproteins consisting of α and β subunits, which attach cell to extracellular matrix or to the neighbouring cells. Some integrins are suggested to be markers for stem cells, such as Integrin α9, β1 and α6. Integrin β1 and α9 are highly expressed in limbal and corneal epithelial cells with much higher expression in limbal basal layer.

C. Mucins

Mucins are critical molecules for the maintenance of ocular surface and tear film. Secretory and membrane bound mucins are two different mucin molecules expressed on human ocular surface. Six different mucins are present on ocular surface, which are produced from epithelial cells or by ocular glands. Epithelial cells of ocular surface produce membrane bound mucins which includes MUC1, MUC4, MUC16 (Gipson *et al.* 2003). Goblet cells in conjunctiva produce a major secretory mucin MUC5AC (Ellingham *et al.* 1999). Asmall soluble mucin, MUC7 is expressed by the lacrimal gland (Ellingham *et al.* 1997).

1.4.1.2 Strategies for Identification of Stem Cell in Oral Mucosa Cultures

- Identification based on *in situ* labelling with cell specific molecular markers.
- Identification and quantitation based on the expression of the enzyme aldehyde dehydrogenase (ALDH).

The hematopoietic system is a well established model for stem cell system with various markers (CD11a, CD11b, CD14, CD28, CD34, CD45 and CD62L) which helps in their easy identification, quantitation and purification. However, the marker profile varies on other systems including the oral mucosa and this mandates a detailed characterisation of these cells in order to optimise their clinical application.

During the last few years, little progress has been made towards identification of oral epithelial phenotypic molecules that may help to identify stem cells *in situ*. A variety of putative oral mucosal stem cell markers like integrin α6β4 (Calenic *et al.* 2010), Oct4 (Dong *et al.* 2010) have been proposed and p75 (CD 271) was identified as the potential putative stem cell marker in non-keratinised oral mucosal epithelial cells in humans (Nakamura *et al.* 2007).

A. Growth Factor Receptors

Growth factor receptors are localised on the cell membrane of cells. Undifferentiated cells in the basal oral mucosal and limbal epithelium have been reported to contain higher levels of epidermal growth factor receptor and low affinity nerve growth factor receptors (p75 or CD271) when compared to suprabasal epithelial cells (Zieske and Wasson 1993; Nakamura *et al.* 2007; Hayashi *et al.* 2008). Expression of high levels of growth factor receptor allows the stem cells to respond to wounding or other regulatory signals.

B. Cytosolic Proteins

Several proteins involved in cellular metabolic functions have been identified to be expressed in stem cell populations. One such enzyme is ALDH in hematopoietic stem cells (Storms *et al.* 2005). ALDH is a major cytosolic protein in corneal epithelial cells (Gondhowiardjo *et al.* 1991) and it has been found to be expressed in cultured oral mucosal epithelium also making it a potential marker for identifying the stem cell population in oral mucosa.

C. Nuclear Transcription Factors

The transcription factor ΔNp63α has been suggested to be a marker for limbal stem cells (Pellegrini *et al.* 2001; Di Iorio *et al.* 2005). This is one of the proteins that is involved in tumor suppression and morphogenesis. It is a p53 homologue and is consistently expressed in the basal cells of stratified epithelium. Two different promoters help in the expression of the p63 gene. Alternative splicing at C-terminal of the protein generates three different isoforms α, β and γ. N-terminal truncation leads to the loss of the trans-activation domain. Switching of p63α expression to p63β and p63γ was observed in corneal wound healing process. ΔNp63α isoform was expressed in the basal epithelial layer and remaining two were expressed only in supra-basal epithelial cells in the corneal wound model and this supports the concept that

alpha isoform is necessary for the maintenance of proliferative potential of limbal stem cells (Di Iorio *et al.* 2005).

D. Transporter Proteins

In hematopoietic stem cells, an active transporter protein is expressed, which binds to ATP and upon hydrolysis of ATP, pumps out toxic molecules. ABCG2, a member of the ATP binding cassette transporters, which localises in the plasma membrane and is actively involved in Hoechst dye efflux from the cytoplasm of cell has been proposed as a universal marker of stem cells from different tissues (Zhou *et al.* 2001).

E. Eye Neuroectodermal and Ectodermal Marker

Pax6 is expressed in every important ocular tissue. Pax6 is expressed in the surface ectoderm that gives rise to the lens and corneal epithelium (Garcia-Villegas *et al.* 2009). Pax6 is also expressed in the neuroectoderm that forms the neuroretina, retinal pigment epithelium and parts of the iris. Pax6 is transitionally expressed in neural crest cells that form the corneal endothelium, keratocytes, parts of the iris and trabecular meshwork. However, Pax 6 plays a crucial role in corneal epithelial cells normal homeostasis, differentiation and wound healing (Davis *et al.* 2003; Ouyang *et al.* 2006; Dora *et al.* 2008). Pax 6 also contributes to the normal development of olfactory system (Grindley *et al.* 1995; Nomura *et al.* 2007) but the Pax 6 role in oral mucosal epithelial cells is not known.

F. Pro-Angiogenic and Anti Angiogenic Factors

The delicate balance between the angiogenic and antiangiogenic factors that maintains the antiangiogenic privilege to the cornea is very essential. Expression of soluble receptor like vascular endothelial growth factor receptor 1 (VEGFR1) expression in corneal epithelial cells contributes to the avascularity of the cornea. Soluble form of the receptor competes with the

membrane bound VEGFR1 and helps in the elimination of its ligand VEGF secreted from the surrounding vascularised tissue. Soluble form of VEGFR1 was proven as one of the major contributor to the corneal anti-angiogenic barrier (Ambati *et al.* 2006; Ambati *et al.* 2007). Pro cultured oral mucosal epithelial cells showed relatively low expression of anti-angiogenic molecules, thrombospondin-1 and sFLT1 in comparison with cultured limbal epithelial cells (Sekiyama *et al.* 2006; Kanayama *et al.* 2007; Kanayama *et al.* 2009).

1.4.2 Labelling Studies

a. Pulse chase experiment: BrdU is a nucleotide analogue which resembles thymine nucleotide in DNA. BrdU incorporates in the DNA (pulse) during cell division (s-phase). Stem cells are slow cycling in nature. When the cells are further grown in BrdU free medium (chase) the slow cycling cells will retain the label (BrdU) while other cells will lose it during subsequent cell divisions. The incorporated BrdU can be detected by immunoflorescence with anti-BrdU antibody (Dolbeare *et al.* 1985; Carayon and Bord 1992).

b. Dye exclusion: Stem cells from different tissues can be isolated based on the ability to exclude Hoechst 33342 dye. Hoechst is able to enter live cells; cells expressing ATP-binding cassette transporters, which include p-glycoprotein and ABCG2 pump out the dye out of the cell. ABCG2 localises on the plasma membrane and actively involved in Hoechst efflux has been used as a conserved marker of stem cells from different tissues. Cell population which are capable of excluding the dye are called as side population cells (SP cells). To evaluate the SP cells, cells can be incubated with the highly specific inhibitor of ABCG2, fumitremorgin C or virapamil along with Hoechst (Kim *et al.* 2002). In human cornea about 0.3-0.5% cells of

total limbal population exhibit the SP phenotype, whereas SP cells were not present in central corneal epithelium (Watanabe *et al.* 2004).

1.4.3 Differentiation Capacity: Previous experiments showed that stem cells have the capacity to differentiate into same lineage cell types or different lineage (transdifferentiation) based on the plasticity of the cells and the *ex vivo* or *in vivo* conditions. Table 1.4 summarises the possible differentiation capacities of stem cells.

Table 1.4: Differentiation capacity of stem cells

Source	**Differentiation (D) or Transdifferentiation (TD)**	**References**
Mesenchymal stem cells	osteocytes, chondrocytes and adipocytes (D)	(Gronthos *et al.* 1994)
Neural stem cells	Neurons, astrocytes and oligodendrocytes (D).	(Gage 2000; Temple 2001)
GIT epithelial stem cells	Absorptive cells, goblet cells, paneth cells, and enteroendocrine cells (D).	(Eder 1969)
Skin epithelial stem cells	Keratinocytes, hair follicle (D)	(Breathnach 1978)
Hematopoietic stem cells	Red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages (D).	(Lewis *et al.* 1968)
Brain stem cells	Muscle cells (TD)	(Song *et al.* 2009)
Mesenchymal stem cells	Neurons (TD)	(Woodbury *et al.* 2000; Cogle *et al.* 2004; Crain *et al.* 2005)
Muscle stem cells	Neurons (TD)	(Schultz and Lucas 2006)

Embryonic stem cells (human, mice)	All the cell types in adult human or mice (D)	(Gutierrez-Ramos and Palacios 1992)

1.4.4 Clonal Assay

Clonal assay is one of the important tool for stem cell biology as cells with precursor character are not always a pure population. *In vitro* cultivation of the isolated cells at low density will helps in the identification and quantitation of stem cells in a tissue. Clonal assay works on indefinite proliferation capacity of stem cells in appropriate conditions.

Cultured epithelial cell from skin showed three different colonies based on the size. These colonies are named as holoclones, meroclones and paraclones (Rheinwald and Green 1975; Barrandon *et al.* 1989). The holoclone has the greatest proliferative capacity and giving rise to less than 5% of final colony number. The paraclones have the less proliferation capacity due to differentiation. Meroclones are the transient amplifying cells formed from the stem cell. Epithelial cells from limbal region of cornea also form three different colonies (Figure 1.5) on feeder cells (Pellegrini *et al.* 1999). Clonal analysis of human corneal tissue showed presence of corneal stem cells in limbus.

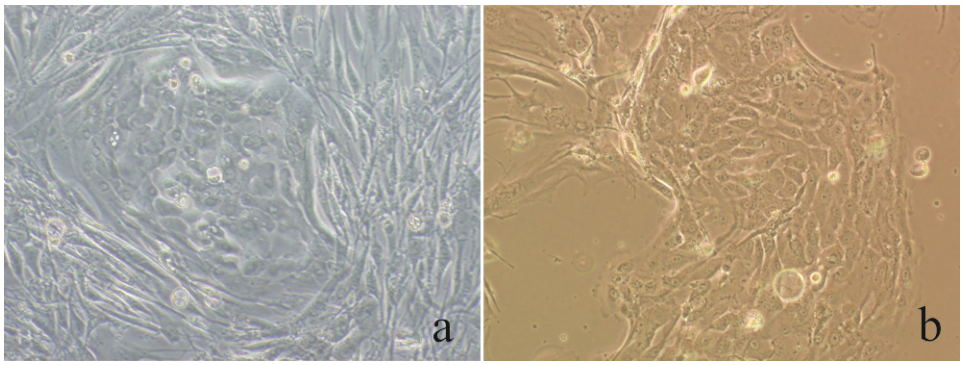


Figure 1.5: Human limbal epithelial cells forming compact holoclone (a) and partially diffused meroclones (b) on mitomycin C treated NIH 3T3 feeder cells.

1.5 Human Eye

1.5.1 Anatomy of the Human Eye

Eyes are light sensory organs, which are present one on each side of the head, in cavities called the orbit (Figure 1.6). They are connected to the brain through the optic nerves. The eye ball has three layers. The outer coat is the sclera, a tough connective tissue. The middle layer is the choroid, which contains rich blood vessels and melanosomes. The melanin pigment of the melanosomes absorbs most of the light and protects the retina. The inner layer is the retina which is transparent and perceives the light signal and provides the sensation of vision. The light rays are focused on to the retina by the transparent cornea (which is the continuation of outer sclera) and the lens. The amount of light entering into eye is controlled by iris, which is pigmented and lies anterior to lens. This portion is called as anterior segment and the space between iris and cornea is called as anterior chamber, which is filled with a clear watery substance called the aqueous humor. The posterior space between the lens and the retina is occupied by vitreous body or the vitreous humor, a clear jelly like substance without any blood vessels or cells. The vitreous body is bounded at its anterior end by the ciliary body, zonular fibers and lens.

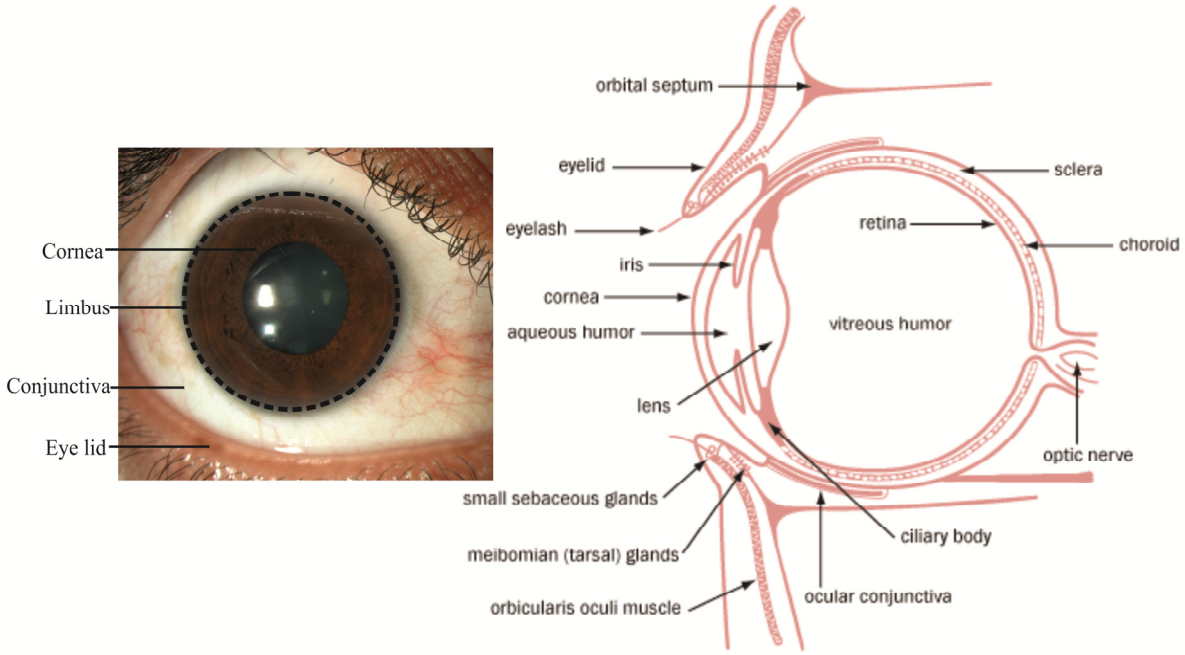


Figure 1.6: Schematic representation of ocular surface and cross section of human eye.

1.5.2 Developmental Biology of Mammalian Eye

Ocular surface epithelial cells originate from the neuroepithelium. During development of the anterior eye segment formation neuroepithelium interacts with neural crest mesenchymal cells. The interaction is under the control of different transcription factors expressed in epithelial or mesenchymal cells or both and orchestrates the organogenesis. The first step during vertebrate eye formation is optic vesicles formation from forebrain. The growing optic vesicle comes in contact with the surface ectoderm. Surface ectoderm responds to the stimulants secreted by optic vesicle and forms the lens placode. The distal part of the optic vesicle invaginates and forms a double layered optic cup. The inner layer of optic cup will form the retina; the outer layer will form the retinal pigmented epithelium.

During the same time of optic cup formation, lens placode enlarges from the surface ectoderm and enter in to the optic cup. The enlarged lens placode is called lens pit, it remains connected to the surface ectoderm (Coulombre and Coulombre 1964). Soon after formation of

lens pit, hyaloids artery passes into the optic cup and supplies nutrients to inner layers of optic cup and lens vesicle. After detachment of lens vesicle from the surface ectoderm the gap between these tissues is filled by mesenchymal cells migrating from the surroundings (6th week). The movement of the mesenchymal cells continues and forms densely packed layers of flat mesenchymal cells along with the fibrillar extracellular matrix. The posterior mesenchymal cells flattens and forms a monolayer of endothelial cells (Reneker *et al.* 2000). At the end of this process, all the layers of cornea have been defined. The surface ectoderm becomes the corneal epithelium, mesenchyme cells between corneal epithelium and endothelium is differentiate in to corneal stromal cells (Hay 1980).

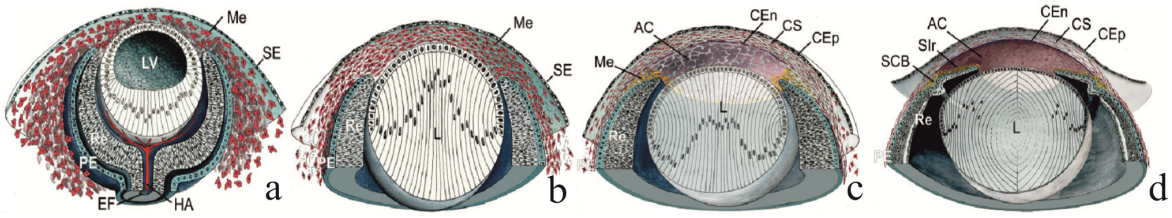


Figure 1.7: Schematic diagram of anterior eye development. (a) Lens vesicle (LV) was detached from the surface epithelium (SE) and optic vesicle was invaginated. Mesenchymal cells (ME) started migration in to the space between the SE and LV. (b) ME cells forms compact layers with fibrillar extracellular matrix. (c) ME cells close to the lens forms the endothelium. After separation of L and SE, anterior chamber is formed between these structures. (d) Anterior chamber (AC) widens and the ME cells flattens to form transparent cornea with epithelium and endothelium.

1.5.3 Ocular Surface Histology

Ocular surface consists of the corneal, conjunctival epithelia and the tear film. The conjunctival epithelium is attached to the sclera loosely and helps in the movement of eye lid

against the ocular surface. Goblet cells in conjunctival epithelium are responsible for mucin production and mucosal immune defence system. Normal tear film is very important to maintain the structural integrity of the ocular surface. Conjunctival epithelium is vascularized and goblet cells secrete mucin which helps in free eye lid movement. Corneal epithelium is avascular and transparent. The ocular surface is flooded with the tear fluid produced by the goblet cells of the conjunctiva (mucin layer), lacrimal glands (aqueous layer) and mebomian glands (lipid layer), which lubricates, nourishes and protects the surface epithelial cells.

1.5.3.1 The Cornea

Cornea is a transparent avascular and alymphatic tissue with a convex surface. The clarity of cornea is due to arrangement of collagen fibres in corneal stroma and due to absence of blood and lymphatic vessels. It is about 11.7 mm in diameter with an axial thickness of 0.52 mm (Donaldson 1966). Cornea contains five tissue layers.

1. Surface epithelium
2. Bowmans layer
3. Stroma
4. Descemet's membrane
5. Endothelium

The corneal epithelium is stratified, squamous and non-keratinized. The basal cells are columnar with rounded heads and flat bases. The cells of the supra-basal layer are called wing cells. This layer contains polyhedral cells, which are differentiated cells of corneal epithelium. Stromal cells secrets the collagen, glycosaminoglycans to maintain the corneal thickness and strength. Endothelial cells are present as monolayer at the posterior part of the cornea.

Endothelial cells helps in the maintenance of corneal hydration and clarity. Descement's membrane is present in between the endothelial cells and corneal stroma.

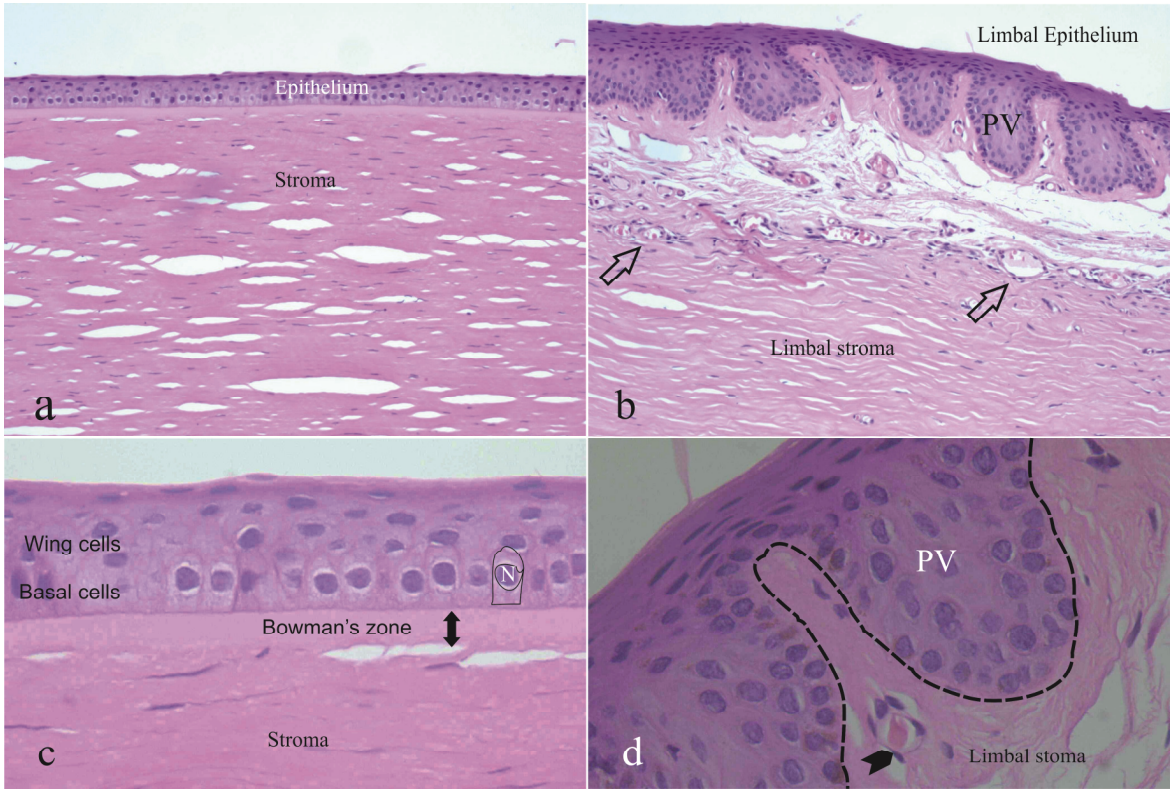


Figure 1.8: Corneal histology. Haematoxylin and eosin stained human cornea. (a) corneal epithelium and the underlying stroma (x100). (b) Limbal epithelium showing finger like extensions deep in to the stromal layer of cornea (x100). These projections are called as palisades of Vogt (PV). Open arrows showing blood vessels at the limbal region (c) Arrangement of columnar basal cells on the Bowman's membrane. On the top of basal cells, wing cells are present (x400). (d) More magnified view of PV. The dotted line is the separation between the limbal epithelium and stromal layer. Arrow head is showing the deep limbal epithelial cells which are called limbal crypts (x400).

1.5.3.2 Conjunctiva

The conjunctival epithelium is well-vascularized. Conjunctiva is also a non keratinised stratified epithelium and contains musin producing goblet cells and epithelial cells. The conjunctival epithelium can be divided into three distinct regions (Figure 1.9).

1. Bulbar conjunctiva, which is contiguous with the corneal-limbal zone and covers the ocular globe.
2. Palpebral, which is contiguous with the epidermis of the eyelid.
3. Forniceal conjunctiva, which is located at the junction of the two regions along the folded margin.

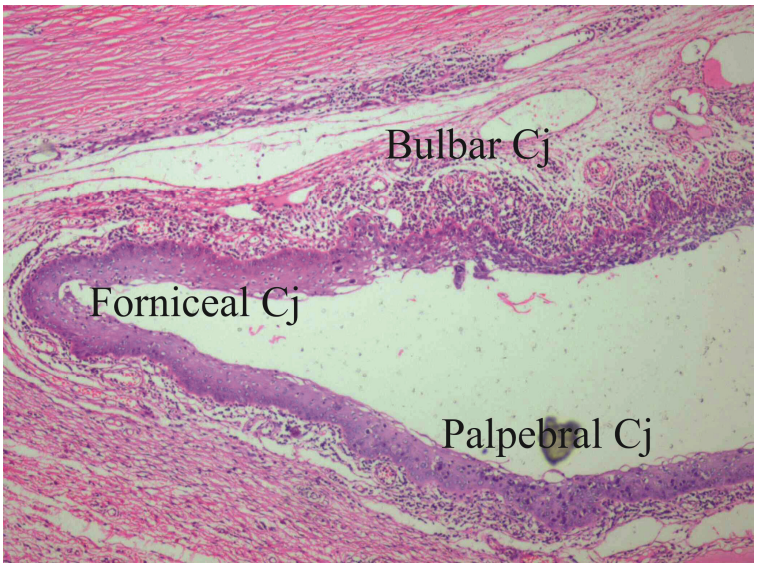


Figure 1.9: Histology of Conjunctiva. Haematoxylin and eosin stained conjunctival tissue showing bulbar conjunctiva on eye ball which is loosely attached, palpebral conjunctiva lining the inner margins of the eye lid. In between the bulbar and palpebral conjunctiva forniceal conjunctiva is present.

1.5.4 Ocular Surface Regeneration

Human ocular surface is covered with two different specialized cell types, conjunctival and limbal-corneal epithelium (Schermer *et al.* 1986). The surface epithelial cells slough off regularly due to eye lid movement and are continuously regenerated from the stem cell population present in the conjunctiva and limbus.

1.5.4.1 Corneal Epithelial Stem Cells

The vascularized corneo-scleral junction is called the limbus and measures about 2 mm in width. Limbus is the niche for the stem cells of corneal epithelium (Davanger and Evensen 1971; Schermer *et al.* 1986; Cotsarelis *et al.* 1989; Thoft 1989; Thoft *et al.* 1989; Lavker *et al.* 1991; Tseng and Tsai 1991). Epithelial cells are continuously renewed from the limbal region and they migrate centripetally on the cornea (Sharma and Coles 1989). Corneal epithelium is renewed in 9 to 12 months (Wagoner 1997) as opposed to every month in case of human epidermis (Green 1980). Although some putative markers for the epithelial stem cell progeny have been proposed (Table 1.5), their role in specifically identifying keratinocyte stem cells is still controversial. Therefore, the identification of stem cells based on either their proliferative capacity *in vitro* or on the identification of slow-cycling cells *in vivo* by ^{3}H or BrdU label retaining cells.

Label retaining and colony forming assay experiments with mouse model and human ocular tissues clearly showed presence of slow cycling, holoclone forming cells in limbal region (Pellegrini *et al.* 1999; Pajoohesh-Ganji *et al.* 2006; Zhao *et al.* 2009). However, recently in mice models, it was shown that the central corneal cells are capable of participating in the regeneration process (Majo *et al.* 2008). In humans, the central island of corneal epithelium was shown to survive for a long time (mean 60 months) in the absence of a

healthy limbus (Dua *et al.* 2009). These results challenge the established fact that the limbus is the only niche for stem cells of cornea (Chang *et al.* 2008; Majo *et al.* 2008).

Table 1.5: Putative limbal stem cell markers

Gene	**Protein function**	**Location**
Vimentin	Structural protein(Lauweryns *et al.* 1993)	Cytoplasm
Cytokeratin 19	Structural protein(Lauweryns *et al.* 1993)	Cytoplasm
α enolase	Glycolysis enzyme(Zieske *et al.* 1992)	Cytoplasm
EGFR	Cell proliferation(Zieske and Wasson 1993)	Cell membrane
p63α	Control of cell division(Pellegrini *et al.* 2001)	Nucleus
Shh	Cell proliferation and survival(Saika *et al.* 2004)	Secretory
ABCG2	Xenobiotic transporter(Budak *et al.* 2005)	Cell membrane

Thoft and Friend proposed an XYZ hypothesis of corneal epithelial cell migration in normal corneal homeostasis. As shown in the figure 1.9, the XYZ hypothesis (X+Y=Z), where X is the anterior migration of the cells from the basal epithelium, Y is the centripetal migration of peripheral cells from the limbus and Z is the loss of cells from the surface, proposed by Richard Thoft, explains the limbus as the source of corneal epithelium (Thoft and Friend 1983).

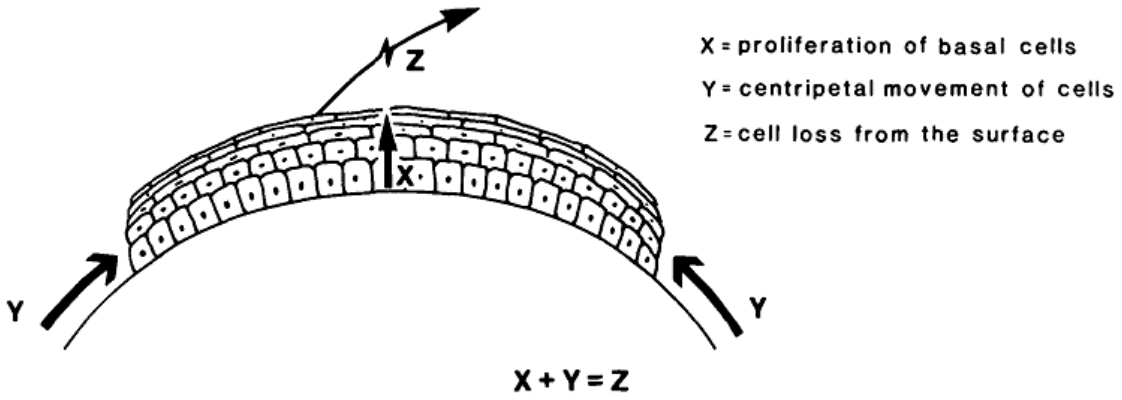


Figure 1.10: The XYZ hypothesis of corneal epithelial maintenance (Thoft and Friend 1983).

The XYZ hypothesis was experimentally tested and measured the centripetal movement of epithelial cells on cornea in mouse model by Buck (Buck 1985). This study proved the centripetal migration of corneal epithelial cells from the limbus to the central cornea.

1.5.4.2 Conjunctival Epithelial Stem Cells

Stem cells were identified all over the conjunctival regions (bulbar, forniceal and palpebral conjunctival epithelia) (Pellegrini *et al.* 1999). Unlike corneal epithelial cells conjunctiva contains two different cell types, conjunctival epithelial cells and the mucin secreting goblet cells. There are also evidences for the presence of conjunctival stem cells in the mucocutaneous junction of the conjunctival epithelium, which is thought to be a source of actively dividing transient amplifying cells that migrate toward the fornix (Ang and Tan 2004).

1.6 Diseases of Ocular Surface

Ocular surface failures can be divided in to two major types depending on the ocular epithelial cell phenotype. The first is the limbal stem cell deficiency, in which the corneal epithelium is

replaced by conjunctival epithelium. The second is the squamous metaplasia, where in the corneal and conjunctival cells shows keratinisation and loss of mucus producing goblet cells from the conjunctiva.

1.6.1 Limbal Stem Cell Deficiency (LSCD)

In patients with corneal epithelial stem cell deficiency, the surrounding conjunctival tissue migrates over the corneal surface and conjunctivalise. This results in photophobia, pain, vascularisation and corneal epithelial defects. Though this process is presumed to be protective, but due to the presence of goblets cells and different cytokeratin profile of the conjunctival epithelial cells, the corneal surface becomes opaque and functionally defective.

