A Study on Corneal Limbal Stem Cells

THESIS

Submitted in partial fulfilment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

B.SUDHA
2003PHXF429

Under the Supervision of
Dr.S. Krishnakumar

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

2007

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN)

CERTIFICATE

This is to certify that the thesis entitled **"A Study on Corneal Limbal Stem Cells"** and submitted by **Ms.Sudha. B** ID No **2003PHXF429** for award of Ph. D. Degree of the Institute embodies original work done by him/her under my supervision.

Signature in full of the Supervisor:

Name in capital block letters: **Dr.S. Krishnakumar**

Designation: **Reader and Pathologist,**
DBT Research Associate,
Vision Research Foundation,
Sankara Nethralaya,
Chennai 600 006.

Date:

ACKNOWLEDGEMENTS

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of three years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

First I would like to give my heartfelt gratitude to almighty without whom the thesis would not have been possible at all.

I want to start expressing a sincere acknowledgement to my supervisor, **Dr. S.Krishnakumar, Reader and Pathologist, DBT Associate, Ocular Pathology Department.** He gave me the opportunity to research under his guidance and supervision. I received motivation; encouragement and support form him during my work. With him, I have learnt writing papers, projects and sharing my ideas to the public. He also provided encouragement, sound advice, good teaching, and lots of good ideas.

I would like to extend my heartfelt gratitude to **Prof. H.N. Madhavan, Director of Research and Vision Research Foundation** for providing me this opportunity to work on this topic. I am extremely grateful for his continuous support during my work.

I would like to thank **Dr.S.S Badrinath, Chairman Emeritus, Sankara Nethralaya, Chennai, India** for providing me the opportunity to pursue my doctoral programme.

I thank **Dr. Lingam Gopal,** Chairman, Medical Research Foundation, **Dr. Tarun Sharma,** Honorary Secretary and Treasurer, Vision Research Foundation, **Dr. T.S Surendran,** Vice-Chairman Medical Research Foundation.

My Sincere Thanks to **Indian Council of Medical Research** for funding the project and also providing my fellowship.

I would like to express my sincere thanks to **Dr. Jyotirmay Biswas**, Head Ocular Pathology Department, for his continuous support and encouragement.

My sincere thanks **Dr. G. Sitalakshmi**, Director Cornea services, **Dr. Geetha Krishnan Iyer,** Cornea Consultant, **Dr. S. Vinay** Cornea consultant, for providing me the limbal biopsies during my study and guiding me in the animal experiments. I also wish to thank all the cornea consultants for their support.
I would also like to thank **Mr.P.Krishnamurthy** of Bio-engineering department who helped in organizing things for the animal experiment

I express my sincere thanks to **Prof. S. Ramakrishnan**, Head of Biochemistry Department, **Dr. Lily Therese,** Reader L &T Microbiology Research Centre, **Dr. G. Kumaramanickavel,** Reader and Head ONGC Department of Genetics and Molecular Biology, **Dr. Angayarkanni**, Lecturer in Biochemistry Research Department, **Dr. J. Malathi,** Lecturer L &T Microbiology Research Centre, Vision Research Foundation for evaluating my practice lecture series.

My sincere thanks to **Prof. S Venkateswaran**, Vice Chancellor, BITS, Pilani and **Prof. L. K. Maheshwari**, Director, BITS, Pilani, for the administrative support. I would also like to thank **Prof. Ravi Prakash**, Dean, Research and Consultancy Division, **Dr. B.R. Natrajan**, Dean, Distance Learning Programme Division, **Dr. Sanjay D. Pohekar** and **Dr. Deshmukh**, Convener Ph.D. for their constant guidance and help.
My sincere thanks to **Dr. Suman Kapur** and **Dr. S.K. Verma DAC members** for kindly reviewing my thesis.

My Sincere thanks to **Dr. Samuel Abraham** Director, Nichi in Biosciences for providing me mebiol gel and **Dr. T. P. Sastry**, Bioproducts Lab, CLRI for providing me Chitosan matrix for growing my cells.

I would like to thank **Ms.Sujatha Arumugam**, Scientist, and CLRI for her constant encouragement and providing me the chitosan scaffolds.

I would also like to extend my thanks to staffs of **Nichin centre of Regenerative Medicine** and **SGS laboratories**, TICEL Biopark for helping me in my animal experiments.

Thanks to **Ms. Srilatha Jasty** dissertation student for her helping me finishing some of my objectives. I would also like to extend my thanks to **Ms. Nalini Venkatesan** for helping in doing the Western Blot.

I would like to thank **Dr. Soumitra**, **Ms. Praveena,** ONGC Genetics and Molecular Biology Department for helping me in doing sequencing.

I would like to thank **Dr. Gunisha Pasricha,** for going through my thesis and giving me valuable criticisms.

Thanks to my friends **Ms.P.Sowmya** and **Ms.A.V.Saijyothi** for their constant support and encouragement throughout my work. I would also like to thank my classmates **Ms.Shyamala Ganesan, Ms.Gayathree Raman, Ms.Sangeetha, Parvathi Verma and JasmineTherese** for their encouragement. I would also like to thank my friend **Mr.Satheesh Kumar** for his help during my final thesis preparation.

Thanks to **Ms. Sowmyalakshmi Kumar** for helping me in formatting my thesis and **Ms. Mouthshy Mitra** who gave me good company during my thesis writing.

I thank my department colleagues **Ms. Vanitha, Ms. Vaijeyanthi, Ms. Adithi Mohan, Ms. Jayanthi, Mr. Mallikarjuna, Ms Seethalakshmi, Ms. Vandhana, Mr. Sudheer** for their constant support.

I would also like to thank **Ms. Bhagyalakshmi, Ms. P.Deepa, Ms. B. Mahalakshmi, Ms. Dhivya, Ms. Leela, Ms. Gayathri, Mr. Samson** of microbiology department **Ms. Coral, Ms. Selvi, Ms. Bharathi, Ms. Bharathselvi** of biochemistry department. **Dr.**

Sripriya, Ms. Uthra, Ms. Mamtha of Genetics department for their support and cooperation.

I would like to thank Librarian **Mr.Shankar** for getting me some articles, and other technical and non technical staff of Sankara Nethralaya.

Last but not least I would like to thank my close friends and relatives who were there with me during each stage of my work.

Finally I would like to dedicate this thesis to my **Appa, Amma** and my sister **Aishwaria** for their continuous support, motivation, love and affection.

TABLE OF CONTENTS

ABBREVIATIONS

ABCG2	**ATP Binding Cassette transporters**
AKT	**Serine-threonine specific protein kinase**
AM	**Amniotic Membrane**
AMT	**Amniotic Membrane Transplantation**
BCRP	**Breast cancer resistant protein**
bFGF	**Basic Fibroblast growth factor**
Brdu	**Bromo deoxy Uridine**
CA	**Carbonic Anhydrase**
CESCs	**Corneal epithelial stem cells**
CS	**Chitosan**
Cx	**Connexin**
DAB	**Diamino Benzidine**
DMEM	**Dulbeccos Minimum essential medium**
DNA	**Deoxyribo nucleic acid**
ECM	**Extracellular Matrix**
EDTA	**Ethylene diamine tetraacetic acid**
EGF	**Epidermal Growth factor**
ERK	**Extra cellular signal related kinase**
ES cell	**Embryonic stem cell**
GAGs	**Glycosaminoglycans**
GAPDH	**Glyceraldehyde 3 phosphate Dehydrogenase**
GSK	**Glycogen synthease 3 Kinase**
GFs	**Growth Factors**
HA	**Hyaluronic Acid**
HCl	**Hydrochloric acid**
HGF	**Hepatocyte growth factor**

HLA	**Human Leucocyte Antigen**
IL	**Interleukin**
K3/K12	**Keratin 3/12**
kDa	**Kilo Dalton**
KGF	**Keratinocyte growth factor**
LC	**Langerhan's cells**
LCK	**Lymphocyte specific protein tyrosine Kinase**
LSC	**Limbal stem cell**
LSCD	**Limbal stem cell deficiency**
MAPK	**Mitogen activated protein kinase**
Mg	**Milligram**
µg	**Microgram**
Ml	**Milliliter**
µl	**microliter**
MQ water	**Milli Q water**
MW	**Molecular Weight**
NGF	**Nerve growth factor**
NIPPAm-co-BMA	**Poly N- isopropylacrylamide-co-n-butyl methacrylate**
PCR	**Polymerase chain reaction**
PDK	**Pyruvate dehydrogenase Kinase**
PI3K	**Phosphatidyl inositol 3 Kinase**
PMC	**Post mitotic cell**
PAGE	**Polyacrylamide gel electrophoresis**
RNA	**Ribonucleic acid**
RT-PCR	**Reverse Transcriptase PCR**
SDS	**Sodium dodecyl sulphate**
SCs	**Stem Cells**
SP	**Side Population**
TACs	**Transient Amplifying cells**

TBE	**Tris Borate and EDTA**
TDC	**Terminally differentiated cell**
TGF	**Transforming growth factor**
TGP	**Thermo Reversible Gelation polymer**
TNF	**Tumor Necrosis factor**
TrkA	**Tyrosine Kinase Receptor A**
UV	**Ultra Violet**

CHAPTER 1 - INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION:

The ocular surface is a complex continuum responsible for the maintenance of corneal clarity, elaboration of a stable tear film for clear vision as well as protection. The integrity of this self-renewing epithelium relies on the existence of limbal stem cells (LSCs). Severe ocular surface injuries such as alkali burn, thermal injury, Stevens-Johnson syndrome, contact lens related epitheliopathy and ocular cicatricial pemphigoid causes limbal stem cell deficiency (LSCD). Severe LSCD can finally result in reduced visual acuity, even blindness. Limbal stem cells deficiency can be caused by a variety of hereditary or acquired disorders. (Dua *et al*, 2000; Thoft *et al*, 1989; Tseng *et al*, 1989). Various surgical techniques have been developed to treat LSCD. Treatment is mainly through the transplantation of ex-vivo expanded corneal limbal stem cells on human amniotic membrane matrix. The diseases and conditions as mentioned above affect the vision of approximately 3000 patients every year in India**.** Usually during such damages, the corneal limbal stem cells present in the limbal area spontaneously multiply and replace the corneal epithelium. However, when the damage due to disease is very severe or when the stem cell themselves are damaged, such repair is not feasible. At present the treatment options available for limbal stem cell damage consists of either full thickness corneal transplant (only one among every 10 person waiting for a transplant gets the corneal transplant done, due to shortage of available cadaver corneas) or limbal autograft from the other normal eye, when the damage is minor or the recent technique of corneal

epithelial transplant, where the limbal explants are which are grown on homologous biological materials like the human amniotic membrane.

The aim of the thesis is to standardize the culture protocols for the cultivation of corneal limbal stem cells and also to understand the biology of the cultured corneal limbal stem cells.

1.1 THE STRUCTURE AND FUNCTION OF OCULAR SURFACE

The cornea forms the front ocular surface of the eye and acts as a window to the world. A clear healthy cornea is very essential for the visual acuity. The development of the cornea is a terminal inductive event in eye formation, with the corneal epithelium derived from the head surface ectoderm overlying the lens after invagination. The complex multi-layered structure of the cornea allows it to fulfill its role, namely, transparency, refraction, photoprotection and protection of internal ocular structures from the external environment.

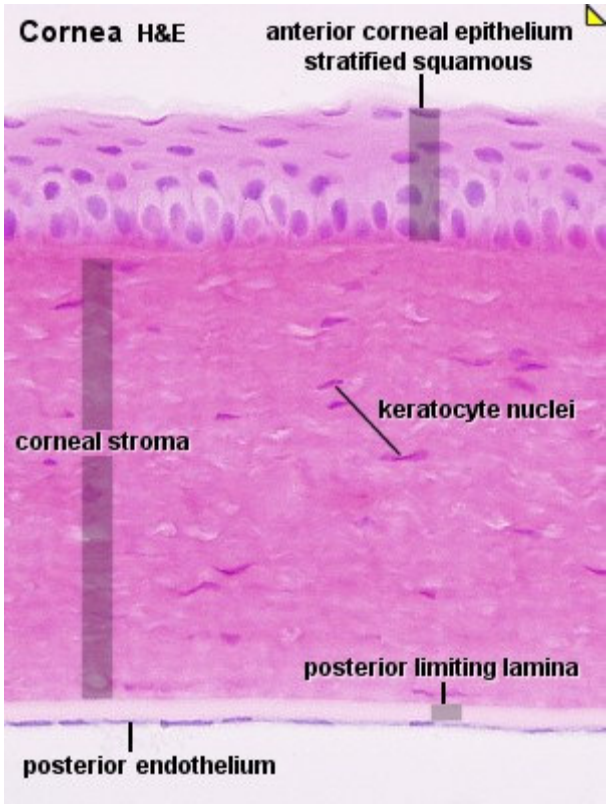
Corneal organogenesis has been extensively reviewed in chicks by Hay (Hay, 1979) and in humans by Sevel and Isaacs (Sevel and Isaacs, 1988), and Barishak (Barishak, 2001).In vertebrates the development of cornea begins as the layer of ectodermal cells covering the lens. This primitive epithelium, two cells in thickness, is first apparent at about five weeks in the human embryo and is contiguous with the surface ectoderm. During the next one to- two weeks in humans, the epithelium stratifies to three-to-four cell-layers thick, the lens completes its formation and detaches from the ectoderm, and

the eyelids form and fuse. Almost immediately after the separation of the lens from the corneal epithelium, waves of neural crest cells migrate into the space between the lens and epithelium. These cells become the corneal endothelium and the stromal keratocytes. This migration of cells appears to be species specific. For example, in reptiles, birds, and primates, including humans, two waves are observed - first, endothelial cells then keratocytes. However, in rodents, cats, rabbits, and cattle, a single migration of cells, resulting in both cell types, is observed (Cintron *et al*., 1983 ;). In the chick, the epithelial cells secrete a collagenous matrix (termed the primary stroma) prior to the influx of neural crest cells (Hay, 1979). This primary stroma is not observed in rabbits, mice, or primates (Cintron *et al*., 1983). Following fusion of the eyelids, the primitive corneal epithelium decreases to two cell layers and remains constant until the time of eyelid opening (twenty-four weeks in humans). During the period of eyelid closure, the cornea gradually enlarges and matures. Upon eyelid opening, a number of developmental events occur that result in a mature cornea.

1.1.1 Cornea

The mature cornea is composed of 5 layers viz., Epithelium, Basement membrane, Bowman's layer, Stroma, Descemet's membrane and Endothelium. (**Figure 1.1**) The role of corneal epithelium which forms the 10% corneal thickness is to; absorb nutrients and oxygen while protecting the eye.

Figure 1.1 Layers of Cornea



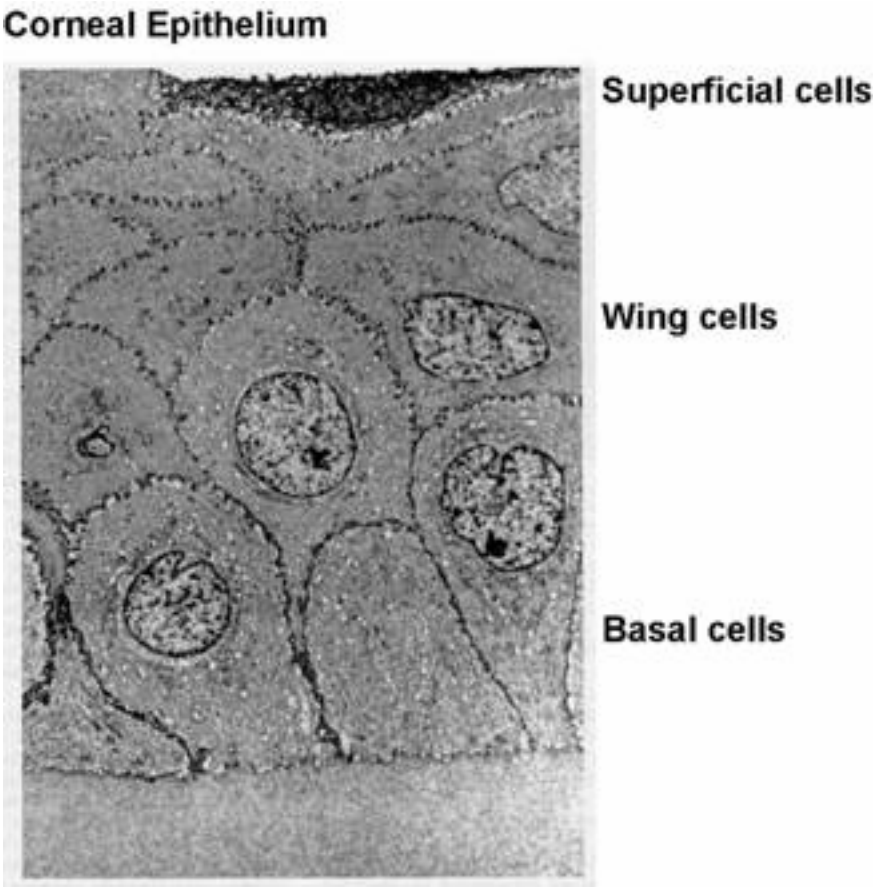
Adapted from www.googleimages.com

This figure shows the Hematoxylin and eosin stained section of the entire cornea.

The corneal epithelium consists of approximately five to seven cell layers. The superficial layer of this epithelium includes a layer or two of squames cells with extensive apical microvilli. Underneath the superficial layer are the suprabasal or wing cells and the final layer is the basal cells. Beneath the corneal epithelium lies the basement membrane and Bowman's layer followed by the stroma. Descemet's membrane is directly below the stroma, which in turn overlies the corneal *endothelium.* The superficial squames of the corneal epithelium have a unique surface with extensive microvilli, called microplicae that serve to increase the cells surface area and to facilitate the close association of the squames with the tear film. The presence of lateral tight junctions between squames prevents the entry of harmful substances into the intraocular tissues. The wing cells are located beneath the apical squames and have lateral, wing-like extensions. These cells are not directly involved in the spreading of the tears and do not undergo frequent cell division; however, recent studies have shown that wing cells do participate in re-epithelialization during wound healing and sometimes can be found at the leading edge in contact with the basement membrane (Danjo and Gipson, 2002). **(Figure 1.2)**

The innermost cells in the corneal epithelium are called basal cells. These cells are a single layer of columnar cells with several important functions in the cornea (Farjo and Soong, 2004). They proliferate to generate new wing and squames, secrete numerous matrix molecules that are incorporated into the underlying basement membrane and stroma, organize the hemidesmosomes that maintain stable attachment to the underlying

Figure 1.2 Corneal epithelium layers



Adapted from www.stemcell.org

Electron microscopic picture showing the layers of corneal epithelium

basement membrane and, finally, organize the more transient cell–matrix attachments called focal complexes that are important in mediating cell migration in response to an injury.