Most commonly, limbal stem cell deficiency is caused by chemical or thermal injury (as shown in the flowchart 1.1), multiple surgeries involving the limbal region (Sridhar *et al.* 2001), contact lens keratopathy (Jenkins *et al.* 1993), Oral medication like hydroxyurea (Ellies *et al.* 2001) and systemic diseases like Stevens-Johnson syndrome. LSCD can be diagnosed by impression cytology of the corneal surface. Presence of conjunctival goblet cells indicates limbal stem cell deficiency or dysfunction (Puangsricharern and Tseng 1995).

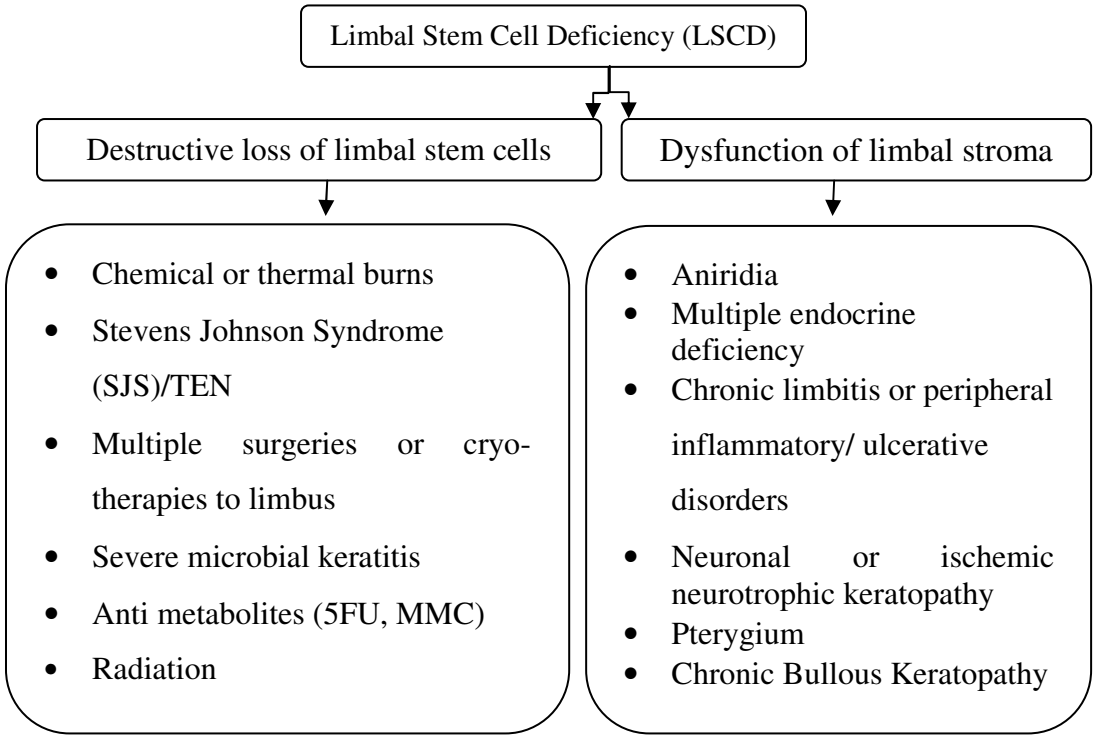
1.6.2 Clinical Presentation and Diagnosis of LSCD

The symptoms of LSCD include decreased vision, photophobia, eye pain, chronic inflammation with redness and watering. The slit lamp examination may include a dull and irregular reflex of the corneal epithelium, which varies in thickness and transparency. Epithelial defects, 360º limbal stem cells deficiency may result in an in growth of thickened fibro vascular pannus, chronic keratitis, scarring and calcification. Epithelial defects can be observed on cornea by fluorescein staining. The pannus growing is more prone to attracts new vessels. In partial stem cell deficiency, a clear line of demarcation is often, but not always,

visible between corneal and conjunctival phenotype of cells. At the line of contact of the two phenotypes, tiny "bud like projections" of normal corneal epithelium can be seen extending into the conjunctivalised area and fluorescein dye tends to pool on the conjunctivalised side of the line of contact, because it is very thin compared with corneal epithelium (Chen and Tseng 1990; Dua and Forrester 1990). Persistent epithelial defects, melting and perforation of the cornea can occur in patients with stem cell deficiency.

LSCD can be best confirmed by taking the impression cytology of cornea (Egbert *et al.* 1977), which can detect presence of goblet cells on the corneal surface. Immunohistochemically, the absence of a cornea-type differentiation (absence of keratin 12) and the presence of goblet cells, has been shown by immunohistochemistry on impression cytology membranes. Conjunctivalised corneal surface may not contain goblet cells, which is evident form the histological observation of pannus removed from the LSCD patients (Fatima *et al.* 2008). Diagnosis of LSCD is crucial because lamellar or penetrating keratoplasty provides only a temporary replacement of the corneal epithelium and the grafted central corneal epithelial cells have a limited proliferative capacity ultimately graft will fail after some time.

Flowchart 1.1: Causes for limbal stem cell deficiency in humans

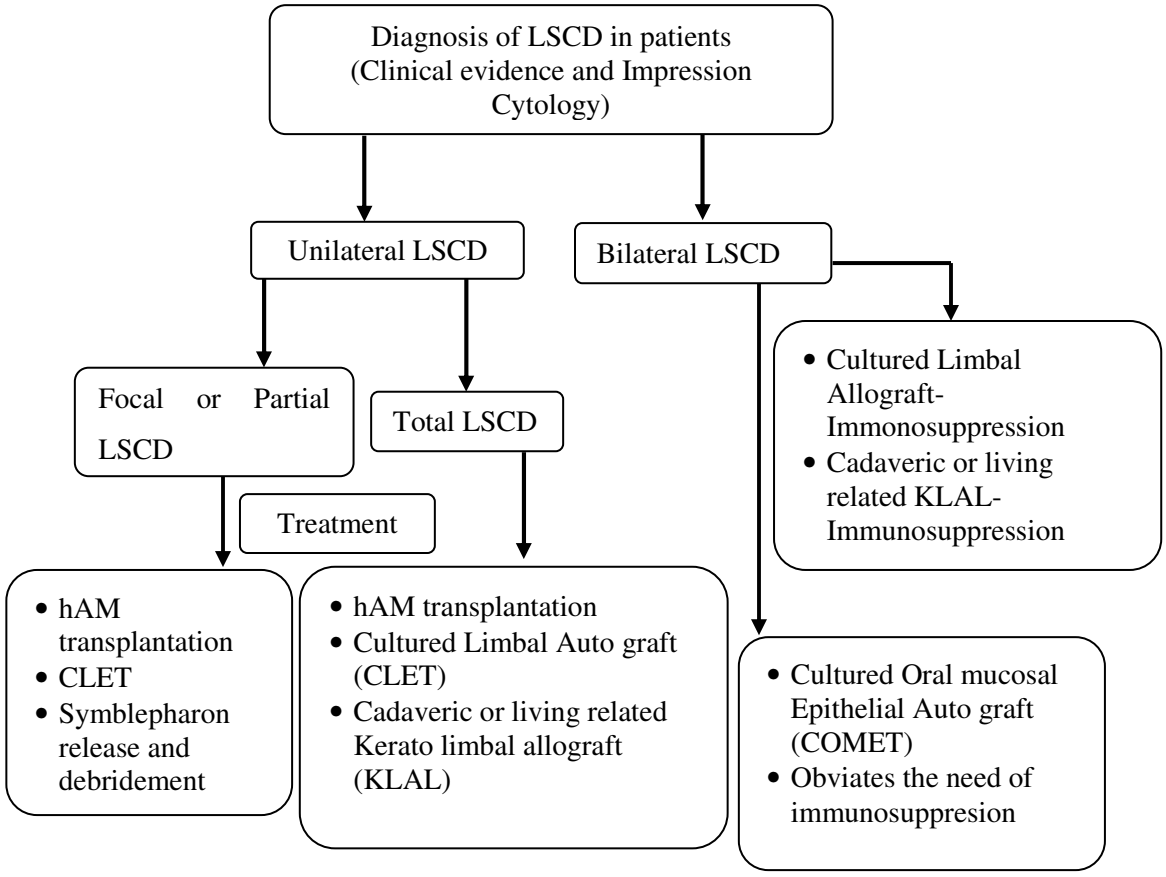


1.6.3 Treatment options for LSCD

A. Symblepharon release and debridement

Symblepheron is a partial or complete adhesion of palpebral conjunctiva of the eye lid to bulbar conjunctiva of the eyeball. It results in the restricted eye lid movement and sometimes covers the cornea. This method involves surgical separation of the palpebral and bulbar conjunctiva, removal of the conjunctival tissue from the corneal surface, called the pannus. This allows the corneal surface to get re-epithelialised from the limbus in patients with partial LSCD. Frequent follow-up of patients with thermal, chemical or mechanical epithelial loss involving the limbus, will allow one to detect any advancing conjunctival epithelial sheet and prevent it from extending on to and beyond the limbus.

Flow chart 1.2: Treatment options for LSCD



B. Human Amniotic Membrane (hAM) Transplantation

Amniotic membrane is the innermost layer of the placenta. It contains avascular stroma covered with single layer of epithelial cells on a thick basement membrane. hAM has anti-inflammatory property, reduces scarring (Solomon *et al.* 2001) and enhance the wound healing capacity. The basement membrane of hAM contains collagens type III and IV and non-collagenous glycoproteins like laminins, nidogen and fibronectin. The use of amniotic membrane in ophthalmology was practiced since the 1940s, when it was used as a patch to cover defects in the conjunctival epithelium caused by trauma and burns (Sorsby and Symons 1946). Decades after the first use of hAM in ophthalmology, Batmanov *et al*. (Batmanov Iu *et al.* 1990) and Kim *et al* in 1995 *(Kim and Tseng 1995)* have reported and reviewed its

ophthalmic use. Thereafter, many groups have used hAM for the treatment of ocular surface in different diseased conditions like LSCD, pterygium, tumour, scar, symblepharon (Duchesne *et al.* 1998; Meller and Tseng 1998), ocular cicatricial pemphigoid, Stevens-Johnson syndrome, chemical and thermal injuries (Tsubota *et al.* 1996; Shimazaki *et al.* 1997; Kruse *et al.* 1998; Meller and Tseng 1998; Azuara-Blanco *et al.* 1999; Meller *et al.* 2000).

C. Direct Limbal Transplantation

The use of autologous conjunctiva for reconstructing chemically burned corneas was described initially by Thoft *et al* in 1977 (Thoft 1977). Failure of trans differentiation of autologous conjunctival cells on the damaged ocular surface (Thoft and Friend 1977) and the identification of corneal stem cells at the limbus by Schermer *et al* (Schermer *et al.* 1986) have made scientists to try limbal tissue for corneal surface reconstruction. Kenyon and Tseng (1989) developed a new surgical procedure termed as "limbal transplantation" (Kenyon and Tseng 1989). In experimental rabbits, Tsai and co-workers (1990) demonstrated that limbal transplantation could restore effectively the corneal epithelial phenotype on severely damaged corneal surface. In contrast conjunctival transplantation results in conjunctival epithelial phenotype (Tsai *et al.* 1990). In case of fibrosis of central corneal stroma resulting in an opaque cornea, the limbal transplantation procedure can be combined with or followed by keratoplasty.

In cases of unilateral LSCD, healthy donor limbal tissues can be obtained from the fellow eye (limbal autograft). The limbal tissue source in bilateral injury can be a live related donors (parents or siblings), or cadaveric tissues (allograft). Limbal transplantation procedures also vary depending on the carrier tissue used for the transfer of the limbal stem cells. A suitable carrier or scaffold is needed in limbal transplants because it is not possible to

culture limbal cells on regular plastic tissue culture dishes and also it is difficult to transfer limbal stem cells as a sheet. Limbal transplant procedures have used either conjunctiva (conjunctival limbal graft) or cornea (kerato-limbal graft) as carrier tissues for limbal stem cells (Holland 1996). This procedure however requires a large limbal graft from the healthy eye. This may increase the risk of limbal stem-cell deficiency in the healthy donor eye (Chen and Tseng 1990).

D. Cultured Limbal Epithelial Transplantation (CLET)

Pellegrini and co-workers first reported that limbal cells can be cultured *in vitro* on lethally irradiated 3T3-J2 cells and can be successfully used for autologous transplantation (Pellegrini *et al.* 1997). The success was defined as restoration of ocular surface integrity and improvement of vision, along with long term maintenance of corneal epithelial cells. Since then various case reports and case series have been reported showing promising results (Rama *et al.* 2010; Sangwan *et al.* 2011). Another study in 2000 showed the use of "bioengineered cornea" for the reconstruction of severely damaged corneal surface with stem cell deficiency (Schwab *et al.* 2000) using limbal epithelial cells cultured on hAM. Limbal epithelial cells were either grown (Table 1.6) on native or de-epithelised hAM (Koizumi *et al.* 2002; Shimazaki *et al.* 2002; Sangwan *et al.* 2003; Harkin *et al.* 2004; Nakamura *et al.* 2004) or on fibrin (Rama *et al.* 2001).

Table 1.6: Different scaffolds used for the limbal epithelial cells culture

S. No	Substrate	Reference	Usage	Source of the scaffold
1	hAM	(Schwab 1999)	Human transplantation (20 eyes)	Human
2	hAM	(Tsai *et al.* 2000)	Human transplantation (6 eyes)	Human
3	De-epithelised hAM	(Schwab *et al.* 2000)	Human transplantation (14 eyes)	Human
4	hAM	(Sangwan *et al.* 2003)	Human transplantation (2 eyes)	Human
5	Fibrin	(Rama *et al.* 2001)	Human transplantation (18 eyes)	Recombinant fibrin
6	Fibrin	(Higa *et al.* 2007)	Characterisation	Recombinant fibrin
7	Temperature Responsive Gels	(Nishida *et al.* 2004)	Characterisation	poly(N-isopropylacrylamide)
8	Fibronectin and other ECM	(Nakagawa *et al.* 1990)	Characterisation	Rabbit plasma fibronectin
9	Collagen III scaffold	(Dravida *et al.* 2008)	Characterisation	Recombinant Collagen III

E. Cultured Oral Mucosal Epithelial Transplantation (COMET)

In patients with bilateral LSCD, autologous limbal transplantation is not possible due to absence of healthy limbal region (Dua and Azuara-Blanco 2000). Limbal-allograft transplantation (from live related or cadaveric donors) can be performed in patients with bilateral deficiencies (Tsubota *et al.* 1999). But this requires long-term immune suppression

that involves high risks of serious eye and systemic complications including infection and liver dysfunction. Also, in case of non-compliance, the allografts fail due to immunological rejection and subsequently results in conjunctivalisation of the corneal surface (Nakamura *et al.* 2010), however, there are reports showing some success in LSCD due to SJS (Koizumi *et al.* 2001; Koizumi *et al.* 2001). To avoid the problems of rejection and other systemic complications, transplantation of autologous oral mucosal epithelial cells cultured on amniotic membranes to damaged ocular surface has been reported. Several groups have explored alternate source of autologous non keratinised epithelial stem cells for treating bilateral ocular surface defects (Table 1.7). In bilateral LSCD patients corneal tissue shows deep scar in the visual axis. After reconstruction of the corneal surface with autologous epithelial cells, it is necessary to transplant corneal tissue for visual rehabilitation.

Table 1.7: Alternative source of autologous epithelial stem cells for treating bilateral LSCD

Alternate sources of epithelium	Study model
Nasal epithelium	Human (Kim *et al.* 2010; Chun *et al.* 2011)
Conjunctival epithelium	Human (Tanioka *et al.* 2006; Ang *et al.* 2010)
Rectal mucosa	Rabbit (Pertuiset *et al.* 2005)
Bone marrow	Rat (Ma *et al.* 2006)
Dental pulp stem cells	Rabbit (Monteiro *et al.* 2009; Gomes *et al.* 2010)
Oral mucosal epithelium	Rabbit (Nakamura *et al.* 2003; Nakamura and Kinoshita 2003; Hayashida *et al.* 2005), Human (Nakamura *et al.* 2004; Nishida *et al.* 2004)
Vaginal epithelium	No animal or human studies

Apart from conjunctival cells, oral mucosal cells have been characterised and reported by different groups for ocular reconstruction (Nakamura *et al.* 2004; Nishida *et al.* 2004; Satake *et al.* 2008). Corneal, oral mucosal epithelial cells share similar phenotypes (stratified, squamous and non-keratinised) and marker expression profiles with limbal epithelium and there by provides autologous alternative to limbal cells. Details of the COMET studies in animal and humans are summarized in Tables 1.8-1.9.

Table 1.8: COMET study in rabbits with limbal stem cell deficiency

Group	Animal model	Culture condition	LSCD model	Follow up time	Outcome	Complications
(Nakamura *et al.* 2003)	Adult albino rabbits (n=8)	Rabbits oral mucosal cells on denuded hAM (Autologous) with mitomycin C inactivated 3T3 feeder cells and air-lifting technique	Lamellar keratectomy	10 days	Epithelial cells sheets adhered well to the host corneal stroma with no evidence of sub epithelial cell infiltration or stromal edema.	Nil
(Nakamura and Kinoshita 2003)	Adult albino rabbits	Rabbits and human oral mucosal cells on denuded hAM (Xeno and autologous transplantation) with mitomycin C inactivated 3T3 feeder cells and air-lifting technique	Lamellar keratectomy	2 days	Intact epithelial sheet	Nil
(Hayashida *et al.* 2005)	New Zealand White rabbits	Suspended culture on poly N-(isopropyl-acrylamide) with mitomycin C treated 3T3 feeder cells without air-lifting	Lamellar keratectomy	4 weeks	Intact epithelial sheet $3.38\pm0.61 \times 10^3$ CFU in primary cells and $1.31\pm0.31 \times 10^3$ CFU in cells harvested from secondary cultures	Nil

Table 1.9: COMET in humans with bilateral limbal stem cell deficiency

Group	Tissue harvesting site	Culture condition	Patient condition	Mean follow up	Vision improvement/other	Complications
(Nishida *et al.* 2004)	Lower lip (Labial mucosa)	Suspended culture on poly N-(isopropyl acrylamide) with mitomycin C treated NIH 3T3 cells for 14 days without airlift	SJS-1 OCP-3 With Symblepharon	14 months	NA/No epithelial defect	Nil
(Nakamura *et al.* 2004)	NA	Suspended co-culture with Mitomycin C treated NIH 3T3 on de epithelised hAM	SJS-Chronic phase-2 (3 eyes) Chemical burn acute-1 (2eyes), Chronic-1	13.8 months (SD2.9) mean time	HM to 4/200-3, 20/30-1, 20/600, 20/200 / No epithelial defect	Nil
(Inatomi *et al.* 2006)	NA	Suspended co-culture with Mitomycin C treated NIH 3T3 on de epithelised Ham	SJS-1 Chemical injury-1	22.5 months	HM to 20/100 after PK, HM to 20/125 after PK	Nil
(Inatomi *et al.* 2006)	NA	Suspended co-culture with Mitomycin C treated NIH 3T3 on de epithelised hAM	Chemical injury-5, Thermal injury-1, SJS-7, Idiopathic ocular surface disorder-1,	20 months	HM-10, CF-2, 20/400-1, 20/500-2 Improved to 20/32 to 20/2000-11,	Epithelial defect in 5 patients

			pseudo cicatricial pemphigoid-1		HM-2, CF-2	
(Nakamura *et al.* 2007)	NA	Suspended co-culture with Mitomycin C treated NIH 3T3 on de-epithelised hAM	SJS-3 Chemical injury-3	NA	NA	Oral cells are present in the PK corneal tissue
(Satake *et al.* 2008)	Inferior buccal mucosa	Suspended co-culture with Mitomycin C treated NIH 3T3 on de-epithelised hAM with airlift	SJS-2 pOCP-2	6, 15, 19 and 24 months	stable ocular surface with 20/125, HM, and 20/200 VA	Increased intraocular pressure in one patient
(Ma *et al.* 2009)	A large biopsy of 6x6 mm	Suspended co-culture with Mitomycin C treated NIH 3T3 on de epithelised hAM without airlift	Chemical injury-3 Thermal injury-2	26 to 34 months	20/40 to 20/400	Microperforation-1, Small epithelial defect-1
(Takeda *et al.* 2011)	NA	Suspended co-culture with Mitomycin C treated NIH 3T3 on de epithelised hAM with airlift	Chemical injury-1 Thermal injury-2	11 months 29.1 months 50.7 months	12/200 NA NA	Nil
(Burillon *et al.* 2011)	Cheek	Suspended co-culture with Mitomycin C treated NIH 3T3 on UpCell®-Inserts	Burns-9 Others-16	12 Months	In 5 patients > 20/200	Vascularised cornea and ED in 9 patients

1.7 Existing Research Gaps

To obviate the need for immunosupression in bilateral LSCD patients, several groups have done COMET to reconstruct ocular surface in heterogeneous groups in the context of LSCD cause. There is no study on patients with complete bilateral LSCD due to ocular burns. Encouraging early and long-term clinical results of *ex-vivo* cultivated oral mucosal epithelial transplantation have also been reported (Nishida *et al.* 2004; Inatomi *et al.* 2006; Inatomi *et al.* 2006; Sahu *et al.* 2007; Satake *et al.* 2008; Ma *et al.* 2009) in SJS and ocular burn patients. However, all the studies have used xeno-biotic materials for cell culture in the form of murine feeder cells (3T3) or fetal bovine serum. Use of xeno-biotic material in cell culture carries the risk of transmitting infections to the transplant recipient (Schwab *et al.* 2006); (Halme and Kessler 2006; Fink 2009) To avoid this, completely xeno-free technique of cell culture is necessary for the cultivation of oral mucosal epithelial cells. Molecular characterisation of the cultured oral mucosal epithelial cells on hAM without any xenobiotics also not reported.

After ocular surface stabilisation by COMET in bilateral LSCD patients for further visual rehabilitation some of the patients may be suitable for corneal transplantation. Previous studies on this excised corneal tissue after COMET demonstrated the persistence of transplanted oral mucosal epithelial cells (Nakamura *et al.* 2007; Chen *et al.* 2009). In this study we want to characterise the excised corneal tissue for cytokeratins (CK3/12, CK14 and CK19), stem cell markers (p63, p75), proliferating marker Ki67 and Pax6. This will help to determine the survival, progenitor cells and polarity of the cell division in corneal tissue that previously underwent autologous cultured oral mucosal epithelial transplantation.

Our group established a xeno free, feeder cell free explant culture system for culturing limbal epithelial stem cells (Vemuganti *et al.* 2004; Mariappan *et al.* 2010),

which has been used successfully to treat over 500 eyes with unilateral LSCD (Sangwan *et al.* 2011). This study extrapolates the same culture system for culturing oral mucosal epithelial cells. Oral mucosal epithelial cultures will be considered for the clinical transplantation after characterisation of these cells for epithelial phonotype, stem cell phenotype. After Institutional Review Board approval, bilateral LSCD patents with vision loss due to ocular burns, without any systemic and oral mucosal diseases will be enrolled after informed consent.

1.8 Hypothesis and Aims of the Study

1.8.1 Hypothesis

In bilateral severe LSCD with no available autologous limbal tissue, cultured oral mucosal epithelium (non keratinised) could serve as an alternative epithelial source, which also obviates the immunosuppresion.

1.8.2 Aims

Phase 1

1. To establish *ex-vivo* cultures of human oral mucosal epithelial cells (explant culture method) on de epithelised human amniotic membrane without any feeder cells.
2. To characterise the cultured oral mucosal epithelial cells in comparison with cultured limbal, conjunctival epithelial cells in terms of histology, immunophenotyping, electron microscopy studies, RT-PCR and microarray.

Phase 2

1. *Ex vivo* expansion of oral epithelial cells for clinical transplantation.
2. To evaluate the clinical outcome of transplantation of cultured oral mucosal epithelial cells on to the damaged corneal surface.

3. To evaluate the *in vivo* survivality of transplanted oral mucosal epithelial cells by immunophenotyping.

CHAPTER 2: MATERIALS AND METHODS

This chapter describes the materials and methods use for the characterisation and cultivation of oral mucosal epithelial cells and its application for reconstructing the ocular surface in patients with severe ocular surface diseases.

2.1 Recruitment of volunteers or patients for oral mucosa biopsy

This study included the patients presenting with bilateral limbal stem cell deficiency (LSCD) and patients with severe ocular surface disease after obtaining the approval of the institutional review board (IRB) and prior informed consent from the subjects.

2.1.1 Inclusion criteria

The study subjects are chosen based on the following criteria

Subjects with bilateral LSCD and sequel of LSCD like corneal opacification, conjunctivalisation, and neovascularisation which leads to loss of vision were included.

2.1.2 Exclusion criteria

1. Subjects with Sjogren's syndrome- an autoimmune disease in which the tubulo-acinar structure of the salivary and lachrymal glands are destroyed as a consequence of infiltration of T-helper-inducer and B-lymphocytes.
2. Subjects having systemic illnesses such as Steven Johnson syndrome (SJS) are excluded.
3. Subjects having a past history of immune-mediated diseases affecting oral mucosa or by clinical examination of oral cavity are excluded.

2.1.3 Recruitment process

The medical records of patients reporting to us with bilateral LSCD were examined by clinicians and suitable subjects for cultured oral mucosal epithelial cell transplantation was

decided based on the inclusion and exclusion criteria as mentioned above. In this pilot study, the autologous oral mucosal biopsy was taken from 18 patients and the tissues received were processed and cultured on hAM.

2.2 Culture of limbal, conjunctival and oral mucosal epithelial Cells

2.2.1 Preparation of Human Corneal Epithelium (HCE) Medium

The epithelial cells (oral mucosa, limbal and conjunctival) are cultured in HCE medium.

Human recombinant epithelial growth factor (hEGF) (100X stock solution-20 µg/mL):

To prepare a 100 µg/mL stock solution, a vial of 200 µg human recombinant EGF (Sigma-aldrich, Cat# E9644) was reconstituted in 2 mL of filter sterilised solution of 10 mM acitic acid containing 0.1 % human serum albumin. Aliquots were made in 100 µL volumes and stored frozen at -20°C for 6 months.

Human recombinant Insulin (hInsulin):

To prepare a 2.5 mg/mL stock solution, a vial of hInsulin (SAFC, Cat# 91077C) was reconstituted in cell culture grade water. pH of the solution was adjusted to 2.0-3.0 with 0.1 N HCl till the solution was becomes clear. The solution was filter sterilised using 0.22 µm syringe filter. Aeration and foaming was avoided during solubilisation. Aliquots were made in 1 mL volumes and stored at +4°C and freezing was avoided.

Penicillin-Streptomycin:

Penicillin-Streptomycin stock solution was procured from Gibco (Cat# 15070). The stock concentration of Penicillin G (sodium salt) is 5000 units/mL and Streptomycin sulphate is 5 mg/mL suspended in 0.85% saline solution. Aliquots were made in 10 mL falcon tubes and stored at -20ºC.

Amphotericin B:

Amphotericin B stock suspension was procured from Sigma (Cat# A 2942). The concentration of stock solution is 250 µg/mL. 10 mL aliquots were made and stored at -20ºC. Working aliquot was stored at 4ºC.

Table 2.1: Ingredients of HCE basal medium

Ingredients	Catalogue number	Quantity
MEM Eagles medium with alpha modification	Sigma, Cat. No. M0644	3.98 g
Nutrient mixture HAM's F12	Sigma, Cat. No. N6760	6.66 g
Sodium bicarbonate	Sigma, Cat. No. S5761	1.38 g
HEPES	Sigma, Cat. No. H4034	5.95 g
Sterile cell culture grade water		Make up to 880 mL

Method of Preparation

Incomplete HCE medium was prepared by dissolving the above mentioned ingredients (Table 2.1) except growth factors and antibiotics in 800 mL of cell culture grade water. The pH was adjusted to 7.2 using 1N HCl. The volume was then made up to 880 mL and sterilised using a 0.22 µm filter assembly connected to a vacuum pump. After filtration, few drops of sterilised medium was inoculated on to chocolate agar plates to check for sterility.

Complete medium was freshly prepared from the basal medium by adding the growth factors (hEGF [10 ng/mL], hInsulin [2.5 µg/mL]), antibiotics (Penicillin [5 U/mL],

Streptomycin [5 µg/mL]) and fetal calf serum (10 %) just before use. The medium was then filter sterilised through 0.22 µm syringe filter and stored at +4ºC. Amphotericin B [2.5 µg/mL] was added after filtration of complete medium.

Sterility Check for Cell Culture Media and Cultures

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

2.2.2 Harvesting of Limbal, Conjunctival and Oral Mucosal Epithelial Tissues

This study was performed with approval of the IRB. Experiments were performed using oral tissue biopsies from healthy adult volunteers (age 18–60 years) or bilateral LSCD subjects after obtaining their informed consent and performing a pre-surgical evaluation of their ocular and oral health. Volunteers with a history of smoking, chewing tobacco, and oral infection/inflammation were excluded. Twenty-five oral biopsies (from volunteers) were used for standardisation of culture conditions and characterisation. Oral hygiene was optimized with preoperative 1 % betadine mouthwash for three days. A mucosal biopsy of 3×3 mm was obtained from the buccal surface of the lower lip using a Bard Parker blade (no:15) under local anaesthesia. The tissue was excised carefully under an operating microscope to exclude the underlying sub-mucosal connective tissue or fat (As shown in Figure. 2.1). The oral tissues were carried to the laboratory in 1x phosphate buffered saline (PBS) containing antibiotics. Processing was done within 4 hours from the time of collection.

Limbal and conjunctival biopsies were taken after obtaining the informed consent from patients undergoing limbal biopsy for cultured limbal transplantation or from patients undergoing cataract surgery. The biopsies were taken using an established technique (Sangwan, *et al*. 2003). Briefly, under local anaesthesia conjunctiva of the eye was incised 3 mm behind the limbus and dissection was continued toward limbus and then into the cornea for 1 mm using a Bard Parker blade (no-15). The conjunctiva was excised at the limbus just behind the pigmented line (palisades of Vogt) and the limbal tissue with 1 mm clear corneal tissue was excised. The tissues were carried to the laboratory in incomplete HCE medium containing antibiotics for further processing. Eighteen limbal and ten conjunctival biopsies were used for the study.

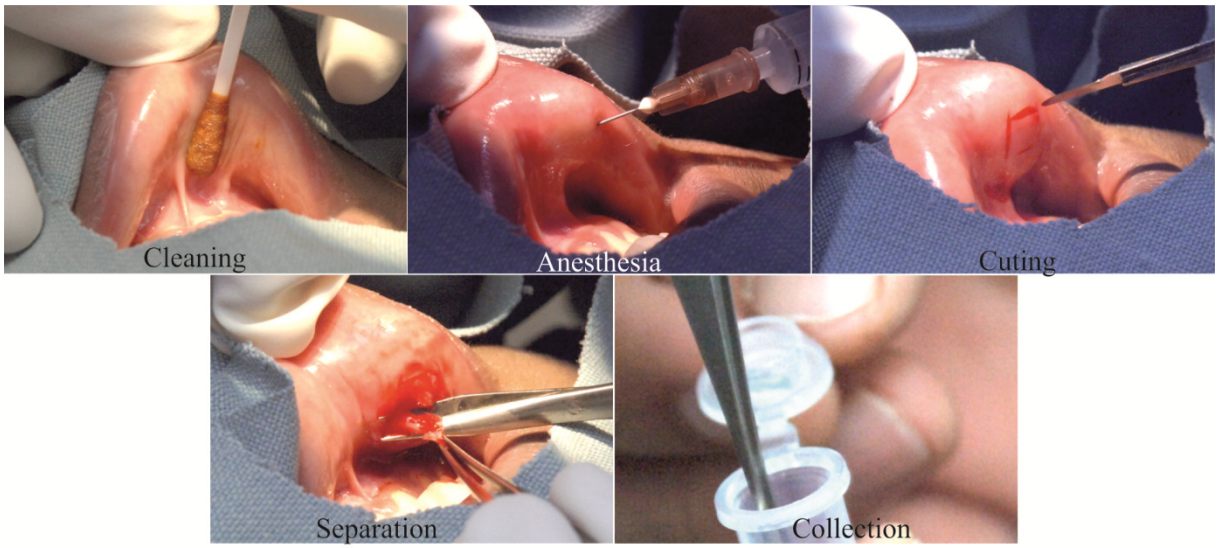


Figure 2.1: Steps involved in the collection of oral mucosal biopsy under local anaesthesia.