While the basal cells generate all of the cells that make up the other layers of the corneal epithelium, they themselves are derived from cells that are located at the limbus, a region between the cornea and conjunctiva.

Bowman's layer, which has unknown functions, is present from limbus to limbus, and consists of both the underlying basement membrane zone and the acellular zone immediately beneath it. Beneath the epithelial basement membrane is the stroma. The cells that reside in the stroma are called corneal fibroblasts and are specialized in producing and organizing the stromal extracellular matrix (ECM). In humans, the stroma makes up over 90% of the bulk of the entire cornea. This dense connective tissue serves primarily as a structural support, but, unlike cartilage and tendon, it is transparent. By mechanisms still not fully understood, stromal fibroblasts regulate the synthesis, organization and spacing of the various types of collagen fibers and proteoglycans that make up the stromal matrix and function to maintain stromal transparency.

In addition, data also show that the stromal fibroblasts produce corneal crystallins that, similarly to the crystallins in the lens, participate in maintaining the clarity of the fibroblast cytoplasm (Jester *et al*., 1999; Piatigorsky, 2000). Without a transparent cornea, light would not come into focus on the retina and vision would be impaired.

Beneath the stroma is another basement membrane known as Descemet's membrane, which overlies the corneal endothelium, a single layer of flattened cells connected by tight junctions. The endothelial cells pump nutrients from the aqueous humour into the stroma and pump excess water out of the cornea. The aqueous humour is made primarily by the cells that make up the ciliary processes and is secreted into the anterior and posterior chambers of the eye.

1.1.2 Conjunctiva:

Surrounding the cornea is the sclera, which is covered by the layer of glandular epithelial tissue called conjunctiva. The conjunctiva is highly vascularized and contains goblet cells that secrete a mucin-rich fluid. This fluid is particularly important in keeping the cornea moist and reflective, as well as giving the avascular tissue some nutrition. It is essential critical to maintaining the integrity of the eye, and allows unrestricted movement of the eye and is reservoir for tears. It also contributes goblet cell-secreted mucin to the tear film and provides a source of immune tissue and antimicrobial agents to protect the ocular surface. Abnormalities of the conjunctiva may lead to restriction of ocular movement, abnormalities of the tear film, and decreased host resistance of infectious agents. In addition, the cornea may ultimately be affected in severe conjunctival disease. This conjunctiva extends from the corneoscleral limbus to the mucocutaneous junction on the eyelids. The conjunctival sac forms a fornix on three sides and an extendible plica medially. Allowing for independent movement of the eye and eyelids. Conjunctival

surface folds increase the surface area of the conjunctiva, decrease the area of contact, and reduce friction between the bulbar and tarsal conjunctiva.

1.1.3 Limbus

The term limbus denotes a border between two different types of tissues. Most commonly it refers to the border zone between the cornea and sclera. Despite its small size the limbus excites everyone interest because it demarcates the optically clear cornea from the conjunctiva and opaque sclera. It maintains the nourishment of the peripheral cornea; it contains the pathways of aqueous humor outflow; and it is the site of surgical incisions into the anterior chamber for cataract and glaucoma**.** It is a specialized region, which is highly vascularized, innervated, and protected from potential damage by UV-light by the presence of melanin pigmentation.

Gross anatomy reveals that, the radius of curvature abruptly changes at the junction of corneal and sclera creating a shallow furrow or sulcus externally, external scleral sulcus. The internal scleral culcus, formed by the scleral spur contains the canal of schlemm and aqueous humour outflow apparatus. Internally, the limbal zone begins at the junction of the peripheral extension of Descemet's membrane and corneal endothelium and most anterior extent of the trabecular meshwork identifiable gonioscopically as Schwalbe's line. (Tero Nishida *et al*, 1997 ;)

The histological limbus relates to the identifiable junction of cornea and sclera observed in histological cross section. Both of them primarily consist of collagen. The corneal collagen fibers typically less than 600Å in diameter and they are arranged in highly

regular lamellar pattern that lends a crystalline quality of the tissue and contributes to its transparency in comparison to scleral fibers which are more thicker in diameter and are not arranged in regular fashion.

The concept of the limbal location of corneal SC was first proposed by Davanger and Evensen (1971) suggesting that the limbal 'palisades of Vogt' (Goldberg and Bron, 1982) contained the proliferative cells that maintain the corneal epithelium. Since then, numerous reports have supported the limbal SC concept **(Figure 1.3)**.

1.1.4 Sclera

It envelops abut 90% of the surface of the eye. It is a dense, white fibrous connective tissue that ensheathes the eye extending from the corneal limbus anteriorly to dural sheath of optic nerve posteriorly. The sclera, like the corneal stroma, is of mesenchymal origin and develops uniformly from the anterior to the posterior region. The exterior side of the anterior region of the sclera is covered by a transparent epithelial tissue the bulbar conjunctiva. The principle function of sclera is to provide a strong eye and to withstand the considerable expansive force generated by the intraocular pressure. The fibrous tissues of the sclera also limit the size and shape of the eye during development. Anatomically, the sclera can be divided into three layers episclera, stroma and lamina fusca. (Foster *et al*, 1994 ;)

Figure 1.3 Palisades of Vogt

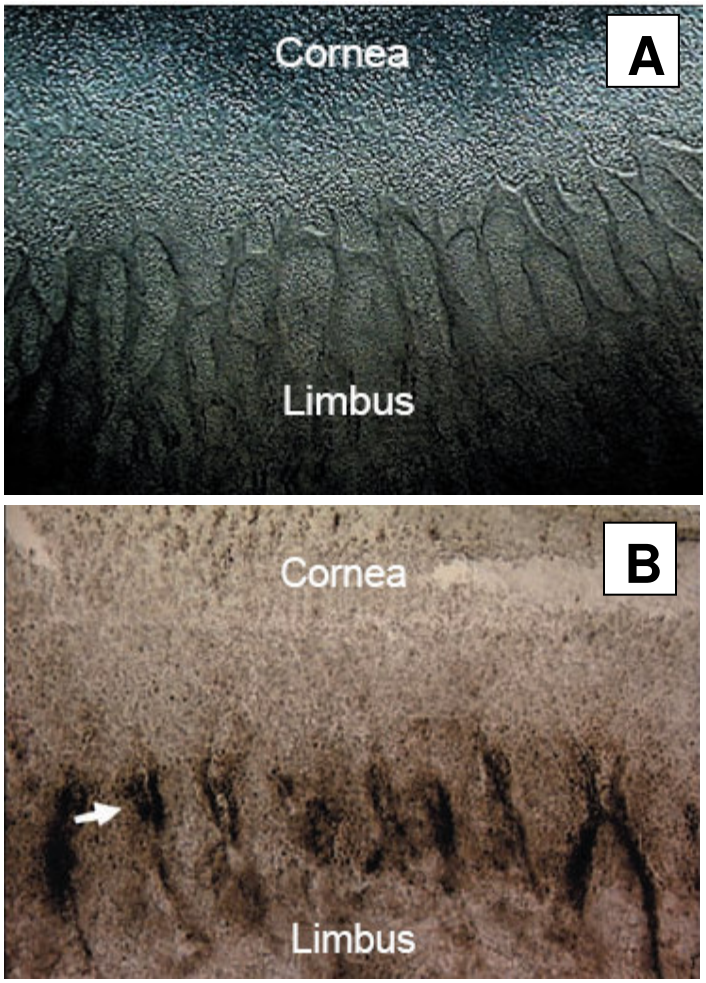


Figure 1.3A shows unique pigmented structure can be identified on the flat mount preparation of Dispase-isolated human limbal epithelial sheets & **Figure 1.3B** shows the presence of the pigmented palisades of Vogt in the darker skinned donor. Adapted from *Cell Research* (2007) **17:**26-36

1.1.5 Tear film

The tear film is constituted by aqueous humor and to a lesser extent by the serum because both contribute fluid to the tear film. The contribution of the aqueous humor to the aqueous component of the tear film was first recognized by the Mishima *et al*, 1968; The hydrostatic pressure exerted by the aqueous and the osmotic gradient that develops across the cornea between the aqueous humor and tears as a result of tear evaporation cause a continuous flow of water from the aqueous humor side of the cornea to the external surface.

Membrane bound subsurface vesicles that contain glycoprotein in the conjunctival epithelium rise to the tear-side surface of the cell and fuse with the cell membrane to distribute glycoprotein over the epithelial cell surface, contributing to the glycocalyx. These glycoproteins are densly packed on the microplicae and microvilli extending from the free surface of the corneal and conjunctival cell; these structures contribute to the maintenance and retention of an evenly distributed mucous layer. (Prydal J *et al*, 1992 ;)

1.2 STEM CELLS:

The term ‘stem cell’ has acquired a rather more restricted meaning in cell biology than in embryology as a result of studies on the growth kinetics of renewing tissues in adult organisms. It is normally used in an embryological context as a synonym for ‘progenitor/founder ‘cell. (Gardner & Beddington, 1988)

In all the renewing tissues the cellular losses are taken care by the discrete subpopulation of cells which are relatively undifferentiated. These stem cells give rise to progeny which progress along a particular lineage such that at the latter stage these progeny can be of limited proliferation prior to differentiation. In early embryonic tissues there is no obvious partitioning of cells into dividing versus differentiating sub-populations

1.2.1 What are stem cells?

Stem cells are undifferentiated or unspecialized cells which have high proliferative potential and they undergo self renewal and able to undergo terminal differentiation which generate mature cells of different cell lineages. Two properties are generally considered to define a stem cell

1. The ability of the cell to undergo numerous cycle of cell division while maintaining the undifferentiated state, which is also know as the ability of **self renewal.**
2. They also have the capacity to differentiate into any mature cell type. They may be either **totipotent or pluripotent**.

Potency specifies the differentiation potential (the potential to differentiate into different cell types) of the stem cell.

1.2.2 Types of stem cells

- Embryonic stem cells come from embryos (<6 weeks). Stem cells from Blastocysts (2 weeks) and they are virtually "immortal".
- Fetal stem cells come from fetuses (> 6 weeks)
- Stem cells are present in some adult tissues, including brain, spinal cord

and bone marrow, eye, etc.

1.2.3 Embryonic stem cell

Embryonic stem cell is defined by its origin. It is derived from the blasotcyst stage of the embryo. The blasotcyst is the stage of embryonic development prior to implantation in the uterine wall. At this stage, the preimplantation embryo of the mouse is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophoectoderm), a fluid-filled cavity (the balstocoel), and a cluster of cells on the interior (the inner cell mass).

Defining properties of embryonic stem cell

- Derived from the inner cell mass/ epiblast of the blastocysts
- Capable of undergoing an unlimited number of symmetrical divisons without differentiating (long term renewal)
- Exhibit and maintain a stable, full (diploid) normal complement of chromosome (karyotype)
- Capable of integrating into all fetal tissues during development
- Capable of colonizing the germ line and giving rise to egg or sperm cells
- Clonogenic that is a single ES cell can give rise to a colony of genetically identical cells or clones, which have the same properties as the original cell.
- Expresses transcription factor Oct-4 which then activates or inhibits the host target genes.
- Can be induced to continue proliferation or differentiation
- Lacks G1 checkpoint in cell cycle.
- Do not show X chromosome inactivation

1.2.4 Adult stem cells

- Stem cells can be derived from various tissues in adults. To date, stem cells have been found in bone marrow, blood, skin, muscle, liver, brain, the cornea and retina of the eye, the lining of the gastrointestinal tract, liver and pancreas.
- The primary role of tissue stem cells is to maintain and to repair the tissue in which they are found.
- Tissue stem cells usually only produce cells specific to the tissue in which they are found. Normally, adult tissue stem cells only have the potential to make a limited range of cell types in the body.
- The possibility that an adult stem cell from one tissue may give rise to cell types of another tissue. This is a phenomenon called "plasticity

Table 1.1 gives the difference between embryonic and adult stem cells.

Table – 1.1

Difference between embryonic and adult stem cells:

Adult stem cells	**Embryonic stem cells**
Easily accessible	Ethical debate
Less potential for proliferation and differentiation	Robust proliferation and differentiation potential
Autologous transplantation	Graft rejection
Limited quality for clinical need	therapeutic cloning
Questionable plasticity & dedifferentiation issue	Scaleable culture for clinical need Teratoma or tumor issue

1.3 CORNEAL STEM CELLS

1.3.1 General features of corneal stem cells

Unlike the rest of the body surface, the ocular surface is designed to be wettable so as to maintain comfort while providing a smooth optical surface. On the ocular surface, the cornea is most unique in being both avascular and transparent so as to allow the light to be transmitted to the retina. Therefore, in order for us to enjoy seeing the outside beautiful world, the corneal epithelium needs to withstand constant attrition caused by exposure-induced dryness and potential light-induced damage.

Stem cells are a subpopulation of cells capable of extensive self-renewal that upon division gives rise to progeny (transit amplifying or TA cells) that have limited renewal capability (Potten and Loeffler, 1990). Additionally, stem cells divide relatively infrequently in mature tissues and are structurally and biochemically primitive. In cases of tissue injury, stem cells can proliferate to repopulate the tissue. The TA cell divides more frequently than the stem cell and ultimately all of the TA cells differentiate in the scheme of ‘‘stem cell $\rightarrow$TA cell $\rightarrow$ terminally differentiated cell’’ (Lavker and Sun, 2000; Potten and Booth, 2002).

The unique properties of stem cells allow their identification in various tissues (Miller *et al*, 1993). In many cases the identification of stem cells provides new insights into the growth and differentiation properties of the tissue in question. In the case of corneal epithelium, this tissue has long been known to have several unusual and puzzling features. For example, almost all corneal epithelial neoplasias are associated with the

peripheral cornea in a rim called the limbus, which represents the transitional zone between the transparent cornea and the white conjunctiva (Waring *et al*, 1984). Another well known and peculiar feature of corneal epithelium is that the peripheral corneal epithelial cells seem to be able to migrate centripetally toward the center of the cornea (Davanger and Evensen, 1971; Buck, 1979). In addition, the basal cells of central cornea are more mature looking than the basal cells of all other stratified squamous epithelia (Kuwabara *et al*, 1976; Buck, 1979; Srinivasan and Eakins, 1979).

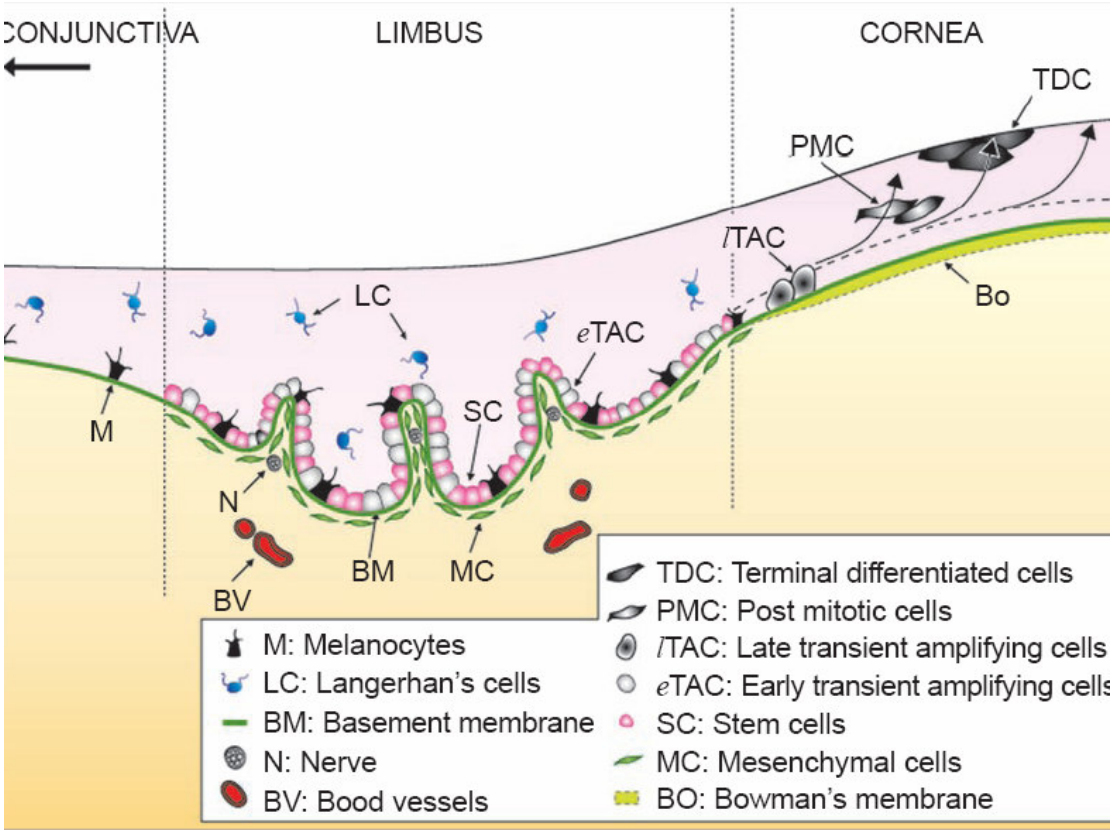
1.4.2 Limbal stem cells niche

Stem cells in all renewable tissues are usually located in a specialized niche that offers protection and nourishment to these essential cells. The corneal epithelial stem cell niche is not an exception. Evidence suggests that the limbus is a potential stem cell niche for the corneal epithelium has been presented in many studies. The cells from the limbal niche have been shown to be slow-cycling (Cotsarelis *et al*., 1989), relatively undifferentiated cells (Chaloin-Dufau *et al*., 1990; Schermer *et al*., 1986), and when isolated have a higher proliferative potential *in vitro* than central or peripheral corneal epithelial cells (Ebato *et al*., 1988; Lindberg *et al*., 1993; Pellegrini *et al*., 1999). Additional support for the limbal stem cell location is the ability of limbal transplants to generate a stratified corneal epithelium (Dua & Azuaro-Blanco, 2000a, b; Koziumi *et al*., 2001a, b) and the inability to establish a permanent corneal epithelium in the absence of limbal epithelium (Chen & Tseng, 1990, 1991; Huang & Tseng, 1991; Kruse, Chen, Tsai, & Tseng, 1990) resulting in abnormal re-epithelialization by the adjacent conjunctiva.

The limbus, physically, offers the stem cells a protective environment as the niche is set in the deep undulations of the Palisades of Vogt, thereby hidden from the hostile external environment. It has both anatomic and functional dimensions. Before functional dimension can be addressed, it is necessary to understand where the limbal niche is. Anatomically, the limbal SC niche is located at the Palisades of Vogt. (**Figure 1.3A & B**) which is highly pigmented because of melanocytes, (Davanger and Evensen *et al* 1971; Coroneo *et al* 1991; Higa *et al* 2005) and is infiltrated with antigen-presenting Langerhan's cells (Baum *et al*., 1970 ;) and suppressor T-lymphocytes (Vantrappen, 1985). The non-uniform junction between limbal epithelium and stroma also protects the cells from shear forces. Melanin accounts for the pigmentation in these cells, offering protection against possible carcinogenic insults of ultraviolet light and the generation of reactive oxygen species. The anatomical closeness to the limbal blood vessels in the underlying stroma ensures that nourishment is available. Stem cell fate, whether it is quiescence, apoptosis, division or differentiation is under the control of interactions between the stem cell and its microenvironment or niche (Morrison *et al*., 1997; Watt and Hogan, 2000).

Unlike that of the cornea, the basement membrane of the limbus is undulating with papillae or 'pegs' of stroma extending upward (Gipson *et al*, 1989;) and fenestrated (Dua *et al* 2005; Shanmuganathan *et al* 2006) these anatomic features in the limbus suggest that limbal epithelial SCs might closely interact with cells in the underlying limbal stroma (**Figure 1.4).** Thus, the underlying stroma and cells within, as well as the local blood supply, are all likely to contain factors that determine stem cell fate. When the stem

Figure 1.4 Hypothetical scheme of limbal stem cell niche



(Adapted from Cell Research (2007) 17: 26-36 Wei *et al*)

cell exits its compartment, the factors that maintain its 'stemness' are no longer available, and the cell is likely to enter the differentiation pathway as a result of new local environmental influences. The precise molecular mechanism by which the stromal niche regulates limbal stem cells is unclear.

The limbal SCs interact with unique extracellular components in the niche. (Turoi *et al*., 1996; Ljubimov *et al*., 1995). These limbal basement membrane components might help determine SC distribution in the niche as suggested in the intestinal crypt villus (Kedinger 1998 ;) Furthermore, like that of other SC niches (Quesenberry *et al* 1998; Peifer *et al* 1999) the limbal basement membrane might help sequester and hence modulate concentrations of growth factors and cytokines that are released from limbal niche cells for efficient and precise targeting onto limbal SCs.

There are evidences that supports that adult germ and somatic stem cells are regulated by their niche, i.e., a special microenvironment consisting of other cellular and extracellular components in the vicinity. (Fuchs *et al* 2004; Moore *et al* 2006; Li *et al* 2005) Therefore, similar to what has been carried out in other types of SCs; one important way of exploring the biological regulation of limbal SCs is to understand how they are regulated by their niche.

Limbal epithelial stem cells (SC) are located at the limbal basal layer. In this epithelial level, there are several other cell types in the vicinity such as the immediate progeny, i.e., early transient amplifying cells (*e*TAC), melanocytes (M), and Langerhan's cells (LC). It

remains to be determined whether these cell types act as niche cells. It is believed that TAC will be destined for progeny production by differentiating into late TACs (*l*TAC) located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiated cells (TDC). The limbal basement membrane (BM) separating the epithelium from the underlying stroma has several unique components. The subjacent limbal stroma contains mesenchymal cells (MC), which may also serve as niche cells. Because the limbal stroma is highly innervated and vascularized, the respective role of nerves (N) and blood vessels (BV) in the niche remains to be defined.

1.3.3 Proliferation and differentiation

The corneal epithelium is a rapidly regenerating stratified squamous epithelium. Homeostasis of corneal epithelial cells is an important prerequisite not only for the integrity of the ocular surface but also for visual function. Under both normal conditions as well as following injury, the maintenance of the corneal epithelial cell mass is achieved by a distinct population of unipotent stem cells (SC) located in the basal epithelium of the corneoscleral limbus (Davanger and Evensen, 1971; Schermer *et al*., 1986; Tseng, 1989). These cells simultaneously retain their capacity for self-renewal and maintain a constant cell number by giving rise to fast-dividing progenitor cells, termed transit amplifying cells (TAC) (Lehrer *et al*., 1998). The highest mitotic rates are seen in the cells in more central cornea away from the limbus, indicating that the majority of proliferation occurs within an intermediate population of transient amplifying cells. (Schermer *et al*, 1986; Beebe and Masters 1996). These TAC make up the majority of the

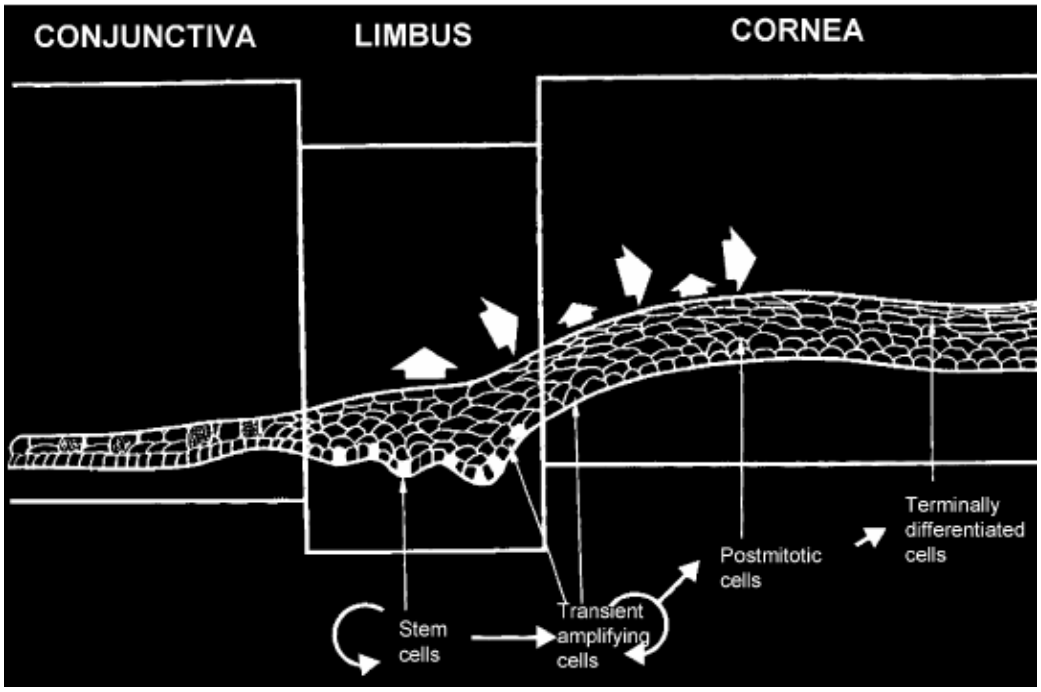
proliferative cell population in the corneal/limbal epithelium. It is believed that corneal TAC can undergo only a limited number of cell divisions before they leave the proliferative compartment to become terminally differentiated **(Figure 1.5)** (Tseng, 1989, 1996; Dua and Azuara-Blanco, 2000; Sangwan *et al*, 2002; Lavker *et al*., 2004; Sun and Lavker, 2004). The stem cells are therefore considered to undergo asymmetric cell division, with one daughter cell remaining within the stem cell microenvironment, and other leaving to become a transient amplifying cell.

Studies of cell movements within the corneal epithelium suggests that the transient amplifying cells moves in the centripetal fashion at the basal level across the cornea and then upward while further differentiating into mature corneal epithelium replacing the older cells lost from the corneal epithelium. (Davanger and Evensen 1971). Thoft and Friend (1983) described a formula to explaining this process in the healthy cornea; X +Y=Z, In this hypothesis X represents the proliferation of the basal cells and Y represents the proliferation and migration of the cells from the limbus and Z represents the loss of the cells from the corneal surface. Therefore the proliferation and migration of cells from the limbus and basal layers normally balance the cell loss on the corneal surface.

1.3.4 Isolation of limbal epithelial stem cells:

Once the limbal niche is identified, the next obvious step is to isolate it from the *in vivo* habitat so that cells in the niche can be further investigated. To this end, intact limbal epithelial sheets can be successfully isolated by digestion with dispase from several species (Espana *et al* 2003a, 2003b; Kawakita *et al* 2005) Single cells rendered by a brief

Figure 1.5: Corneal limbal stem cell Homeostasis



(Adapted from Experimental eye research, 2005; 247 – 264)

treatment with trypsin and EDTA of such isolated limbal epithelial sheets have been used by many investigators as the primary source of limbal epithelial SCs for subsequent manipulations including fluorescent-activated cell sorting (FACS) and culturing. It remains unknown whether different protocols of dispase digestion actually remove the entire limbal epithelial SCs, niche cells or both. This concern is raised because a recent study showed that there is invading 'crypt-like" structures in human limbal palisades of Vogt (Dua *et al* 2005). Furthermore, this concern is justified because as described above limbal basal progenitor cells can invade into the limbal stroma (Kawakita *et al*, 2005) If indeed both limbal SCs and their niche cells were completely removed from the *in vivo* habitat by dispase digestion, it remains unknown whether subsequent trypsin/ EDTA treatment might disrupt their intrinsic intercellular connections, and whether such disruption might then affect the success of subsequent cultivation/expansion. If however only limbal SCs were isolated, there has not been any attempt made to isolate niche cells from the remaining limbal stroma.

Based on the principle that many adult somatic SCs preferentially express Breast cancer resistant protein 1 (Bcrp1) /ABCG2, a member of ATP binding cassette transporters, they can be isolated as the side population (SP) using FACS via the unique property of effluxing Hoechst 33342 dye (Zhou *et al*, 2001; Goodell *et al* 1996; 1997). Using this method, SP cells have been isolated from human (Watanabe *et al*, 2004; Budak *et al*, 2005; de Paiva *et al*, 2005), rat (Umemeto *et al*, 2005;) and rabbit (Budak *et al* 2005, Umemoto *et al* 2006 Park *et al*, 2006;) limbal tissues. The frequency of SP cells from the freshly isolated limbal epithelium varied from 0.2% to 0.64% in humans and from 0.4%

to 1.21% in rabbits, while no SP cells were detected in human and rabbit central corneas. Interestingly, a recent study showed that 4.6% of SP cells are isolated from the rat central corneal epithelium, which is significantly higher than 0.4% from the rat limbus (Umemeto *et al*, 2005). Future studies are needed to determine whether ABCG2 is expressed not only in the limbal tissue, whether other members of ABC transporter family may be expressed by some corneal cells, and whether cells other than SCs can also express ABCG2. Although epidermal SP cells have been proven devoid of melanocytes or dendritic cells (Redvers *et al*, 2006), a preliminary study did show that both human and rabbit limbal SP cells contain non-epithelial cells such as lymphocytes, raising the question whether it is valid to use SP as a source of limbal SCs.

1.3.5 Identification and Characterization of stem cells

Due to the lack of an SC marker, the characterization of limbal SC has been based on the following criteria, which also apply to other types of SC:

i) **Low level of differentiation with a primitive phenotype:** Initial immunohistochemical data suggested that limbal basal cells have the lowest level of differentiation among all corneal epithelial cells. In particular, it was noted that both cytokeratins K3 and K12, specific markers for the corneal epithelial phenotype, were not expressed in limbal basal epithelial cells (Schermer *et al*., 1986; Kurpakus *et al*., 1990). Since then, numerous studies have confirmed the undifferentiated phenotype of limbal basal cells (Kiritoshi *et al*., 1991; Liu *et al*., 1993; Kurpakus *et al*., 1994; Matic *et al*., 1997).

ii) **Slow cell cycle and the ability to retain labelled DNA precursors over a prolonged period:** Experimental evidence has been provided by demonstrating the presence of slow-cycling and label-retaining cells preferentially in the basal layer of the limbal basal epithelium in several animal models (Bickenbach, 1981; Cotsarelis *et al*., 1989; Lavker *et al*., 1991; Tseng and Zhang, 1995; Lehrer *et al*., 1998).

iii) High proliferative potential after activation by wounding or *in vitro* culture conditions: Studying the proliferative potential with and without wounding and in the presence of differentiation-inducing agents has further supported the concept of the limbal location of corneal epithelial progenitor cells (Ebato *et al*., 1988; Cotsarelis *et al*., 1989; Lavker *et al*., 1991, 1998; Lindberg *et al*., 1993; Kruse and Tseng, 1993a, 1994; Kruse, 1994; Tseng and Zhang, 1995; Tseng, 1996; Lehrer *et al*., 1998; Hernandez Galindo *et al*., 2003). Further support of the limbal SC concept has come from clinical observations of abnormal corneal epithelial wound healing after removal of the limbal epithelium (Chen and Tseng, 1990, 1991; Huang and Tseng, 1991) and from the transplantation studies pioneered by Tseng and colleagues, who demonstrated that the limbal epithelial cell population can be used to reconstitute the entire corneal epithelium in cases of severe ocular surface injury (Kenyon and Tseng, 1989; Tseng, 1989; Tsai *et al*., 1990). Finally, the limbus represents the predominant site of corneal tumour formation (Waring *et al*., 1984). These observations are in good agreement with earlier data concerning centripetal migration of epithelial cells originating from the limbus (Davanger and Evensen, 1971; Buck, 1985).

iv) **Capacity for unlimited self-renewal:** Studies investigating the clonogenic potential of human corneal epithelial cells, mostly in the presence of feeder cells, have shown that cells that generate holoclones and survive SC transplantation are present only in the limbus (Lindberg *et al*., 1993; Pellegrini *et al*., 1999).

Other features that distinguish SC from TAC refer to morphologic criteria, e.g. differences in cell size. Confocal microscopy and flow cytometry have been used to demonstrate that the smallest cells are located in the limbal basal epithelium as compared to the basal corneal epithelium (10.1G0.8 vs. 17.1G0.8 mm) (Romano *et al*., 2003, Arpitha *et al* 2005). Another striking morphological feature of limbal basal cells is their pigmentation, which results from intrinsic melanogenesis and protects the cells from solar damage (Davanger and Evensen, 1971; Wolosin *et al*., 2000). Electron microscopy shows that the basal cells of the limbal epithelium express features of immature cells such as small cell size with a cytoplasm rich in tonofilaments, euchromatin- rich nuclei, barely detectable nucleoli, and a high nuclear-cytoplasmic ratio (Chen *et al*., 2004, Arpitha *et al* 2005). Along with morphological criteria, limbal basal cells have been also described to possess basal cytoplasmic processes to connect with the underlying matrix (Gipson, 1989; Chen *et al*., 2004).

Little is known about the spatial arrangement of limbal SC within the basal epithelial cell layer. As in most stratified epithelia, SC is believed to be interspersed throughout the basal cell layer, most likely in small clusters. From the percentage of radiolabelled thymidine retaining cells present in the limbal zone, it has been concluded that SC may

represent less than 10% of the total limbal basal cell population (Lavker *et al*., 1991). Lehrer *et al*. (1998) showed that the limbal basal epithelium contains both slow-cycling SC and early TAC, which may display some characteristics of SC, in close neighborhood. As cells move centripetally across the limbal–corneal demarcation, they may concomitantly develop late TAC features and initiate de novo expression of differentiation markers. Lehrer *et al*. also demonstrated a hierarchy of TAC in the corneal epithelium with cells in the periphery capable of multiple divisions and cells in the central cornea dividing only once.

By transmission electron microscopy, Kruse *et al*, 1994; has observed groups of small, roundish, densely packed cells at the bottom of the epithelial papillae forming the limbal palisades of Vogt. Within these clusters, they have demonstrated two different cell types that can be distinguished: one to few small, primitive appearing putative SC, which are surrounded by larger melanin-containing early progenitor cells (putative TAC). The putative SC rest on a delicate basement membrane without forming cytoplasmic processes and are characterized by a high nuclear-cytoplasmic ratio, heterochromatin-rich nuclei without distinct nucleoli, and a sparse cytoplasm containing minute melanin granules, few mitochondria, ribosomes, and few intermediate filaments; hemidesmosomes and intercellular junctions are largely absent. In contrast, the putative TAC disclose nuclei with increased euchromatin and distinct nucleoli, prominent melanin granules and tonofilament bundles, cell processes interdigitating with the underlying matrix, and numerous (hemi) desmosomes. Groups of similar types of cells, but without pigment granules, can be additionally found in the transition zone between peripheral

cornea and limbus. Two centrioles, associated with the process of mitosis, can often be observed in these cells, which might correspond to late TAC or the TC described by Lauweryns *et al*. (1993a).

Collectively, there is several convincing evidence documenting the existence of adult limbal SC in close spatial relationship to their early progenitor cells within the palisades of Vogt. However, comparing findings from different studies, species-specific differences should be taken into account. For instance, the location of human corneal epithelial SC is different from the area where mouse SC are located (Moore *et al*., 2002; Hsueh *et al*., 2004). In contrast, a shortage of consistent data exists regarding the molecular characteristics and protein expression patterns in this tissue-specific SC population, making identification and purification of these cells variable and difficult.

1.3.6 Markers for identification of corneal stem cells:

Although there are several putative stem cell markers for the identification of corneal limbal stem cells but there are several controversies still exists. But several workers have contributed to the identification of the corneal limbal stem cells. The markers used for the identification are listed in the table 2.

There is no standard criterion for the establishment of a protein or group of proteins as a marker(s) for a specific cell type or disease state. For the Corneal Epithelial Stem Cells, it was proposed that the following as criteria for markers.

(1) While the markers do not have to be present exclusively on cells of the ocular surface, their presence or absence alone or in combination must provide a reliable means for the enrichment and/or isolation of the CESCs.

(2) The markers must also allow for the identification of the CESCs cells within their native tissue in healthy individuals.

(3) The number of CESCs bearing markers must remain relatively constant throughout the life of the organism in healthy individuals. Treatments or conditions that increase cell proliferation in the corneal epithelium should not increase the numbers of CESCs expressing markers in the long term.