2.2.3 Preparation of Human Amniotic Membrane

The hAM was prepared from the placenta obtained during full term caesarean deliveries with the patients consent and processed at the Ramayamma International Eye Bank of L. V. Prasad

Eye Institute as reported earlier (Vemuganti, *et al*. 2004). Briefly, the placenta was washed repeatedly with Ringer's solution containing antibiotics under sterile conditions. The amniotic membrane was peeled off from the chorion, rinsed using Ringer's solution, and placed on a sterile nitrocellulose paper, keeping the epithelium side up. Nitro cellulose sheet with hAM were then cut to fixed dimensions (5x5 cm) and stored in sterile glass vials containing DMEM with 50% glycerol and then stored at −70°C. Prior to use, frozen vials were thawed at 37°C for 30 minutes. The hAM was then peeled off from the nitrocellulose membrane using sterile forceps, and placed on a glass slide (3.5x4 cm) with the epithelium side up. The membrane was then de-epithelialised using 0.25 % trypsin-EDTA at 37°C for 30 minutes followed by mechanical scraping with sterile glass slides and washing with 1x PBS. The excess membrane was gently tucked beneath the glass slide using a blunt forceps to hold the membrane on top of the glass slide.

2.2.4 Explant Culture of Limbal, Conjunctival and Oral Mucosal Epithelial Tissue

The oral biopsy was washed three times with 1x PBS containing antibiotics (penicillin, streptomycin, gentamicin, and amphotericin B). The tissue was then chopped into small pieces (1-2 mm) using a sterile Bard Parker blade (No-21) and the pieces were picked and placed on de-epithelialised hAM using a sterile needle. Tissues were then allowed to adhere to the de-epithelialised hAM and cultured in 4 mL of HCE medium supplemented with 10% fatal calf serum or human serum (autologous) for a period of three to four weeks in a humidified incubator at 37°C with 5% CO_2 supply. The medium was partially replaced (2 mL) on every alternate day. Limbal and conjunctival biopsies were cultured in a similar manner.

2.3 Characterisation of Cultured Oral Mucosal Epithelial Cells

2.3.1 Preparation of paraffin blocks and tissue sectioning

Reagents

1. 10 % Buffered formalin
2. Paraffin
3. Isopropanol
4. Xylene

Procedure

2-3 week old epithelial cultures were fixed in 10 % buffered formalin for overnight at room temperature. Tissues were impregnated with molten paraffin and embedded in appropriate moulds with molten paraffin. The paraffin moulds were allowed to solidify and transferred on to ice blocks.

Microtome (Leica, RM2035) was used to take 5 µm thick serial sections of the tissues. Paraffin block was placed on mounting stage of microtome. The blade angle was adjusted and sections were taken. Tissue sections were floated on diluted methanol to remove folding in tissue slice. The sections were then transferred to 40ºC water bath containing deionised water mixed with gelatine and were transferred on to silane (Sigma, Cat# A3648) coated glass slide.

2.3.2 Hematoxylin and Eosin staining

Reagents

1. Mayer alum haematoxylin
2. Acid isopropanol (0.3 % v/v)

Procedure

Slides to be deparaffinised were placed in a hot air oven set at 110ºC for 15-20 minutes. The slides were then washed with xylene for 5 minutes to dissolve the wax. The slides were then washed in gradients of alcohol from 80 %- 100 % and then washed under a running tap water. Tissue sections were dipped in to haematoxylin solution for 2-5 minutes. After washing in running tap water, non specific staining was washed off with 1% acid alcohol for a very brief time (2 dips). The slides were then rapidly dipped in to weak ammonia water and washed thoroughly under running tap water. The specimen was then counter stained in eosin solution for 2 minutes. The tissue sections were then dehydrated and cleared through 2 changes each of 95% ethanol, absolute ethanol and xylene for 5 minutes each. Slides were then dried and mounted with resinous medium (DPX) and observed under a light microscope. By this staining method, the nucleus of the cell was stained blue while the cytoplasm was stained in pink to red colour.

2.3.3 Periodic acid Schiff staining

Reagents

1. 0.5% Periodic acid solution
2. Schiff reagent
3. Mayer's haematoxylin solution

Procedure

Slides to be deparaffinised were placed in a hot air oven set at 110ºC for 15-20 minutes. The slides were then washed with 100 % xylene for 5 minutes to dissolve the wax. The slides were then washed in gradients of alcohol from 80 %- 100 % and then washed under a running tap water. Tissue sections were oxidized in 0.5 % periodic acid solution for 5 minutes and rinsed

in distilled water. After washing, the slides were placed in Schiff's reagent for 15 minutes and washed in tap water for 5 minutes. Slides were counter stained with haematoxylin for 1 minute and washed in tap water for 5 minutes. Slides were then dehydrated and mounted with (DPX) a synthetic mounting medium, and observed under a light microscope. Using this staining method the cellular glycogen, mucin and some basement membrane components are stained red/purple colour and the background was stained in blue.

2.3.4 Transmission electron microscopy

Cells cultured on hAM were fixed in 2.5% glutaraldehyde in1x PBS for 24 h and then post-fixed in 1 % osmium tetroxide. Samples were dehydrated in a series of alcohol grades and embedded in Spurr's resin. Ultra thin sections were cut using a Leica Ultra Cut UCT-GAD/E-1/00 ultra-microtome (Leica, Wetzlar, Germany), stained with uranyl acetate and counter-stained with 4% lead citrate. Sections were scanned in a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan) at 80 kV.

2.3.5 Immunohistochemistry

Reagents

1. 1x PBS
2. 10 mM citrate buffered solution (pH-6.0)
3. H_2O_2 (30 % v/v)
4. 2.5 % Bovine serum albumin (BSA)
5. Hematoxylin solution

Serial paraffin embedded sections (5 μm thick) of cultured epithelial sheets were deparaffinised by heating at 110°C, rehydrated and blocked for endogenous peroxidase using 3% H_2O_2 in methanol for 30 min. Antigen retrieval was done using 10 mM citrate buffer (pH 6.0) and heated in a microwave oven for 15 minutes (3x5 minutes) and then allowed it to cool to room temperature. Nonspecific sites were blocked by incubation with 2.5 % BSA in 1x PBS after which the sections were incubated overnight at 4°C with the primary antibody diluted to appropriate concentrations. Detection of the bound antibody was performed using a Super Sensitive Non-Biotin HRP detection system (BioGenex, San Ramon, CA), according to the manufacturer's instructions. The sections were counterstained with hematoxylin, mounted in a resinous (DPX) mounting medium and observed using a light microscope.

2.3.6 Immunoflorescence

For immunofluorescence analysis, cells were fixed with 4 % formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 minutes and then washed with 1x PBS. Non specific sites were blocked using 10 % FCS diluted in 1x PBS for 60 min. The sample was then incubated with the primary antibodies at appropriate dilutions (Table 2.2) for one hour at room temperature. Unbound antibodies are washed by 1x PBS rinses. Slides were then incubated with an appropriately diluted secondary antibody (Biotinylated goat anti-mouse or anti-rabbit antibody conjugates [Invitrogen, USA]) for 45 minutes, followed by avidin conjugated to Alexafluor 488 for 45 minutes at room temperature. Unbound antibodies are thoroughly washed with1x PBS rinses. The nucleus was counterstained with propidium iodide (PI) and the sample was then mounted on a glass slide using a glycerol mountant. The slides were then observed and imaged using Carl Zeiss LSM510 confocal microscope.

Table 2.2: Antibody information

S.No	Antibody	Make (Cat#)	Dilution (IHC or IF)
1	Keratin K3/K12	Millipore (CBL218)	1:100 (IHC)
2	Keratin K12	Santa Cruz (SC25722)	1:100 (IHC)
3	Keratin K19	Dako (M0888)	1:50 (IHC)
4	Keratin K14	Biogenex (AM146)	Pre-diluted (IHC)
5	p63	Thermo (MS1081)	1:100 (IHC/IF)
6	p63α	Santa Cruz (SC5301)	1:100 (IHC/IF)
7	E-Cadherin	Chemicon (MAB3199)	1:100 (IHC/IF)
8	p75	Abcam (AB3125)	1:100 (IHC/IF)
9	PAX6	Abcam (AB5790)	1:100 (IHC/IF)
10	CD31	Dako (IR610)	1:50 (IHC)
11	CD34	Dako (M7165)	1:75 (IHC)
12	Ki67	Dako (IR626)	Pre diluted (IHC/IF)
13	BrdU	Dako (M744)	1:200 (IHC/IF)

2.3.7 Flow Cytometric Measurement of Cell Cycle

After cultures become confluent on hAM, cells were harvested by treatment with 0.25% trypsin-EDTA for 10 minutes at 37ºC. Cells were fixed in 70% ethanol at -20°C 1 hour. Cells were then pelleted and resuspended in 250 µL of 1x PBS containing 0.2% Triton X-100 solution and ribonuclease A (1µg/µL) for 30 min at 37ºC temperature, followed by the addition of 130 µL propidium iodide (PI) at 0.1 mg/mL. Cells were incubated in the dark for

10 minutes and analyzed by flow cytometry using a Becton Dickinson FACS Aria (San Jose, CA). After excluding cell debris and doublets, DNA content and cell cycle were analyzed using Cellquest-Pro software. Cells showing DNA content less than the peak for G_1/G_0 were considered sub-diploid and apoptotic.

2.3.8 Reverse Transcription-polymerase Chain Reaction

Total RNA was extracted from 2-3 weeks old cultures using Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Moloney Murine Leukaemia Virus reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) was used to prepare cDNA from mRNA. During the first strand synthesis, in order to avoid reverse transcriptase inactivation, two sets of reaction mixtures were prepared.

A. Reaction mix 1

One microgram of the total RNA was taken in an autoclaved 0.2 mL tube and 1 µL (500 pg/µL) of oligo dT primer was added and kept for denaturation at 65°C for 10 minutes to breakdown secondary conformations of RNA.

Total RNA	5 µL (1 µg)
Oligo dT primer	1 µL (500 pg)
	6 µL

The reaction mix was then quick chilled on ice to avoid re-anealing and formation of secondary structures.

B. Reaction mix 2

5X Reverse transcriptase buffer	10 µL
dNTPs	5 µL (10 mM stock)
Reverse Transcriptase enzyme	1 µL (20 U/µL)
RNase Inhibitor	1 µL (40 U/µL)
RNase free water	27 µL
	44 µL

Reaction mix 2 was added to the mix-1 and a PCR reaction was carried out for cDNA synthesis at the following amplification conditions.

1. 25°C – 10 minutes (For primer annealing)
2. 42°C - 1 hr (Extension)
3. 80°C – 10 minutes (Inactivation)

Second strand synthesis and PCR amplification conditions:

1. 94°C -2 minutes (Denaturation)
2. 94°C -50 sec
3. Annealing varies with primers (50°C – 65°C)
4. 72°C – 50 sec (Extension)
5. Steps 2-4 are repeated for 34 cycles
6. 72°C - for 10 minutes (Final extension)

Annealing temperature and magnesium chloride concentrations were optimized for specific primers. PCRs were performed, using the cDNA template and primers shown in table 2.3. The PCR products were resolved on a 0.8 % agarose gel, stained with ethidium bromide and imagined using a gel documentation system (BioRad, Gel Doc-XR+).

Table 2.3: Primer sequences and PCR conditions used for gene expression characterisation.

Gene	Sequence (5'-3')	Annealing tempt (°C)	Product size (bps)
Cytokeratin 3	F-GGCAGAGATCGAGGGTGTC R-GTCATCCTTCGCCTGCTGTAG	60	145
Cytokeratin 4	F-GCCATGATTGCCAGACAGCAGTGT R-GGGGGTGAGCAAGCTATGGTTG	58	408
Cytokeratin 12	F-ACATGAAGAAGAACCACGAGGATG R-TCTGCTCAGCGATGGTTTCA	60	150
Cytokeratin 13	F-GATCCAGGGACTCATCAGCA R-AAGGCCTACGGACATCAGAA	58	289
Cytokeratin 15	F-GGAGGTGGAAGCCGAAGTAT R-GAGAGGAGACCACCATCGCC	64	193
Connexin 43	F-CCTTCTTGCTGATCCAGTGGTAC R-ACCAAGGACACCACCAGCAT	60	145
PAX6	F-ATAACCTGCCTATGCAACCC R-GGAACTTGAACTGGAACTGAC	55	208
ΔNp63α	F-GGAAAACAATGCCCAGACTC R-ATGATGAACAGCCCAACCTC	60	1389
ΔNp63β	F- GGAAAACAATGCCCAGACTC R-CAGACTTGCCAGATCCTGAC	60	1376
ΔNp63γ	F- GGAAAACAATGCCCAGACTC R-GGGTACACTGATCGGTTTGG	60	1168
p75 (CD271)	F- TGA GTG CTG CAA AGC CTG CAA R-TCTCATCCTGGTAGTAGCCGTAG	54	230
GAPDH	F- GCCAAGGTCATCCATGACAAC R-GTCCACCACCCTGTTGCTGTA	57	498
EGFR	F-TCTCAGCAACATGTCGATGG R-TCGCACTTCTTACACTTGCG	55	474
MUC5AC	F- TCCACCATATACCGCCACAGA R- TGGACGGACAGTCACTGTCAAC	55	103

ABCG2	F-AGTTCCATGGCACTGGCCATA R-TCAGGTAGGCAATTGTGAGG	56.5	379
Integrin 9α	F- TGGATCATCGCCATCAGTTTG R- CCGGTTCTTCTCAGCTTCGAT	55	123

2.3.9 Agarose Gel Electrophoresis

Required amount (0.8 %) of agarose was added to 1x Tris-Acetate-EDTA buffer (TAE). Agarose was melted in microwave with swirling to ensure even mixing. Molten agarose was cooled to luke warm temperature (~45°C). Ethidium bromide was added to the final concentration of 0.5 µg/mL from a stock solution of 10 mg/mL and mixed thoroughly. The resulting gel was poured in a casting tray with an inserted comb. The gel was allowed to stand till it gets solidified. The comb was then removed gently from the gel tray and placed inside the electrophoresis tank contain 1x TAE buffer enough to cover the gel. 5 µL of PCR products were mixed with 1 µL of loading buffer (6x) (MBI Fermentas) on parafilm and loaded into the wells using a micropipette. A known molecular weight marker (100-bp/1kb DNA ladder) (MBI Fermentas) was also loaded in a separate well in order to compare and estimate the size of PCR products.

2.3.10 Microarray

Microarray slides

Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array slides were used for this experiment. Each chip comprised of 1,300,000 unique oligonucleotide features covering over 47,000 transcripts and variants, which, in turn, represent approximately 39,000 of the best characterized human genes. The majority of the probe sets used in the design of the array was selected from GenBank®, dbEST, and RefSeq.

Total RNA isolation

Total RNA was extracted from cultured limbal, conjunctival and oral mucosal epithelial cells by using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The A260/A280 ratio of all RNA samples was estimated based on spectrometric readings (Bio photometer and Spectra max). The quality of RNA preparation was checked on a 1 % agarose gel.

One-Cycle cDNA Synthesis

Preparation of poly-A RNA controls for one cycle cDNA synthesis (spike-in controls)

The controls were amplified and labelled together with the samples. Examining the hybridisation intensities of these controls on Gene Chip arrays helped to monitor the labelling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of lys < phe < thr< dap internal controls quantity.

Prepare the poly-A RNA dilutions for 5 µg of total RNA

From the poly A-RNA stock solutions working solutions were prepared by serial dilutions. 2 µL of the poly-A Control Stock was added to 38 µL dilution buffer for the first dilution (1:20). Diluted sample was mixed thoroughly by quick vortexing and spinning. 2 µL of the first dilution was added to 98 µL of poly-A control dilution buffer to prepare the second dilution (1:50). Diluted sample was mixed thoroughly by quick vortexing and spinning. 2 µL of the second dilution was added to 18 µL of poly-A control dilution buffer to prepare the third dilution (1:10) and mixed thoroughly. 2 µL of this third dilution was added to total RNA (5 µg). The first dilution of the poly-A RNA controls can be stored up to six weeks in a non-frost-free freezer at –20°C and frozen-thawed up to eight times.

First strand cDNA synthesis

One-Cycle cDNA Synthesis Kit was used for this step. A thermal cycler was used for all incubations. The following program (Table 2.4) was used as a reference to perform the first-strand cDNA synthesis reaction.

Table 2.4: Program for cDNA synthesis

Tempt	Incubation time
70°C	10 minutes
4°C	Hold
42°C	2 minutes
42°C	1 hour
4°C	Hold

Table 2.5: RNA/T7-Oligo(dT) primer mix preparation for 5 µg of total RNA

Component	Volume
Sample RNA	Variable
Diluted poly-A RNA controls	2 µL
T7-Oligo(dT) Primer, 50 µM	2 µL
RNase-free Water	Variable
Total Volume	12 µL

5 µg equivalent total RNA sample was taken in a 0.2 mL PCR tube. 2 µL of the appropriately diluted poly-A RNA controls was added to each tube followed by 2 µL of 50 µM T7-Oligo (dT) primer. RNase-free water was added to a final volume of 12 µL (see Table 2.4). The reaction cocktail was mixed by gentle flicking and the tubes were then centrifuged briefly (~5 seconds) to collect the reaction mix at the bottom of the tube. The tube was then incubated for 10 minutes at 70°C. The sample was cooled at 4°C for at least 2 minutes. In a separate tube,

first-strand master mix was prepared enough for all of the RNA samples (Oral mucosa, limbal, conjunctival-each 3 samples) as per the recipe shown in table 2.6.

Table 2.6: Preparation of first-strand master mix

Component	**Volume**	**For ten reactions**
5x 1st Strand Reaction Mix	4 µL	40 µL
DTT, 0.1M	2 µL	20 µL
dNTP, 10 mM	1 µL	10 µL
Total Volume	7 µL	70 µL

First-Strand master mix (7 µL) was transferred to each RNA/T7-Oligo(dT) primer mix for a final volume of 19 µL. The PCR tubes were centrifuged briefly (~5 seconds) and immediately placed on the PCR block maintaining 42°C and incubated for 2 minutes. 1 µL of Super Script II was then added to each RNA sample for a final volume of 20 µL. Reaction solution was mixed well by gentle flicking and centrifuging. The tubes were then placed immediately on the PCR block maintaining 42°C and incubated for 1 hour. The sample tubes were then cooled to 4ºC before proceeding to second-strand cDNA synthesis.

Second –strand synthesis

One-Cycle cDNA Synthesis Kit was used for this step. The following program was used to perform the second-strand cDNA synthesis reaction in a thermal cycler.

Tempt	Time
16°C	2 hours
4°C	Hold
16°C	5 minutes
4°C	Hold

In a separate tube, second-strand master mix was prepared enough for all samples, as per the following recipe (Table 2.7).

Table 2.7: Preparation of second-strand master mix

Component	**Volume/reaction**	**Volume/10 reactions**
RNase-free water	91 µL	910 µL
5X 2nd strand reaction mix	30 µL	300 µL
dNTP, 10 mM	3 µL	30 µL
E. coli DNA ligase	1 µL	10 µL
E. coli DNA polymerase I	4 µL	40 µL
RNase H	1 µL	10 µL
Total volume	130 µL	1300 µL

Second-Strand Master Mix (130 µL) was added to each first-strand synthesis sample (20 µL) and mix thoroughly. The samples were then incubated for 2 hours at 16°C and 2 µL of T4 DNA polymerase was then added to each sample and incubated for 5 minutes at 16ºC. The reaction was stopped by adding 10 µL of 0.5 M-EDTA and the samples were then processed for double-stranded cDNA cleanup.

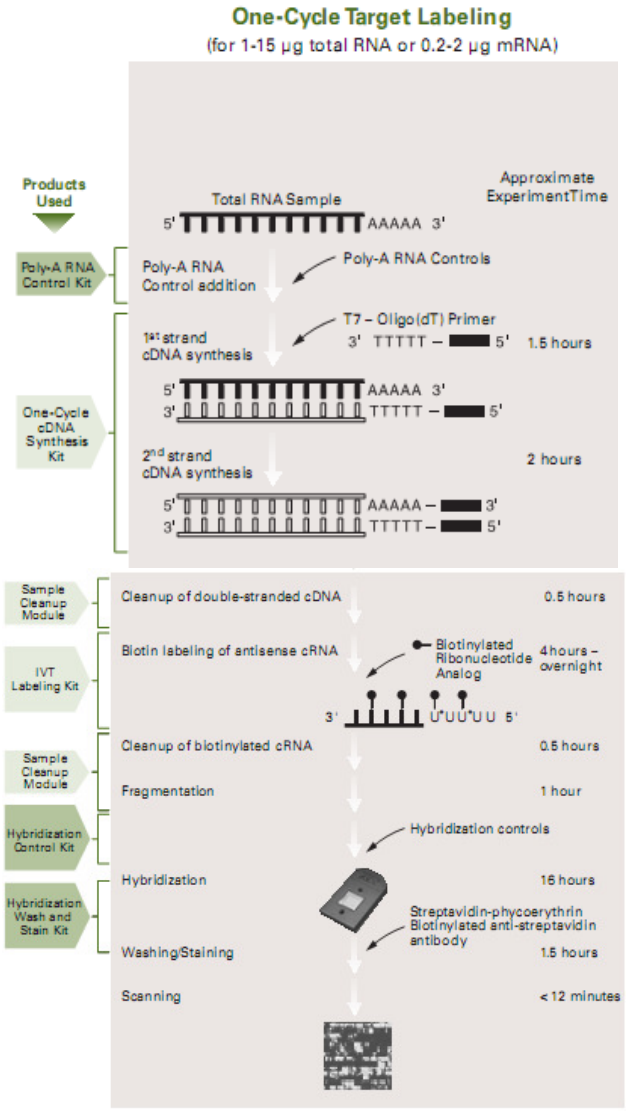


Figure 2.2: Schematic representation of work flow of GeneChip® Eukaryotic Labelling Assays for Expression Analysis

Cleanup of Double-Stranded cDNA

Affymetrix Sample Cleanup Module was used for cleaning up of the double-stranded cDNA. All other components needed for cleanup of double-stranded cDNA are supplied with the Gene Chip sample cleanup module. cDNA wash buffer was supplied as a concentrate. Before

using for the first time, 24 mL of ethanol (96-100%) was added, as indicated on the bottle, to obtain a working solution. All steps of the protocol were performed at room temperature.

About 600 µL of cDNA binding buffer was added to the double-stranded cDNA preparation. From this sample solution, 500 µL was added to the cDNA cleanup spin column sitting in a 2 mL collection tube, and centrifuge for 1 minute at ≥ 8,000g. Flow-through was discarded. The remaining solution was reloaded in to the same spin column and centrifuged. Flow-through and collection tubes were discarded. Spin column was transferred into a new 2 mL collection tube. 750 µL of the cDNA wash buffer was added onto the spin column and centrifuged for 1 minute at ≥ 8,000 g. Spin column was opened and centrifuged for 5 minutes at maximum speed (≤ 25,000 g). The flow-through and collection tubes were discarded. Columns were placed into the centrifuge with open caps. The tubes were positioned in such a way that they are oriented opposite to the direction of rotation (i.e., if the micro centrifuge rotates in a clockwise direction, the caps are oriented in a counter clockwise direction). Centrifugation with open caps allows complete drying of the membrane. Spin columns were then transfer to a fresh 1.5 mL collection tube and 14 µL of cDNA elution buffer was added directly onto the spin column membrane and incubated for 1 minute at room temperature and centrifuged for 1 minute at a maximum speed (≤ 25,000g) to elute the bound DNA. Care was taken to dispense the cDNA elution buffer directly onto the membrane. The average volume of elute recovered was 12 µL. After the cleanup procedure, the samples were processed for preparing biotin-labelled cRNA.

Synthesis of Biotin-Labeled cRNA

Gene Chip *in vitro* transcription (IVT) Labelling Kit is used for this step. The entire cDNA cleanup product was used for each IVT reaction. The cDNA preparation was transferred to

RNase-free microfuge tubes and the following reaction components were added in the order indicated in the table (Table 2.8) below. If more than one IVT reaction is to be performed, a master mix can be prepared by multiplying the reagent volumes by the number of reactions. The sample mixture was prepared at room temperature, since spermidine in the 10X IVT labelling buffer can lead to precipitation of the template cDNA at lower temperatures.

Table 2.8: Preparation of master mix for IVT

Component	**Volume 1 reaction**	**10 reactions**
Template cDNA	~12 µL	-
RNase-free water	8 µL	80 µL
10X IVT labelling buffer	4 µL	40 µL
IVT labelling NTP mix	12 µL	120 µL
IVT labelling enzyme mix	4 µL	40 µL
Total volume	40 µL	280 µL

Samples were incubated at 37°C for 16 hours. To prevent condensation that may result from water bath-style incubators, incubations were performed in oven incubator for even temperature distribution. The labelled cRNA was stored at –70°C.

Cleanup and Quantification of Biotin-Labeled cRNA

Sample Cleanup Module was used for cleaning up the biotin-labelled cRNA. 60 µL of RNase-free water was added to the IVT reaction and mixed by vortexed for 3 seconds. 350 µL IVT cRNA binding buffer was added to the sample and mixed by vortexed for 3 seconds. 250 µL of ethanol was then added to the mixture and mixed well by pipetting. The resulting solution (700 µL) was loaded on to IVT cRNA cleanup spin column sitting in a 2 mL collection tube. The samples were centrifuged for 15 seconds at ≥ 8,000 g. The spin columns were transferred

into a new 2 mL collection tube and (500 μL) IVT cRNA wash buffer was added onto the spin column. The tubes were centrifuged at ≥ 8,000 g for 15 seconds and. the flow-through was discarded. 500 μL 80% (v/v) ethanol was then added onto the spin column and centrifuge again for 15 seconds at ≥ 8,000 g. The flow-through was discarded and the spin columns were then centrifuged for 5 minutes at maximum speed (≤ 25,000 g) with an open cap. The columns were then transferred into a new 1.5 mL collection tube and 11 μL of RNase-free water was added directly onto the spin column membrane. The tubes were centrifuged at a maximum speed (≤ 25,000 g) to elute the bound cRNA. The purified cRNA preparations was quantified by diluting the cRNA with RNase free water (1:200) and were stored at –20°C immediately.

Quantification of the cRNA

cRNA was quantitated by using spectroscopic method. Concentration was calculated by applying the convention that 1 absorbance unit at 260 nm equal to 40 μg/mL RNA. The absorbance was measured at 260 nm and 280 nm to determine sample concentration and purity. In the quantification process the starting total RNA template may be carried over and add to the absorbance of the cRNA preparation. To correct for this, the formula below was used to determine the adjusted cRNA yield:

Adjusted cRNA yield = RNAm - (total RNAi) (y)

RNAm = amount of cRNA measured after IVT (μg)

Total RNAi = starting amount of total RNA (μg)

y = fraction of cDNA reaction used in IVT

Fragmenting the cRNA for target preparation

The cRNA fragmentation is critical in obtaining optimal assay sensitivity. Affymetrix recommends that the cRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure.

The fragmentation buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis. The following table shows suggested fragmentation reaction mix for cRNA samples at a final concentration of 0.5 µg/µL. The total volume of the reaction may be scaled up or down dependent on the amount of cRNA to be fragmented.

Table 2.9: Sample fragmentation reaction by array format

Component	**49/64 Format**
cRNA	20 µg (1 to 21 µL)
5X Fragmentation buffer	8 µL
RNase-free water (variable)	To make up to 40 µL
Total volume	40 µL

The reaction was prepared in RNase free tube. The tubes were incubated at 94°C for 35 minutes. Immediately tubes were transferred to ice. The undiluted, fragmented sample cRNA tubes were stored at –20°C.

Hybridisation and washing

Reagents

- GeneChip® Hybridisation, Wash and Stain Kit: Affymetrix, P/N 900720 (30 reactions)
- Hybridisation module
- Pre-Hybridisation mix
- 2x Hybridisation mix
- Dimethyl sufoxide (DMSO)

- Nuclease-free water
- Gene Chip Eukaryotic Hybridisation Control Kit: Affymetrix, P/N 900454 (30 reactions), both contain control cRNA and control Oligo B2
- Control oligo B2, 3 nM

Instruments

- Hybridisation Oven
- Sterile, RNase-free, micro centrifuge vials, 1.5 mL
- Micropipettors, (P-2, P-20, P-200, P-1000)
- Sterile-barrier pipette tips and non-barrier pipette tips
- Heating block

Eukaryotic Target Hybridisation

For the 49/64 probe array format (Table 2.10) we used 20 µg cRNA. The reagents were mixed for each target as shown in Table 2.10. Probe arrays were equilibrated to room temperature immediately before use. Hybridisation cocktail was heated to 99°C for 5 minutes in a heating block. In the meantime, the arrays were filled with the pre-hybridisation mix through one of the septa. The probe arrays filled with the pre-hybridisation mix was incubated at 45°C for 10 minutes on a rotating shaker. The hybridisation cocktail was transferred to a 45°C heating block for 5 minutes and then centrifuged at a maximum speed to get a clear solution. The arrays were removed from the hybridisation oven and pre-hybridisation mix was removed using a micropipette. The array was then filled with 200 µL of the clarified hybridisation cocktail. The probe array was again placed inside the hybridisation oven (45°C) and incubated at 45ºC with 60 rpm rotation for 16 hours. During the latter part of the hybridisation period,

washing and staining solutions were prepared as per the manufacturer's instructions. Washing and staining processes were automated.

Table 2.10: Hybridisation cocktail for single probe array

Component	**49 Format**
Fragmented and labelled cRNA	15 µg
Control oligonucleotide B2 (3 nM)	5 µL
20X Eukaryotic hybridisation controls (bioB, bioC, bioD, cre)	15 µL
2X Hybridisation mix	150 µL
DMSO	30 µL
Nuclease-free water	To make up the volume to 300 µL
Total volume	300 µL

2.3.11 Microarray Scanning and Analysis

The Affymetrix® GeneChip® Scanner 3000 was controlled by the Affymetrix® GeneChip Command Console (AGCC). The probe array was scanned after the wash protocols are completed. The laser was warmed up by turning it on at least 10 minutes prior to scanning. The glass surface of the probe array was cleaned with a non-abrasive tissue before scanning. Tough-Spots® were adhered on probe array vents to prevent the leakage of fluid. The probe array was inserted into the autoloader. Data collection was started after successful completion of autofocus. After the scan was completed, AGCC did the following processes:

- Saves the image data.
- Aligns a grid on the image to identify the probe cells.
- Computes the probe cell intensity data.
- Ejects the probe array.