(4) The markers should identify fewer CESCs in individuals presenting with LSCD. Once markers are identified, the isolated cells need to show proof of their inherent 'stemness'. This could be done for CESCs as rigorously as it is for other adult stem cell populations, and should include demonstrating longterm repopulation, restoration of a clear cornea, expression of cornea-specific markers such as K3 and K12, and showing evidence for clonogenicity, *in vitro.*

Table 1.2: Semiquantitative immunohistochemical localization of putative stem cell marker in human ocular surface epithelia

Markers	**Corneal epithelium**		**Limbal epithelium**		**Conjunctival epithelium**	
	Basal	**Suprabasal**	**Basal**	**Suprabasal**	**Basal**	**Suprabasal**
Cytoplasmic and nuclear markers						
Keratin 3/12	++	++	-	+	-	-
KeratrinK5/14	- or (+)	-	+	(+)	++	
Keratin 19	-	-	++	-	(+)	++
Vimentin	-	-	++	(+)	-	-
α enolase	(+)	-	++	(+)	++	-
Metallothionine	-	+	(+)	+	-	+
P63	(+)	-	++	(+)	(+)	-
Nestin	++	++	-	-	-	-
Cell surface markers	++	+	-	+	-	-
Connexin 43						
E-cadherin	++	++	(+)	++	(+)or+	++
P-cadherin	(+)	-	-or (+)	-	(+)	-
β catenin	++	++	++	++	++	++
Integrin α2	++	+	-or ++	+	++	++
Integrin α3	++	+	-or ++	+	++	+
Integrin α6	++	+	-or ++	+	++	+
Integrin αv	++	+	++	+	++	+
Integrin β1	++	+	++	+	++	+
Integrin β2	+	+	+	+	+	+
Integrin β4	++	+	-or ++	+	++	+
Integrin β5	+	-	+	(+)	+	-
Integrinα3β1	++	+	- or++	+	++	+
EGFR	++	+	++	+	++	+
KGF-R bek	-	-	(+)	-	-	-
HGF-R met	(+)	-	(+)	-	-	-
NGF-R TrkA	+	(+)	+	-	- or (+)	-
Transferrin R CD71	-or (+)	+	- or (+)	+	- or (+)	-
TGF-β-R1	++	+	++	+	++	(+)
TGF-β-R11	++	+	++	+	++	-
ABCG2	-	-	++	-	-	-

(Adapted from Experimental Eye Research 81 (2005) 247–264)

A number of markers have been proposed as potential LSC markers. Nevertheless, a definitive marker remains elusive. All methods presently used for the identification of LSCs are indirect.

Currently available molecular markers for LSCs can be classified as:

(i) Cytoskeletal proteins

(ii) Cytosolic proteins

(iii) Nuclear proteins

(iv) Cell surface proteins

(v) Cell to cell and cell to matrix interaction molecules

(vi) Growth factor receptors

(vii) Transporter molecules

(viii) Cell surface glycoconjugates

(ix) Neuronal marker

Some of the important markers which are commonly used for the identification of the LSCs are mentioned here.

p63

p63 has recently been proposed as a keratinocyte stem cell marker. p63 is a transcription factor belonging to the p53 gene family. (Lohrum *et al*, 2000) However, unlike p53, it is not a tumour suppressor gene. p63 is normally expressed in the nuclei of keratinocytes with proliferative potential, including skin, cervix, prostate and cornea. (Pellegrini *et al*, 2001; Yang *et al*, 1998) It plays a key role in morphogenesis by regulating epithelial development and differentiation. (Yang *et al*, 1999) p63-knockout mice have a

remarkable absence of stratified squamous epithelia and their derivatives, which may be related to a failure to maintain stem cells. (Yang *et al*, 1999) Pellegrini *et al*, 2001, investigated the expression of p63 in human corneal and epidermal cells using clonal analysis and western blotting. They demonstrated that p63 was expressed strongly in epidermal and limbal holoclones (stem cells) and weakly in meroclones (young TACs), but was undetectable in paraclones (TACs). Immunohistochemistry showed abundant expression in the limbal basal layer, where numerous p63-positive cells were interspersed with fewer p63-negative cells. Corneal epithelium did not show any detectable levels of expression although very low levels of expression were observed in occasional basal cells of the peripheral cornea adjacent to the limbus. Most of the cells expressing p63 in the limbal basal layer also expressed proliferating cell nuclear antigen (PCNA), a specific marker of proliferating cells. (Bravo *et al*, 1987 ;) However, not all the cells expressing PCNA also expressed p63, and these were often found adjacent to p63-positive cells. Furthermore, cells expressing high levels of p63 frequently did not express PCNA. Thus to conclude that p63 is expressed by keratinocytes that possess proliferative potential rather than by keratinocytes that are actively proliferating. Therefore, although much higher levels of p63 expression are found in the stem cell compartment, it is not expressed exclusively by stem cells. It appears likely that young TACs also express p63. Galindo *et al* 2003. investigating the expression of p63 in human limbal epithelial cells expanded on intact amniotic membrane, concluded that p63 expression was localized to cells with high proliferative capacity, including both LSCs and TACs. Dua *et al*, 2003, found that in addition to limbal basal cells, most of the basal cells of central cornea in adult humans also expressed p63. They concluded that p63 was too ubiquitous to be a

stem cell marker. In their work on human corneal epithelial cells expanded *ex vivo*, Kim *et al.* reported that smaller cells that resembled the phenotype of limbal basal cells expressed higher levels of p63 than larger cells that were thought to represent more differentiated phenotypes. However, they could not definitely state that the smaller cells were indeed stem cells. Kim *et al.* also made the observation that p63 was expressed by groups of cells rather than by individual cells, suggesting that p63 was expressed not just by stem cells but also by TACs. Thus p63 is expressed by both the limbal stem cells and early TACs.

Isoforms of p63:

The functional significance of p63 in regulating cell proliferation in various stratified epithelial cells has previously been proposed. More than six isoforms have been reported for this protein; however, it is not yet clearly understood how functionally different these isoforms are. Investigations of these isoforms were done in the ocular surface epithelia. (Kawasaki *et al*, 2005). He has studied the spatial distribution of the p63 isoforms within the human ocular surface epithelia. In his study he has concluded that ΔNp63α most dominant isoform within the human ocular surface epithelia. This isoform may contribute, at least in part, to the maintenance of cell proliferative capacity within the ocular surface epithelia. Di Iorio *et al* (2005) has studied the isoforms of ΔNp63 and studied their role in the migration of the limbal cells in human corneal regeneration.

Integrins

Integrins are a group of transmembrane proteins that play a pivotal role in cell-to-basement membrane adhesion. In mice, integrin α9 has been localized to the basal cells of epidermis, conjunctiva and limbus, but not in central cornea. (Stepp *et al*, 1995) In human corneas, integrin α9 has been localized to a small subset of cells in the limbal basal epithelium. (Chen *et al*, 2004) This expression pattern may provide strong adhesion of limbal basal cells to the underlying basement membrane and help to explain its considerable resistance to shear forces. The specificity of integrin α9 as a marker of LSCs in humans requires further investigation. Integrin β1 has been proposed as keratinocyte stem cell marker.(Jones *et al*, 1995) In cultures of human corneal epithelial cells expanded *ex vivo*, more small cells (presumed to be stem cells) have been found to express integrin β1 as compared with larger cells (representing more differentiated cells). In human cornea, integrin β1 is uniformly expressed by basal cells of both limbus and cornea and lacks any real specificity in distinguishing human limbal from corneal basal cells. (Chen *et al*, 2004)

ATP-binding cassette subfamily G, member 2

ATP-binding cassette subfamily G, member 2 (ABCG2), otherwise known as breast cancer resistance protein 1 (BCRP1), is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters. It has been identified as a molecular determinant for haematopoietic stem cells (HSCs), and has been proposed as a universal marker of stem cells. (Zhou *et al*, 2001) Authors have demonstrated that this protein is immunolocalized to the cell membrane and cytoplasm of some human limbal basal epithelial cells, but not

in most limbal suprabasal cells and corneal epithelial cells.(Chen *et al*, 2004; de Paiva *et al*, 2005; Wolosin *et al*, 2004; Budak *et al*, 2005; Schrehard *et al*, 2005;) ABCG2-positive cells possess LSC-like characteristics, such as higher colony-forming efficiency than ABCG2-negative cells and greater expression in primary cultures of human limbal compared with corneal epithelium. ABCG2 expression is thought to be a common attribute of stem cells to protect them against drugs and toxins. The validity of ABCG2 as a marker of LSCs remains to be clarified.

Nestin

Nestin is an intermediate filament protein and has been proposed as a neural stem cell marker. As limbal epithelium and central nervous system are both derived from neural ectoderm, investigators have explored the possibility that LSCs express this neural progenitor. Seigel *et al.* 2003 observed that p63 and nestin colocalized to a small subset of cells derived from limbal explants cultured *in vitro*. These cells were also shown to have functional neuronal properties.

Nerve growth factor receptor:

Nerve growth factor (NGF) is a neurotrophin that has been shown to play a role in the proliferation and differentiation of human corneal epithelium. (You *et al*, 2000;) The high-affinity receptor for NGF is known as TrkA. Stimulation of TrkA is thought to promote cell survival. TrkA expression has been demonstrated in the basal epithelial cells of normal cornea and conjunctiva with little or no expression in their corresponding stroma. (Lambiase *et al*, 1998) The low-affinity NGF receptor is known as p75NTR.has

been postulated as a neural stem cell marker (Stemple *et al*, 1992) and its stimulation in the absence of TrkA is thought to promote apoptosis. Grueterich *et a l* 2003. noted high expression of TrkA in both limbal and corneal basal and suprabasal epithelia, with greater expression noted in the limbus. In contrast, p75NTR was expressed in the suprabasal limbal epithelium and entire corneal epithelium, but absent from the limbal basal epithelium. The selective expression of TrkA but not p75NTR in limbal basal epithelium suggests that NGF signalling favours LSC survival. Their findings also demonstrate that TrkA lacks the specificity to be considered a LSC marker, whereas p75NTR can be considered a differentiation marker of corneal epithelium.

α E*nolase:*

The glycolytic enzyme α-enolase is a multifunctional protein, which may exert a variety of cellular functions in addition to its primary role in the glycolytic pathway. (Pancholi, *et al*, 2001). Although originally considered a cytosolic protein, it can be also expressed on the cell surface of various cell types serving as a plasminogen-binding receptor. It has been reported that the expression of α-enolase increases in mitotically active, growing cells, but remains almost at an undetectable level in quiescent cells. Alpha-enolase was originally reported to immunolocalized preferentially to limbal basal cells of rat, rabbit and human corneas and was proposed as a marker of limbal SC. Zieske *et al.* 1992 initially developed a monoclonal antibody (4G10.3) that bound a 50-kDa cytoplasmic protein immunolocalized to limbal basal cells in rat corneas. This protein, later identified as α-enolase, (Zieske *et al*, 1992;) demonstrated reciprocal binding with the differentiation marker K3, suggesting that α-enolase was a marker of undifferentiated

corneal epithelial cells. However, it seems unlikely that α-enolase is stem cell-specific, as the majority of basal cells (non-stem cells) of other stratified squamous epithelia also express this protein, including basal cells of the peripheral cornea.

Metabolic enzymes

Several metabolic enzymes such as Na+/K+ ATPase, carbonic anhydrase and cytochrome oxidase have been found in higher concentrations in limbal basal cells than in basal cells of the central corneal epithelium (Zieske *et al*, 1994;). This would suggest that limbal basal cells are more metabolically active than central corneal basal cells, a notion discordant with the concept that stem cells are slow-cycling and biochemically primitive. However, it is more likely that the higher concentrations of enzymes are associated with the more proliferative early TACs of the limbal basal layer, rather than with LSCs. This would imply that LSCs comprise only a small percentage of the total limbal basal cell population. This is in keeping with current thinking that stem cells comprise only a small percentage of the total cell population they serve, ranging from 0.01% for HSCs to 10% for epidermal stem cells.(Alison *et al*, 2002;) Indeed, only about 10% of limbal basal cells are thought to be stem cells. (Wolosin *et al*, 2000; Cotasarelis *et al*, 1989 ;) Therefore, there appears to be a gradient of differentiation within the limbus itself, with the more differentiated and actively proliferating TACs responsible for expressing higher levels of metabolic enzymes.

Keratin 3 and 12

The absence of the cornea-specific differentiation markers K3 (Schemer *et al*, 1986) and K12 (Kurpakus *et al*, 1990) from the limbal basal epithelium indicates that limbal basal cells are the least differentiated cells of the corneal epithelium, consistent with the known characteristics of stem cells in general. K3 and K12 form a 'keratin pair' and are considered markers of corneal epithelial differentiation.

Connexins:

Cells communicate with each other through gap junctions. In the corneal epithelium, only two gap junction proteins have been identified so far: connexin 43 and connexin 50. (Dong *et al*, 1994) Connexin 43 is expressed by the corneal basal epithelium but not by limbal basal epithelium.(Dong *et al*, 1994) This implies that Connexin 43 expression is acquired during the process of TAC differentiation. It has been suggested that the absence of intercellular communication helps maintain the stem cell niche, (Matic *et al*, 1997) reflecting the need for LSCs to maintain a unique intracellular environment. The absence of gap junctions could also be a protective mechanism as it makes an individual stem cell less vulnerable to insults affecting its neighbour. Limbal basal cells completely devoid of Connexin 43 are thought to be stem cells whereas those that stain weakly for it are thought to be early TACs.(Matic *et al*, 1997) In contrast, connexin 50 is expressed by the suprabasal cells of both limbal and corneal epithelium, but not by the basal cells. It is therefore absent from LSCs and TACs. (Wolosin *et al*, 2000) Both proteins are considered useful markers of advanced corneal differentiation.

Involucrin:

Involucrin is the protein precursor of a cross-linked envelope that is a prominent component of the stratum corneum, the most superficial layer of epidermis. (Rice *et al*, 1979 ;) All stratified squamous epithelia make this protein whether or not they make a stratum corneum. Involucrin is not found in basal epithelial cells but appears in the course of their superficial migration. In human corneal epithelial cells expanded *ex vivo* from limbal explant and single-cell cultures, Kim *et al,* 2004 found that a greater percentage of large cells (i.e. more differentiated cells) expressed involucrin, compared with small cells (stem-like cells), in both culture systems. In adult human corneas prepared for immunohistochemistry, Chen *et al.* found that involucrin was present in all the layers of the limbal and corneal epithelium except in the limbal basal layer. (Chen *et al*, 2004) These studies suggest that involucrin can be considered a differentiation marker of stratified squamous epithelium.

1.3.7 Embryonic stem cell marker in corneal stem cells:

Stem cells exist in most adult organs, being best characterized in the bone marrow and gut. They are defined as cells that undergo symmetric and asymmetric division to give rise to daughter cells needed for self-renewal and amplification or to a daughter cell that acts as a progenitor cell for the purpose of producing specific differentiated lineages, respectively. Given the recent interest in the multiple uses of embryonic and adult stem cells for basic and applied research (i.e. reproductive cloning or regenerative tissue therapy), attempts have been made to characterize markers that would identify these stem cells. Oct3/4 or Oct4 (also referred to as Pou5f1), a transcription factor, was discovered in

1990 (Okamoto *et al*, 1990; Scholer *et al*, 1990; Rosner *et al*, 1990). It was found in ovulated oocytes, mouse pre-implantation embryos, ectoderm of the gastrula (but not in other germ layers) and primordial germ cells, as well as in embryonic stem cells but not in their differentiated daughters (Solter *et al*, 2000;) More recently, OCT4/Pou5f1 has been shown in cells isolated from human amniotic fluid (Prusa *et al*, 2003). Subsequent studies seemed to suggest that *Oct4* might be a specific gene marker for totipotency or a gene required for totipotency (Pesce *et al*, 1998; Deyev *et al*, 2004;). In fact, it is thought that the success or failure of cloning depends on expression of this gene during reprogramming of the genome of a nucleus transferred to an enucleated oocyte (Boiani *et al*, 2002;) It has also been reported that the expression of this embryonic stem cell marker is also observed in the corneal limbal stem cells. (Baharvand et al, 2007) The three transcription factors that are expressed at high levels in embryonic stem cells (ESCs) are Nanog, Oct-4 and Sox-2. These transcription factors regulate the expression of other genes during development and are found at high levels in the pluripotent cells of the inner cell mass. The downregulation of these three transcription factors correlates with the loss of pluripotency and self-renewal, and the beginning of subsequent differentiation steps. (Carlin et al, 2006 ;) The roles of Nanog, Oct-4 and Sox-2 have not been fully elucidated. They are important in embryonic development and maintenance of pluripotency in ESCs. There are no much studies on the expression of this embryonic stem cell markers expression on the cultivated corneal limbal stem cells.

1.4 Limbal stem cell deficiency:

Damage to or dysfunction of the limbal SC population results in partial or total limbal SC deficiency, which has severe consequences for corneal wound healing and ocular surface integrity (Chen and Tseng, 1991; Huang and Tseng, 1991; Dua *et al*., 2003). Limbal SC deficiency is characterized by conjunctival epithelial ingrowth, vascularization, chronic inflammation, recurrent erosions and persistent ulcer, destruction of the basement membrane, and fibrous tissue ingrowth leading to severe functional impairment (Puangsricharern and Tseng, 1995; Holland and Schwartz, 1996). In these cases, renewal of the SC population, e.g. by autologous or homologous transplantation of limbal tissue, is required for regeneration of the entire corneal surface and for restoration of visual function (Kenyon and Tseng, 1989; Kruse and Reinhard, 2001; Tseng *et al* 1987; Zieske 1994; Lavker *et al*, 2004; Dua *et al*, 2000 ;) Patients can subsequently suffer tearing, severe phtophobia, and decreased visual acuity. When the cytological evidence of conjunctivalization is used as a clinical tool for diagnosing limbal SC deficiency (Puangsricharern *et al*, 1995 ;) these diseases can further be subdivided into two major categories (Lavker *et al*, 2004 ;).

i. The first category is characterized by the destructive loss of limbal SCs by chemical/thermal burns, **(Figure 1.6 A, 1.6B & C)**

- Stevens-Johnson syndrome
- multiple surgeries
- extensive microbial infection
- Radiation, and anti-metabolite uses

Figure 1.6

Limbal stem cell deficiency

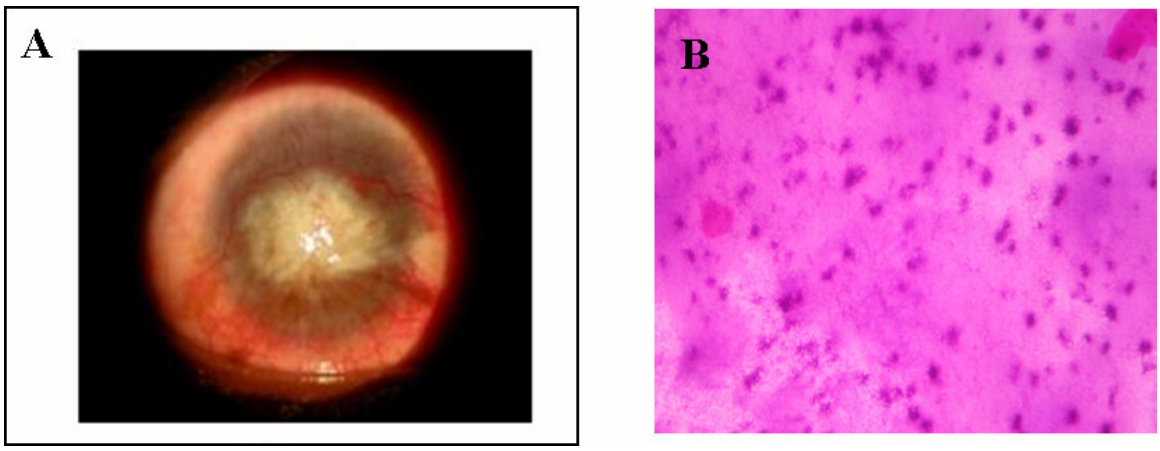


Figure 1.6A shows corneal surface of the patient injured with soap nut powder showing corneal opacity and limbal stem cell deficiency. Figure 1.6 B Imprint cytology showing numerous goblet cells suggestive of limbal stem cell deficiency.

ii. Intriguingly, diseases in the second category do not have such a destructive loss, and yet with time, also manifest the same phenotype of limbal SC deficiency.

- Aniridia (due to allele mutation of PAX 6)
- Heterozygous Pax 6- null mice (small eye) also show the pathologic features of limbal SC deficiency (Ramaesh *et al*, 2005a;) It has been speculated that the limbal niche is dysfunctional in these mouse limbal deficient corneas (Ramaesh *et al*, 2005b)
- Endocrine deficiencies and diverse diseases affecting the peripheral cornea and limbus

1.4.1 Therapeutic strategies:

The current treatment strategy available for the treating mild cases is the application of EGF (Epidermal Growth Factor) or fibronection to induce proliferation of the remaining viable cells in severe cases requires limbal graft. (Akpek and Foster, 1999) In the conventional method, corneal graft in the central corneal part is replaced by the similar sized by button obtained from the cornea of the deceased donor. In the above procedure only the central portion of the cornea is replaced because it does not contain blood vessel and the possibility of the tissue rejection is reduced. But long term success depends on the condition of the stem cell population present in the patient's limbus. Corneal limbal grafts can be attempted if stem cell deficiency is suspected but this requires treatment of the patients with the extended course of immunosuppressive drugs such as steroids and

cyclosporine A. (Tsai and Tseng 1994; Tsubota 1999 ;) Such drugs assist the acceptance of the graft but increase the risk of microbial infection. Therefore many ophthalmologists consider the possibility of performing autologus limbal stem cell graft.

An increasingly popular surgical procedure for ocular surface reconstruction in individuals with severe thermal or chemical burns or serious ocular surface disorders, such as Stevens–Johnson syndrome, ocular cicatricial pemphigoid, and recurrent pterygium, (Tsubota *et al* 1996; Lee *et al* 1997; Prabhasawat 1997;) involves the use of preserved human amniotic membrane as a biologic drape to dress the bare stroma after the removal of abnormal conjunctival tissue. **Figure 1.6A&B** shows the limbal stem cell deficiency picture.

The use of Amniotic membrane is based on the rabbit model reported by Kim and Tseng, (1995) in which the amniotic membrane is thought to inhibit conjunctival overgrowth and provide a good substrate for normal epithelial migration. The results of ocular surface reconstruction with amniotic membrane are generally good. Moreover, many workers have found that (Tsubota 1996; Shimazki *et al* 1997; Tseng *et al* 1998) in ocular surface disorders with stem cell deficiencies the use of limbal transplantation and keratoepithelioplasty in conjunction with amniotic membrane transplantation is often highly successful. Thus, it seems evident that the combination of amniotic/corneal epithelial cell transplantation is a potentially powerful one, (Sangwan *et al*, Vemuganti *et al*, 2004) and also, recently, it was demonstrated in rabbits the feasibility of cultivating corneal epithelial cells on amniotic membrane and transplanting them onto injured eyes

with epithelial stem cell deficiencies. (Koizumi *et al* 2000 ;) **(Figure 1.6C** shows the picture of limbal stem cell deficiency before and after treatment)

1.5 Substrates used for the ex-vivo expansion corneal limbal epithelial cells:

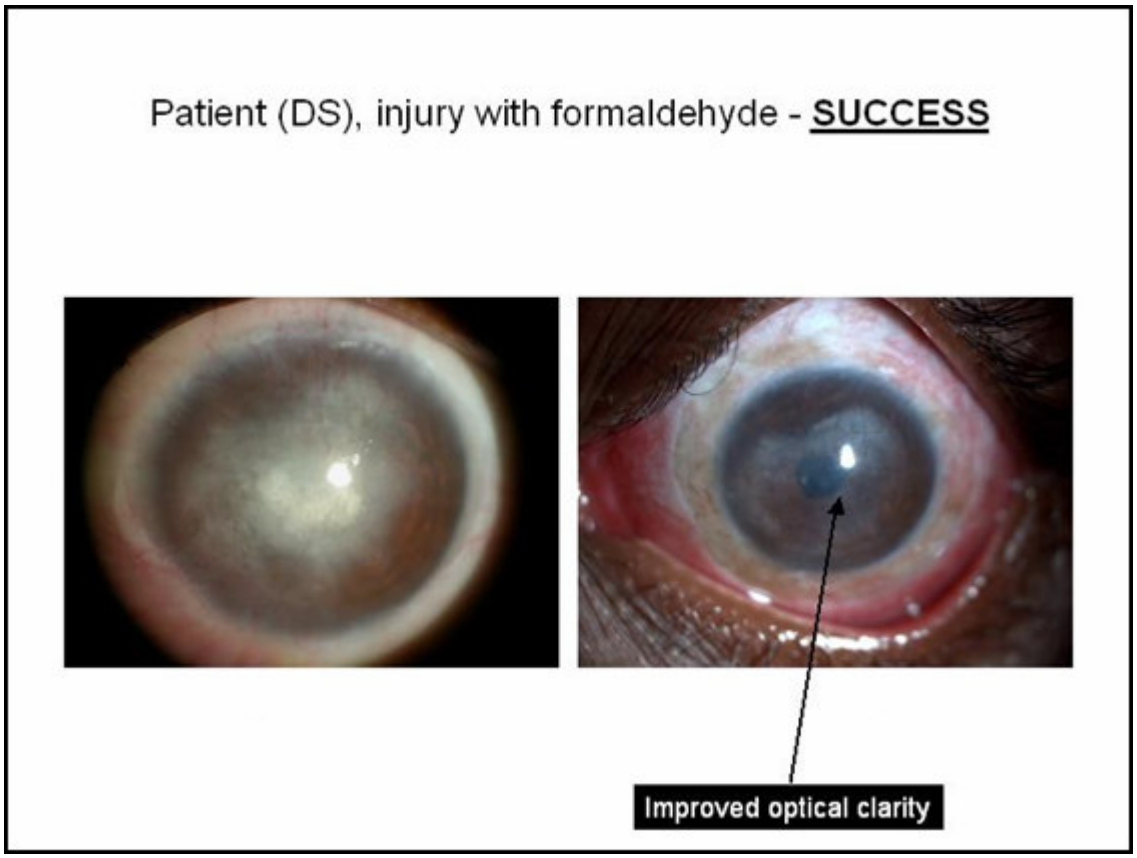
1.5.1 Amniotic membrane

The amniotic membrane is the innermost layer of placental membrane. It was first used in 1910 in skin transplantation. (*Davis et al* 1910 ;) Since then, the use of amniotic membrane has expanded in many surgical fields, including brain and genito-urinary tract surgery. The first ophthalmic application to treat ocular surface disorders was reported in the 1940s. (De Rotth 1940 ;) The application of amniotic membrane transplantation in the management of ocular surface disorders is ever increasing.

Amniotic membrane, which is derived from fetal ectoderm, is a translucent membrane consisting of five layers starting from the innermost layer: (1) epithelium; (2) basement membrane; (3) compact layer; (4) fibroblast layer; and (5) spongy layer. Its thickness varies from 0.02 to 0.50 mm. It lacks blood vessels or a direct blood supply. (Bourne *et al* 1960 ;) **(Figure 1.7)**

Amniotic membrane produces a large number of cytokines, of which Interleukins 6 and 8 are predominant. (Reisenberger 1998 ;) Expression of these cytokines increases in the presence of IL-1ß, TNF-alpha, and bacterial lipopolysaccharide. Studies reveal that human amniotic membrane preserved at -80°C for 1 month retains the presence of EGF, TGF-alpha, KGF, HGF, bFGF, TGF-ß1, TGF-ß2, endothelin-1, leukotrines, and carbonic anhydrase isoenzymes CA-1 and CA-2. These proteins are hypothesized to contribute to

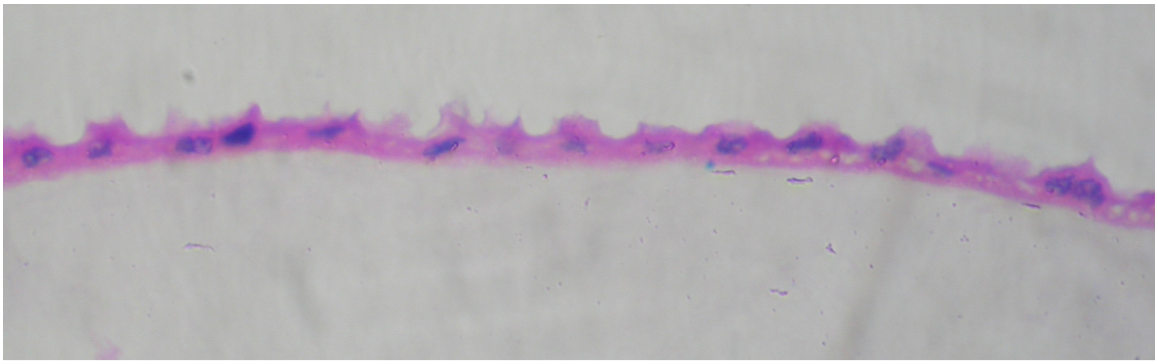
Figure 1.6C



This picture left shows the patient with limbal stem cell deficiency after injury with formaldehyde. Picture in the right shows the clear corneal surface after treatment with *ex vivo* expanded limbal epithelial cells.

Figure 1.7

Amniotic Membrane



This picture shows the H&E staining of the Amniotic epithelium after processiong

immune-mediated defense mechanisms during pregnancy. (Zhang *et al* 2001) According to the literature, amniotic membrane does not express HLA-A, B, or DR antigens, so immunological rejection after transplantation is rare. (Houlihan *et al* 1995) Additionally, amniotic membrane is also believed to have antimicrobial and antifibroblastic activity properties and cell migration/growth promoting activity. (Talmi *et al* 1991; Tseng *et al* 1998).

1.5.2 Properties of Amniotic membrane:

1.5.2.1Promoter of Re-epithelialization

The presence of a normal substrate is essential for normal proliferation, differentiation, and migration of epithelial cells. Lee and Tseng (Lee and Tseng 1997) reported successful re-epithelialization of 10 out of 11 cases of persistent epithelial defect by AMT. They postulated that amniotic basement membrane plays an important role in epithelial differentiation, the migration of epithelial cells, and the adhesion of basal epithelial cells.

1.5.2.2 Inhibitor of Inflammation and Scarring

Amniotic membrane executes anti-inflammatory and antifibrotic effects. Studies show that amniotic membrane induces a downward regulation of transforming growth factor ß, which is an important fibroblastic activator in wound healing. (Tseng *et al*, 1998) Amniotic membrane may also act as an anatomical barrier because its stromal layer is avascular, and blocks new vessels from growing. Additionally, antiangiogenic and antimicrobial properties of amniotic membrane are reported in the literature.(Kim and

Tseng *et al*, 1995) Kim and Tseng (1995) demonstrated that rabbit corneas with limbal stem cell deficiency and total keratectomy were less likely to become revascularized if covered with amniotic membrane.

The Kim and Tseng study (Kim and Tseng 1995 ;) has led to successful applications in various ocular surface diseases. Cicatrizing diseases of the ocular surface associated with acute or chronic stem cell loss constitute an indication for AMT. These ocular surface disorders include chemical or thermal burns, Stevens-Johnson syndrome, and cicatricial pemphigoid. Shimazaki *et al* (1997) reported the first clinical applications of the membrane in patients with the aforementioned conditions in 1997. Tseng *et al* (1998) applied the membrane in the treatment of 31 eyes of 26 patients with partial and total stem cell deficiency. The majority of these patients had chemical burns or Stevens-Johnson syndrome. Other patients presented with contact lens-induced keratopathy, aniridia, atopy, and iatrogenic stem cell disease.

AMT was reported to be successful in patients with persistent epithelial defects.(Azura *et al* 1999) Heiligenhaus *et al* (2003) reported that AMT was applied to promote the healing of acute ulcerative and necrotizing herpetic keratitis. In these studies, AMT shows great promise in acute ulcerative and necrotizing ocular surface diseases. Thus Amniotic membrane has many unique properties that can aid the treatment of different ocular surface disorders. AMT can promote normal epithelialization of the cornea and conjunctiva and prevent excessive fibrosis during ocular surface reconstruction. It is an effective alternative in the treatment of many challenging ocular surface disorders.

Although AMT holds great promise and its application is expanding in many ophthalmologic fields.

1.5.3 Role of feeder layer in culturing corneal limbal stem cells:

In fact, many types of adult somatic SCs have limited functions when detached from their *in vivo* niche. To circumvent this problem, one common approach is to cultivate them on a feeder layer made primarily of growth-arrested mesenchymal cells as a surrogate niche. For many types of epithelial progenitor cells, *ex vivo* expansion resorts to co-culturing on γ irradiated or mitomycin C-treated murine 3T3 fibroblast feeder layers first pioneered by Rheinwald and Green in 1975. There are several studies which have previously reported the use of the underlying inactivated fibroblasts for *in vitro* expansion of the corneal epithelium which has shown to be promoting the growth of the corneal epithelial cells and preventing differentiation. (Lindberg *et al* 1993; and Wei *et al* 1993 ;) similar results have been obtained using the fibroblast conditioned medium (Tseng *et al*; 1996) but the basic mechanisms remain unclear. (**Figure 1.8** shows the confluent growth of mitomycin C treated 3T3 feeder layer)

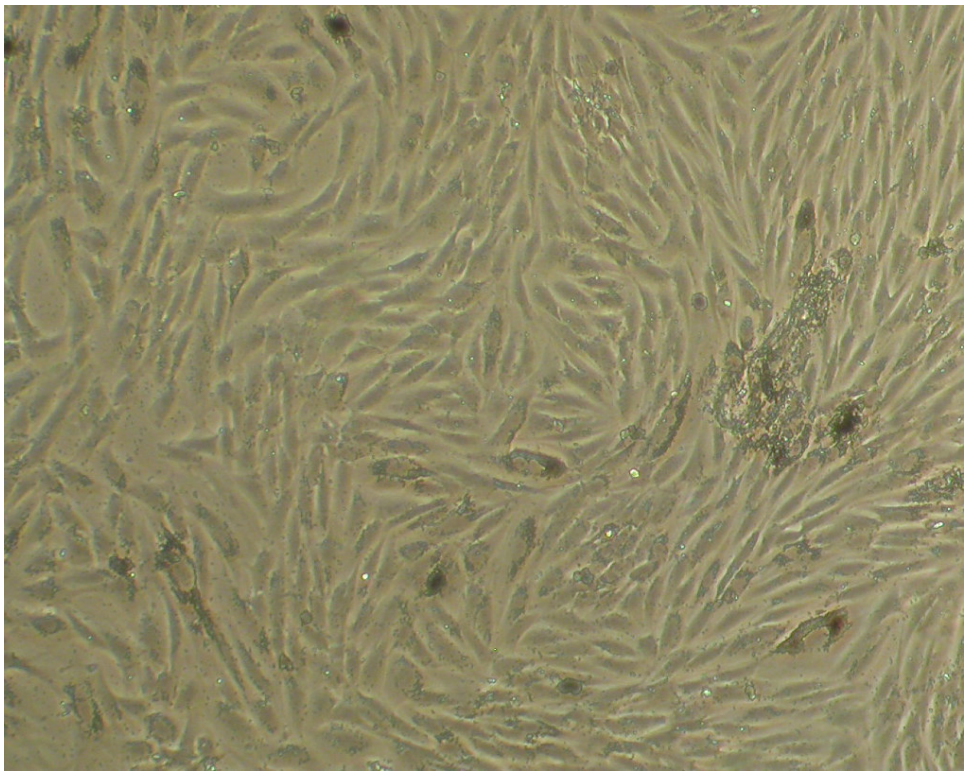
1.5.4 Role of extracellular matrix [ECM]

1.5.4.1 Extracellular matrix role in cell adhesion and proliferation

ECM is the natural medium in which cells proliferate, differentiate and migrate, and therefore is the gold standard for tissue regeneration (Meredith *et al*, 1993; Bosman *et al*, 2003;) Cell-ECM interaction is specific and biunivocal. Cells synthesize assembly and degrade ECM components responding to specific signals and, on the other hand, ECM

Figure 1.8:

3T3 Feeder layer:



This figure shows the monolayer of the mitomycin C inactivated 3T3 feeder layer at the end of 24 hours

controls and guides specific cell functions. This continuous cross-talk between cells and ECM is essential for tissue and organ development and repair.

In physiologic conditions, ECM composition derives from homeostasis, a fine dynamic balance of regeneration, differentiation and programmed cellular death (apoptosis), which continuously remodel ECM through protein breakdown and synthesis (Meredith *et al*, 1993). Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network formed by an intricate agglomerate of weaves, struts and gels interconnecting cells with matrix proteins. Dynamic properties of ECM are controlled by Proteoglycans, and a number of signalling molecules, such as growth factors (GFs), which mediate cell-ECM and cell-cell interactions. All these molecules are embedded in an amorphous, fundamental substance represented by glycosaminoglycan` chains, which form the highly hydrated gel structure imbibing the matrix. (Ungaro *et al*, 2004) The following **table 1.3** shows the various extracellular matrixes and their role in tissue engineering.