Once the probe intensity data (.cel file) was generated, probe summarisation was performed in Expression Console. Microarray data analysis was done using Avadis 4.3 software (Strand Life Sciences Pvt. Ltd., India). Grouping of .cel files was done depending on the tissue source (three biological tissues of cultured oral mucosa, limbal and conjunctival epithelial cells). In primary analysis data was normalised. Statistical significance was assessed using unpaired t-test and ANOVA. Fold changes were calculated and genes showing more than 1.5 fold difference between the test and control samples were selected. Filtering and compilation of the data was done using Microsoft Excel.

2.3.12 Lithium chloride Treatment and Immunoflorescence for 5-bromo-2-deoxyuridine for Human Corneal Epithelial Cells

HCE cells were plated at a density of 1×10^5 cells/22 mm^2 cover slip in a 6 well tissue culture plate. Cells were treated for overnight with 10 mM LiCl in complete HCE medium. After treatment, 10 μM 5-bromo-2-deoxyuridine (BrdU) was added and incubate for 20 minutes. The cells were then washed with 1x PBS and fixed with~3.5 % formaldehyde at room temperature for 10 min and then washed with 1x PBS for three times. The DNA of the cells was then denatured using 2N HCl solution for 60 minutes at room temperature and then neutralized with 0.1 M sodium borate solution for 10 min. The cells were then washed with 1x PBS for three times and then processed for immune-staining as explained previously. The cells were then observed and counted using Corel DRAW software and percent of BrdU

positive cells were calculated for each treatment conditions. The experiments were done on three biological repeats and the averages were represented as mean ± standard deviation.

2.3.13 Collection of patient information and analysis of COMET treatment outcome

Patient information collected prospectively includes, preoperative characteristics, previous surgical procedures and postoperative course, examination findings, medical and surgical management. Data regarding postoperative outcomes were collected at intervals of 1 month, 6 months, 12 months and 24 months after COMET. The data were entered onto a Microsoft Excel spreadsheet for compilation and subsequent analysis.

2.3.14 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to estimate the sFLT1 concentration in the spent medium of oral mucosal and limbal epithelial cultures using rabbit polyclonal sFLT1 antibody. For ELISA assays, wells of costar high binding (Corning, Acton, MA) microtiter 96 well plates were rinsed with de-ionized water to remove polystyrene fragments. After that, they were coated with 200 µL of the spent media from the cultures. The plate was incubated for 8 hours at 4ºC. After incubation, the media was discarded and plates were washed three times with phosphate-buffered saline with Tween-20 (1xPBST, pH 7.4, 0.1 % Tween-20). The free surface was blocked with 2% bovine serum albumin for 1 hour at room temperature. Antibody was diluted (1:300) in 1xPBS and 200 µL of the antibody solution was added to each well. The plate was incubated for 2 hours at 37ºC. After incubation, the antibody solution was discarded and plates were washed three times with 1xPBST. Anti rabbit antibody tagged with Horseradish peroxidise was added after appropriate dilution. Plate was incubated at 37ºC for 1 hour. After washing with 1xPBST for three times, chromogenic substrate tetra methyl benzemidine was

added and incubated for 5 minutes. Reaction was stoped by adding 100µL of 1 N hydrochloric acid. Absorbence was measured by ELISA plate redus at 450 nm.

2.3.15 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Reagents

1. Tris-HCl (1.5 M –pH 8.8)
2. Tris-HCl (1.0 M-pH 6.8)
3. Acryl amide (30%W/V)
4. Sodium dodecyl sulphate (SDS) (10 % W/V)
5. Ammonium persulphate (APS) (10 % W/V)
6. Running buffer (TGS) (5x)
7. Sample buffer (2x)
8. Commassie stain solution
9. Destaining solution

Procedure

The glass plates, spacers and combs were cleaned with methanol and allowed for drying. Glass plates with spacers were assembled and clipped. Resolving gel solution (for 0.75 mm spacers 5.0 mL and for 1 mm spacers 6.0 mL) was prepared as directed in table 2.11. APS and TEMED were added just before pouring. After pouring the resolving gel, about an mL of butanol was gently added to create an organic layer on the top. Care was taken not to produce any air bubbles within the gel column. The gel was then allowed to polymerize for 30 minutes at room temperature

Table 2.11: Resolving gel recipe (For 10 mL)

Stock to be added	**10%**	**12%**	**14%**	**15%**
30% Acrylamide (mL)	3.3	4.0	4.7	5.0
1.5M Tris-HCl- pH8.8 (mL)	2.5	2.5	2.5	2.5
10% SDS (µL)	100	100	100	100
H_2O (mL)	4.0	3.3	2.6	2.3
10%APS (µL)	100	100	100	100
TEMED (µL)	10	10	10	10

Table 2.12: Stacking gel recipe (For 5mL)

Stock to be added	**Volume**
30% acrylamide	830 µL
1M Tris-HCl (pH-6.8)	630 µL
10% SDS	50 µL
H_2O	3.4mL
10%APS	50 µL
TEMED	5 µL

After the resolving gel got polymerized, the layer of butanol is poured off and blotted out with a tissue strip. Stacking gel solution was prepared as directed in table 2.12 and gently poured on to the resolving gel layer till the top of the glass plate. A suitable comb was then inserted in to the layer of stacking gel solution and allowed to polymerize for 30 minutes. Comb was then carefully removed without disturbing the wells. The wells were then filled with water or with 1x TGS running buffer to wash off any un-polymerised acrylamide.

Sample Preparation and Loading:

Protein samples were diluted with equal volume of SDS sample buffer (2x) and heated in a boiling water bath for 3-5 minutes (spin for few sec before loading). The gel plate sandwich was assembled to the PAGE unit (Hoefer *Inc*, Holliston, MA). Lower and upper tanks were filled with 1x TGS running buffer. Protein samples were loaded into each well carefully using a 50 µL pipette. Care was taken to avoid spill over in to adjacent wells while loading. 5 µL of a protein molecular weight marker was loaded in one of the wells.

Power supply was switched on and ran at 50-75V till the dye front reaches the resolving gel (30 minutes). The voltage was then increased to 100V. When the dye front reached the bottom of the resolving gel, power was switched off and the gel plates were removed from electrophoresis unit. The gel was removed from the plate and processed either for direct staining or for semi dry transfer of the resolved proteins on to a suitable membrane.

Western blotting

Transferring the resolved proteins on to the membrane

6 pieces of Whatmann 3 paper and a polyvinylidene fluoride (PVDF) membrane of the same dimension as the gel were cut out and soaked in the 1x transfer buffer for 10 minute for equilibration. The resolved proteins on the gel were then transferred on to the PVDF membrane using a semidry transferring apparatus (Amersham). This apparatus works on the same principle as SDS-PAGE. In SDS-PAGE all the proteins acquire negative charge and when subjected to a electric field they will move towards the anode. SDS-PAGE gel with the resolved proteins and PVDF membrane were sandwiched between pre-soaked Whatmann 3 filter papers. Gel was oriented towards the cathode and PVDF membrane towards the anode.

Protein transfer was carried out at 15 V constant voltage for 1 hour to enable complete transfer of proteins from the gel to the PVDF membrane.

Blocking

50 mL of 5% non fat milk (NFM) was prepared by dissolving 2.5 g of NFM powder in 40 mL of 1x PBST (PBS +0.1% Tween 20). The membranes were incubated in this solution for 1 hour at room temperature or overnight at 4ºC.

Primary antibody incubation

Antibodies were diluted in appropriate volumes of blocking buffer and pipetted in to a polybag containing the membrane and sealed without any air bubbles and incubated for overnight at 4ºC or 1 hour at room temperature with constant shaking, depending on the conditions standardised for each antibody. Membranes were then washed three times with 1xPBST for 5 minutes each.

Secondary antibody incubation

The washed membranes were then incubated with appropriately diluted secondary antibody in blocking buffer (Secondary antibody-Anti Rabbit or mouse HRP conjugated- from Sigma-Cat#A9169, A9044 respectively) for 1 hour at room temperature. The membranes were then washed thoroughly with 1xPBST 3 times at 5 minutes intervals.

Immunoblot detection system

Amersham (Cat# RPN2132) ECL kit was used for this purpose. Solution A and B from the kit was mixed in 40:1 ratio just before developing. The membrane was placed on a polythene sheet with the protein side on the top and the ECL mixture was added and incubated for 1 minute. The reagent was then removed by lifting the membrane using a forceps and touching the edges against a tissue paper. The blot was then placed inside a dry polybag in proper

orientation and smoothened out to remove any trapped air bubbles. The wrapped blot with the protein side up was then placed in an X-ray cassette and a sheet of X-ray film (Hyperfilm) was placed on top of the blot. This was carried out in a dark room under a red light. Depending on the intensity of the signal, the film was exposed for 15 seconds or more time. Exposed film was replaced with a fresh film for longer exposures. The films were developed and fixed and allowed to dry before imaging.

CHAPTER 3: CULTURE AND CHARACTERISATION OF ORAL MUCOSAL EPITHELIAL CELLS

3.1 Introduction

Corneal epithelial stem cells are present at the limbal region and constantly renew the epithelial cells and maintain the clarity of cornea. Damaged ocular surface can be observed in a number of ocular surface disorders such as chemical/ thermal injuries, Steven-Johnson syndrome, pterygium or severe ocular microbial infections. In these conditions, the conjunctival epithelial cells cover the corneal surface. The re-epithelialisation of the corneal surface by the conjunctival epithelium is followed by chronic inflammation, stromal scarring, neovascularization and persistent epithelial defects of cornea. This condition is referred to as limbal stem cell deficiency (LSCD) (Shapiro *et al.* 1981; Chen and Tseng 1991).

The treatment option available for LSCD is limbal transplantation using auto or allograft limbal tissues (Kaufman 1984; Thoft 1984) or *ex-vivo* cultured limbal transplantation (Pellegrini *et al.* 1997). However, in bilateral LSCD cases there is no autologous source for limbal stem cells therefore either a living or a cadaveric allogeneic donor is required (Fernandes *et al.* 2004). An alternative to allogeneic limbal grafting which necessitates long-term systemic immunosuppression is the transplantation of autologous epithelium from non-ocular sources. Therefore, sources of autologous tissue that can functionally replace the corneal epithelium have been considered as a potential alternative to allogeneic limbal transplants. Since the corneal epithelium is of the stratified squamous type, autologous epithelial cells such as oral, conjunctival, nasal, rectal and vaginal epithelia (Table 1.7), which have a similar morphology, could be considered as an alternative to allogeneic limbal transplants. Autologous conjunctival cultures can be used for the reconstruction of damaged

conjunctiva but not suitable for the reconstruction of corneal surface (Sangwan *et al.* 2003). Animal trails have been performed to check the feasibility of using oral mucosal epithelium for this purpose as it is easily available and can be harvested without invasive surgery. These studies suggest that oral mucosal epithelium is a feasible alternative for allogeneic limbal transplants (Nakamura *et al.* 2003; Nakamura and Kinoshita 2003; Hayashida *et al.* 2005).

The human oral cavity is lined by a mucous membrane (the oral mucosa) consisting of a stratified squamous epithelium, which may or may not be keratinized and an underlying connective tissue layer, the lamina propria. A stratified squamous non-keratinized epithelium called lining mucosa covers the oral surface of the lips, cheeks, floor of mouth and ventral surface of the tongue. A stratified squamous keratinized epithelium is found on surfaces which are subjected to abrasion that occurs due to mastication such as the roof of the mouth and gums. The epithelium is constantly replaced by cell division in the basal layers, and turnover time is less in the non-keratinised mucosal epithelium than in the masticatory mucosa (Squier and Kremer 2001). The differences between keratinized and non keratinised epithelial cells are 1) size of tonofilaments in cytoplasm 2) presence of keratinised upper layer. The human corneal epithelium is non-keratinised and stratified; the lining oral mucosa which contains same type of cells (stratified, non-keratinised) may be suitable for reconstruction of damaged ocular surface in LSCD patients.

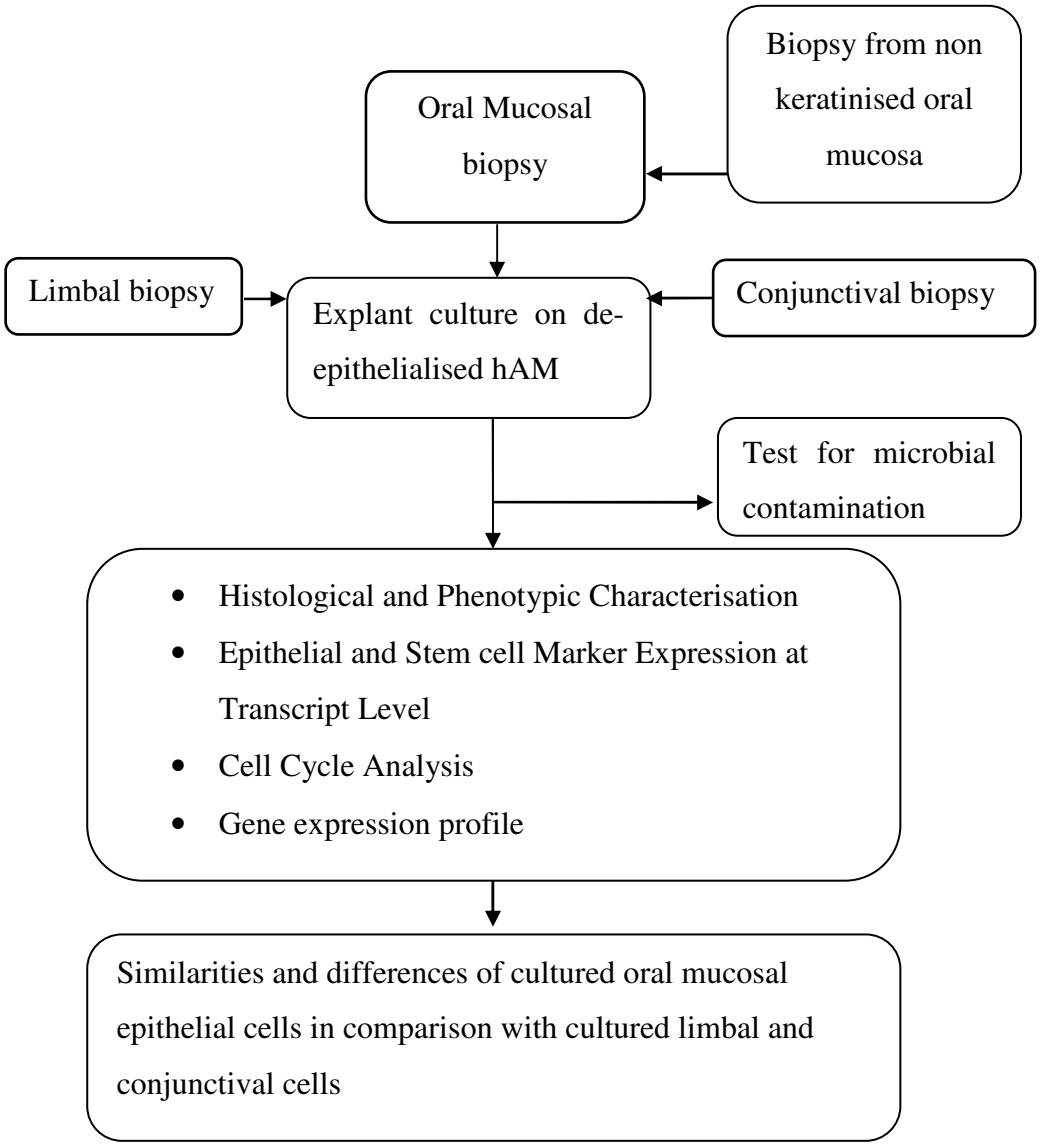
Transplantation of *ex vivo* cultured epithelial cells in regenerative medicine was started in 1975 by Green and co-workers for the reconstruction of skin damaged due to thermal injuries (Rheinwald and Green 1975). The same concept was applied to damaged ocular surface reconstruction by Pellegrini and co-workers (Pellegrini *et al.* 1997). The *in vitro* proliferation capacity of oral mucosal epithelial cells was demonstrated by many groups

(Fleiner and Ewers 1989; Zhou *et al.* 2000; Zhou *et al.* 2001). Thus a study was attempted at developing a culture system of oral mucosal epithelial cells by explant culture method without any xenobiotics, which could possibly be used for corneal surface reconstruction in patients with bilateral LSCD. The oral mucosal epithelial cells in the culture were characterised with different strategies like semi quantitative RT-PCR, immunohistochemistry and microarray (Flow chart 3.1) to check for cytokeratin profile (K3, K12, K14, K19), stem cell markers (p75, p63α, β, γ and ABCG2), mucins and enzymes (Muc5AC, ALDH), angiogenic factors (FGF1, FGF2, VEGF-A, TGFβ and ANG1) and adhesion molecules (Cx43, E-cadherin and integrins).

To compare the global gene expression profile of cultured oral mucosal epithelial cells with that of cultured limbal epithelial cells, microarray was carried out using Affymetrix U133 plus gene chip. Since gene expression is strongly influenced by cell culture methods, it is necessary to compare the gene expression profile of these cells after culturing them on de-epithelialised hAM. As the oral mucosal epithelial cells are from vascularised source, investigating the levels of potential angiogenic factors in comparison with cultured limbal epithelial cells will help in understanding the mechanism of corneal neovascularisation after COMET. Three different age-matched biological replicates were selected for each group and the cells were cultured under similar conditions.

Analysis of cell replication state can be achieved by labelling the cell nuclei by fluorescence dye. Quiescent and G1 cells will have one copy of DNA and will therefore have 1x fluorescence intensity. Cells in G2-M phase of the cell cycle will have two copies of DNA and accordingly have 2x intensity. Since the cells in S phase synthesize DNA, they will have fluorescence values between 1x and 2x population (Lo *et al.* 2005).

Flow chart 3.1: Culture and characterisation of oral mucosal epithelial cells



3.2 Hypothesis

Oral mucosal epithelial cells can be expanded to generate an epithelial cell sheet and could be used as a substitute for ocular epithelium in certain clinical conditions of severe bilateral LSCD. While morphology/function of oral mucosa appears to be similar to ocular tissue,

specific differences in terms of stemness, gene expression is not known. Hence this study attempts to culture oral mucosa in a manner similar to limbal culture using feeder cell free, explant culture system and evaluate the similarities and differences with limbal and conjunctival cultures in *in-vitro* condition.

3.3 Aims

- To cultivate oral mucosal epithelial cells from human oral mucosal tissues on de-epithelialised hAM in a feeder cell free explant culture technique (similar to limbal culture system).
- An attempt to evaluate the oral specific morphological/molecular phenotypic/gene expression profiles in comparison with *ex vivo* cultured limbal and conjunctival epithelial cells.

3.4 Results

3.4.1 Sterility Check of Chemicals and Spent Medium

All the reagents used for the culture of oral mucosal epithelial cells were tested for the presence of aerobic and anaerobic microorganisms. After incubation for 7 days in a bacterial incubator, they were negative for microorganisms and hence were approved for culture purpose.

3.4.2 Culture of Oral Mucosal Epithelial Cells

A total of 25 human oral, limbal and conjunctival epithelial cells were cultured as explants on de-epithelialised hAM using HCE medium supplemented with 10% fetal calf serum or autologous serum and recombinant growth factors (hInsulin, hEGF). Growth of the cultures was monitored under phase-contrast microscope (Olympus CK40). Initiation of growth was slow in oral mucosal epithelial cells when compared to the limbal and conjunctival cultures.

Oral mucosal epithelial cell migration from the explants was noticed on day 4 after the initiation of culture, whereas this can be observed within 1-2 days in limbal and conjunctival cultures (Figure 3.1). Epithelial cell sheet formation was observed in all the cultures by day 5-6 in oral mucosal epithelial cultures (Figure 3.1) as compared to 4-5 days in case of limbal and conjunctival cultures. Entire hAM was covered within a period of 3 weeks as compared to 2-3 weeks in limbal and conjunctival cultures. The hAM cultured oral cells appeared to be slightly smaller in size compared to the cultured limbal or conjunctival cells. During standardisation, no problems (microbial contamination, hAM tares and inadequate growth) were encountered. The epithelial cell monolayer spread all over the hAM in the oral mucosa, limbal and conjunctival cultures.

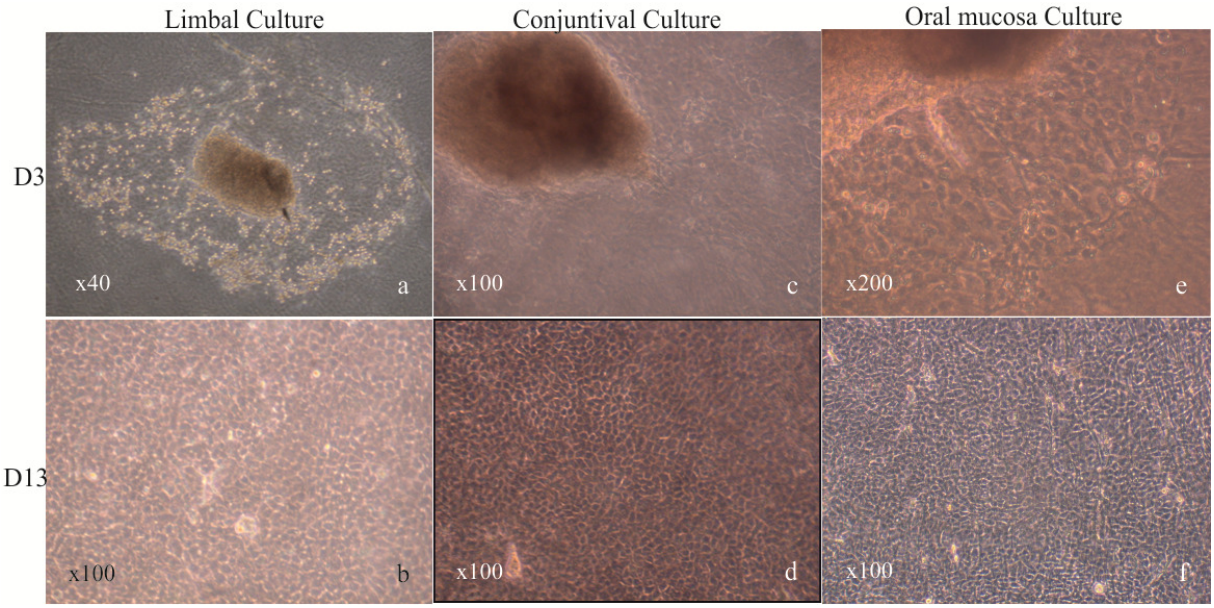


Figure 3.1: Epithelial cell cultures as seen under phase contrast microscope. Cultured limbal, conjunctival and oral mucosal epithelial cells on day 2 (a, c, e) and day 13 (b, d, f) after starting the culture

3.4.3 Characterisation

3.4.3.1 Histological and Phenotypic Characterisation of Cultured Oral Mucosal Epithelial Cells

In order to observe the cell morphology of oral mucosal epithelial cultures, hematoxylin and eosin (H and E) staining was done on formaldehyde fixed paraffin sections. Confluent oral epithelial cell cultures underwent stratification in places and formed one to two layers of cells as seen after H and E staining (Figure 3.6). No goblet cells were seen in these cultures by periodic acid Schiff (PAS) staining (Figure 3.6).

Electron microscopy was done to check for the presence of cell-cell junctions and cell-basement membrane junctions. As shown in figure 3.2 C, cultured oral epithelial cells showed gap junctions and desmosomes, which is similar to limbal and conjunctival cultures. However, hemidesmosomes-the junctions between cells and the amniotic membrane substrate were not clearly visible in any of these cultures under this resolution, although cells from all the three cultures were in close apposition with the hAM.

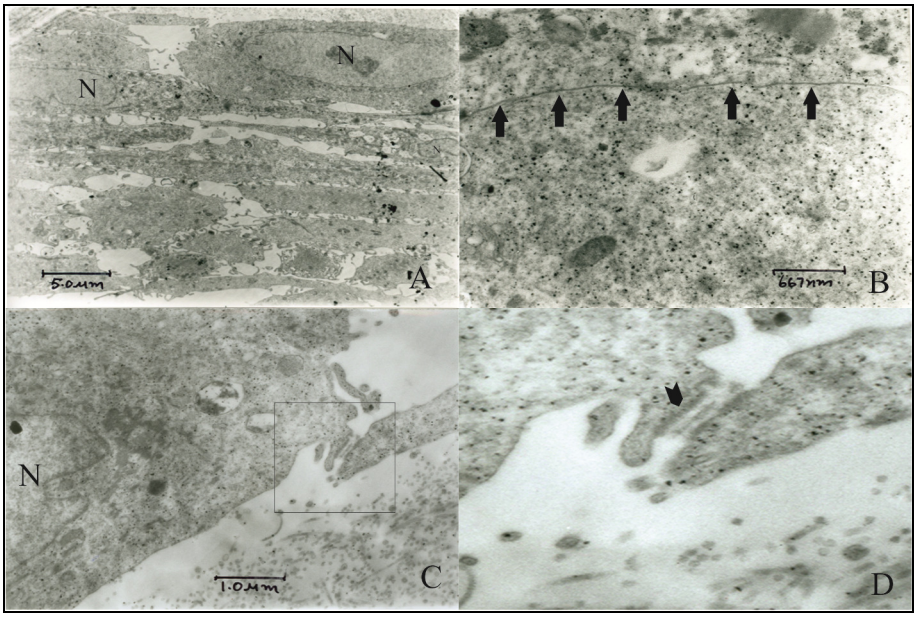


Figure 3.2: Transmission electron microscopic pictures of cultured oral mucosal epithelial cells on human amniotic membrane. (A) Stratified epithelial cells are shown on hAM, N represents the nucleus of the epithelial cell. (B) Intercellular belt desmosome present between two stratified cells (arrows) (C) Gap junction present in between microvillus of two epithelial cells (D) the gap junction at higher magnification.

Immunohistochemical characterisation of the cultured epithelial cells was done by using fixed and processed paraffin sections and fresh cultures. Using the antibody clone-AE5 that recognizes cytokeratin 3, it was shown that cytokeratin 3 was expressed in all the three cultures (Figure 3.3, 3.5). Immunohistochemistry using anti-p63 antibody, which recognizes all isoforms of p63, showed that all the three cultured cell types expressed this marker (Figure 3.5) in all cells. Immunohistochemistry with p75 antibody showed (Figure 3.3, 3.5) a very high level of expression in all the cultured oral cells and oral tissue (Figure 3.4) in contrast to the limbal tissue (Figure 3.4), cultured limbal and conjunctival cultures. Cultured oral epithelial cells also showed the expression of E-cadherin, vimentin and CD29 (Figure 3.5).

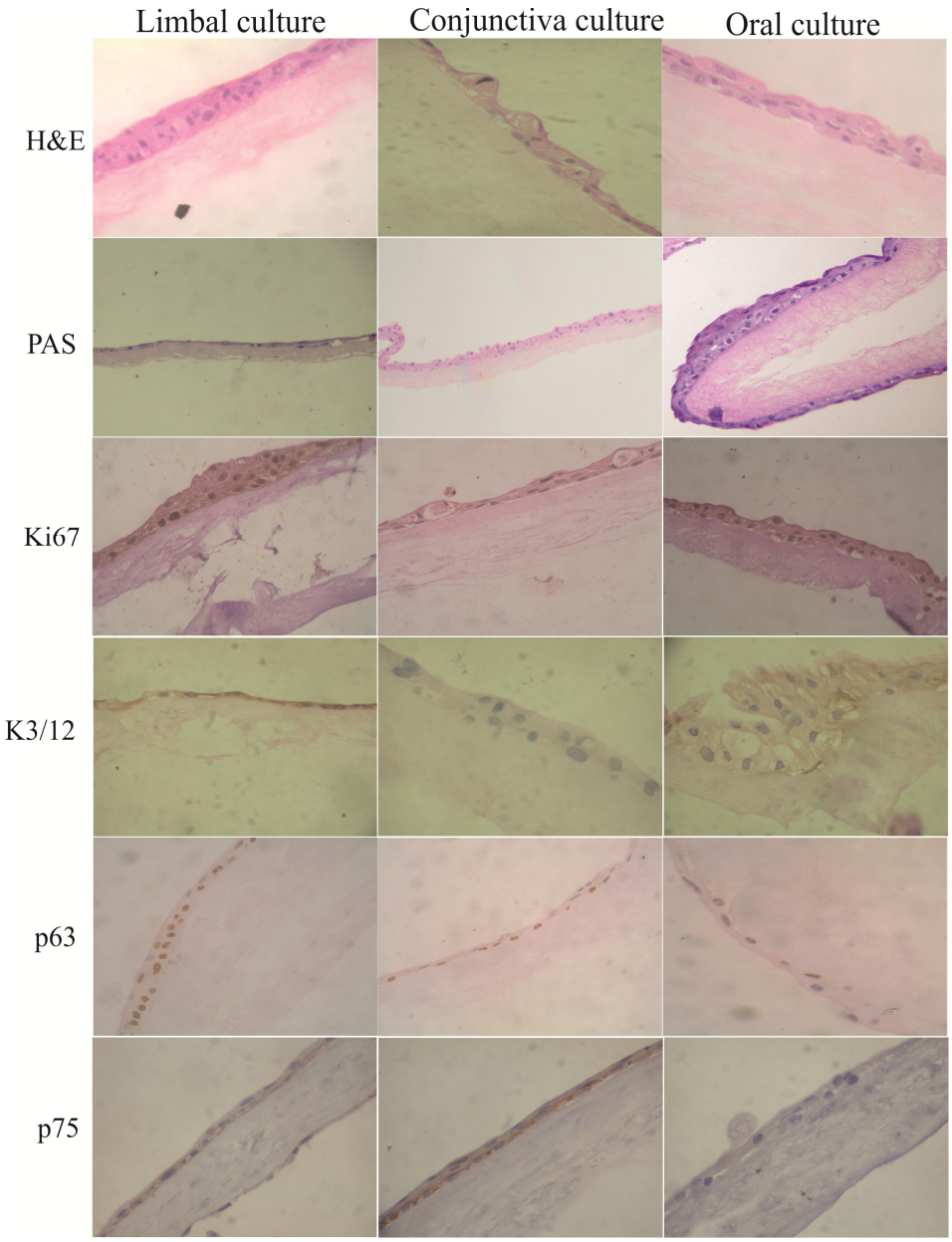


Figure 3.3: Histological and phenotypic characterisation of cultured oral mucosal epithelial cells. Cultured oral mucosal epithelial cells in comparison to the limbal and conjunctival epithelial cells cultured on de-epithelialised human amniotic membrane shows actively dividing differentiated cells with stem cells. All figures are at X400 magnification.