Table – 1.3

ECM components and their role:

Component	Function	Location
Collagen	Tissue scaffolding, tensile strength Cell-ECM interactions Cell-cell interactions Fibroblast activation	Ubiquitous
Proteoglycans	Collagen embedding Tissue resistance to compressive forces Transport of nutrients Fibroblast and chondrocyte proliferation Endothelial and epithelial cell differentiation	Ubiquitous
Hyaluronic acid	Transport of metabolites and nutrients Tissue resistance to compressive forces Cell migration Cell proliferation	Ubiquitous
Laminins	Intracellular signalling Cell differentiation Cell shape/movement	Basement membranes
Fibronection	Cell attachment to ECM Cell migration Cell proliferation	Ubiquitous
Growth factors	Cellular signalling	Ubiquitous

Adapted from Topics in Tissue Engineering 2004, volume 2. Eds. N. Ashammakhi & R.L. Reis

The sequestration of Growth Factors within ECM in inert form is necessary for rapid signal transduction, allowing extracellular signal processing to take place in time frames similar to those inside cells. In addition, GF storage in ECM is crucial to maintain homeostasis through continuous GF activation upon ECM degradation. ECM, therefore, serves as a sustained release reservoir for GFs, this aspect being crucial for molecules that are released over a short period but able to stimulate processes involved in tissue regeneration, such as angiogenesis, that take extended periods to be carried out.

1.5.4.3 ECM and GFs: a complex interplay controlling tissue growth and repair

For the formation of complex tissues from single cells, and for tissue maintenance, large amounts of information are needed and must be transported from cell to cell and from cells to ECM. GFs are protein molecules specific for intercellular and cell-ECM signalling, which are involved in ECM dynamic properties through specific surface receptors, driving GFs regulatory activity (Flaumenhaft *et al*, 1992; Gumbiner *et al*, 1996; Taipale *et al*, 1997).

GFs are released by many cell types for immediate signalling and they activate specific pathways controlling cell migration, differentiation and proliferation. They are locally secreted by ECM, in which they are stored in insoluble/latent forms, and can elicit their biological activity once released. During tissue morphogenesis the presence of soluble GFs guides cellular behaviours, thus governing neo-tissue formation and organization. GFs are normally synthesized as membrane-bound or high molecular weight precursors that must be modified to release the active form. GFs are often bound to ECM molecules,

such as glycosaminoglycans (e.g. heparins). The interaction with these molecules alters GF action, by retaining the active/latent forms near cells and modifying GF transport properties. (Ungaro *et al*, 2004)

1.5.5 Role of polymers and scaffolds in bioengineering cornea:

Critical step of all tissue engineering techniques is the use of a three-dimensional structure which, mimicking the extracellular matrix (ECM), serves as scaffold which is able to promote and guide actively the tissue regeneration process. The cells are generally implanted or seeded into an artificial structure capable of supporting three-dimensional tissue formation. These scaffolds are often critical, both *ex vivo* as well as *in vivo*, to recapitulating the in vivo milieu and allowing cells to influence their own microenvironments. Such devices, usually referred to as scaffolds, serve at least one of the following purposes:

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify the behaviour of the cell phase

1.5.5.1 Scaffolds in tissue engineering:

Beyond simply improving tissue integration for synthetic implants, functional tissue regeneration within artificial matrices or on artificial surfaces is now possible. This is where the new science of tissue engineering diverges from conventional biomaterials

research. Tissue engineering combines elements of engineering and materials science with genetics, molecular, cell, and developmental biology in organ replacement and organ regeneration. (Langer *et al*, 1993, 1999 ;) Engineered replacement tissue constructs are already in development for a variety of tissues including skin, cartilage, nerve, liver, kidney, muscle, heart valves, and blood vessels.(Bell *et al*, 1981; Yannas *et al*, 1982; 1987; Cao *et al*, 1997;)

To achieve the goal of tissue reconstruction, scaffolds must meet some specific requirements. A high porosity and an adequate pore size are necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients. Biodegradability is essential since scaffolds need to be absorbed by the surrounding tissues without the necessity of a surgical removal. The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually it will break down leaving the neotissue, newly formed tissue which will take over the mechanical load. Injectability is also important for clinical uses. (Chaignaud *et al*, 1997)

Naturally-derived molecules, such as proteins and polysaccharides, find wide application in tissue engineering. Collagen has been used especially for regeneration of soft tissues either alone or in combination with other agents (Pachence *et al* 1996;) Naturally derived polymers in general, and collagen in particular, though having poor mechanical properties, are interesting because they do not induce a host response, and may enhance

the biological recognition in the growing neo-tissue, encouraging the normal cellular functions (Chaignaud *et al*, 1997;). For the replacement of soft tissues, there are many strategies employing Hyaluronic Acid [HA] as a scaffold material. Through chemical modifications of HA it is possible to obtain HA derivatives, that exhibit better mechanical features but not be recognized by cells, thus impairing biological activity (Brun *et al*, 1999) On the other hand, semi-interpenetrating (semi-IPN) gels made of collagen and HA, have been demonstrated suitable to realize scaffolds for tissue engineering applications because the structures of collagen-HA gels strongly resemble the organization of ECM in soft tissues (Xin *et al*, 2004). Table 1.4 shows the several synthetic and natural polymers which has wide application in Tissue Engineering field.

Table 1.4:

Bio-degradable Polymers used in tissue engineering:

Type	Class	Polymer
Natural polymers	Polysaccharides	Alginates
		Chitosan
		Dextrans
	Polypeptides and proteins	Albumin
		Collagen
		Gelatin
Synthetic polymers	Polyanhydrides	Poly (SA)
		Poly (FAD-SA)
		Poly (SA-co-CHP)
	Polyesters	Poly (ε-Caprolactone)
		Poly lactide
	Polyorthoesters	Block copolymers
	Polyphosphazenes	
	Polyphosphoesters	Poly (DAGP-EOP)

Adapted from Topics in Tissue Engineering 2004, volume 2. Eds. N. Ashammakhi & R.L. Reis

1.5.5.2 Biodegradable microspheres for the controlled release of growth factors:

Microspheres are particulate delivery systems which can incorporate small drugs or macromolecules (Davis *et al*, 1988; Okada etal, 1995; Freiberg *et al*, 2004; Varde *et al*, 2004). Particles are made of different polymers, which inherent physico-chemical

characteristics (e.g. chemical nature, composition, molecular weight, hydrophilicity, degradability), obviously affect final microsphere properties. As well-known, biodegradable polymers have attracted increasing attention in microsphere development because, differently from non degradable systems, they do not require further manipulation after introduction within the body.

1.5.5.3 Thermo reversible gelation polymer:

There are several polymers which are being used in the tissue engineering; thermo reversible polymer is one such polymer which has the property of being liquid at low temperature and becoming gel at the higher temperature. Mebiol Gel is a copolymer composed of the thermo responsive polymer block [poly (N-isopropylacrylamide-co-n-butyl methacrylate) (poly NIPAAm-co-BMA)] and the hydrophilic polymer block [polyethylene glycol (PEG)]. This polymer block is hydrophilic at temperatures below 20^0C and hydrophobic at temperatures above 20^0C forming cross-linking points and homogenous three-dimensional (3-D) network of Mebiol Gel in water. Cells or tissues can be embedded in a liquid Mebiol Gel solution at lower than 20^0C and culture three-dimensionally in a hydrogel state at 37^0C. The sol-gel transition temperature can be controlled by chemical composition of Thermo Reversible Gelation polymer (TGP). (Yoshioka *et al*, 1994a, 1994b, 2003).

1.5.5.4 Chitosan:

Chitosan, a member of the family of glycosaminoglycans (GAGs), has been studied as a substrate and scaffold for tissue engineering of skin. (Zhu *et al*, 2005;) GAGs are major

components of skin dermis and cornea, and play a critical role in the process of wound healing. (Chandy *et al*, 1990; Suh *et al*, 2000; Sechriest *et al*, 2000 ;) Chitosan has been found to have a beneficial role on wound healing *in vitro* and in vivo. (Chandy *et al*, 1990) In addition, chitosan has already proven to be useful in ophthalmology, where it has been developed for contact lens fabrication and ocular bandage lenses. However, a pure chitosan substrate would be too stiff for application on the curved ocular surface. Therefore, gelatin, a soft, elastic natural material, can be introduced into the Chitosan membrane to improve its chemical and physical properties. Integration of gelatin into chitosan will reduce the stiffness of a membrane and may also improve its biological properties. Gelatin, a biodegradable and biocompatible polymer, is a processed type I collagen so there are no immune properties remaining. In addition, collagen is of course the primary component of the extracellular matrix in the eye and skin. Gelatin, as a denatured collagen, may be expected to have useful biological properties on cell attachment, migration, proliferation and differentiation.

1.6 Signal Transduction Pathways Involved In The Self Renewal And Survival Of Stem Cells:

In biology, **signal transduction** refers to any process by which a cell converts one kind of signal or stimulus into another, most often involving ordered sequences of biochemical reactions inside the cell, that are carried out by enzymes and linked through second messengers resulting in what is thought of as a "second messenger pathway". Such processes are usually rapid, lasting on the order of milliseconds in the case of ion flux, to minutes for the activation of protein and lipid mediated kinase cascades. In many signal

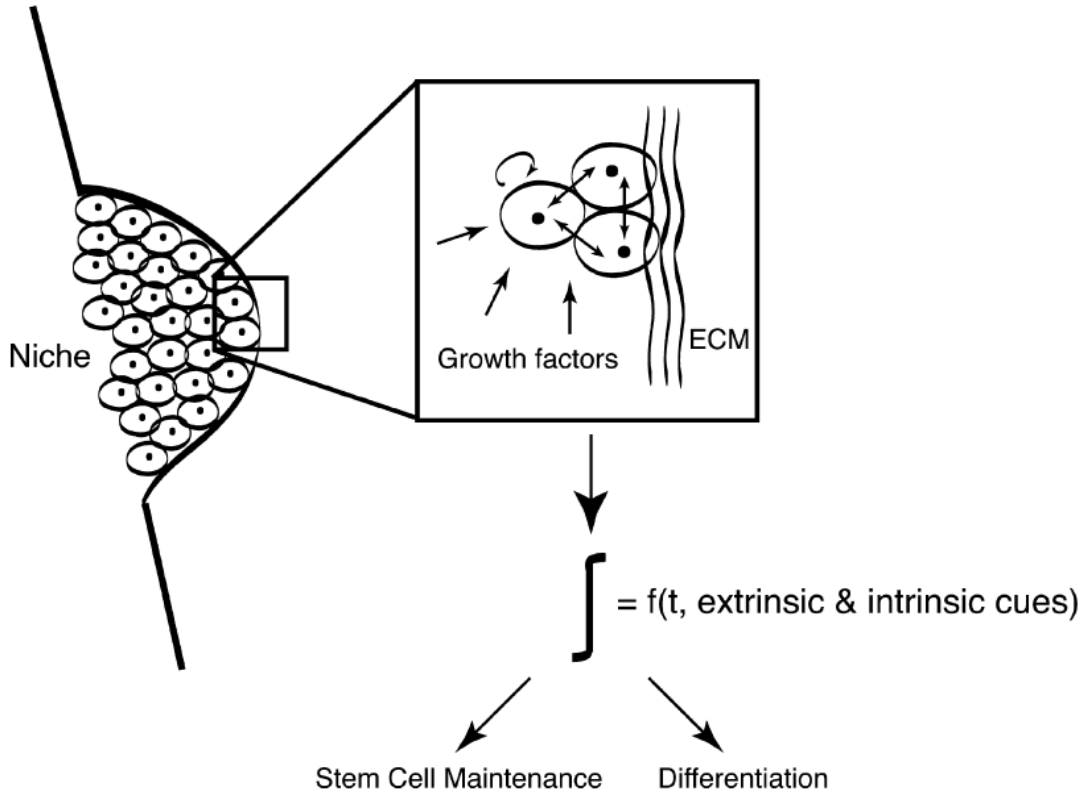
transduction processes, the number of proteins and other molecules participating in these events increases as the process eliminates from the initial stimulus, resulting in a "signal cascade" and often results in a relatively small stimulus eliciting a large response.

Figure 1.9 shows the how stem cells are maintained in the niche through signaling pathway.

One of the most important issues in stem cell biology understands the mechanisms that regulate self-renewal. Self-renewal is crucial to stem cell function, because it is required by many types of stem cells to persist for the lifetime of the animal. Moreover, whereas stem cells from different organs may vary in their developmental potential, all stem cells must self-renew and regulate the relative balance between self-renewal and differentiation. Understanding the regulation of normal stem cell self-renewal is also fundamental to understanding their biology.

Many stem cells reside in a spatially restricted compartment called a niche. This niche provides an environment that supports the survival of the multipotent stem cell without induction of differentiation. Neighboring differentiated cell types secrete factors and provide a milieu of extracellular matrix that allows stem cells to self-renew and to maintain the capacity to respond to differentiation programs. Physical contact between stem cells and their non-stem cell neighbors in the niche is critical in keeping the stem cells within this compartment and in maintaining stem cell character. Often, stem cells within the niche are quiescent or slow-cycling, but proliferation might be induced by injury. The stemness of the cells in the niche is maintained by the intrinsic self renewal

Figure -1.9 Signaling in the niche



The niche provides an environment that attracts stem cells and keeps them in an undifferentiated state by supporting self-renewing cell divisions. Accordingly, differentiation may be initiated when the stem cell leaves the niche. The balance of quiescence, self-renewal, and cell commitment is influenced by secreted growth factors that initiate intracellular signaling cascades and activate distinct sets of transcription factors. Further, the extracellular matrix (ECM) plays an important role in retaining the stem cells in the niche. Thus, self-renewal versus lineage specification and differentiation are the result of the capacity of a stem cell to integrate multiple signals that vary with location and time.

factors and extrinsic self renewal factors. The intrinsic self renewal factors include the cell surface markers. The extrinsic self renewal factors include various signal transduction pathways.

All stem cells and precursors respond to multiple growth factors, and their effects can be modulated by extracellular matrix components (Dutton *et al*, 2000). Several different integrins that bind to the extracellular matrix seem to be differentially involved in the regulation of proliferation, cell migration and differentiation. Binding to extracellular matrix proteins such as laminin activates an intracellular signaling pathway via phosphatidylinositol3 (PI3) kinase and Akt kinase (Armullik *et al*, 2004). In sum, stem cell development is controlled by the combinatorial activity of multiple factors, acting in signaling networks (Fuchs *et al*, 2004; Sommer *et al*, 2002). The composition of such networks is dynamic, changing with time and location. To unravel the complete mechanism contribution of individual signal transduction pathways should be studied, knowing that this contribution is likely to be modulated by the crosstalk with other pathways.

The second largest category of transcripts enriched in human embryonic stem cells was comprised by the genes involved in the signaling pathways. A significant number of them fell within three major signalling categories belonging to the RAS/MAPK/ERK signalling pathway (G3BP, RASAL2, SOS1 and ITGB1BIP3), the PI3K/AKT (PI3KCB and PTEN), and NFκβ signalling (LCK, PELLINO 1 and TNSF11).

In corneal stem cells the PI3K mediated signaling pathway is studied by He *et al*, 2006). These pathways are mainly involved in self renewal and prevent the cells from undergoing apoptosis. ***PI3K*** enzymes are normally regulated by growth factors and serve to phosphorylate phospholipids at the plasma membrane. Activated PI3K phosphorylates PIP2 and generates PIP3 which acts as a second messenger. AKT interacts with PIP3 and subsequently translocates to the plasma membrane. AKT also referred as PKB plays a critical role in controlling the balance between survival and apoptosis and is activated by phospholipids binding and activation loop phosphorylation at threonine 308 by PDK1(Pyruvate dehydrogenase Kinase) and phosphorylation at the car boxy terminus at serine 473. In addition it regulates glycogen synthesis through phosphorylation and inactivation of GSK3β and GSK3α. Activation of AKT survival signalling pathways leads to phosphorylation of Iκβ, the cytoplasmic inhibitor of nuclear factor κβ (NFκβ). Phosphorylation of Iκβ initiates proteasome-dependant degradation and allows NFκβ.

CHAPTER 2 - OUTLINE OF THE WORK

2.1 Introduction:

Stem cells in all renewable tissues are usually located in a specialized niche that offers protection and nourishment to these essential cells. The corneal epithelial stem cell niche is not an exception. Evidence that the limbus is a potential stem cell niche for the corneal epithelium has been presented in many studies. The cells from the limbal niche have been shown to be slow-cycling (Cotsarelis *et al*., 1989), relatively undifferentiated cells (Schermer *et al*., 1986), and when isolated have a higher proliferative potential *in vitro* than central or peripheral corneal epithelial cells. Hence in this study, I have tried to isolate the cells from and limbus, and understand the biology of the cells by culturing and characterizing those using specific makers.

Objectives:

1. **To find out the usefulness of the intact (Amniotic membrane [AM] with devitalized epithelium) and denuded (Amniotic membrane without epithelium) for the *ex-vivo* expansion of the Limbal stem cell cultivation.**
    - To standardize culturing limbal epithelial cells from the limbal explants obtained from cadaveric donor eyes.
    - To compare the expression of the stem cell associated and differentiation markers on the cells cultured over the intact and denuded AM by RT-PCR and Immunohistochemistry
    - To find out the label retaining cells on the cells expanded over the intact and denuded AM by Brdu labelling
    - To confirm the expression of the p63 and ABCG2 by western blotting

2. To culture and characterize the limbal epithelial cells on the Non-polymeric Matrices

- To culture the limbal epithelial cells on the chitosan matrix and its derivatives by explant culture technique from limbal biopsies obtained from cadaveric donor eyes
- To characterize the cells using stem cell associated and differentiation markers by RT-PCR
- To study the proliferative index using Brdu labelling

3a. To culture limbal epithelial cells in a thermoreversible gelation polymer

- To culture the limbal epithelial cells in thermoreversible gelation polymer
- To characterize the cultured cells using marker study by RT-PCR and immunohistochemistry
- To study the proliferative capacity using tritiated thymidine uptake study

3b. *Ex vivo* expansion or rabbit limbal epithelial cells in thermoreversible gelation and transplantation of the same into the limbal deficient rabbits.

4. To study the effect of 3T3 feeder layer in ex-vivo expansion of the corneal limbal stem cells

- To culture the limbal epithelial cells on the denuded AM with and without 3T3 feeder layer

- To compare the expression of the stem cell associated and differentiation marker cultured on the cells cultured in the presence and absence of 3T3 feeder layer

5. **To study the expression of p63 isoforms limbal epithelial cells cultured over the intact and denuded amniotic membrane by RT-PCR**

6. **To Study the signal transduction pathway mediated by NGF on the cells cultured over the intact and denuded amniotic membrane which is involved in proliferation and self-renewal of corneal stem cells**

7. **To study the embryonic stem cell markers (OCT4, Nanog and Pax6) expression on the limbal epithelial cells cultured over the intact and denuded amniotic membrane.**

CHAPTER 3 - METHODOLOGY

3.1 Collection Preparation and Preservation of Amniotic Membrane:

An elective caesarian delivery helps in the correct choice of a consenting donor and planned collection and processing of AM. Placenta collected after natural vaginal delivery may have structural defects associated with stretching of the membrane during labour and delivery, and may be contaminated by normal vaginal flora, Herpes, *Chlamydia* or other contaminant bacteria. (Adds *et al*, 2001)

The following steps were followed to finally obtain quality AM for transplantation. The required transport medium, the washing solutions and preservative medium, properly checked for sterility it was made available at least a day before the collection and preparation of the membrane. The working solutions and media should have been prepared 7 - 10 days ahead with complete verification of pH and sterility. The required reagents are prepared freshly before use. The steps involved in the preparation are described below.

- The detailed medical history and clinical condition of the potential donor was taken to exclude the risk of tissue-transmissible infections and unsuitability of the donors.
- The consent of the donor was obtained for the donation (and subsequent use of AM). Donors are screened for human immunodeficiency virus (HIV) type 1 and 2, hepatitis B virus (HBV), hepatitis C virus (HCV) and *Treponema pallidum* infections. Competent laboratories had performed these tests and the records were

maintained for 11 years post-transplantation. It is necessary to preserve all the records where the review of the records may become necessary if HIV or any slow virus infections develop in the recipient(s).

- Consent for subsequent screening of blood to determine the HIV status after the "window" period of 3-4 months was also obtained from the donors.
- The AM was obtained under sterile conditions after elective caesarian section.
- The obstetrician had placed the placenta in a sterile stainless steel 12-inch diameter basin, preferably covered with a sterile lid. During placement the clamp from the cord was not removed, this is to avoid the AM getting covered with blood.
- In the clean atmosphere of the operating room or the clean laminar flow workbench, the AM was dissected from the placenta in two large bits. As much of the chorion as possible was peeled out before the bits are dropped into a sterile, wide mouthed 125 ml screw-capped reagent bottle containing 50 ml transport medium. The transport medium generally used is the commercially available Dulbecco's minimum essential medium (DMEM) supplemented with 3.3% L-glutamine and antibiotics (50 µg/ml gentamicin, 100 units/ml penicillin, 200 µg/ml ciprofloxacin and 1 mg/ml Amphotericin B). The media and the antibiotic preparations used were of recognized quality manufactured as per ISO 9002 standards as certified by a competent certifying authority. The membrane was transported immediately to the laboratory.
- In the laboratory, under the laminar flow hood, the AM is washed free of blood clots with DMEM containing antibiotics. Any leftover chorion attached to the AM

and blood clots were gently peeled off the epithelial cell layer using round-ended forceps.

- With the epithelial / basement layer surface up, the AM is spread uniformly without folds or tears on individually sterilized 0.22 μm nitrocellulose membranes of the required size (47 mm or 25 mm, commercially available - Millipore or Sartorius). The AM around the nitrocellulose membrane was cut and allowed to adhere to the cellulose membrane.
- If in doubt, a small piece of about 1 cm^2 of the membrane was examined on a microscopic slide under a phase contrast microscope to ascertain the epithelial side.
- The filter membrane along with the adherent AM is fully placed carefully in the preservative medium in 50-ml wide mouthed screw-capped irradiated transparent plastic bottles. The preservative medium used is 1:1 (vol/vol) ratio of sterile glycerol (sterilized by autoclave) and DMEM with 3.3% L-glutamine, 25 μg/ml gentamicin, 50 units / ml penicillin, 100 μg/ml ciprofloxacin and 0.5 mg/ ml Amphotericin B.
- The bottles were labeled with the appropriate size and date of preparation.
- A random bottle from the batch is left over the work bench at room temperature for about an hour and about 5 ml of the same was inoculated into 100 ml of brain heart infusion medium and 100 ml of thioglycolate broth medium to check the bacterial and fungal sterility. These media were incubated for 21 days and if there was no growth of bacterium or fungus is observed, the batch should be considered as preserved free of cultivable microbial agents.

- The AMs were stored at -80°C to facilitate the devitalization of the epithelial cells.
- The HIV negative status of the donor was checked by repeat serology done after 3-4 months after collection of AM. After the confirmation the membranes were used for the culture/therapeutic purpose.

3.2 Denudation of Amniotic membrane:

Immediately before use, the amniotic membrane was thawed, washed three times with sterile PBS, and cut into pieces approximately 2.5 X 2.5 cm in size. Membranes were then deprived of their amniotic epithelial cells by incubation with 0.02% EDTA (Hi-Media, India) at 37°C for 2 hours to loosen the cellular adhesion, followed by gentle scraping with a cell scraper (BD biosciences). Preliminary experiments on hematoxylin–stained, ethanol–fixed tissues confirmed that this protocol effectively removed epithelial cells from the amniotic membrane. Tissues were then washed twice more with sterile PBS

3.3 Collection of limbal biopsy:

All the eye tissues were obtained from the C.U Shah eye bank of Medical Research Foundation, Sankara Nethralaya, Chennai. The tissue samples were handled according to the declaration of Helsinki**.** Corneal limbal biopsy of 2 mm^3 from the cadaveric donor eye was collected in Dulbecco's Minimum Essential Medium (DMEM) with 3% Fetal Calf Serum (FCS) and antibiotics as the transport medium and was transported to the cell biology laboratory for further processing. The biopsy tissue bit was gently washed three

times using tissue culture growth medium (DMEM with 10% FCS and antibiotic mixture). After careful removal of excessive sclera the tissue bit was cut into multiple bits using sterile sharp curved scissors / Bard-Parker blade.

3.4 Explant Culture Technique:

Amniotic membrane with the epithelial side facing upwards was fastened on the 6 well plate culture insert (BD biosciences,). On the centre of the either intact AM or EDTA treated deepithelialized amniotic membrane, chitosan the explants were placed. After placing the biopsy about 0.5ml of the medium containing equal volume of DMEM and F12 supplemented with 10% FBS, 50µg/mL of streptomycin, 1.25 µg/mL of Amphotericin B 2ng/ml of mouse epidermal growth factor (EGF), 5µg/mL of insulin, 5µg/mL of Transferrin, 5ng/mL of selenium, 5mg of Keratinocyte growth supplement, 0.5mg/mL of Hydrocortisone was added just to cover the explants and plate was incubated at 37^0C under 95% humidity and 5% CO_2 for about half an hour to forty five minutes. After incubation remaining 1.5ml of the medium was added to cover the entire well completely. The medium was changed once in three days and cell growth was monitored daily for 3 weeks with an inverted phase contrast microscope (Nikon, Tokyo, Japan). Once they reach confluence the cells were harvested for the marker study by immunophenotyping and molecular characterization.

3.5 Immunocytocemistry/Immunohistochemistry:

3.5.1 Preparation of cultures for staining:

Once the cells reached confluence the cultures for immunostaining was fixed in 10% Neutral buffered formaldehyde for paraffin embedded sections and for the immunofluorescent staining the amniotic membrane with the cultured cells were fixed in Optimum Cutting Temperature medium (Bright, Germany) and frozen sections were taken. For paraffin embedded sections the cultured were fixed in buffered formal saline and processed in graded alcohol then sections were taken on the silane coated slides.

3.5.2 Immunofluorescence staining*:*

For Immunofluorescence staining the frozen sections which was taken on the silane coated slides were air dried at room temperature for half an hour and fixed in cold acetone for half an hour and then preceded for staining. The slides were blocked with 5% serum for 1 hour. The primary antibodies were diluted in 1X casein solution in Tris Borate EDTA buffer (TBE buffer) for one hour to over night. Then the cultures were washed thrice with the TBE buffer and incubated with corresponding secondary antibody diluted in 1X casein diluted in Tris Borate EDTA buffer for 1 hour at room temperature. Once again the cutures were washed with 1X TBE and counter stained with 0.5% Evans Blue. Finally the cultures were washed with 5 times with 1X TBE and mounted in Phosphate Buffered Saline (PBS) in glycerol.

3.5.3 Immunoperoxidase staining:

Slides with the sections were deparaffinized and antigen retrieval was done by trypsinization method and then washed with Tris HCl buffer. Endogenous peroxidase was quenched 0.3% H_2O_2 for 30 mins and then slides were washed thrice with Tris HCl buffer. Then slides were incubated with the primary antibody for 2 hours – overnight again washed thrice with Tris HCl buffer. Then the slides were stained with link and streptavidine provided DAKO (Germany,) kit for 1hour each and then washed with the Tris buffer according to the manufacturers instructed protocol. Visualization of the reaction was done by incubating with DAB (3'di-amino-benzidine) for 5 minutes provided in the kit, then washed with the Tris buffer and counter stained with Harris Hematoxylin for 30seconds. Slides were washed and mounted with glycerol.

3.6 RNA analysis:

3.6.1 Total RNA extraction:

Cell samples were harvested and washed with the PBS and total RNA was extracted with the Qiagen RNA easy mini kit. (Germany,) Approximately 1 X 10^7 cells were collected in the Rnase free vial centrifuged at 3000 rpm for 5 mins. Then the supernatant was discarded, to the pellet 350µl of RLT buffer reconstituted with β mercaptoethanol was added which is provided in the Kit, the vial was vortexed after adding the RLT buffer and tube was centrifuged at maximum speed for about 3 mins. Then the supernatant was carefully transferred to a new vial, to that one volume (350µl) of ethanol was added to the lysate and it was mixed nicely by pipetting. Then the sample was applied to the minielute spin coloumn and centrifuged at 10,000 rpm for 15 secs. The flow through was discarded,

350µl of RW1 (provided in the kit) was added to the coloumn and centrifuged at 10,000rpm for 15 secs, the flow through was discarded to the coloumn *DNase I* treated for 15 mins, again 350ml of Rw1 was added and centrifuged at 10,000rpm for 15 secs. The flow through was discarded after the centrifugation and the coloumn was transferred to a new collection tube and 500µl of RPE buffer was added onto the coloumn and the tube was centrifuged at 10,000 rpm for 15secs. Then again the coloumn was treated with 500µl of 80% ethanol centrifuged at 10,000rpm for 2mins. Both the flow through and collection tube was discarded and the coloumn was transferred to the new collection tube the cap was kept opened and centrifuged at maximum speed of 14,000rpm for 5 mins. Then the coloumn was placed on a news vial 30ml of Rnase free water was added and centrifuged at 10,000rpm for 1 min and the vial was labelled and stored at -80^0C until use.

3.6.2 Reverse Transcription and Amplification:

Reverse Transcription was performed using sensiscript reverse transcripatase (Qiagen), with a recombinant heterodimeric enzyme. PCR amplification of the first – strand cDNAs were performed using specific primer pairs, designed from published human gene sequences along with House keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as internal control. Table 3.1 & Table 3.2 give the published primer sequence for which amplification was done. Amplification was performed in the Eppendorf PCR systems (personal). PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide with molecular marker *Hinf I* Φ digest/ 100bp ladder (Bangalore genei, India) to confirm the size of the resultant product.

3.6.3 Semiqunatitative RT-PCR analysis:

Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control, the mRNA expression of different molecular markers was analyzed by semiquantitative RT-PCR as described previously. (Li *et al*, 2001, 1995 ;) RT-PCR was performed using sensiscript reverse transcripatases, which is recombinant heterodimeric enzyme. PCR amplification of the first – strand cDNA synthesis was performed using Qiagen Sensiscript reverse transcriptase kit. Briefly first strand cDNA synthesis was carried out from 0.3mg of the extracted RNA for different markers in Eppendorf PCR systems. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control, the mRNA expression of different molecular markers was analyzed by semiquantitative RT-PCR. PCR amplifications were performed with the specific primers designed from published human gene sequences. (Chen *et al*, 2004 ;) Semiquantitative RT-PCR the bands were analyzed Quantity G software Bio-Rad gel documentation system. Semi quantitation was established by terminating reactions at appropriate intervals for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide with molecular marker *Hinf I* φ digest/100 bp ladder to confirm the size of the resultant product. of the amplification curve. The fidelity of the RT-PCR products was verified by comparing their size with the expected cDNA bands and by sequencing the PCR products.

Table 3.1:

Primer sequence and Reaction condition for the Reverse transcriptase PCR

Gene	Primer sequences	Annealing temp. ^{0}C	PCR product size
ΔNp63	**FP: CAGACTCAATTTAGTGAG** **RP: AGCTCATGGTTGGGGCAC**	**54^{0}C**	**440 bp**
ABCG-2	**FP: AGTTCCATGGCACTGGCCATA** **RP: TCAGGTAGGCAATTGTGAAGG**	**62^{0}C**	**379 bp**
Connexin 43	**FP: CCTTCTTGCTGATCCAGTGGTAC** **RP: ACCAAGGACACCACCAGCAT**	**66^{0}C**	**154 bp**
K3	**FP: GGCAGAGATCGAGGGTCTC** **RP: GTCATCCTTCGCCTGCTGTAG**	**64^{0}C**	**145 bp**
K12	**FP: CATGAAGAAGAACCACGAGGATG** **RP: TCTGCTCAGCGATGGTTTCA**	**63^{0}C**	**150 bp**
GAPDH	**FP: GCCAAGGTCATCCATGACAAC** **RP: GTCCACCACCCTGTTGCTGTA**	**63^{0}C**	**498 bp**

Table 3.2

Primer pairs for the p63 isoforms:

Gene	**Primer sequences (3'-5')**	**Annealing temp. ^{0}C**	**PCR product size**
Isoform-determining Regions TA domain	FP: TCGTAGAAACCCCAGCTCAT RP: TTGTTTGTCGCACCATCTTC	64^{0}C	199 bp
ΔNp specific region	FP: CTGGAAAACAATGCCCAGAC RP: TGGAGAGAGAGCATCGAAGG	64^{0}C	192bp
α specific region	FP: AGGGGCTGACCACCATCTAT RP: GTCTCACTGGAGCCCACACT	64^{0}C	196bp
α,β sharing region	FP: CCACAGATTGCAGCATTGTC RP: GTGAATCGCACAGCATCAAT	64^{0}C	304α bp, 210β bp
γ specific Region	FP: CCCGGAGAGAAACTCCAAA RP: TTGGGTCTCTGAGCCAAAGT	64^{0}C	211bp
TAp63α	FP: GAAGATGGTGCGACAAACAA RP: ATGATGAACAGCCCAACCTC	63^{0}C	1436bp
TAp63α,β	FP: GAAGATGGTGCGACAAACAA RP: ATCGCATGTCGAAATTGCTC	63^{0}C	1547α bp, 1453β bp
TAp63γ	FP: GAAGATGGTGCGACAAACAA RP: TTCCTGAAGCAGGCTGAAAG	63^{0}C	1130bp
DNp63α	FP: CTGGAAAACAATGCCCAGAC RP: ATGATGAACAGCCCAACCTC	63^{0}C	1390bp
DNp63α,β	FP: CTGGAAAACAATGCCCAGAC RP: ATCGCATGTCGAAATTGCTC	63^{0}C	1499α bp, 1405β bp
DNp63γ	FP: CTGGAAAACAATGCCCAGAC RP: TTCCTGAAGCAGGCTGAAAG	63^{0}C	1082bp

3.7 DNA sequencing

1. Amplification of specific sequence from DNA,
2. Electrophoresis of amplified products in 2% agarose gel,
3. Elution of DNA from agarose gel,
4. Cycle sequencing,
5. Purification of extension products,
6. Sequence analysis.

3.7.1 Gel Elution Procedure:

The PCR amplified products were eluted by Qiagen mini gel elution kit as per the instructions given in the kit insert.

- The DNA fragments were excised from the gel with a clean, sharp scalpel.
- 3 volumes of Buffer QG in the kit to 1 volume of gel.
- The vial is incubated at 50^0C for 10minutes/until the gel slice is completely dissolved. To help gel to dissolve, mix by vortexing the vial every 2-3 minutes during the incubation.
- Once the gel slice is completely dissolved, the color of the mixture is checked (should look similar to the Buffer QG without dissolved agarose).
- 1 gel volume of Isopropanol was added to the sample and mixed by inverting the tube several times.
- To bind the DNA the sample was applied to a mini elute column with a 2ml collection tube and centrifuged for 1minute.

- The flow through was discarded and the mini elute column is placed back in the collection tube.
- 500µl of Buffer QG was added to the spin column and centrifuged for 1minute.
- The flow through was discarded and the mini elute column is placed back in the collection tube.
- 750µl of Buffer PE was added to the mini elute column to wash and centrifuged for 1minute.
- The flow through was discarded and centrifuged the column for additional 1minute at 10,000 rpm.
- The mini elute column was placed into a clean 1.5ml vial.
- 10µl of Buffer EB (10mM Tris-chloride, P^H8.5 or water to the centre of the membrane in the column, and let it stand for 1minute and then centrifuge for 1minute.

3.7.2 Cycle Sequencing:

Cycle sequencing combines amplification and DNA sequencing using 5' dye labeled terminators.

3.7.3 Requirements for cycle sequencing:

- Forward primer or Reverse primer at the concentration of 2 pmol/µl.
- Big Dye Terminator cycle sequencing Ready reaction kit (ABI PRISM, USA)

3.7.4. Reaction Protocol:

- Ready reaction mix (RR mix) =1.0 µl
- 5x sequencing buffer = 3.0 µl
- II round forward/reverse primer (1pmol/µl) = 2.0 µl
- Milli Q water = 1.5 µl
- Eluted DNA = 2.5 µl

Total reaction: 10.0 µl

3.7.5. Reaction condition for cycle sequencing:

- Initial Denaturation - 96˚C for 1 minute.

  Followed by 25 cycles of

- Denaturation: - 96˚C for 10 seconds.
- Annealing: - 50˚C for 5 seconds.
- Extension: - 60˚C for 4 minutes.