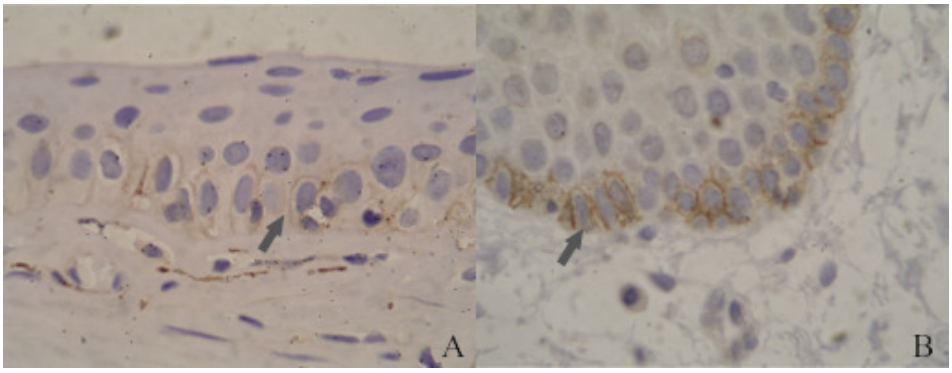


Figure 3.4: Expression pattern of p75 in limbal and oral mucosal epithelial tissues. Limbal and oral mucosal epithelial tissues basal cells show membrane staining for p75 (CD271).

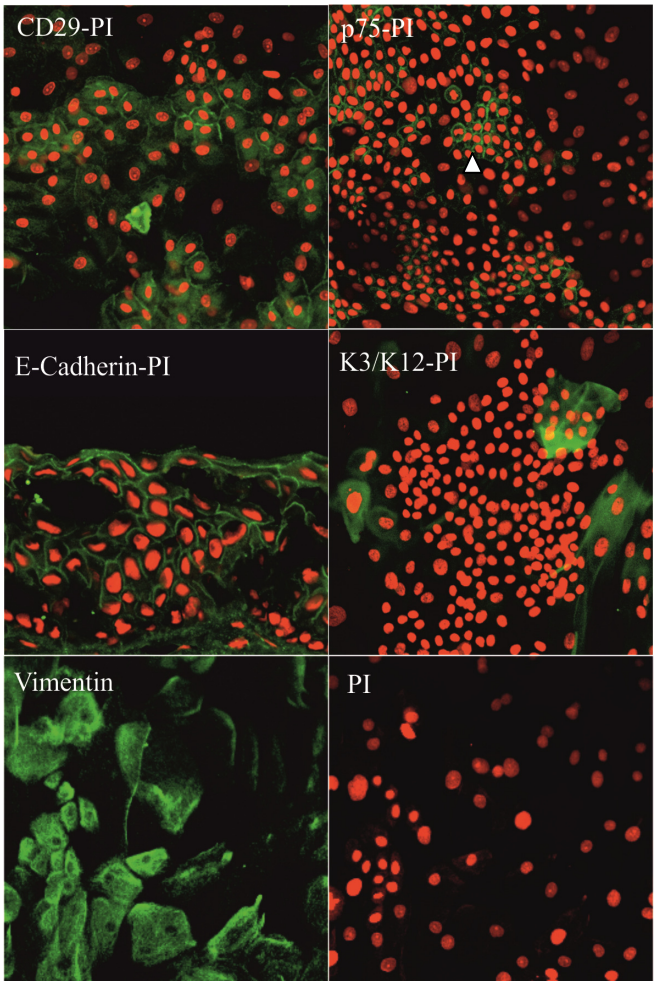


Figure 3.5: Phenotypic characterisation of cultured oral mucosal epithelial cells for p75, CD 29, E-cadherin, K3/K12 and Vimentin. Staining of cultured oral mucosal epithelial cells on hAM showing the expression of cytokeratin 3, E-cadherin, CD29 (differentiation markers), vimentin and p75 (stem cell marker). Staining was done on intact hAM bound cells. All images are at X200 magnification.

3.4.3.2 Characterisation of Cultured Oral Mucosal Cells for Epithelial Specific and Putative Stem Cell Markers (at Transcript Level)

In order to check for the expression of tissue specific markers, differentiation and stem cell markers of oral epithelial cells in comparison to the limbal and conjunctival epithelial cells, reverse-transcription polymerase chain reaction (RT-PCR) was carried out. cDNA was synthesized from total RNA isolated from confluent cultures and was used for RT-PCR using the primer sets shown in table 2.2. Both limbal and oral epithelial cells expressed cytokeratin 3 (Figure 3.3). However, the oral mucosal epithelial cultures did not express cytokeratin 12. Conjunctival cultures also expressed cytokeratin 3 and cytokeratin 12. The oral mucosal epithelial cells also expressed cytokeratin 4 and cytokeratin 13. These cytokeratins were also expressed by cultured limbal and conjunctival cultures. Cytokeratin 15 expression was observed in all three cultured cells.

Connexin 43 was expressed by all the differentiated epithelial cells and all the three cultured cell types. Cultured oral cells also express basal levels of Pax6, a marker for ocular tissues. Conjunctiva specific Muc5AC is expressed by oral cells but not the limbal epithelial cells. All the three cultures expressed epidermal growth factor receptor and integrin 9α.

RT-PCR analysis was also performed to check for the presence of stem cell markers, namely, isoforms of p63, ABCG2 and p75. All the three cultured cells expressed ΔNp63α,

ΔNp63β and ΔNp63γ (Figure 3.6). No expression of transactivating isoforms of p63 was seen in any of the cultures (data not shown). As shown in figure 3.3, all three cell types expressed putative stem cell markers p63, ABCG2 and p75.

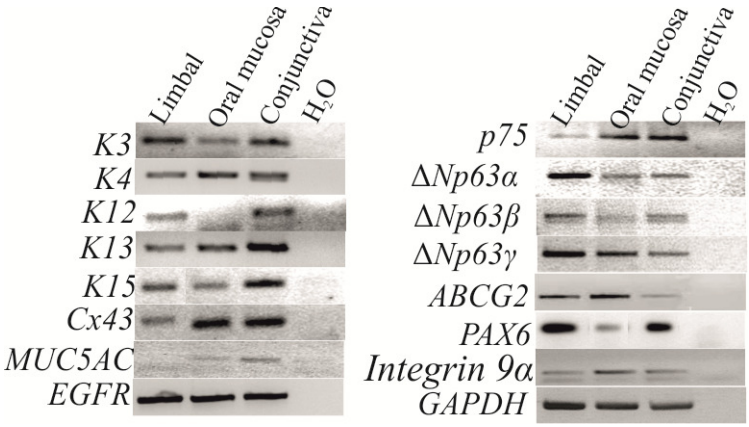


Figure 3.6: Semi quantitative RT-PCR for cytokeratins, mucin, putative stem cell marker for cultured limbal, oral mucosal and conjunctival epithelial cells. *Cytokeratin12* was not present in cultured oral mucosal epithelium. All other cytokeratins, *Cx 43*, *Integrin 9 α*, *EGFR*, putative stem cell markers (*p63*, *ABCG2 and p75*), *Pax6* were present in all the three cultures. *GAPDH* was used as loading control. PCR was performed for 35 cycles.

3.4.3.4 Cell Cycle Analysis

Cultured oral mucosal epithelial and limbal epithelial cell cycle analysis showed 67.1% and 70.4% G0-G1 cells respectively. In both the cultures, equal numbers of cells were found to be in S-G2-M phase (20%). Confluent cultures of oral and limbal epithelial cells showed 5% of apoptotic cells (Figure 3.7).

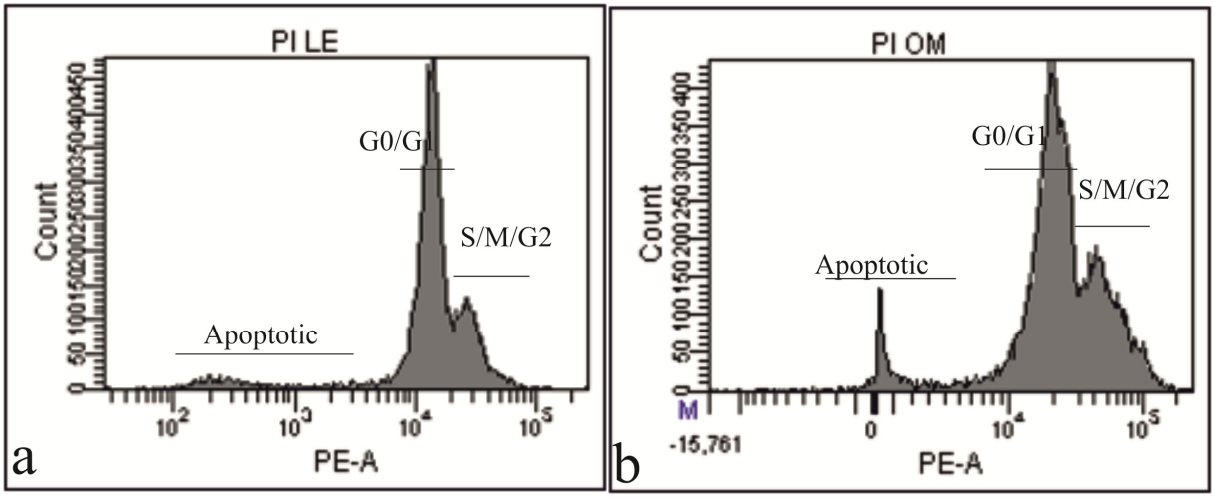


Figure 3.7: Cell cycle analysis of cultured epithelial cells. Histogram showing the PI intensity and the cell count. Cultured limbal epithelial cells (a) and oral mucosal epithelial cells (b) show same number of cells at different stages of cell cycle.

3.4.3.5 ALDH Activity in Cultured Epithelial Cells

FACS analysis showed that 0.1% of cells were ALDH positive in freshly isolated oral cells from the hAM cultures. Oral epithelial cells passaged on culture dish without hAM did not show any ALDH activity (Figure 3.8).

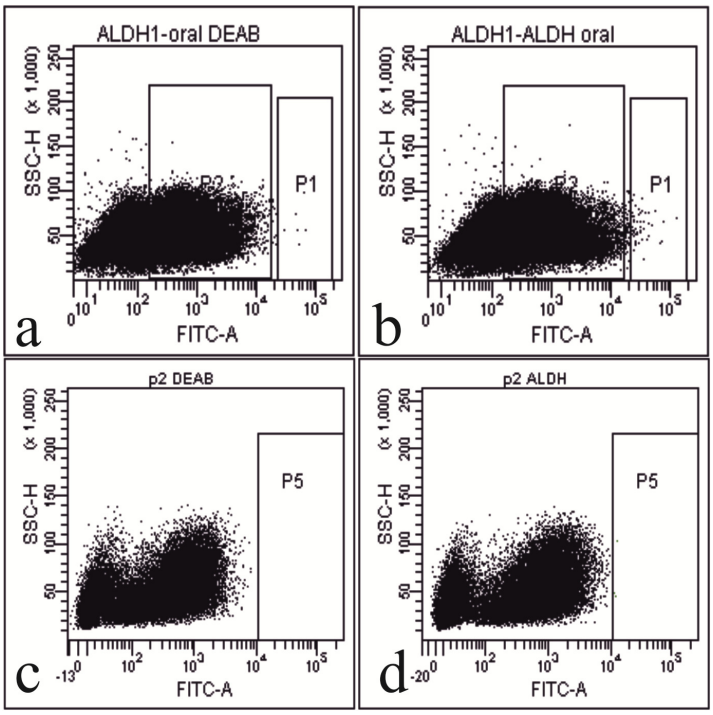


Figure 3.8: ALDH activity in cultured oral mucosal epithelial cells. Dot plot showing the presence of 0.1% ALDH positive cells in freshly isolated oral mucosa epithelial cells (b) in comparison with the cells incubated with DEAB, a specific ALDH inhibitor (a). Passaged (P2) cells did not show ALDH activity (c, d).

3.4.3.6 Gene Expression Profile

i) Total RNA Isolation and Quantitation

Total RNA was isolated using Trizol reagent and was purified by Qiagen-RNeasy purification kit. RNA was eluted in 20 µL of water. RNA was quantitated by Bio photometer. Absorbance and quantity of RNA obtained is mentioned in table 3.2.

Table 3.1: Absorbance and quantity of RNA in each sample (Dilution-1:50)

Bio photometer	**260 nm**	**280 nm**	**230 nm**	**260/280**	**260/230**	**Concentration (µg/µL)**
CLEC 1/2/3	0.453/0.421/ 0.598	0.258/0.239/ 0.336	0.205/0.230/ 0.296	1.75/1.76/ 1.78	2.2/1.83/2 .02	0.905/0.84/ 1.19
COMEC 1/2/3	0.66/1.673/0 .559	0.364/0.899/ 0.313	0.390/0.813/ 0.334	1.83/1.86/ 1.78	1.71/2.06/ 1.67	1.33/3.34/1 .11
CCJEC 1/2/3	0.51/0.833/0 .496	0.296/0.457/ 0.278	0.239/0.503/ 0.242	1.73/1.82/ 1.79	2.14/1.66/ 2.05	1.02/1.66/0 .99

ii) Data Analysis and Interpretation

Affymetrix human gene chips HGU 133 plus 2.0 containing 47,000 transcripts and variants which represent 39,000 characterised human genes were used in this study. There were 554 transcripts upregulated and 610 transcripts downregulated in the oral mucosal epithelial cells with fold change ratios >2, p value less than or equal to 0.05. A comprehensive analysis of the microarray data revealed that the genes belonging to the TGF beta pathway (p=1.5E-2), p53 pathway (p=8.7E-4) are upregulated in cultured oral mucosal epithelial cells when compared to the cultured limbal epithelial cells. Cytokeratin 4 and cytokeratin 13 are upregulated in oral mucosal cells. Among the upregulated genes, 10 were eye specific genes (crystalline-beta, crystalline-gamma, chemokine ligand 14, SRY-box2, Aldehyde dehydrogenase 4A1, Ankyrin, CDC6, SRP2, EHHADH and ELMOD1). Some of the cell surface associated mucin coding genes (Mucin 4, Mucin 20) were downregulated in oral mucosal epithelial cells. Top 50 upregulated and downregulated genes with the fold changes were shown in table 3.2 and table 3.3 respectively. Gene clustering data for these genes were shown in table 3.4 and 3.5.

Table 3.2: Top 50 upregulated genes in oral mucosal epithelial cells in comparison with limbal epithelial cells

Gene Name	Fold Change Absolute(OM Vs Limbal) p>0.05
odontogenic, ameloblast associated	131.0806
keratin 4	86.57607
selenoprotein P, plasma, 1	65.18908
Cornulin	64.22633
paired-like homeodomain transcription factor 2	43.30433
chloride channel, calcium activated, family member 4	41.70231
chemokine (C-X-C motif) ligand 14	37.35994
chemokine (C-X-C motif) ligand 14	36.35967
flavin containing monooxygenase 2 (non-functional)	33.70321
flavin containing monooxygenase 2 (non-functional)	31.62174
SRY (sex determining region Y)-box 2	26.55086
cytochrome P450, family 3, subfamily A, polypeptide 5	23.40173
paired-like homeodomain transcription factor 1	23.23189
paired-like homeodomain transcription factor 1	23.16656
cytochrome P450, family 3, subfamily A, polypeptide 5	21.23953
aldo-keto reductase family 1, member B10	20.02733
serpin peptidase inhibitor, clade B (ovalbumin), member 3	16.71289
crystallin, gamma S	16.65183
serpin peptidase inhibitor, clade B (ovalbumin), member 3	15.80069
leucine-rich repeat-containing G protein-coupled receptor 6	14.07634
calmodulin-like 3	13.82757
sclerostin domain containing 1	13.18687
BCL2-like 14 (apoptosis facilitator)	13.01475

histatin 1	12.67302
dopamine receptor D5	12.64796
leucine rich repeat containing 32	12.61106
serpin peptidase inhibitor, clade B (ovalbumin), member 4	11.63529
NK2 transcription factor related, locus 3 (Drosophila)	11.58127
prostaglandin E receptor 3 (subtype EP3)	11.14232
hypothetical LOC389328	11.133
metallophosphoesterase domain containing 2	11.07724
bone marrow stromal cell antigen 2	10.82503
iroquois homeobox protein 1	10.47895
cytochrome P450, family 4, subfamily B, polypeptide 1	10.23192
Selenoprotein P, plasma, 1	10.16211
solute carrier family 13, member 5	9.959248
Transmembrane protein 165	9.812299
serine peptidase inhibitor, Kazal type 6	9.556814
cytochrome P450, family 3, subfamily A, polypeptide 7	9.436494
cytochrome P450, family 3, subfamily A, polypeptide 5	9.390591
solute carrier family 24, member 3	9.357933
Guanylate binding protein family, member 6	9.28297
family with sequence similarity 123A	8.850794
fibronectin type III and SPRY domain containing 1-like	8.843391
keratin 13	8.810053
ribonucleotide reductase M2 polypeptide	8.708057
Fibronectin type III and SPRY domain containing 1-like	8.634509
iroquois homeobox protein 4	8.3323
Nuclear receptor subfamily 2, group C, member 2	8.133679
secreted frizzled-related protein 2	8.043871

Table 3.3: Top 50 downregulated genes in oral mucosal epithelial cells in comparison with limbal epithelial cells

Gene Name	**Fold Change Absolute (Limbal Vs OM) p>0.05**
paired box gene 6 (aniridia, keratitis)	158.2694
paired box gene 6 (aniridia, keratitis)	133.7709
keratin 12 (Meesmann corneal dystrophy)	109.8793
pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	69.85954
kynurenine 3-monooxygenase	42.53125
kynurenine 3-monooxygenase	42.17531
mucin 16, cell surface associated	35.02665
carcinoembryonic antigen-related cell adhesion molecule 7	35.01554
carcinoembryonic antigen-related cell adhesion molecule 7	31.1291
uroplakin 1B	26.38823
guanine deaminase	26.30825
carcinoembryonic antigen-related cell adhesion molecule 7	26.00149
indoleamine-pyrrole 2,3 dioxygenase	25.88408
solute carrier family 34 (sodium phosphate), member 2	24.84836
dickkopf homolog 4 (Xenopus laevis)	23.01859
chloride intracellular channel 5	22.3166
calmodulin-like 5	20.98231
aldehyde dehydrogenase 1 family, member A1	20.18477
uroplakin 1B	19.61169
matrix metallopeptidase 7 (matrilysin, uterine)	17.51268
MRNA; cDNA DKFZp727C211	16.29499
breast carcinoma amplified sequence 1	15.49527
cyclin A1	14.15117
non-metastatic cells 5	13.72221

odd-skipped related 2 (Drosophila)	13.18119
family with sequence similarity 3, member D	13.00445
serine peptidase inhibitor, Kazal type 1	12.53197
solute carrier family 44, member 4	11.86704
mucin 4, cell surface associated	11.8567
mucin 20, cell surface associated	11.36181
transglutaminase 2	11.31414
cyclin-dependent kinase inhibitor 2A	11.20875
V-set domain containing T cell activation inhibitor 1	11.09969
chromosome 8 open reading frame 47	10.96276
solute carrier family 44, member 4	10.89518
chemokine (C-X3-C motif) ligand 1	10.87166
tetraspanin 12	10.2695
mucin 20, cell surface associated	10.111
hypothetical protein FLJ10781	10.07652
adrenergic, beta-1-, receptor	9.86226
chemokine (C-X3-C motif) ligand 1	9.690893
242005_at	9.355881
cyclin-dependent kinase inhibitor 2A (melanoma)	9.096191
kallikrein-related peptidase 6	8.961943
dehydrogenase/reductase (SDR family) member 9	8.838155
zinc finger protein 42 homolog (mouse)	8.668344
dehydrogenase/reductase (SDR family) member 9	8.584971
transglutaminase 2	8.406888
Calcyphosine	8.302089
transglutaminase 2	8.223345

Table 3.4: Functional classification of upregulated genes in cultured oral mucosal epithelial cells.

Cell division	**Keratins**	**Cell cycle**
CENPN, CDC6, NEK2, NUF2, NCAPG, AURKB, KIF2C, CDCA3, NUSAP1, KIF11, FAM83D, CEP55, CASC5, OIP5, MAD2L1, ZWINT, CKAP2, NCAPH, CCNB2, NAV1	*KRT16, KRT75, KRT15, KRT33A, KRT31, KRT4, KRT8, KRT13*	*MAD2L1, UBE2C, CCNB1, CDC20, CDCA3*
Serine protease inhibitor	**Oral Mucosa specific genes**	**Chromosome maintenance**
SERPINB13, SPINK6, SERPINE2, SPINK7, SERPINB3, SERPINB4	*ODAM, THSD1, IGFL1, C10orf99, PI15, SOSTDC1, FAM20C, FAM20A*	*MCM5, MCM7, MCM6*
Homeobox genes	**Transcription factor**	**DNA binding inhibitors**
IRX4, ISL2, IRX3, DLX3, LASS3, IRX1, PITX2, PITX1, DLX5	*MAF, SEC14L2, E2F1, TFCP2L1, MAFB, ETS2, PAX9, FOXE1*	*ID3, HEY1, ID2, ID4*
Calcium binding proteins genes	**Cell-cell adhesion and signal transduction**	**Xenobiotic detoxification**
VSNL1, CRNN, CAPNS2, CALML3, PLS1, S100A14,	*LYPD3, GPC1, BST2, LY6D, FLRT2, FXYD6,*	*CYP3A5, CYP4B1, CYP2J2, CYP1B1, FMO2*

CAPN14	*CLCA2, THSD1, ITGA6, CDH3, CA12, KCNK10, TM4SF1, DSG3, ADAM23, CLCA4, FAT2, PRIMA1, CLIC6, KCNJ15, LOC388630*	
Cell proliferation	**Protein kinase**	**Transcription factors**
CRIP2, LPXN, PDLIM4, SP6, MOBKL2B	*PDK3, CSNK1G3, TK1, MAP3K4, CDC6, PBK, TTK, NEK2, PFKFB3, CHEK1, EEF2K, MELK, AURKB, CDK6, PAK3*	*ATF7, BNC1, ZNF367, MOBKL2B, BCL11B, RBM20, ZNF750, BCL11A, SP6*
Transmembrane proteins	**GTPase**	
ITM2A, C21orf63, TMPRSS11E, TMPRSS11B, LGR6, PRRG4, CLEC2B, GALNTL4, BST2, HS6ST2, SLC2A12, MRGPRX3, TM4SF1, LRRC8E, HAS2, FXYD6, LOC388630, UGT8, TPST1, LRRC32	*GBP6, RAB7B, RAB31, GBP2*	

Table 3.5: Functional classification of upregulated genes in cultured limbal epithelial cells.

Cytokines	**Secretary ligands**	**Cell adhesion and receptors**
FAM3D, GDF15, LIPH, C6orf58	*INSL4, NMB, GDF15, IL17C*	*SLCO4A1, B4GALT5, GPR110, FRY, PCDHA11, TRA TRD, ST3GAL4, CASC4, PCDHA6, PCDHA8, C6orf105, SLC44A3, GALNT6, C20orf54, ICAM2, SLC15A1, SLCO3A1, ST6GALNAC5, CDH6, ENPP4, RAMP1*
Cysteine protease	**Ion Transport**	**Keratins**
CAPN5, CTSS, CTSO, CTSL2, CTSC	*SLC34A2, KCNE3, KCNQ1, SLC12A2, SLC12A7, KCNMB4, SLC4A11*	*KRT12, KRT23, KRT17, KRT80, KRT78, INA*
Nucleosome components	**Vesicle trafficking**	**Calcium binding**
HIST1H2AC, HIST1H2BE, HIST1H2BI, HIST1H2BD, HIST1H2BO, HIST1H2BH, TSPYL5	*CADPS2, SYTL2, MLPH, SYTL4*	*CALML5, CAPS, EFCAB2, RPTN*
Enzymes	**Transcription factors**	**Transcriptional activator**
C11orf54, APOBEC3B,	*THRB, ESRRG,*	*HSF2, GATA3, BACH2,*

NANOS1, PNKD, GDA, QPCT	*MEOX1, GATA6, ABLIM3, KLF4, HES5, NR2F2, RARB, CITED2, CLOCK, PAX6*	*VGLL1, NFE2L3, ELF3*
Transcriptional regulation		
ZNF542, ZSCAN4, GFI1, ZNF404, DMRTA2, ZNF506, ZNF677, ESRRG, OSR2, ZNF667, ZFP42, GATA3, BCL11B		

Both microarray as well as semi-quantitative RT-PCR results did not show any angiogenic genes that were differentially expressed between the two cell types. The semi-quantitative RT-PCR for some of the angiogenic markers showed that these markers were equally expressed in both the limbal and oral cells (Figure 3.9).

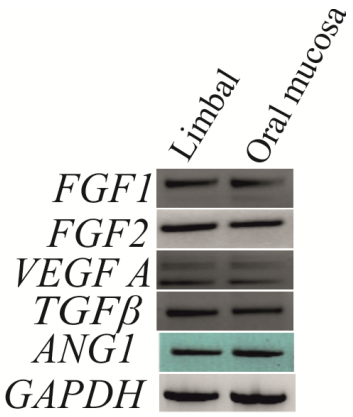


Figure 3.9: Semi-quantitative RT-PCR for pro-angiogenic factors. Angiogenic molecules *FGF, VEGF-A, TGF-β* and *ANG1* are expressed in same levels in oral and limbal epithelial cultures.

Expression levels of sFlt1 by oral and limbal epithelial cells were measured by ELISA. The results showed that sFlt1 was highly expressed by the limbal cultures (Figure 3.10).

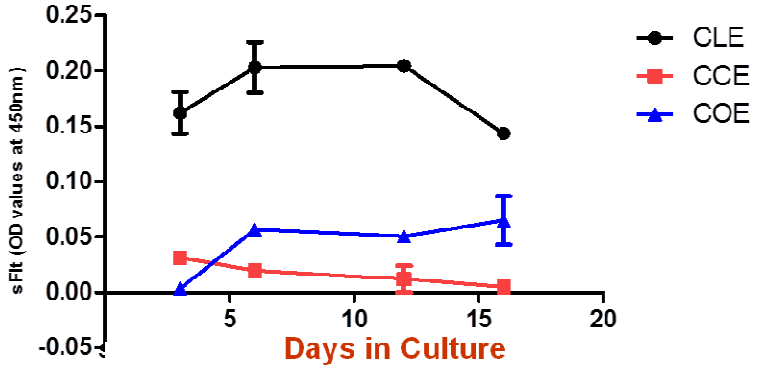


Figure 3.10: sFLT1 quantitation in spent media of cultures by ELISA. Cultured limbal epithelium secretes higher levels of soluble VEGF receptor 1 than the cultured oral and conjunctival epithelium.

3.5 Discussion

Oral Mucosal Epithelial Cell Culture on De-epithelialised hAM without Feeder Cells

Oral mucosal epithelial cells were cultured successfully on de-epithelialised hAM by explant culture method without the use of any feeder cells. The cultures were healthy, became confluent on de-epithelialised hAM in three to four weeks and underwent stratification in areas close to the explants. The advantage of this technique is that it precludes the use of any feeder cells which are of animal origin. Although feeder cell-free cultures of oral epithelial cells have been established using a temperature-responsive culture surface (Murakami *et al.* 2006), such a technique has not been reported so far using explant culture method on the amniotic membrane for reconstructing severely injured (Grade 4) human ocular surface.

Oral mucosal epithelial cells can be cultured in the presence of autologous serum as reported earlier (Ang *et al.* 2006). It was observed that the growth rate was faster when

cultured with autologous serum as opposed to the cultures with fetal calf serum (data not shown). Autologous serum used for growing the epithelial cultures makes xenobiotic free and suitable for clinical applications.

Cultured Oral Mucosal cells Share Some of the Epithelial Markers with Cultured Limbal Epithelial Cells

Comparison of the morphological and molecular phenotypic characteristics of cultured oral mucosal and limbal epithelial cells indicate that the cultures expressed cytokeratins (K3, K4, K13, K15), adhesion molecules (integrin α9, connexin 43), receptors (EGFR) and Lineage marker (Pax6). H and E staining of cultured cells showed monolayer of epithelial cells in all cultures. TEM showed the presence of gap junctions and desmosomes between cells in all cultures. As expected corneal epithelial specific cytokeratin 12 expression was absent in cultured oral epithelial cells as has been reported earlier by other groups (Nakamura *et al.* 2003; Hayashida *et al.* 2005). Cytokeratins 3 and 12 are the hallmark of differentiated corneal epithelium. The presence of clusters of corneal progenitor cells in conjunctiva has been reported and it is possible that the conjunctival expression of cytokeratin 3 and cytokeratin 12 observed in this study could be due to the presence of some corneal cells near the conjunctival margin (Kawasaki *et al.* 2006). Expression of Pax6, an embryonic ocular/neural lineage marker in oral mucosal epithelial cells is the new observation. This observation was further supported by expression of Pax6 in ectodermal placode which forms nasal processes during human embryonic development (Grindley *et al.* 1995). Universal gene expression profile shows 150 fold more Pax6 expression in limbal epithelial cells when compared with the oral mucosal epithelial gene expression. Expression of Pax6 in oral mucosa showed by molecular

phenotyping and universal gene expression analysis is contradictory due to the presence of Pax6 isoforms and the probes used in microarray chip (Affymetrix U133 Plus) may not be able to recognise the oral mucosa specific Pax 6 isoform. Cultured oral and limbal epithelial cells showed expression of similar cytokeratin profile (K3, K4, K13, K15), adhesion molecules (integrin α9, connexin 43), receptors (EGFR) and lineage markers (Pax6).

Putative Stem Cells Marker Expressing Cells are Present in Cultured Oral Mucosal and Limbal Epithelial Cells

The oral cells also expressed the stem cell-associated markers such as isoforms of ΔN p63, ABCG2 and p75 as observed using RT-PCR analysis and IHC. When the cultures underwent stratification, p63 expression was observed in the basal layer. Based on the observations, clusters of p75 positive small cells in the cultures could postulate p75 as an appropriate marker for oral epithelial stem cells. Limbal tissue basal cells and cultured epithelial cells expressed p63 and p75 as reported earlier (Touhami *et al.* 2002; Qi *et al.* 2008). RT-PCR results demonstrated that all three cultures expressed the three isoforms of ΔNp63 (α, β and γ). p75 positive oral mucosal cells might help in the renewal of epithelial cells on the ocular surface. Unlike the corneal epithelial cells which are homed at the limbal region and migrate towards the central cornea in a centripetal direction upon proliferation and differentiation, the oral cells are expected to be at the basal layers of stratified epithelium throughout the corneal surface and maintain normal homeostasis. Absence of ALDH activity in these passaged cells demonstrates that ALDH may not be an appropriate marker for the oral mucosal epithelial stem cells as opposed to bone marrow stem cells (Storms *et al.* 2005) Oral, conjunctival and

limbal epithelial cells expressed the stem cell-associated markers such as isoforms of ΔN p63, ABCG2 and p75.

Cell Cycle Analysis of Oral and Limbal Cultures Show Presence of Actively Dividing Epithelial Cells

Cell cycle analysis of limbal and oral mucosal epithelial cells grown on de-epithelialised hAM showed that the majority (~70%) of the cells are in G0/G1 phase. The cells are primed for cell division and some of the cells may be in the quiescent G0 phase or undergoing asymmetric division as evident from the immunostaining pattern of p75 in these cells. The number of actively dividing cells and stem cells is crucial for implementation of successful cell based therapy. Co-segregation of a putative epithelial stem cell marker like p75 along with cell cycle analysis could shed some light on the quantity of stem cell population in the actively proliferating oral cultures.

Gene Expression Profile of Cultured Oral Mucosal Epithelial Cells

The current gene expression profile study of the cultured oral mucosal epithelial cells in comparison with cultured limbal epithelial cells showed upregulation of tissue specific genes (Table 3.3 and 3.4), but did not show any differential expression of epithelial specific genes or pro-angiogenic genes.

Mucins are very important components of the ocular surface tear film. The ocular surface expresses membrane associated and soluble mucins. Mucin 1 and 16 are membrane associated mucins expressed on corneal surface and are required for the maintenance of the tear film (Argueso *et al.* 2003; Gipson *et al.* 2003). Hori *et al* have shown that the native oral

mucosal tissue does not express mucin 16, whereas cultured oral epithelial cells start expressing mucin 16 (Argueso *et al.* 2003; Hori *et al.* 2008). Through microarray analysis, (Table 3.4) we showed that mucin 16 is expressed at lower levels in cultured oral epithelial cells when compared to the cultured limbal epithelial cells. Even though the culture induced mucin 16 expression is comparatively less to the limbus derived epithelial cells, it may persist after COMET helping to maintain the tear film on the ocular surface.

In the present gene expression study comparing limbal and oral mucosal epithelial cells, no differential expression of any epithelial specific or stem cell specific markers. Gene expression profile of limbal epithelial cells in comparison with limbal stromal cells (non epithelial cells) showed complementary role related to cytokine, growth factors and also it showed upregulation of epithelial stem cell markers (ABCG2, vimentin, cytokeratin 14 and cytokeratin 19) and differentiation markers (cytokeratin 3/12, cadherin 1) (Polisetti *et al.* 2010). Differential expression of epithelial specific stem cell and differentiation markers in this experiment may be due to comparison between limbal epithelial cells and stromal cells of limbus. However, the current gene expression profile study of cultured oral mucosal epithelial cells in comparison with cultured limbal epithelial cells showed differential expression of tissue specific genes (Top 50 genes are given in table 3.3 and 3.4) but did not show any differential expression of epithelial specific genes or stem cell specific genes (For *eg*. p63).

The microarray analysis did not show any differential expression of angiogenic pathway specific genes in limbal and oral mucosal cultures. This may be due to the expression of pro-angiogenic factors at similar levels in the limbal and oral mucosal cells. This interpretation was further supported by the semi quantitative RT-PCR results for angiogenic genes (Figure 3.11), which showed similar expression levels of pro-angiogenic genes *FGF2,*

VEGF-A, TGF-β and *ANG1* in cultured oral mucosal and limbal epithelial cells. However, the limbal region has a vascular bed to nourish the stem cells. Therefore, it is possible that they express similar levels of angiogenic markers when compared to oral epithelial cells. The levels of angiogenic factors in the spent medium of cultured oral and limbal epithelial cells are also the same with some exceptions like the absence of bFGF cytokine in cultured limbal and oral mucosal epithelial cells (Sekiyama *et al.* 2006). Though oral mucosal cells were shown to express the anti-angiogenic molecule *CXCL14* in this microarray analysis, it may not be a strong antagonist like sFLT1 (Ambati *et al.* 2006). Secretory sFLT1 protein level in spent medium is less in oral mucosal epithelial cultures compared with the limbal epithelial cell cultures (Figure 3.10). This was further supported by results obtained by estimating the levels of sFLT1 in oral mucosal cultures (Kanayama *et al.* 2009). Other groups have shown that the anti-angiogenic protein thrombospondin-1 (TSP1) was expressed in the cultured limbal epithelial cells whereas, it was absent in oral mucosal epithelial cultures (Kanayama *et al.* 2007).

In summary, these results suggest that oral mucosal epithelial cells can be cultured as epithelial sheets on de-epithelialised hAM by explant culture method for potential clinical transplantation (extrapolation of culture system used for limbal epithelial cell culture). Cultured oral mucosal epithelial cells are morphologically similar to cultured limbal epithelial cells on hAM. Characterisation studies demonstrate that cultured epithelial cells contain a distinct population of stem cells and differentiated cells which serve to reconstruct and replenish the stem cell pool when transplanted to the patient's cornea with LSCD. Oral and limbal cultures share some of the markers like p63, connexin 43, cytokeratin 3, ABCG2 and p75. Gene expression and semi quantitative RT-PCR studies demonstrated similar levels of

pro-angiogenic factor expression in limbal and oral mucosal epithelial cultures. On the other hand, it was shown that the limbal cultures expressed more of the anti-angiogenic factor, namely sFLT1 when compared to the conjunctival and oral mucosal epithelial cells. Cultured autologous oral mucosal epithelial cells could be used for reconstruction of bilateral LSCD patients to get a stable ocular surface. The characteristic properties of oral epithelial cells after cultivation (baseline data) help to trace the changes in oral mucosa after transplantation to the damaged ocular surface.

In the absence of healthy limbal tissue as in cases of patients having bilateral LSCD, autologous COMET is a good alternative choice for the ocular surface reconstruction as it obviates the need for immunosupression. Long-term immunosuppression imposes secondary complications such as kidney dysfunction, dry eye syndrome and systemic infections. Whereas, non-compliance may induce the risk of graft rejection in case of limbal allografts (Tsubota *et al.* 1999; Ilari and Daya 2002; Samson *et al.* 2002). According to the available literature, short-term outcomes of both limbal allografts and COMET treatment were encouraging (Pauklin *et al.* 2010). However, the long term results of limbal allografts show relatively very little success rate and increased secondary systemic complications (Miri *et al.* 2010). Based on the literature evidence and similarities found in the present study, a decision was taken to conduct a proof-of-principle pilot study of using autologous COMET for the treatment of patients with bilateral LSCD with the approval of IRB and funding agency (DBT).

In conclusion, this study demonstrates that it is possible to generate a sheet of oral epithelium from oral epithelial tissue as explant culture system without feeder cells. The cultured oral mucosal epithelial cells from the explants grown on de-epithelialised hAM with

a heterogeneous population of putative progenitor cells/transient amplifying cells/differentiated epithelial cells show similar characteristics to cultured limbal epithelial cells. While the native tissues of limbal, oral and conjunctival epithelial tissues show morphological, phenotypic and genotypic differences, the cultured cells do not show such differences. Limbal, oral mucosal epithelial cultures are derived from vascularised tissues have similar pro-angiogenic factors, however we speculate that cornea sustains as an avascular milieu which could be due to secretion of anti angiogenic factors like sFLT1 or other factors.

CHAPTER 4: CLINICAL TRANSPLANTATION AND POST COMET GRAFT SURVIVAL

4.1 Introduction

In cases of bilateral LSCD, healthy limbal tissue is totally absent for considering a treatment option with autologous CLET. Autologous COMET offers a better alternative for ocular surface reconstruction as it does not require postoperative immunosuppressive therapy. Also, COMET requires only a small amount of oral mucosal tissue for *in vitro* culture expansion. Moreover oral epithelium shows phenotypic and morphological semblance with corneal epithelial cells and thereby does not pose any risk to the donor site (Madhira *et al.* 2008; Krishnan *et al.* 2010). Transplantation of cultured oral mucosal epithelial monolayer onto the damaged ocular surface can act as a substitute for corneal surface epithelium and help in the stabilisation of damaged ocular surface.

Animal trials and preliminary human trials have demonstrated that the *ex-vivo* cultured oral mucosal epithelium could be a suitable therapeutic alternative to limbal epithelium. Safety and efficacy of autologous cultured oral mucosal epithelial cells in heterogeneous group of patient eyes with ocular surface diseases (LSCD due to SJS, ocular burns and OCP) have been shown by many groups (Nakamura *et al.* 2004; Inatomi *et al.* 2006). However, the cell culture protocols described for COMET for animal and human transplantation utilised various animal derived / xenobiotic materials (Nakamura *et al.* 2003; Nakamura *et al.* 2004; Nishida *et al.* 2004; Chen *et al.* 2005; Hayashida *et al.* 2005; Ang *et al.* 2006; Inatomi *et al.* 2006; Ma *et al.* 2009; Burillon *et al.* 2011). Use of xenobiotic materials in cell culture for clinical transplantation is undesirable as it carries the risk of transmitting known or unknown infections to the transplant recipient (Schwab *et al.* 2006). To avoid xenobiotic usage, oral mucosal epithelial cultures were grown as explant cultures on de-epithelialised hAM using

autologous serum, adopted from the standardised limbal epithelial cell culture protocol (Mariappan *et al.* 2010). Clinical pilot study of COMET treatment for patients with bilateral LSCD was conducted. COMET was done on 19 eyes of 18 patients who had severe, bilateral ocular surface dysfunction due to chemical or thermal injuries. This study has been reviewed and approved by our institutional review board (IRB) and ethics committee.

Criteria for Successful Transplantation

Based on the clinical appearance of the corneal surface an impression of success or failure of therapy was made. Success was defined as a totally epithelialised, stable and avascular corneal surface. Failure was defined as appearance of any superficial corneal vascularisation (even if the corneal surface was epithelialised and stsable), epithelial defects lasting more than two weeks and conjunctival overgrowth on the cornea (conjunctivalisation). The secondary clinical outcomes were improvement in best corrected visual acuity (BCVA) from baseline and ocular as well as oral complications.

Subsequent Surgical Management of COMET Cornea

Either penetrating keratoplasty (PK) or Boston type 1 keratoprosthesis (An acrylic plastic replacement for the central area of an opacified cornea) was performed in eyes with a stable ocular surface (irrespective of superficial vascularisation), but poor visual improvement attributed to corneal stromal scarring. The corneal tissue excised during PK or keratoprosthesis surgery was fixed in 10% formaldehyde and processed for histopathology and immnuohistochemistry analysis as described below. Sequential management of bilateral LSCD with COMET is shown in the figure 4.1 and flow chart 4.1.

Phenotypic Characterisation of the Corneal Tissue after COMET

Nakamura *et al.* first reported the phenotypic characterisation of epithelial cells followed by COMET (Nakamura *et al.* 2007). This group analysed 6 corneal tissues from 5 patients who underwent COMET and subsequent PK. From their study, it was concluded that the autologous cultivated oral mucosal epithelial cells survived on the corneal surface and maintained ocular surface integrity. Chen *et al*. demonstrated the presence of transplanted oral epithelial cells in corneas. The basal compact epithelial cells expressed stem cell markers p63, ABCG2 and p75 (Chen *et al.* 2009).

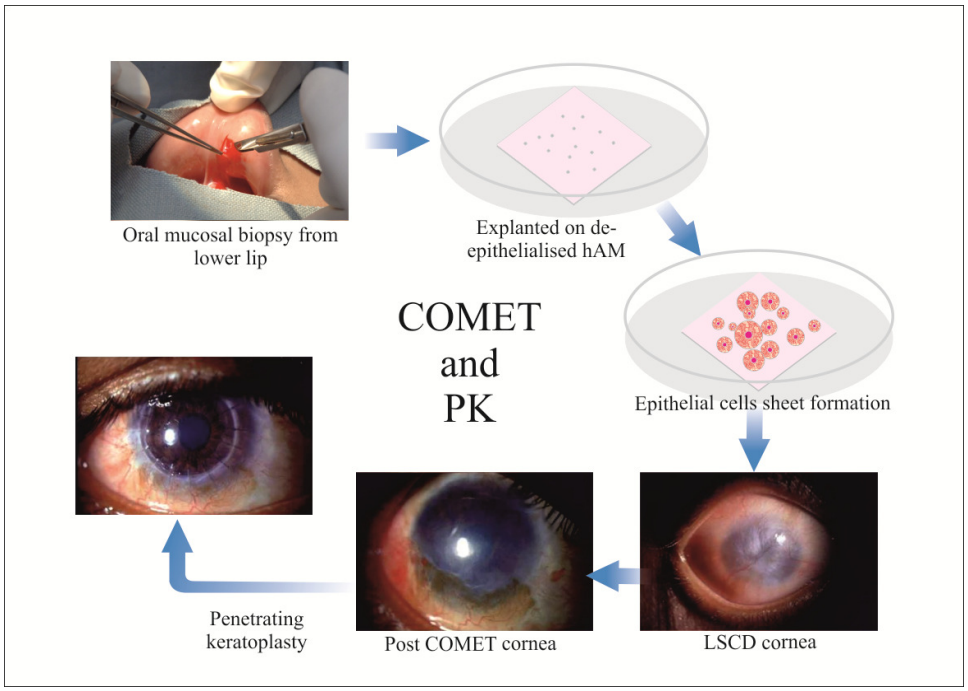
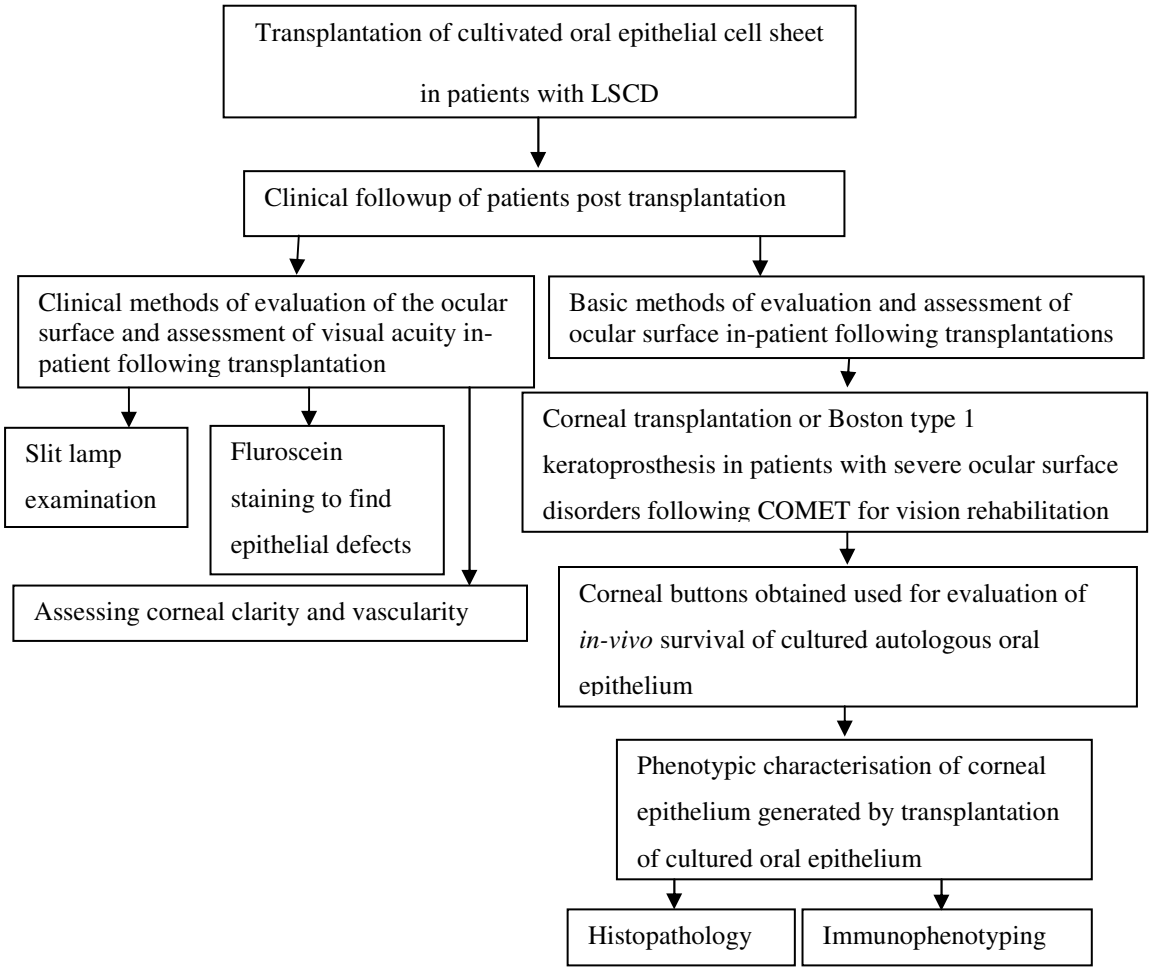


Figure 4.1: Schematic representation of the different steps involved in the management of COMET. Oral mucosal epithelial biopsy was harvested and grown on hAM as explants. COMET was done after 20 days of culture in sterile conditions. Damaged ocular surface was epithelialised with transplanted oral epithelial cells within one month. Epithelial cells are

transparent on avascular corneal bed. Due to severe chemical injury, the stromal region of cornea becomes opaque. For visual rehabilitation PK (Corneal transplantation) was done.

Flow Chart 4.1: Investigation of *in-vivo* survival of transplanted oral mucosal epithelial cells



4.2 Hypothesis

In severe bilateral LSCD, the ocular tissue reconstruction could possibly be achieved by transplantation of *ex vivo* cultured non ocular autologous oral mucosal epithelium. Upon stabilisation of ocular surface, further visual rehabilitation could be achieved by corneal transplantation.

4.3 Aim

- To evaluate the clinical outcome in terms of transplanted (oral) epithelial cells stability, corneal vascularity and symptomatic relief of patients who underwent cultured oral epithelial cell transplantation.
- To evaluate the secondary outcome measures like visual acuity after COMET, complications at donor site (oral mucosa) and transplanted site (ocular surface).
- To evaluate the transplanted epithelial characters in excised corneal tissue taken during corneal transplantation or Boston type 1 keratoprosthesis.

4.4 Results

4.4.1 Sterility Check for Chemicals and Spent Medium

All the chemicals used for the culture of epithelial cells and spent medium were tested for the presence of aerobic and anaerobic microorganisms. After incubation for 7 days in a bacterial incubator, they were negative for microbial contamination.

4.4.2 Cultured Oral Mucosal Epithelial Transplantation

After IRB approval, a total of 19 cultures (38 membranes) were established using healthy oral mucosal biopsy taken from bilateral LSCD patients with low vision. All cultures were successful and reached confluency between 16-20 days, covering ≥6.25 cm^2 area of the hAM. COMET was performed in 19 eyes of 18 patients during October 2007 to May 2009 at L.V Prasad Eye Institute, Hyderabad. The transplantations were performed by one experienced ocular surface surgeon. Brief summary of all patient's history who underwent COMET treatment is shown in appendix-1.

4.4.3 Post COMET Evaluation of Clinical Outcomes in Treated Patients

During the entire study period, COMET was conducted on 19 eyes of 18 patients with bilateral and total LSCD following ocular surface burns and evaluated by the ocular surface specialist. The mean age at the time of surgery was 23.7± (12.5) years with male to female ratio of 2.8:1. The median time period between the initial injury and autologous COMET was 34 months (Range: 6 to 240 months). Three patients underwent biopsy and transplantation under general anaesthesia, whereas others were operated under local anaesthesia. No anaesthetic or intra-operative complications occurred during either biopsy or transplantation. No donor site complications were noted. The mucosal defect created on the lower lip following the oral biopsy completely healed by one week.

The data was collected prospectively from the medical records of the patients after 48 months of the first COMET surgery. The mean follow-up was 22.3 (Range: 7 to 48) months. Post-operatively on day one and one week, fluorescein staining (to evaluate the epithelial integrity) was negative over the grafted area and no folding or loosening of the hAM was noted. At six weeks, 16 (84%) of eyes showed completely epithelialised and stable corneal surface without peripheral superficial corneal vascularisation. However, peripheral superficial corneal vascularisation was seen in all eyes by three months. Therefore none of the eyes met the clinical criteria of success at 3 months and thereafter. In 7 (36.8%) eyes, the peripheral vascularisation did not progress and the corneal surface was completely epithelialised and stable at 12 months after COMET (Figure 4.2). In the remaining 12 (63.2%) eyes recurrence of conjunctivalisation occurred in 2 eyes and persistent epithelial defects occurred in 10 eyes. The central cornea of these eyes became progressively vascularised or developed persistent

epithelial defects with recurrence or worsening of symblepharon between 3 and 9 months of COMET (Figure 4.2 and Table 4.2).

Prior to COMET, the BCVA ranged from hand movements to perception of light in all eyes. On the last date of follow-up or before undergoing keratoplasty or keratoprosthesis surgery, the BCVA had not improved in 12 (63%) eyes, had improved to counting fingers in 6 (32%) eyes and to 20/125 (5%) in one eye.

Table 4.1: Characteristics of cases and clinical outcome of patients with oral mucosal epithelial culture reconstruction.

CHARACTERISTIC	N (%)
Age	
8 years or younger	2
9 to 16 years	1
Older than 16 years	16
Visual Acuity	
Light Perception	11 (58)
Hand Movements	8 (42)
Etiology of Ocular Surface Burns	
Lime	8 (42)
Sulphuric Acid	4 (21)
Fire-cracker	2 (11)
Sodium Sulphate	1 (6)
Ammonium Nitrate	1 (6)
Liquid Ammonia	1 (6)
Titanium Oxide	1 (6)
Formic Acid	1 (6)
Previous Ocular Surface Surgery	
None	6 (32)
Amniotic Membrane Grafting	8 (42)
Penetrating Keratoplasty	5 (26)
Allogeneic Limbal	5 (26)

Transplantation	
Symblepharon Release	3 (16)
Ocular Surface Status	
Conjunctivalisation	15 (79)
Persistent Epithelial Defect	4 (21)
Symblepharon	8 (42)

Of the 7 eyes with a stable ocular surface, PK was done on one eye and four eyes were subjected to Boston type 1 keratoprosthesis surgery for visual improvement. Following PK the corneal graft developed repeated epithelial defects and a permanent tarsorrhaphy (the eyelids are partially sewn together to protect the cornea) had to be performed three months later. Three years after PK, the BCVA with an intact tarsorrhaphy was hand movements. The final BCVA in the four eyes that underwent Boston type 1 keratoprosthesis ranged from 20/20 to 20/30 with a maximum follow-up of 26 months.

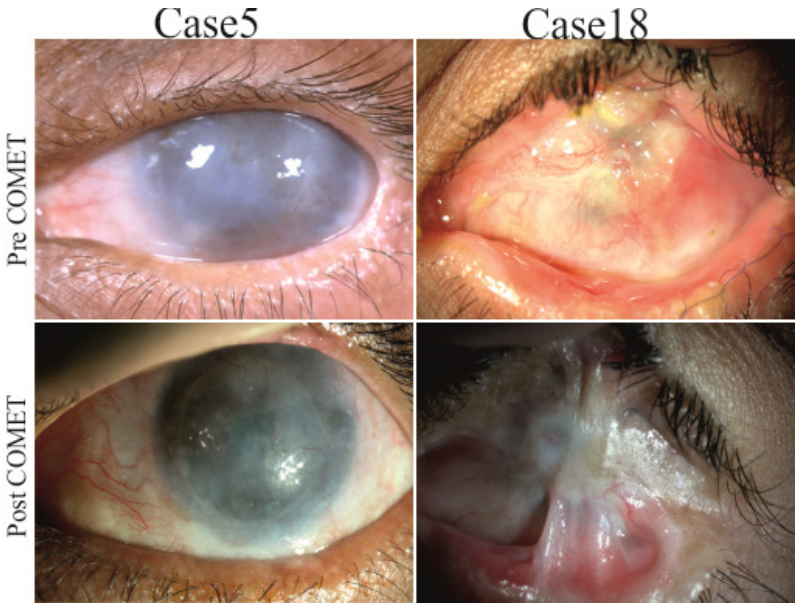


Figure 4.2: Clinical photographs of the ocular surface before and after autologous cultivated oral mucosal epithelial transplantation (COMET) in eyes with ocular burns. Case 5: A 34 year old male artisan with history of bilateral acid burns and failed living related conjunctival limbal allografting and penetrating keratoplasty underwent COMET in his left

eye. One year following COMET the ocular surface was stable with non-progressive peripheral corneal vascularisation, corneal scarring and contact lens corrected vision of 20/125. Case 18: An 8 year old male child suffered bilateral fire-cracker injury following which he underwent COMET in the left eye. One year after COMET the ocular surface showed severe symblepharon formation with conjunctivalisation of the cornea

4.4.4 Assessment of Transplanted Cells on Post-PK Corneal Tissues from COMET Patient.

In patients who underwent COMET treatment, penetrating keratoplasty and Boston type 1 keratoprosthesis was done to improve the visual acuity after the ocular surface was reconstructed with transplanted oral mucosal epithelial cells. This provides an unique opportunity to evaluate and document the fate of the transplanted cells on the excised corneal tissue. Ocular surface of the LSCD patient who underwent COMET followed by PK is shown in figure 4.3.

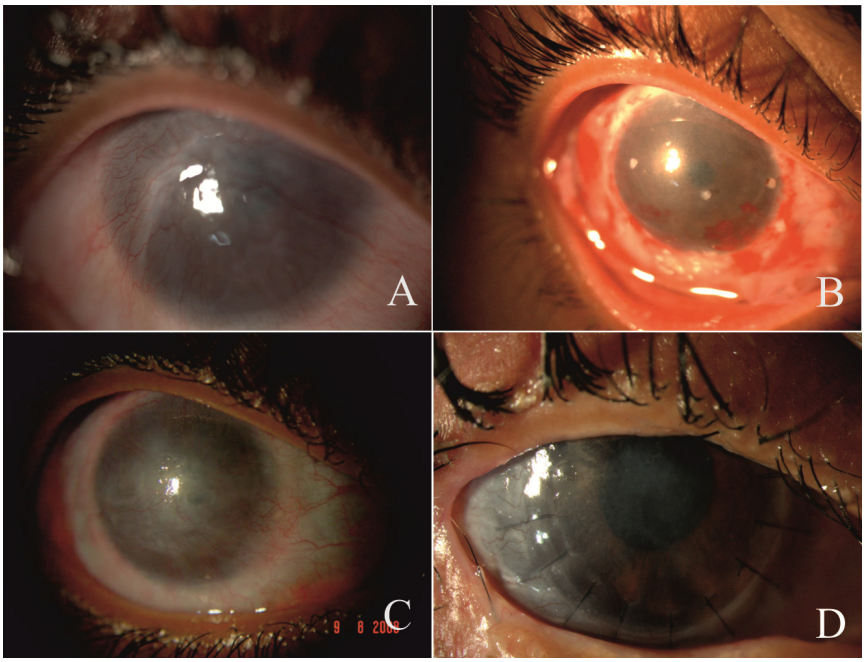


Figure 4.3: Ocular surface after the COMET and optical-PK of Case 1. Slit lamp pictures showing the pre COMET (A), Post COMET day 1 (B), Pre-optical PK (C) and post PK (D) cornea.

4.4.4.1 Histological examination of the post-COMET corneal tissue

Hematoxylin and eosion staining of the corneal buttons excised during penetrating keratoplasty or Boston type 1 keratoprosthesis of the COMET treated eye showed a five to six cell thickness stratified epithelium with the formation of a basement membrane. The amniotic membrane was not visible at the base of the epithelial layer. The basement membrane was formed. Goblet cells were not observed in PAS staining. Epithelial cells appear non keratinised. Few sub-epithelial vasculatures were also seen in close proximity to the basement membrane (Figure 4.4).

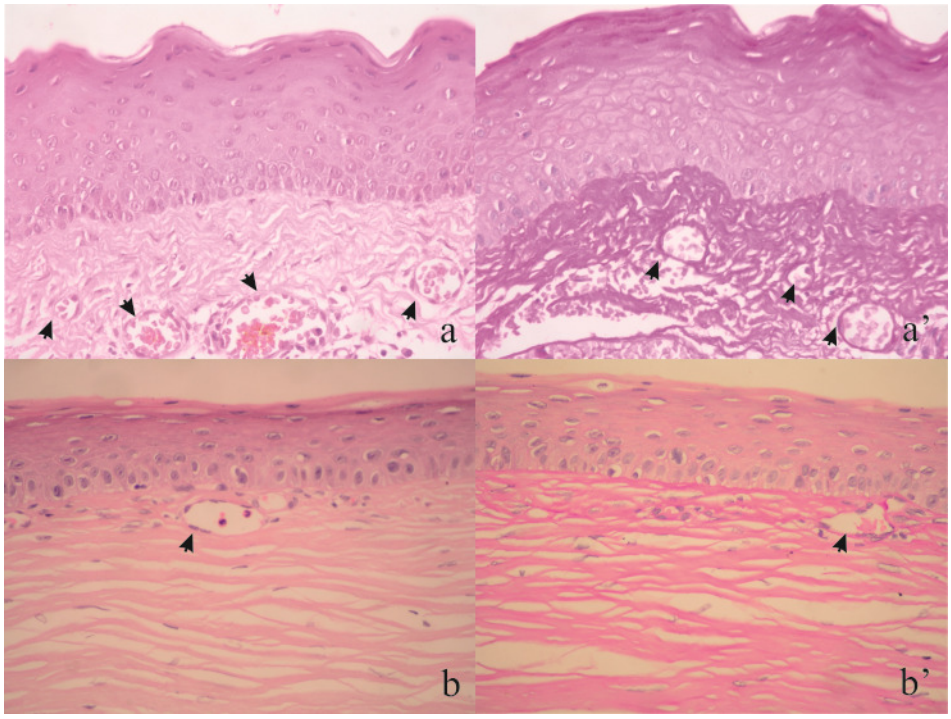


Figure 4.4: Histological pictures of post COMET cornea. Hematoxylin and Eosin (a, b), PAS (a', b') stained post COMET-PK corneal tissue showing hyperplasia of epithelial cells in two patients. Sub epithelial vasculatures with blood cells (arrow head). PAS staining shows no goblet cells in the stratified epithelium.

4.4.4.2 Immunohistochemical examination of the post-COMET corneal tissue

Immunophenotypic characterisation of post COMET corneal tissue was done in comparison with native corneal, oral mucosal and conjunctival epithelial tissues.

Immunohistochemical examination of the post-COMET corneal tissues and control corneal and conjunctival specimens showed (Figure 4.5) cytokeratin 19 being expressed in the basal layer of the epithelial cells of post-COMET corneas, in the basal layer of the limbal epithelium in control corneas, in all layers of the conjunctiva and in the basal cells of the oral

mucosa. Expression of cytokeratin 14 was not seen in post-COMET corneas, control corneas and oral mucosa and it is present in the basal cells of conjunctiva. Cytoplasmic cytokeratin 3/12 expression was seen in all epithelial layers of post-COMET corneas, control corneas and oral mucosa but not seen in conjunctiva. Cytoplasmic cytokeratin 12 staining was seen only in the control corneal epithelium and was not seen in oral, conjunctival and post COMET corneal epithelium. Ki-67 expression was seen in the supra-basal layer of all specimens. p63 expression was seen in basal and supra-basal layers of the post-COMET corneas, control corneas, conjunctiva and oral mucosa. p75 expression was seen in basal epithelial cells of post-COMET corneas, basal epithelial cells of the limbus in control corneas, basal cells of conjunctiva as well as oral mucosa. CD31 and CD34 expression (Figure 4.6) was seen in sub-epithelial layers of the central and peripheral post-COMET corneas.

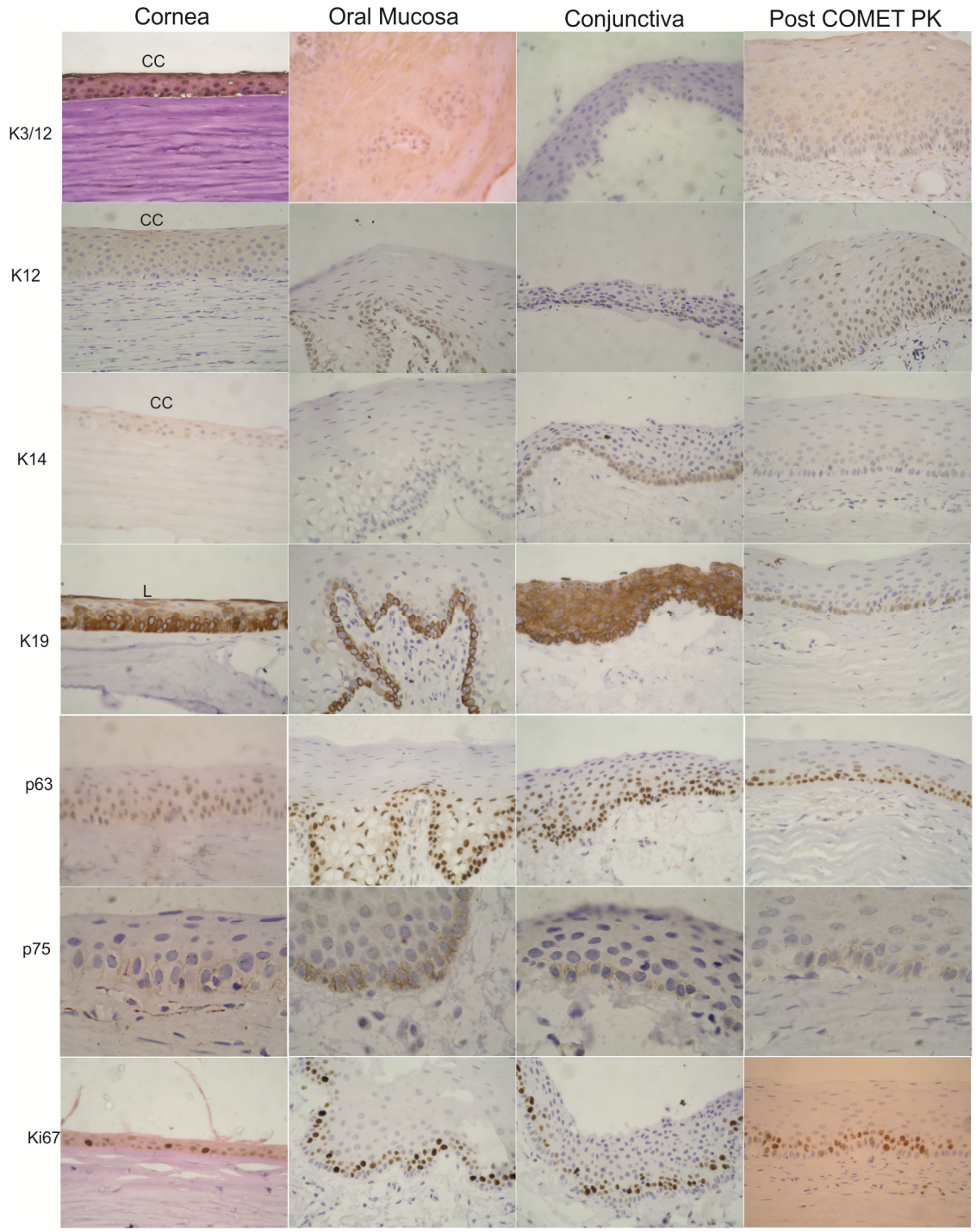


Figure 4.5: Immunohistochemistry for cytokeratin, proliferative and stem cell marker profile. Cytoplasmic cytokeratin 3/12 staining is present in central corneal epithelium, oral mucosal epithelium and post COMET corneal epithelium but not present in conjunctiva.

Cytoplasmic cytokeratin 12 staining was seen throughout the central corneal epithelium and was not seen in oral mucosa, conjunctiva and post-COMET corneal epithelium. Cytokeratin 14 was not expressed by the epithelial cells of the central cornea, oral mucosal epithelium and post COMET corneal tissue, but was expressed by the basal conjunctival epithelial cells. Cytokeratin 19 is expressed in all the layers of the limbal and conjunctival epithelium but not expressed in the central cornea (data not shown). The basal cells of oral mucosal epithelium and the post COMET PK tissue showed cytokeratin 19 expression. p63 immunostaining showed nuclear staining in the basal and supra-basal cells of all the tissues tested. p75 immunostaining showed membrane staining only in the basal epithelial cells of the cornea, oral mucosa, conjunctiva and post COMET PK tissue. Ki 67 staining in corneal, oral mucosal, conjunctival epithelium and post COMET corneal tissue showed clear nuclear expression by the proliferating supra-basal cells. (All pictures magnification- x400, p75 stained tissues- x1000).

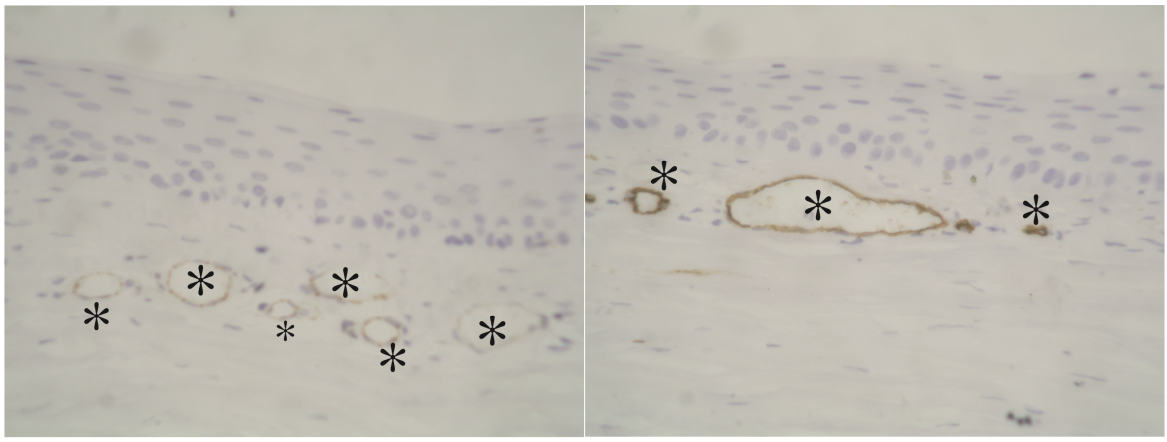


Figure 4.6: Immunohistochemical staining for vascular endothelial cells markers CD31 and CD34: Bright field microscopy pictures of post COMET corneal tissue stained for

vascular endothelial markers CD31 and CD34 showing positive and conforms the presence of sub epithelial blood vessels.

4.5 Discussion

COMET emerged with the promise of being an autologous alternative to allogeneic cell based therapy in eyes with bilateral and total LSCD. In this study, 19 eyes of 18 patients which underwent COMET symptomatic relief was found in all the transplanted eyes. In seven eyes corneal surface was completely epithelised and stable. Marginal visual improvement was observed in 6 eyes, clinically significant visual improvement was seen in one eye. However at the end of three months after COMET, all the corneas showed peripheral superficial vascularisation which proceded to complete vascularisation in 12 eyes but remain stable in 7 eyes. In the later group, an additional procedure of PK/BKP was attempted for visual rehabilitation. The histological and phenotypic characterisation of excised corneal tissues revealed that transplanted oral epithelial cells maintained the oral phenotype (Cytokeratins 19 in basal cells *etc*.) with no evidence of conjunctivalisation or transdifferentiation to the corneal phenotype. Further oral derived epithelial cells established a vascularised bed which was seen clinically and histologically suggesting that it is an important factor which dictates the outcome of non homologous epithelial graft on ocular surface. This surely warrants further studies.

A comparison of this study with the indications, laboratory techniques and clinical outcomes of previous studies on autologous COMET with a sample size of 9 or more eyes as summarised in table 1.9 (Nakamura *et al.* 2004; Ang *et al.* 2006; Inatomi *et al.* 2006; Burillon *et al.* 2011; Nakamura *et al.* 2011; Satake *et al.* 2011). It is noteworthy that: a) none of the

previous studies used a xeno-free culture technique; b) the indications for COMET varied widely among different studies; c) all studies used clinical criteria for assessing the outcome of therapy; d) success rates with regards to ocular surface stability ranged from 28.5% to 100% with mean follow-up durations ranging from 12 months to 55 months; and e) all studies reported appearance of peripheral superficial corneal vascularisation after COMET. In the context of this heterogeneous data, ocular surface stability achieved in our study (7 [37%] in 19 eyes) compares well with that reported by Satake and associates 18 (4 [36%] in 11 eyes) and Burillon and associates 19 (4 [44%] in 9 eyes) in eyes with ocular surface burns. A comparison between this study and previous studies on COMET with those on allogeneic limbal transplantation is again difficult, because the indications and sample sizes vary among different studies. Indeed, there are no comparable published studies (with a sample size of five eyes or more) of allogeneic cultivated limbal transplantation in eyes with ocular burns (Shortt *et al.* 2010; Baylis *et al.* 2011).

With regards to keratolimbal allografts, in two series of 16 and 17 eyes with ocular burns among other indications, Solomon and associates (Solomon *et al.* 2002) and Maruyama-Hosoi and associates (Maruyama-Hosoi *et al.* 2006) reported long-term corneal epithelial stability in 71.3% and 58.8% eyes respectively. Similar to ocular surface stability, the proportion of patients who gained 20/200 or better vision, after keratolimbal allografting (43.5% to 44.6%) was also greater as compared to that after COMET (7% to 30%, Table 1.9) (Nakamura *et al.* 2004; Ang *et al.* 2006; Inatomi *et al.* 2006; Burillon *et al.* 2011; Nakamura *et al.* 2011; Satake *et al.* 2011). This limitation of COMET is particularly significant because unlike patients with unilateral LSCD, who usually have good vision in the unaffected eye and may be satisfied with a stable and symptom free ocular surface in the affected eye, the

primary need of a patient with bilateral blindness is improvement in vision. Therefore the benefit of COMET of being an autologous therapy not requiring immunosuppression, does not outweigh its poor clinical outcomes. In view of these results, currently we do not offer COMET to patients with bilateral LSCD.

Our phenotypic study of the excised corneal tissue revealed its phenotypic similarity to the native oral mucosal tissue (Figure 4.5). It confirmed that the transplanted cultured oral mucosal epithelial cells do survive, stratify and integrate without undergoing any change onto the ocular surface. These results were similar to Chen and associates and Nakamura and associates who performed histopathology and immunohistochemical analysis in four and six post-COMET eyes, respectively (Nakamura *et al.* 2007; Chen *et al.* 2009). On histopathology, they found the transplanted epithelium to be five to twelve layers thick without goblet cells and basal columnar cells. On immunohistochemistry, they also found that cytokeratin 3 was present in all epithelial layers, cytokeratin 12 was present occasionally at the peripheral portion of corneal tissue (Residual native corneal tissue), p63 and p75 was present in the basal epithelial layers. While the corneal epithelial stem cells are located at the limbal region, oral epithelial stem cells are located throughout the basal layer of epithelium (like native tissue). The staining pattern of p75 on excised cornea showed that presence of stem cells scattered all over the cornea at epithelial basal region. Additionally this study showed expression of vascular endothelial markers CD31 and CD34 in the sub-epithelial region of the post-COMET corneas to corroborate with the clinical findings of superficial vascularisation.

One of the important finding in this study is the secretion of sFLT1 by limbal epithelial cells not by oral mucosal cells or conjunctival cells (Figure 3.10). sFLT1 is an

antiangiogenic factor which sequesters the VEGF molecule was reported by Ambati *et al* (Ambati *et al.* 2006) to be a crucial factor in maintaining corneal avascularity. These two evidences in combination with the clinical and histopathological findings of corneal vascularisation following COMET suggest that limbal derived epithelium is specially primed to survive on the avascular corneal stroma. It also suggests that abrupt angiogenic to antiangiogenic milieu of the limbus possible plays an important role. Further studies in this direction would through light not only on corneal avascularity but other avascular-vascular region of the body like cardiac valves and cartilage.

These findings suggest that the transplanted oral mucosal epithelium maintains its original phenotype without any trans-differentiation to the corneal phenotype. This is the first study on transplantation of oral mucosal cells cultivated using a xenobiotic free technique of oral epithelial cell culture. Strength of this study is the homogeneity of the patient cohort; being the largest such study in cases with bilateral ocular burns. Unlike others studies an explant culture technique was used and transplanted at a monolayer stage.

CHAPTER 5: OFFSHOOT PROJECT OF THE THESIS

During the course of the work on the oral mucosal epithelial cell culture and transplantation, experiments were done on some interesting related projects with relevance to the subject and its application. This chapter briefs the novel findings of the offshoot project, undertaken along with the central idea of the thesis.

5.1 Corneal Epithelial Signalling Network

5.1.1 Introduction

Several studies suggest that corneal epithelial cells express molecules involved in Notch (Ma *et al.* 2007; Ma *et al.* 2011), Bone morphogenetic proteins (You *et al.* 1999), Wnt (Fokina and Frolova 2006) and Shh (Takabatake *et al.* 1997) pathways. Among these signalling mechanisms, Wnt signalling is playing a major role from embryonic stage to adult corneal homeostasis. Wnt signalling cascade execute its effects by two different pathways. One is β-catenin dependent canonical path way, other one is non-canonical Wnt signalling. The first one is well characterised compared to non-canonical pathway. Wnt's (Wnt 1-16) are secreted cysteine rich glycol proteins, upon binding with Frizzled receptors, lead to a cascade of signalling events that result in the stabilization and cytoplasmic accumulation of β-catenin. Cytoplasmic β-catenin translocates into the nucleus, where it interacts with the Lef1/Tcf transcription factors and together regulates the expression of Wnt target genes. In the absence of Wnt signalling stimulation, β-catenin will undergo proteasomal degradation by the destruction complex (APC, GSK-3β and Axin) resulting in the inhibition of Lef1/Tcf-mediated gene transcription (Figure 5.1).

In avian (Chicken Wnts are homologues to 18 human Wnts) models, Wnt signalling regulates embryonic development in the presence of Wnt antagonists which are expressed in distinct temporal-spatial patterns (Jin *et al.* 2002). Out of the 18 Wnt family genes, 11 are expressed in the anterior eye of chicken. In cornea Wnt 3a, Wnt 6 and Wnt 9b expression might play an important role in anterior eye development (Fokina and Frolova 2006). Wnt7a and matrix metalloproteinase-12 (MMP-12) expression increases immediately after corneal wound and induce cell proliferation and helps in wound closer. MMP-12 inhibition leads to delayed wound closure. Upregulated Wnt7a was shown to activate both β-catenin and the Rho GTPase protein, Rac. Rac in turn induces MMP-12 expression (Lyu and Joo 2005). Thus both β-catenin dependent canonical pathway and the Rac-dependent non-canonical pathway synergistically promote corneal wound healing.

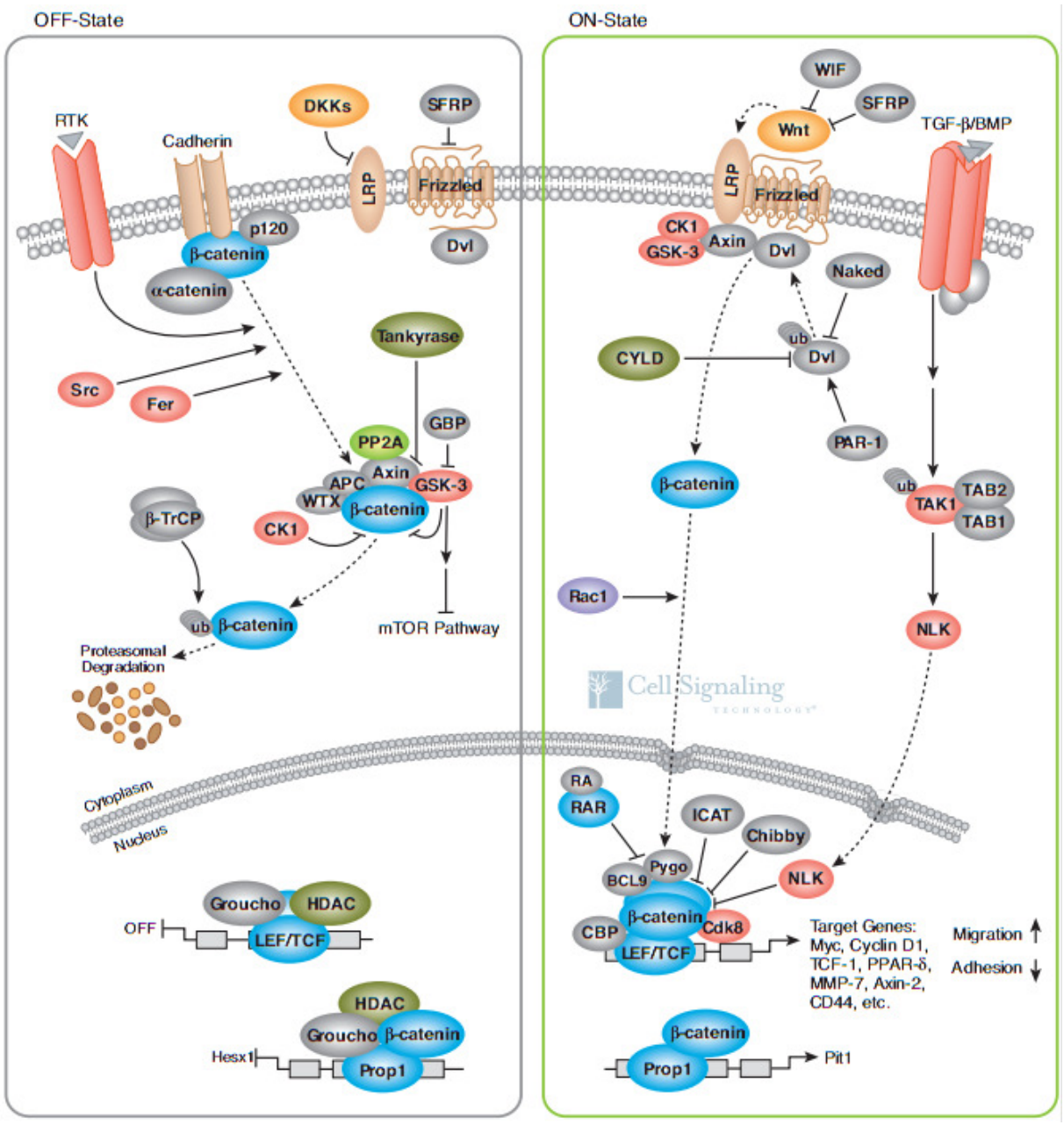


Figure 5.1: Schematic representation of Wnt/β-catenin signalling. In the absence of Wnt ligand (off state) β-catenin is degraded by distraction complex (GSK3-Axin-APC) and the target genes are in repressed state. In presence of Wnt ligands (on state), the destruction complex disassembles leading to the accumulation of β-catenin in the cytoplasm and later it

enters in to the nucleus and start activating Wnt target genes like c-m*yc, Cyclin D1, Axin2 etc*. Source- http://www.cellsignal.com

To elucidate the roles of regulatory networks and the molecular mechanism of adult corneal epithelial differentiation and homeostasis, knock out mouse models serves as a powerfull tool. For e.g. Secreted Wnt inhibitor protein Dickkopf-2 (DKK2) was recently shown to play an important role in corneal development. In $DKK2^{-/-}$mice, the corneal surface was opaque as opposed to a transparent cornea in normal mice. Also the presence of hair follicles, sebaceous glands, conjunctiva specific goblet cells and down regulation of corneal epithelial specific markers like Pax6 and K12 clearly indicates a role for Wnt signalling in corneal epithelial development. This may be due to unregulated activation of Wnt throughout the cornea (Mukhopadhyay *et al.* 2006). This regulatory mechanism was further elucidated using PITX2 and DKK2 null mice. DKK2 is directly regulated by the canonical Wnt target gene PITX2. The levels of PITX2 protein is tightly regulated and the fluctuations causes Axenfeld-Rieger Syndrome (Gage *et al.* 2008). This is also evident with the gain or loss of function PITX2 models (Doerdelmann *et al.* ; Amendt *et al.* 1998; Xia *et al.* 2004) and was further confirmed by PITX2 gene dosage experiments in mice models (Gage *et al.* 1999; Holmberg *et al.* 2004; Diehl *et al.* 2006).

Upon activation of canonical Wnt signalling PITX2 gets activated which in turn activates DKK2 and there by establishes a negative feedback loop and helps in the regulation of Wnt signalling. Also, the master regulatory gene, Pax6 is crucial for vertebrate and invertebrate eye formation and maintenance. Insufficiency of Pax6 in heterozygous condition (Ou *et al.* ; Ramaesh *et al.* 2003; Ou *et al.* 2008) or over expression (Schedl *et al.* 1996; Aalfs

et al. 1997; Davis and Piatigorsky 2011) lead to eye abnormalities including epithelial defects. A transgenic mouse with *Pax6* gene under the control of cornea specific promoter Aldh3a1 over expresses Pax6 specifically in corneal epithelial cells. In this model, during early stages of development, the barrier function was compromised and blood vessels were seen all over the cornea. In late phases, the cornea becomes opaque and ulcerated. Immunohistochemistry of these corneas showed down regulation of corneal epithelial specific keratin 12 and relative decrease in Pax6 protein expression in transgenic corneal epithelial cells. *In vitro* studies showed up-regulation of Wnt inhibiter factor 1 (wif1) and FLT1 promoter activity. Suppression of Wnt signalling by Wnt regulators like DKK2 is needed for the corneal epithelial differentiation (Zhang *et al.* ; Gage *et al.* 2008). In presence of high levels of Pax6, it should favour the suppression of Wnt pathway through activation of wif1 and promote differentiation by K12 expression in corneal epithelial cells. Expression of sFlt1 in corneal epithelial cells should inhibit blood vessel formation. But it is not very clear as to why Pax6 over expression inhibits differentiation and promotes angiogenesis in this mice model. To address the role of Wnt signalling and its gene regulatory network involving Pax6 gene, we used RT-PCR approach to compare the differences in gene expression between the normal and diseased ocular surface tissues. Sub cellular localisation studies were done in HCE cell line and primary corneal epithelial cells using IHC for corneal specific markers and Wnt regulators.

5.2 Results

5.2.1 Expression of Wnt Ligands and Wnt Target Genes in Normal and Diseased Conditions

In order to check whether Wnt signalling is deregulated during pathogenesis or differentially regulated in cornea, limbal and conjunctival epithelium, we looked at the gene expression profiles of different Wnt signalling markers by RT-PCR using gene specific primer sets (*Wnt4, Wnt 5A, Wnt 11, Wnt16, PITX2, CCND1, CCND2, CDC42, LEF 1* and *VEGF-A*). The total RNA was extracted from the human limbal cultures, conjunctival and pterygium tissues collected from different human donors. RNA inputs were normalised using the house keeping gene, *GAPDH* as loading control and semi-quantitative RT-PCR was performed for appropriate cycles where the amplification is in log scale. RT-PCR results indicates that some of the Wnt pathway genes (*Axin2, DKK2, PITX2, Wnt5A* and *Wnt16A*) are over expressed in pterygial cells and are also differentially expressed among the limbal, conjunctival and corneal epithelial cells. However, we found that the results were not consistent due to variation in the age and conditions of the donor/cadaveric tissue. Also, due to limited availability of human cadaveric/donor tissues, we went ahead and performed experiments using human corneal epithelial cell line (Araki-Sasaki *et al.* 1995). We induced canonical Wnt pathway by LiCl treatment of HCE cells *in vitro* and checked for its effect on some of the Wnt signalling related genes (Figure 5.3) by RT-PCR.

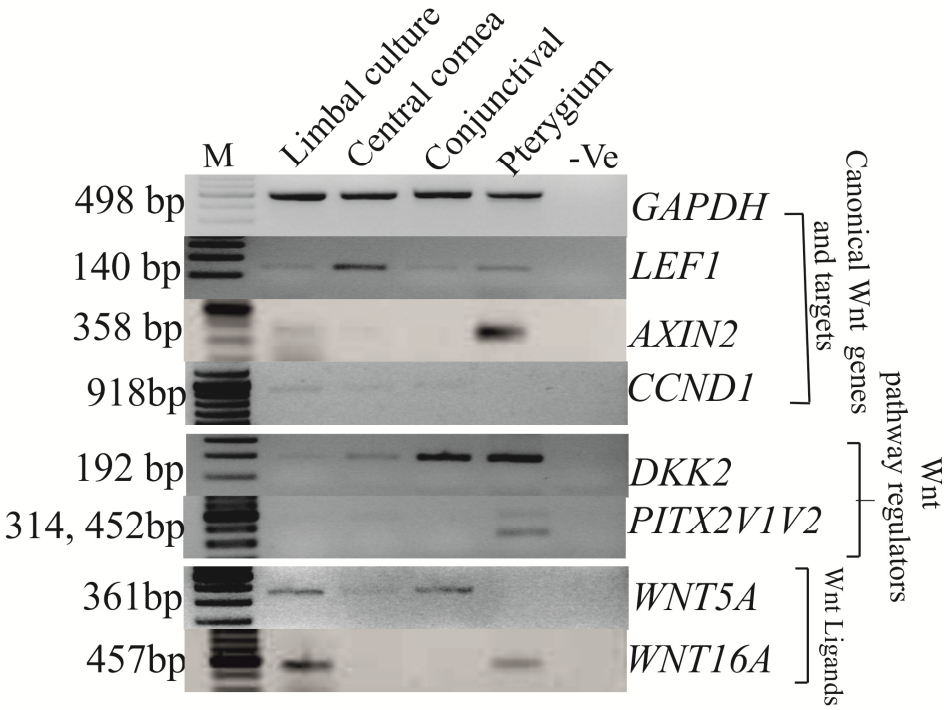


Figure 5.2: Wnt gene expression in human ocular tissues (normal and diseased). Expression levels of Wnt and Wnt downstream genes are screened by semi quantitative RT-PCR.

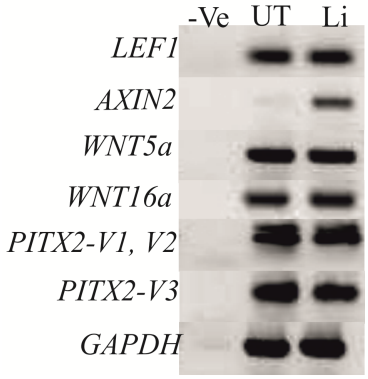


Figure 5.3: Semi quantitative RT-PCR with cDNA from HCE cell line treated with LiCl (Li) along with untreated control (UT) and negative control (-Ve) showing expression of Wnt genes and up regulation of *AXIN2* expression.

5.2.2 Activation of Wnt Signalling Helps in Symmetric Proliferation of Stem Cells

Activation of Wnt signalling in HCE cell line and primary limbal epithelial cells was done by treating the cells with LiCl, a well known inhibitor of glycogen synthase kinase 3β, which results in the disassembly of β-catenin destruction complex, cellular accumulation and nuclear translocation of β-catenin. Epithelial cells isolated from primary limbal cultures on hAM were passaged on to mitomycin C treated NIH 3T3 feeder cells and cultured for 10 days. Both the HCE cells and primary cells were treated with 10mM LiCl for 12 hrs. Untreated cells were kept as controls. No increase in cell death was observed in LiCl treated cultures by gross examination. Immunocytochemistry with Ki67 antibody showed that cell proliferation was high in LiCl treated cells when compared to untreated controls (Figure 5.4a). BrdU labelling and IHC with anti BrdU antibody also confirmed that cell proliferation rate is increased in LiCl treated cells (Figure 5.4a, c).

Also, the expression of putative stem cell marker ΔNp63α was elevated in the LiCl treated limbal primary cells when compared to the untreated cells (Figure 5.4b). We have used an antibody that identifies the ΔNp63α isoform specifically and can differentiate between transient amplifying cells and the stem cells. These results demonstrated that the activation of Wnt/β-catenin signalling promoted proliferation and expansion of limbal epithelial stem cells as opposed to differentiation. This experiment was done in primary limbal cells as the HCE cell line does not express the ΔNp63α protein both under treated and untreated condition. This observation is in confirmation with earlier reports that suggest the role of Wnt/ β-catenin signalling in the regulation of limbal stem cells (Nakatsu *et al.* 2011).

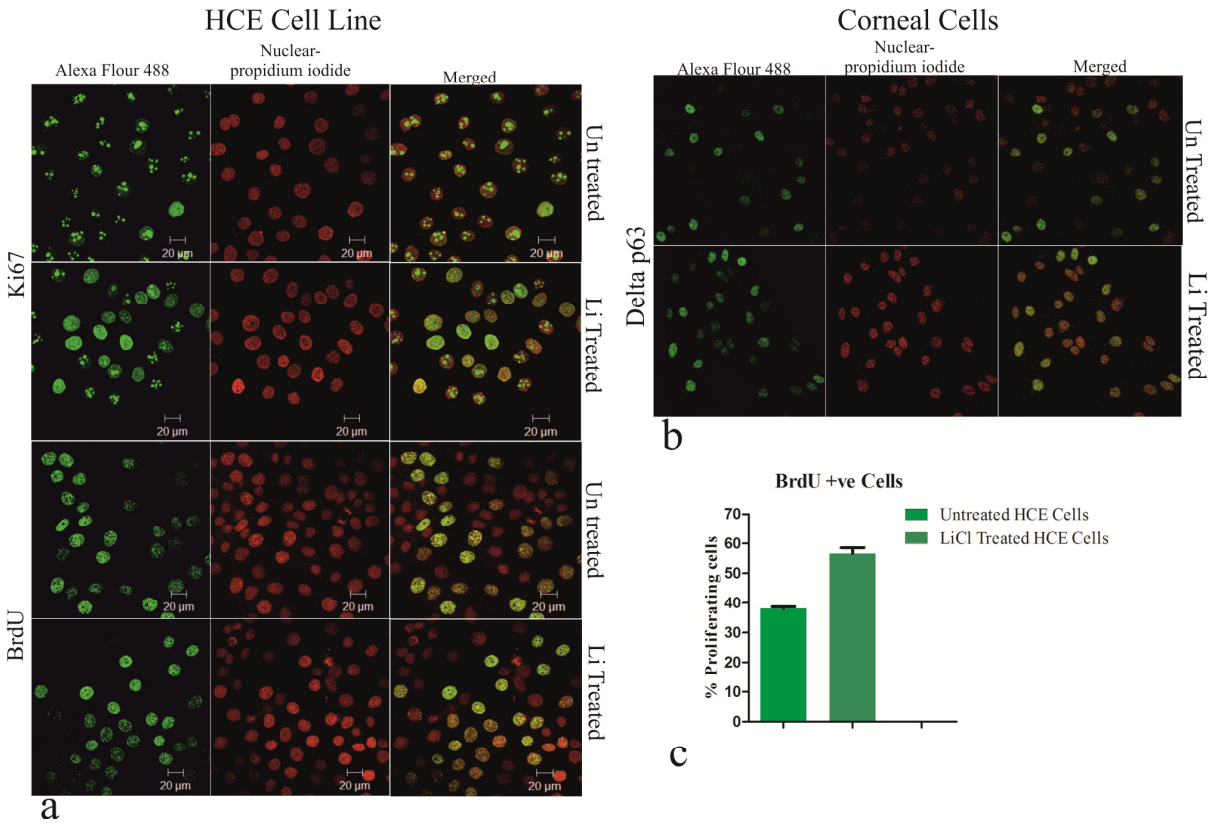


Figure 5.4: Activation of Wnt/β-Catenin signalling increased the proliferation of HCE cell line and primary limbal epithelial cells. An increase in Ki67 positive cells was observed in LiCl treated cells compared to untreated cells (a). Labelling with BrdU after treating with LiCl showed more BrdU positive cells when compared to untreated cells (a) Graphical representation of BrdU labelled cells after treatment with LiCl showing ~20% more proliferating cells (c). Expression of ΔNp63α was increased after treatment with LiCl (b). Scale bars: 20 µm.

5.2.3 Effect of Wnt/β-catenin Signalling on Pax6 Expression

To examine whether the activation of Wnt/β-catenin signalling alters the expression of the ocular master regulatory gene, Pax6, we evaluated both Pax6 and β-catenin expression in treated and untreated cells. Upon LiCl treatment, Pax6 expression was up-regulated and found to be localised both in the nucleus and cytoplasm at very high levels when compared to untreated cells (Figure 5.5a, b). Upregulation of Pax6 was also confirmed by western blotting (Figure 5.6). In the absence of LiCl, beta-catenin was localised to the membrane in all the cells of HCE cell line and primary cells. After treatment with LiCl, a fraction of β-catenin was detected in the nucleus indicating nuclear translocation and activation of Wnt target genes (Figure 5.5a, b). However the total cellular β-catenin levels did not change as shown in figure 5.6.

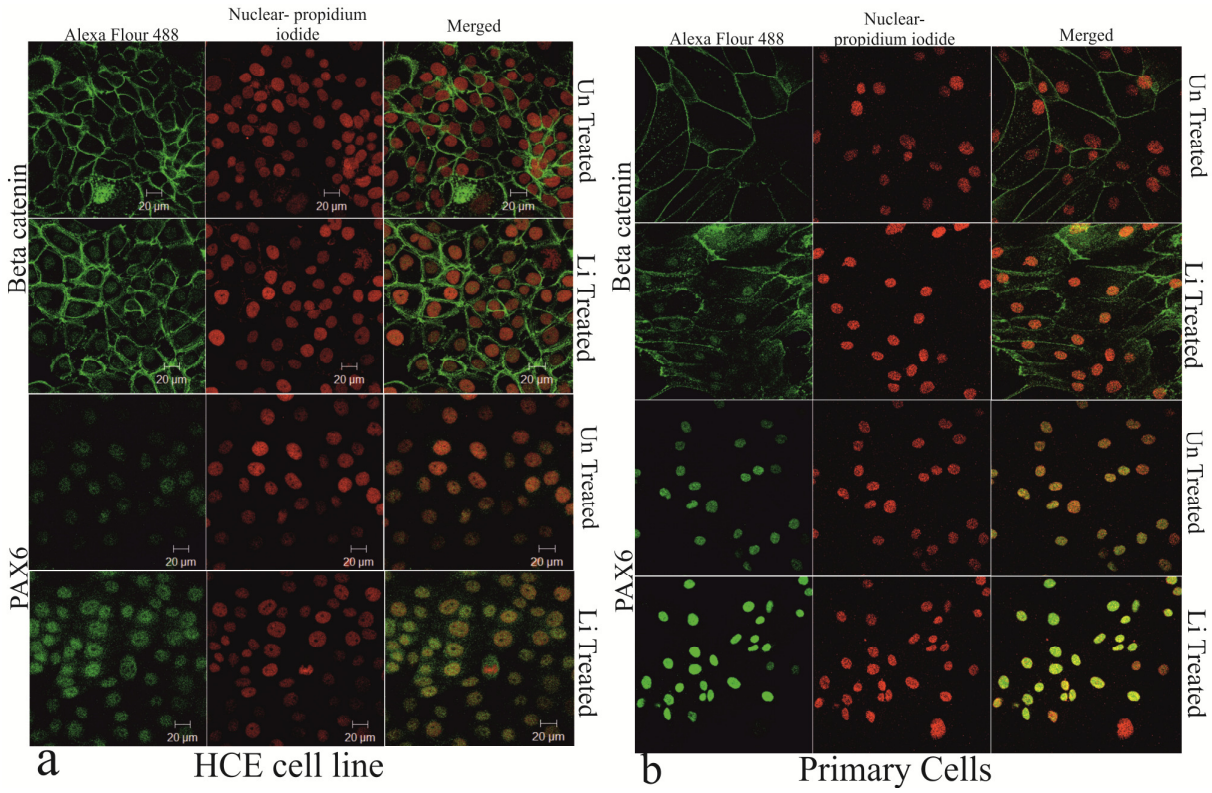


Figure 5.5: Activation of Wnt/β-catenin signalling by LiCl treatment of corneal epithelial cells. Treatment with 10 mM LiCl in complete HCE medium for 12 hours leads to nuclear localisation of β-catenin. In the absence of LiCl, β-catenin was localised predominantly to the cell membrane in (a) HCE cell line and (b) primary limbal epithelial cells. Also LiCl treatment resulted in increased expression of Pax6 which was localised both in the nucleus and cytoplasm in HCE cell line and primary limbal epithelial cells. Nuclei of the cells were counter stained with propidium iodide. Scale bars: 20 µm

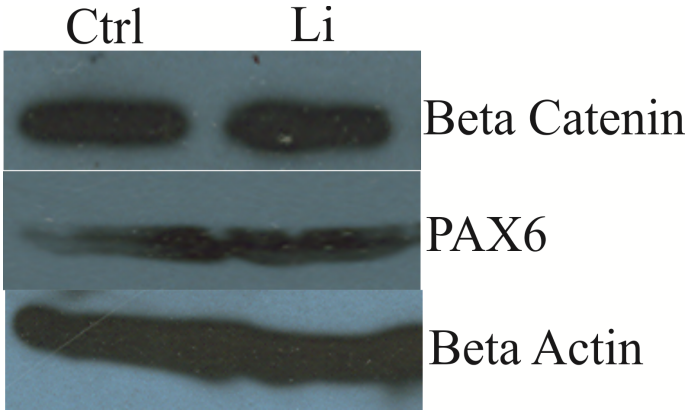


Figure 5.6: Effect of LiCl treatment on the levels of β-catenin and Pax6 proteins in cultured HCE cell line. 12 hour treatment with LiCl increases cellular Pax6 protein levels. β-catenin expression levels remained constant. β-actin is the loading control.

5.3 Discussion

We observed that Wnt signalling pathway genes are active in ocular surface tissues and corneal epithelial cell line. In proliferating corneal cells, β-catenin is membrane bound, so the Wnt/beta catenin signalling is kept inactive. Wnt signalling is known to regulate self renewal and differentiation of several stem cells. Interestingly, after treatment with LiCl, the number of ΔNp63α positive cells and proliferating cells (as evident from BrdU, Ki67 labelling index) has increased significantly when compared to untreated controls. This agrees with the recent findings that activation of Wnt/β-catenin signalling increased the proliferation and colony forming efficiency of primary human limbal stem cells. The stem cell phenotype was maintained as shown by higher expression of putative stem cell marker, ΔNp63α (Nakatsu *et al.*).

Pax6, a homeobox protein, is the master regulator for ocular development and corneal surface maintenance. In corneal epithelium, the differentiation marker, cytokeratin 12 is

directly regulated by Pax6 to promote differentiation. In contrast, Wnt induced cell proliferation upregulates Pax6 and ΔNp63 proteins. To further elucidate this gene regulatory network, it becomes important to understand the interactions between Pax6 and β-catenin-Lef1 protein complexes in limbal stem cells before and after differentiation.

In conclusion, these findings demonstrate that Wnt signalling is active in corneal epithelial cells. Activation of Wnt/β-catenin increases cell proliferation rate and also increased the expression of ΔNp63α and Pax6 protein levels and thereby could play an important role in corneal epithelial homeostasis. Interestingly, we found that the activation of Wnt/catenin pathway also upregulated the Pax6 expression, while promoting cell proliferation and stem cell expansion. This was contradictory to the earlier report which suggested that EGF mediated down regulation of Pax6 expression is important for corneal epithelial cell proliferation (Li and Lu 2005). Though, Wnt activation has increased Pax6 levels both mRNA and protein levels of Pax6, we also observed an increased cytoplasmic redistribution. It remains to be tested whether the increased Pax6 protein is transcriptionally active and how it regulates the balance between cell proliferation and differentiation during corneal homeostasis.

CONCLUSIONS AND LIMITATIONS

Conclusions

In this study, an attempt was made to provide non homologous but autologous cell therapy for patients whose corneal epithelial stem cells in both eyes are totally damaged due to chemical injuries leading to a pathological condition termed as limbal stem cell deficiency. The technique reported here is highly reproducible and easily adaptable. The study demonstrates that using a simple explant culture technique, it is possible to generate a sheet of oral mucosal epithelium from a small piece of oral tissue as demonstrated in limbal epithelial cell culture. This technique was simple, cost effective and xeno-free. Characterization of the cells revealed their similarity to corneal epithelium and its suitability for ocular surface reconstruction.

In summary,

- Developed a highly reproducible feeder-cell free, xeno free technique of culturing oral mucosal epithelial sheet within 18-21 days.
- Phase contrast and bright field microscopic observation of cultured cells showed honeycomb pattern on de-epithelised hAM. Paraffin embedded stained sections showed monolayer of epithelial cells attached to hAM.
- Electron microscopy demonstrated that the cells formed gap junctions and desmosomes.
- Genotypic and phenotypic (RT-PCR and immunohistochemical) analysis showed that *ex-vivo* cultured oral epithelial cells expressed markers of epithelial differentiation such as cytokeratins (K3, K4, K13, K15), integrins (CD 29), E-cadherin and connexin 43. These cells did not express cytokeratin 12, a corneal epithelial-specific keratin. Demonstrating that explant cultured oral mucosal epithelium retain their phenotypic character.
- The cultured oral epithelial cells expressed stem cell markers of epithelial cells such as ΔN isoforms of p63 as well as p75 (CD271).
- Universal gene expression profile of cultured oral mucosa and limbal epithelial cells showed tissue specific gene up-regulation or down-regulation, no significant difference was seen in pro-angiogenic factor profile between cultured limbal and oral epithelial cells at transcript level.

- Cultured oral epithelial cells secret less sFLT1 protein compared with limbal epithelial cells.
- Cultured oral mucosal epithelial transplantation (COMET) to bilateral LSCD patients resulted in immediate symptomatic relief with no pain, irritation and redness in all the transplanted eyes.
- Long term follow-up of COMET patients indicated peripheral neo-vascularisation which further progressed to the central cornea and affected the visual outcome. However in 7 eyes (36.8%) the peripheral vascularisation remains same and the corneal epithelium was completely epithelised and stable at 12 months after COMET.
- The evaluation of the corneal buttons from patients undergoing COMET followed by PKP showed, transplanted oral epithelial cells integrated, survived and maintains oral phenotype. Histological examination revealed an intact, multilayered stratified epithelium indicating *in vivo* stratification of oral mucosal epithelial cells. Thus a monolayer of cultured oral epithelial cells is capable of stratification *in-vivo*.
- Successful reconstruction of ocular surface of LSCD patients showing relief from symptoms like pain, photophobia, redness and watering, with marginal visual outcome. However the issue of angiogenesis needs to be addressed for expanding the scope of this treatment modality.

Limitations

- While cultured oral epithelial cells showed sufficient cells after *ex vivo* culture, the exact quantity of stem cell population in oral mucosal cultures was not known at the time of transplantation. This could be overcome by evaluating the stem cells by using markers like p75, p63α and by clonal assay in parallel oral mucosal cultures.
- While designing the microarray gene expression experiment, comparison of cultured cells along with the native tissues on a competitive hybridisation platform may give a better result.

FUTURE SCOPE OF THE WORK

- Elucidating the regulatory mechanisms in normal and wounded corneal epithelium will help to understand the characters (transparency, avascularity and maintenance of limbal barrier) of cornea.
- Priming of non ocular oral epithelial cells to ocular cells compensates for limbal barrier function. This could be overcome by co culturing the oral epithelial cells with limbal stromal cells.

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APPENDIX-1

(1) Phosphate Buffered Saline

PBS (1X) was used for washing and other purposes. The following chemicals were added to distilled water. After dissolving the chemicals thoroughly the pH was set to 7.2 using 0.1M NaOH or 1N HCl with pH meter and the final volume made to 100ml with distilled water.

NaCl	0.8g
KCl	0.02g
KH_2PO	4 0.012g
Na_2HPO_4	0.091g

(2) Trypsin-EDTA

Trypsin enzyme was used for processing the human amniotic membrane. It was prepared by reconstituting the lyophilized powder of trypsin in 1x PBS, to make 0.25% trypsin solution. To this 0.1 mM EDTA solution was added and filter sterilized (0.2µm syringe filter) and stored at 4°C. This solution is stable for 15 days at 4°C.

(3) Fetal Bovine Serum (FBS)

FBS was obtained from Sigma Aldrich. After filter sterilization aliquots of 50mL were made and stored at –80°C.

(4) Hematoxylin and Eosin staining

Formalin fixed tissue sections (6 micron) ware taken on a clean glass slide. Deparaffinize the slides by heating at 60°C and subsequent xylene dips each for 5 min-three changes. Dehydrate

with isopropyl alcohol and rehydrate to distilled water. Stain in freshly filtered Harris' hematoxylin for 6 to 10 minutes. Wash in running tap water for2 to5 minutes. Differentiate in 1%acid alcohol, 1 to 2 dips. Wash briefly in tap water. Place the slides in weak ammonia water until sections are bright blue colour. Wash in running tap water, place in 80% ethyl alcohol for 1 to 2 minutes. Counter stain with Eosin solution for 2 minutes, dehydrate and clear with xylene, each 2 minutes. Mount with resinous medium.

1. Mayer alum haematoxylin

Haematoxylin	5g
100% Ethanol	50mL
Potassium or Ammonium alum	100g
Distilled water	1000mL
Mercuric oxide, Red	2.5g

The alum was dissolved in water with gentle warming on magnetic stirrer. In another flask haematoxylin and ethanol was mixed. The strong alcoholic solution of haematoxylin was mixed with alum solution and stirred to ensure all the haematoxylin powder is dissolved. The mixture was heated rapidly until it boils for 1 min and mercuric oxide was slowly added to the haematoxylin-alum mixture and allowed the solution to come to room temperature. To

intensify the nuclear stain 20mL of glacial acetic acid was added. Staining solution was stored at room temperature until used.

2. Acid alcohol (0.3%V/V)

Ethanol (99%)	700mL
Distilled water	300mL
Conc hydrochloric acid	3mL

Acid alcohol was prepared by adding 3 mL of concentrated HCl to 300 mL of water. Mix the solution slowly and 700 mL absolute ethanol was added slowly. This is prepared freshly before use.

(5) Periodic acid Schiff staining

Solutions

1. 0.5% Periodic acid solution

Periodic acid	0.5g
Distilled water	100 mL

(6) SDS-Poly acrylamide gel electrophoresis (SDS-PAGE) Reagents

A. Tris-HCl (1.5M –pH 8.8)

Weigh 36.3g of Tris HCl and dissolve in approximately 150mL of deionised water. Adjust the pH with HCl to 8.8 and makeup the final volume to 200mL. Autoclave the buffer prior to use. This is stable for one month at room temperature.

B. Tris-HCl (1M-pH 6.8)

Weigh 12.21 g of Tris-HCl and dissolve in approximately 50 mL of deionised water adjust pH to 6.8 and make up to100mL. Autoclave buffer prior to use.

C. Acryl amide (30%W/V)

Weigh 87g of acrylamide and 3g of bis-acrylamide and dissolve in approximately of deionised water 170 mL and makeup the final volume to 300mL. Solution should be stored in brown bottle at 4 degree C. After the preparation stock should be filtered with Whatmann filter. Both acrylamide and bis-acrylamide are neurotoxin and can be absorbed through the skin. Wear the gloves while making the stock. This is stable for one month at 2-8 degree C.

D. sodium dodesyl sulphate (SDS) (10%W/V)

Weigh 5g of SDS and suspend in approx. 20mL of deionised water and dissolve it at 60 degree C till it dissolves completely and then make up the volume to 50mL.

E. Ammonium persulphate (APS) (10%W/V)

Weigh 1g of APS and dissolve in 10 mL of deionised water. This can be aliquot (500 µL) and can be stored at -20 degree C. Once the aliquot is thawed it should not be refreeze.

F. Running Buffer (TGS) (5x)

Tris HCl	75.5g
SDS	25g
Glycine	470g

SDS should be dissolved prior at 60 degree C in approx. 500 mL and then made up to the volume to 5 litres with deionised water. The pH of the buffer should be 8.3. This is

stable for over one month at room temperature. 5X can be diluted to 1X /2X according to the need with deionised water.

G. Sampling Buffer (2X)

Solutions to be added	Volume	Concentration (2X)	1X
1M Tris HCl pH 6.8	2mL	100mM	50mM
80% Glycerol	5mL	20%	10%
10% SDS	8mL	4%	2%
β Mercapto ethanol	0.4mL	2%	1%
Bromophenol Blue	0.04g	0.2%	0.1%
Water	5mL		
Final Volume	20mL		

For non-reducing PAGE loading buffer should be without β Mercapto ethanol.

H. Commassie Stain Solution (1litre)

Commassie brilliant blue	0.25%	2.5g

G250		
Acetic Acid	10%	100mL
Methanol	45%	450mL
H_2O		450mL

Dye should be completely dissolved in acetic acid and methanol (stirring is better) then only it should make up to the volume with deionised water. This can be reused for at least 5 times.

I. De-stainer (5 litres)

Methanol	20%	1 litre
Acetic Acid	10%	500 mL.

Make up the final volume to 5 liters with deionised water.

Clinical history and outcome of patients

Case	Age (Yr)	Sex	Eye	Cause	Duration (M)	Previous Ocu. Sx	VA Pre-COMET	Lid Abnormalities	Symblepharon	PED	VA-3M	VA 6M	OS stability 6Mth	Outcome ≤ 12M of after pk/kpro	Subsequent surgeries (COMET-Sx time gap in M)	FU (COMET to last visit)	Outcome	VA @Final FW
1	18	F	OD	Lime	34	None	HM	None	None	None	CF 1M	CF 1M	Stable	Stable	PK (12)	27	Stable	20/400
2	18	F	OS	Lime	37	Allo-LT, PK	PL	None	None	None	CF 1M	CF 1M	Stable	Stable	Kpro (27)	32	Stable	20/25
3	26	M	OS	Acid	41	None	PL	None	None	None	HM	HM	Stable	Stable	Kpro (42)	46	Stable	20/30p
4	48	M	OD	Liq Ammonia	34	AMG	HM	None	None	Yes	HM	HM	Fail	Fail	None	20	Fail	PLPR
5	34	M	OS	Acid	89	AMG, Allo-LT (2), PK	PL	None	None	None	20/125	CF 1M	Stable	Stable	None	38	Stable	CF 1M
6	22	M	OD	Titanium Oxide	7	None	PL	None	None	Yes	HM	HM	Stable	Stable	None	12	Fail	HM
7	30	F	OD	Acid	108	PK-2	PL	Lag	Yes	Yes	PL	PL	Fail	Fail	Tarso (10)	10	Fail	PL
8	17	F	OS	Lime	12	Allo-LT	PL	None	Yes	None	PL	PL	Fail	Fail		48	Fail	HM
9	23	M	OD	Ammonium nitrate	10	AMG	HM	None	None	Yes	PL	HM	Stable	Stable	TA+BCL (17)	41	Fail	CF 1 M
10	24	M	OD	Acid	6	AMG	HM	Lag	Yes	None	HM	HM	Stable	Stable	None	15	Stable	CF 1M
11	30	M	OD	Na2SO4	53	AMG (2)	HM	None	None	None	HM	HM	Fail	Fail	None	12	Fail	HM

Case	Age (Yr)	Sex	Eye	Cause	Duration (M)	Previous Ocu. Sx	VA Pre-COMET	Lid Abnormalities	Symblepharon	PED	VA-3M	VA 6M	OS stability 6Mth	Outcome ≤ 12Mth pkp/kpro	Subsequent surgerys (COMET-Sx time gap in M)	FU (COMET to last visit)	Outcome	VA @Final FW
12	16	M	OS	Lime	120	No	PL	None	None	None	HM	CF 1M	Stable	Stable	Kpro (12)	38		20/20
13	18	M	OD	Cracker Injury	60	AMG (1),Tarsoraphy (6), Allo-LT, PK (1), SR (2)	PL	None	None	None	PL	HM	Stable	Stable	Kpro (12)	36	Stable	20/30
14	49	F	OD	Formic acid	13	AMG	PL	None	Yes	None	PL	PL	Fail	Fail	None	9	Fail	PL
15	18	M	OS	Lime	105	ALLO-LT(2), PK, SR	PL	None	Yes	None	PL	PL	Fail	Fail	None	13	Fail	HM
16	35	M	OD	Unknown	240	None	HM	None	None	None	HM	CF 1M	Cataract	Fail	None	12	Fail	HM
17	3	F	OS	Unknown	18	None	HM	None	Yes	None	PL	PL	Fail	Fail	None	10	Fail	HM
18	8	M	OS	Cracker Injury	12	SR	PL	None	Yes	None	HM	HM	Fail	Fail	None	7	Fail	HM
19	8	M	OD	Lime	6	SR, Tarsorrhaphy, AMG	HM	None	Yes	None	HM	HM	Fail	Fail	None	8	Fail	HM

LIST OF PUBLICATIONS

1. Madhira SL, Vemuganti G, Bhaduri A, **Gaddipati S**, Sangwan VS, Ghanekar Y. Culture and characterization of oral mucosal epithelial cells on human amniotic membrane for ocular surface reconstruction. *Mol Vis* 2008;14:189-196.

2. Dravida S*, **Gaddipati S***, Griffith M, et al. A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. *J Tissue Eng Regen Med* 2008;2:263-271. * These authors contributed equally.

3. Mariappan, I., Maddileti, S., Savy, S., Tiwari, S., **Gaddipati, S**., Fatima, A., Sangwan, V. S., Balasubramanian, D. and Vemuganti, G. K. In vitro culture and expansion of human limbal epithelial cells, *Nat Protoc* 2011 5/8 (Aug): 1470-9.

4. Sangwan, V. S, Basu, S., Vemuganti, G. K., Sejpal, K., Subramaniam, S. V., Bandyopadhyay, S., Krishnaiah, S., **Gaddipati, S**., Tiwari, S., and Balasubramanian, D. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. *Br J Ophthalmol 95*, 1525-1529.

5. **Gaddipati S**, Muralidhar R, Sangwan VS, Mariappan I, Vemuganti G, Balasubramanian D. Oral Epithelial Cells Transplanted on to Corneal Surface Tend to Adapt to the Ocular Phenotype, *Indian J Ophthalmol* (Accepted on May 2012)

6. Basu, S., Fernandez, M. M., Das, S., **Gaddipati, S**., Vemuganti, G. K., and Sangwan, V. S. Clinical outcomes of xeno-free allogeneic cultivated limbal epithelial transplantation for bilateral limbal stem cell deficiency. Br J Ophthalmol. (Accepted on August 2012)

Book Chapters

1. Mariappan I, **Gaddipati S**, Das T, Vemuganti GK , Sangwan VS. Stem Cells and Ocular Disorders: Basic Science to Clinical Applications, *Bentham OPEN eBooks* (In press)

PRESENTATIONS

Presented a paper (**Oral**) entitled "Culture and Characterization of Oral Mucosal Epithelial Cells on Human Amniotic Membrane" at the ASIA ARVO International Conference, Hyderabad, 2009.

Presented a paper (**Poster**) entitled "A biomimetic scaffold for culturing limbal stem cells: promising alternative for clinical transplantation" at the ASIA ARVO International Conference, Hyderabad, 2009.

Presented a paper (**Poster**) entitled "Fate of Transplanted Oral Mucosal Epithelial Cells on the Ocular Surface of a patient with Bilateral Severe Limbal Stem Cell Deficiency" at the XXXIII All India Cell Biology Conference and International Workshop, Hyderabad, 2009

Presented a paper (**Poster**) entitled "Fate of Transplanted Oral Mucosal Epithelial Cells on the Ocular Surface of a patient with Bilateral Severe Limbal Stem Cell Deficiency" at the GRC CORNEA International Conference, CA, USA, 2010.

Presented a paper (**Poster**) entitled "Fate of Transplanted Oral Mucosal Epithelial Cells on the Ocular Surface of a patient with Bilateral Severe Limbal Stem Cell Deficiency" at the IERG National Conference, Hyderabad, 2011.

LIST OF AWARDS

- Council of Scientific and Industrial Research- Senior Research Fellow-2009
- Best poster presentation in Asia ARVO-2009
- Nature travel fellowship for attending Gordon research conference (Biology and Pathobiology of the Cornea)-2010

BRIEF BIOGRAPHY OF THE CANDIDATE

SUBHASH GADDIPATI

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ACADEMICS:

2006-present **Ph.D** **Birla Institute of Technology and Science,** Rajasthan, India

Thesis: Characterization of cultivated oral mucosal epithelium and transplantation to human ocular surface in severe bilateral limbal stem cell deficiency.

2004-2006 **M.Tech** **University of Hyderabad,** Hyderabad, India

Medical Biotechnology (CGPA-8.93)

2005-2006 **PGDCAQM** **University of Hyderabad,** Hyderabad, India

Chemical analysis and quality management (64.8%)

2002-2004 **M.Sc** **Andhra University,** Vizag, India

Biochemistry (65.7%)

1999-2002 **B.Sc** **Nagarjuna University,** Guntur, India

Microbiology, Biochemistry, Chemistry (79.0%)

PUBLICATIONS

1. Madhira SL, Vemuganti G, Bhaduri A, **Gaddipati S**, Sangwan VS, Ghanekar Y. Culture and characterization of oral mucosal epithelial cells on human amniotic membrane for ocular surface reconstruction. *Molecular Vision* 2008;14:189-196.

2. Dravida S, **Gaddipati S**, Griffith M, et al. A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. *J Tissue Eng Regen Med* 2008;2:263-271.

3. Mariappan I, Maddileti S, Savy S, Tiwari S, **Gaddipati S**, Fatima A, Sangwan VS, Balasubramanian D and Vemuganti G K. In vitro culture and expansion of human limbal epithelial cells, *Nature Protocols* 5/8 (Aug): 1470-9.

4. Sangwan VS , Basu S, Vemuganti G, Sejpal K, Subramaniam SV, Bandyopadhyay S, Krishnaiah, **Gaddipati S**, Tiwari S, Balasubramanian D. Clinical Outcomes of Xeno-Free Autologous Cultivated Limbal Epithelial
Transplantation: A Ten Year Study, *Br J Ophthalmol* 2011 (Accepted on 09 Jul 2011).

5. **Gaddipati S**, Muralidhar R, Sangwan VS, Mariappan I, Vemuganti G, Balasubramanian D. Oral Epithelial Cells Transplanted on to Corneal Surface Tend to Adapt to the Ocular Phenotype, *Indian J Ophthalmol* (Communicated)

Book Chapters

1. Mariappan I, **Gaddipati S**, Das T, Vemuganti GK , Sangwan VS. Stem Cells and Ocular Disorders: Basic Science to Clinical Applications, *Bentham OPEN eBooks* (In press)

PROJECTS

- Autologous limbal epithelial cell cultivation for reconstruction of damaged ocular surface with unilateral limbal stem cell deficiency
- Characterization of limbal epithelial cells cultured on human amniotic membrane in comparison with other cultivation techniques
- Collagen scaffolds for cultivation of human limbal epithelial cells
- Autologous cultivated human oral mucosal epithelial cells for reconstruction of ocular surface damage with bilateral limbal stem cell deficiency

PRESENTATIONS

- Presented a poster in **Gorden research conference**-Biology and pathobiology of cornea March, 2010, CA, USA.
- Presented an oral talk in the **Asia ARVO** meeting, January 2009, Hyderabad, India.
- Presented a poster in the **Asia ARVO** meeting, January 2009, Hyderabad, India.
- Appeared for an oral presentation in the **Indian Eye Research Group** Annual Conference, July 2008. Aravind Eye Care Systems, Madurai, India.

AWARDS and ACHIEVMENTS

- Awarded **Nature travel fellowship** for attending GRC-Cornea coference-2010
- Awarded **best poster** in Asia ARVO-2009 conference.
- Awarded senior research fellowship from **Council of Scientific and Industrial Research,** Gov of India-2008.
- Qualified **GATE-2004** with 96.4 percentile score
- Achieved national level **1st rank** in the M.Tech entrance, University of Hyderabad, Hyderabad, India-2006.

PRACTICAL SKILLS:

Cell Biology:

Primery limbal, corneal, oral mucosal epithelial cells, stromal cells cultivation on human amniotic membrane and collagen scaffolds for corneal bioengineering. Transfection and protein localization studies by confocal microscopy (Ziess LSM-510), Pulse chase and labeling experiments.

Molecular Biology Techniques:

DNA and RNA extraction from WBC, animal tissues and bacteria, plasmid isolation, agarose gel electrophoresis, primers design, PCR, gene cloning, shot gun cloning, yeast transformation.

Immunology

ELISA, FACS, western blotting, RIA, Immunodiffusion, Immunohistochemistry, Immunofluorescence, Hemagglutination, affinity purification of antibody, coupling of antibody to enzymes, FITC and latex beads, rising antibody in rabbit and mice.

Computer skills

Corel draw, Photoshop CS, MS Office, EndNote, GraphPad Prism, ImageJ.

REFERENCES:

1. Prof. GEETA K.VEMUGANTI,
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BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Geeta Kashyap Vemuganti graduated from Ajmer medical college in Rajasthan. She acquired MD and DNB degrees in pathology from Nizam's Institute of Medical Sciences, Hyderabad and joined LV Prasad Eye Institute as the Head of Ophthalmic Pathology Service, and is currently designated as Head of Ophthalmic Pathology Laboratory and Sudhakar and Sreekanth Ravi Stem Cell Biology Laboratory.

She underwent training in ocular pathology at various schools in the USA. She is a visiting pathologist at Eye and Ear Infirmary, and Department of Pathology, University of Illinois, Chicago.

Her areas of research interest include oculo-adenexal tumors; especially retinoblastoma, ocular surface squamous cell carcinoma. She is also interested in corneal pathology, specially related to corneal infections & dystrophies. Genotype-phenotype co-relation in hereditary diseases of the eye is another area of her special interest.

Her work in the area of stem cells has brought the breakthrough in 'Stem Cell Therapy' in India. The team led by her developed a technique of culturing limbal stem cells, which has helped in treating more than 450 patients with severe ocular surface disease. She has also developed a novel technique of cultivating a "composite culture" of central limbal and peripheral conjunctival epithelium.

She has published over 100 papers in peer reviewed National and International journals of repute. She is on the editorial board and a reviewer for several national and international scientific journals. She is the recipient of a number of national and international awards; including Col Rangachari Gold Medal of the All India

Ophthalmological Society, CHEMTECH PHARMABIO "Outstanding Contribution" Award (Biotech), 2005, "National Bioscience Award for Career Development", 2003-04.