3.7.6. Purification of Extension Product:

The extension products were purified to remove the unincorporated dye terminators before the samples were analyzed.

- To the 10 µl of cycle sequenced products, added 10 µl of Milli Q water in a 0.5ml microfuge vial.
- Then added 2 µl of 125mM EDTA (1 µl of 0.5M EDTA + 3 µl Milli Q water).

- To this added 2 µl of 3M-sodium acetate (pH 4.6) and 50 µl of chilled ethanol.
- Kept at room temperature for 15 minutes.
- Centrifuged at 12,000 rpm for 20 minutes.
- Discarded the supernatant and the pellet was washed twice with 200 µl of 70% ethanol at 12,000 rpm for 10 minutes.
- The vials were then dried at 37˚C and added 15 µl of formamide.

Again denatured at 90˚C for 3 minutes and immediately snap freezed

3.7.7 Loading of samples:

The sequence of the PCR amplified DNA is deduced with the help of the ABI Prism 3100 AVANT (Applied Biosystems, USA) genetic analyzer that works based on the principle of Sanger di deoxy sequencing.

The amplified products with the dye at the terminated 3'end were subjected to capillary electrophoresis by an automated sample injection. The emitted fluorescence from the dye labels on crossing the laser area were collected in the rate of one per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing. The sequences were then analyzed by software called as the sequence analysis softwares such as Bio Edit sequence alignment software and Chromas software. The sequencing was confirmed by www.ncbi.nlm.nih.gov/genbank.

3.8 Western Blot analysis:

The first step in a western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product. The light output can be captured using film. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.

3.8.1 Sample preparation:

The cultured cells once they reach the cells were harvested by gentle scraping using cell scraper and the protein was extracted using Pro-Prep protein extraction kit (InTron biotechnology, USA).

3.8.2 Protein extraction protocol:

- The cells are harvested from the cultures and collected in a vial.
- The harvested cells are centrifuged at 10000rpm for 5-10 minutes and the supernatant is discarded using a pipette.

- The obtained cell pellet is washed thrice with phosphate buffered saline at 10000rpm for 5minutes.
- The cell pellet is re-suspended in 400µl-500µl of the PRO-Prep solution and sonicated at 50-60 pixels for1min (thrice).
- The lysate was incubated at -20^0C for 10-30 minutes.
- The lysate was then centrifuged at 4^0C at 13000rpm for 45mins and the supernatant was collected in a fresh 1.5ml vial.
- The protein concentration in the collected supernatant was measured by the lowery method and the concentration was noted.

3.8.3 Protein Estimation:

Protein estimation was done by conventional Lowry method. The assay was run along side a standard curve BSA ranging from 0 to 2 mg/mL. The absorbance was read at 660 nm.

3.8.4 SDS-PAGE:

- SDS-PAGE was performed first- For this purpose 10% separating gel was prepared and poured in the assembled glass plates sealed with 2% agarose gel and left it without disturbing.
- The separating gel was covered with saturated butanol while left for polymerization.

- After the separating gel was polymerized 4% stacking gel was prepared and poured above the separating gel by removing the butanol layer and the comb was placed carefully with out any air bubble and left it a side for solidification.
- After the gel has been solidified the comb was removed care fully.
- The protein samples are mixed with 3x sample loading buffer to final volume of 50µl and incubated it at 95^0C for 2 to3mins.
- Then the sample was loaded on the gel and run till the dye reaches the bottom of the gel.
- The gel was taken care fully from the glass-assembled plates. The loading order was noted by giving a small cut at one end.

3.8.5 Immunodetection:

The proteins were transferred to nitrocellulose of Polyvinilidine Fluoride membrane at 100V for one hour in cold transfer buffer. Once transferred the membrane was stained with 0.5% Ponseau to the check whether the transfer was done properly. Then the blots were washed with Milli Q water and blocked with 10% blocking solution. Primary antibodies were incubated 0.5mg/ml in 1X casein solution for 2 hours to overnight at room temperature. The blots were washed 3 times with TBST buffer 30 minutes each. The presence of the specific proteins was then shown by chemilumniscence of HRP via ECL + plus western blot detection system (Pierce, US) The blot was exposed to X-ray (Kodak X ray film) film for about 5 to 10 mins to record the result and developing was done.

3.9 Proliferation kinetic study by BrdU labeling

3.9.1 BrdU incorporation and chasing experiment:

Thymidine or BrdU labeling has been successfully used to identify 'label retaining' stem cells that are slow cycling or mitotically quiescent (Cotsarelis *et al*., 1989; Lauweryns *et al*., 1993). The substitution of an endogenous DNA base, thymidine, with the analogue BrdU allows specific labeling of only the dividing cells. Once the slow cycling cells have been labeled, they should retain this label for much longer period while other more mitotically active cells will loose the label through multiple mitosis. The cultured cells for which the label retaining property has to be studied were incubated with 10mM of Brdu continuously for 24 hours the cultures were chased for 1–21 days by switching to BrdU free medium. Then the presence of the label retaining cells was detected by the following method.

1. Flat mount preparation was done for the cultures which was chased for the Brdu and the slides were fixed in ethanol fixative
2. The fixed slide was washed with the wash buffer thrice provided in the kit. The wash buffer was diluted 1:10 dilution with the double distilled water.
3. The slides were then incubated with anti BrdU antibody (1:10) dilution with the incubation buffer provided in the kit. The slides were then incubated for 30 mins at 37^0C in the moist chamber.
4. After incubation the slides were washed thrice with wash buffer.
5. IgG fluorescein antibody 1:10 diluted with PBS was added and incubated at 37^0C for 30 mins

6. The slides were washed thrice with the wash buffer and mounted with glycerol and observed under the phase contrast microscope.

The BrdU labeling indices were assessed by the counting through a microscope using 40X objective. A total of 500 to 911 nuclei were counted in 6–8 representative fields. This number (500 counted nuclei) was considered as a minimum requirement to obtain representative figures. The labeling index was expressed as the number of positively labeled nuclei/the total number of nuclei X 100%.

4.0 Impression cytology

Impression cytology of cornea/conjunctiva using millipore paper:

This technique is useful in evaluating patients with dry eyes, whose samples are examined for the presence or absence of goblet cells and changes of other epithelial cells. During this process, bits of cellulose acetate filter paper /millipore paper (pore size of 0.2μ) is applied to the cornea/ conjunctival surface, gently pressed for about 30 seconds and removed. Superficial epithelial cells that adhered to the paper is then stained after it is fixed in 95% alcohol either by H&E, PAS or by modified technique as required

Staining procedure

Haematoxylin-eosin stain Modified Technique:

0.5% Periodic acid- -----2minutes

Rinse in tap water-

Schiff reagent freshly diluted in distilled water 1:3in -2 minutes

D.W …….10 dips

Tap water-----2minutes

0.5% Sodium Metabisulphite ----2 minutes

Tap water……..10 dips

Haematoxylin……..2 minutes

Scott's Tap water substitute……..10 dips

D.W ……2 minutes

Tap water……..10 dips

Dehydration in 95% Alcohol- 10 dips

Modified Orange G solution……2 minutes

95% Alcohol……3 minutes

Eosin Y solution…….2 minutes

95% Alcohol……5minutes

Absolute alcohol…….2 minutes

Xylene…….5 minutes

The slides were then dried and mounted

CHAPTER 4 - USE OF INTACT AND DENUDED AMNIOTIC MEMBRANE FOR LIMBAL STEM CELL CULTIVATION

4.1 Introduction

Human amniotic membrane is an important substratum for the growth of corneal stem cells and also for ocular surface reconstruction (Dogru and Tsubota *et al* 2005; Gomes *et al*, 2005; Koizumi *et al* 2001) due to many properties such as (a) AM basement membrane facilitates epithelialization and maintains an epithelial phenotype; (b) the avascular stroma of AM exerts anti-inflammatory, anti- angiogenic and anti-scarring effects. AM with its epithelium removed is used as substrata for culturing corneal stem cells. However, intact AM (i.e., retaining devitalized AM epithelium) appears to offer more favorable microenvironment for the expansion of stem cell-containing limbal epithelium than denuded AM (i.e., exposed AM basement membrane) (Meller *et al* 2002; Grueterich and Tseng *et al* 2002a, 2002b)

Clinically, limbal epithelial cells from the limbal explants expanded over AM in cell cultures can restore the corneal surface with partial and total limbal stem cell [SC] deficiency.(Tseng *et al*, 2001; Grueterich *et al*, 2003; Tsai *et al*, 2000) There are 2 different approaches for using AM as a matrix for corneal stem cell cultures. In the first method, AM with its epithelium removed (Denuded AM) is used as substrata for culturing corneal stem cells. (Meller *et al*, 2002 ;) In the second method, intact AM (i.e., retaining devitalized AM epithelium) is used to grow the corneal stem cells. (Grueterich *et al*, 2002a 2002b ;) There are numerous studies showing the advantages of *ex vivo* expanding the limbal explants on the intact AM as it provides a more favorable

microenvironment for expansion of corneal stem cells. (Koizumi et 2000; Galindo *et al*, 2003; Grueterich *et al*, 2002b ;) Previous studies (Galindo *et al*, 2003; Grueterich *et al* 2002a) have shown that the limbal explants expanded on intact AM retain a limbal phenotype, i.e. they do not express the gap junction markers connexin 26 and connexin 43, which allows the cells to be segregated from the transient amplifying cells. (Wolosin *et al*, 2000) and also do not express the cornea phenotype markers, K3/ K12 (Galindo *et al* 2003; Grueterich *et al*, 2002b ;) And moreover that when we use human amniotic membrane with intact epithelial cells immunity is not an issue because very small amount primary human amniotic epithelial cells contain class I A and class II HLA antigens, consistent with a low risk of tissue rejection.

Similarly ABCG2 is a member of the ATP-binding cassette (ABC) family of cell surface transport proteins that includes more than 50 members and mediates the transfer of a diverse array of substrates across cellular membranes. ABCG2 expression occurs in a variety of normal tissues and is relatively limited to primitive stem cells. De Paiva *et al* identified this transporter protein in the population of the clonogenic human limbal epithelial cells. (Cintia *et al*, 2005 ;) There is not much information available on the expression of the putative stem cell marker ABCG2 on the limbal epithelial cells expanded over intact AM and denuded AM.

Therefore in this study, we investigated the expression of the Putative stem cell marker, ABCG2 on the limbal epithelial cells expanded on the cryopreserved intact and epithelially denuded membrane. In addition we also compared the p63, connexin 43 and K3/K12 expression on these cultured cells over the intact and denuded AM.

4.2 Design of experiments:

4.2.1 Culturing of corneal limbal cells on the intact and denuded Amniotic membrane:

Preparation of Human Amniotic Membrane

Human amniotic membrane was prepared according to procedure mentioned in the methodology section and preserved and used for the cultivation of limbal stem cells.

Limbal Biopsy preparation

Corneal limbal biopsy of 2 mm^3 from the cadaveric donor eye was collected in DMEM with 3% FCS and antibiotics as the transport medium and was transported to the cell biology laboratory for further processing. The biopsy was processed as per the procedures mentioned in the methodology section. A total of 20 limbal biopsies were obtained from the cadaveric donor eyes.

Human Limbus culture on Amniotic membrane

The limbal explants were placed on the intact and denuded human amniotic membrane as mentioned in the methodology and the explants were covered with the DMEM and F12 reconstituted with growth factors.

4.2.2 Proliferation study by Brdu labeling

Once the outgrowth has reached a confluence the cultures were incubated with fresh medium containing 10-µM of Brdu for 24 hours (n = 8) for the cells cultured over the intact AM and n = 8 for the cells over the denuded AM). The cultures were shifted to the BrdU free medium and chased at the end of 24hours, 7^{th} day, 14^{th} day and 21^{st} day. All samples in triplicate were fixed in cold methanol at 4^0C for 10 min and processed for

BrdU immunofluorescent staining as previously described (Li and Tseng, 1995; Li *et al*., 2001). Brdu labeling indices were assessed according to the procedure mentioned in the methodology.

4.2.3 Comparison of the expression of markers by Immnunohistochemistry, RT-PCR and Western blot

After 3 weeks of incubation the cells were harvested for the marker study. The cultures were processed for the immnunohistochemistry for p63 and ABCG2, RT-PCR was carried out for ΔNp63, ABCG2, Connexin 43 and K3/K12. Further the expression of the markers p63 and ABCG2 was confirmed by western blot. All the procedures were followed according to the protocol given in the Methodology section.

4.3 Results:

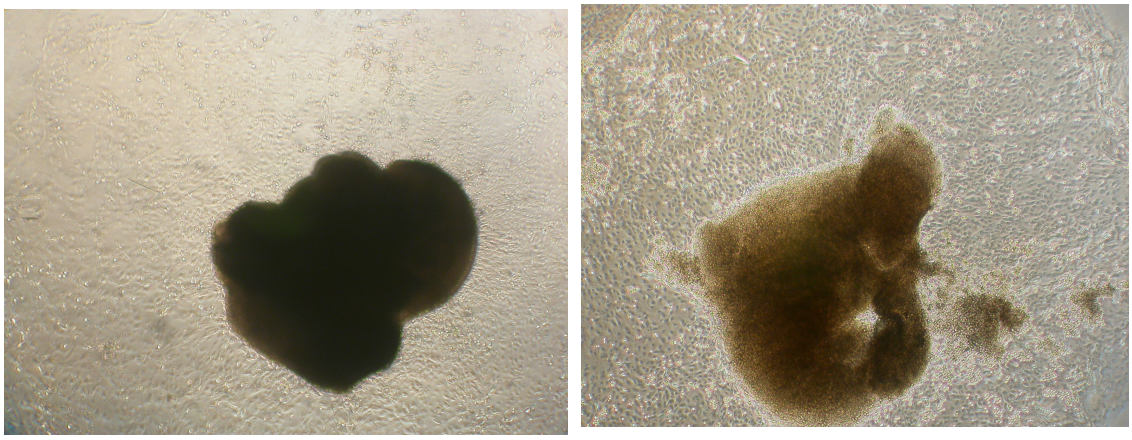
4.3.1 Culture results:

Cultivation of corneal limbal biopsy on the intact and denuded membrane:

A total of 20 limbal biopsies were obtained during the 6 month period between November 2005 – April 2006. The limbal biopsies were collected from the donor aged 8 – 85 enucleated within 6 to 8 hours after death, and were collected in the Transport medium. (DMEM with 3%FBS and the antibiotic mixture, Penicillin, Streptomycin and amphotericin) All the 20 specimens were used for the culture over the intact and denuded membrane. **Figure 4.1** shows the growth of the cells on the intact and denuded membrane. The outgrowth rate of the 10 cultures was photographed every 2nd day and the image was transferred to the computer and analyzed using the quantity G area

Figure 4.1

Growth of limbal epithelial cells on the intact and denuded Amniotic membrane



4.1 A **4.1 B**

This figure shows the growth of the limbal epithelial cells from the explants at the end of two weeks of incubation. Figure **4.1A** shows the growth on the intact amniotic membrane and **4.1B** on the denuded Amniotic membrane. The expanded cells appear as a monolayer of small uniform with nucleus cytoplasmic ratio of approximately 1:1 (20X Mag)

measurement software. Mean radius of the all the cultures was at each day was plotted and the culture medium was changed every 3rd day until they reached confluence. The cells cultured over the denuded AM showed significantly a higher growth rate than those on the intact membrane and reached almost a confluent growth after 12 days. The growth rate was faster on the cells cultured over the denuded AM when compared to the intact. ($p<0.05$) **(Figure 4.2)** The cultures on the denuded membrane showed from day 2 onwards whereas those on intact membrane began to grown only from day 4.

4.3.2 Immunohistochemistry results:

ABCG2 and p63 expression by immunohistochemistry:

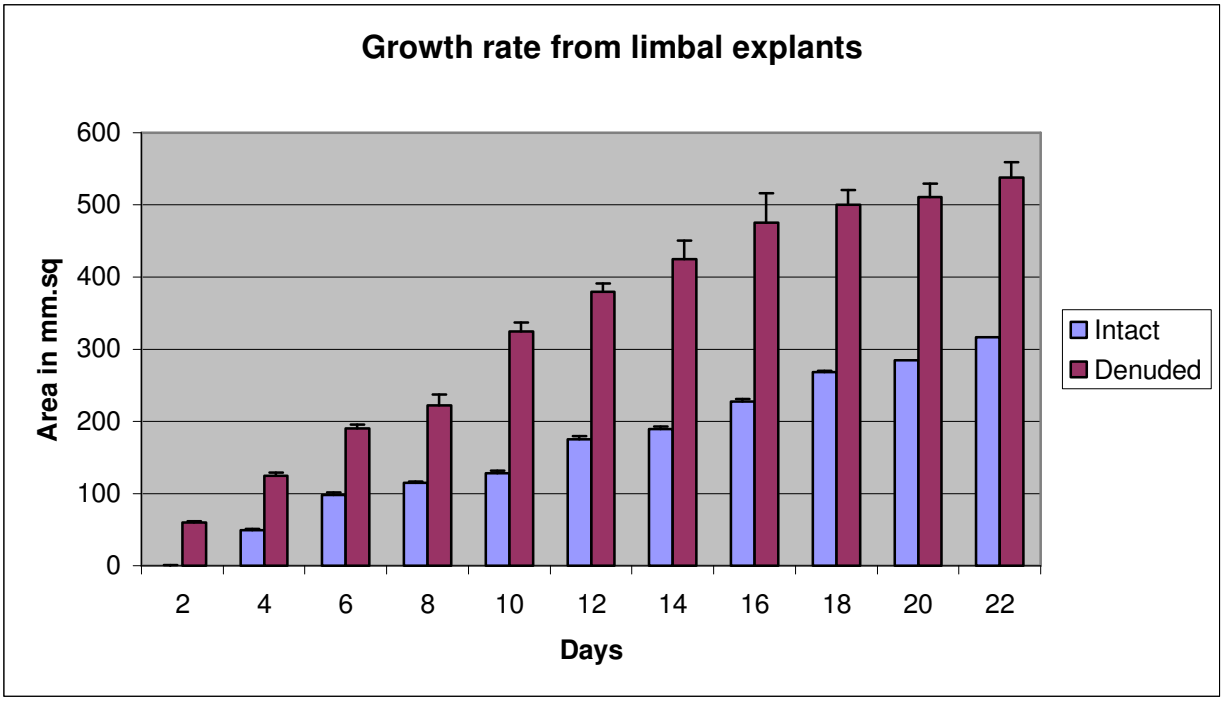
After 3 – 4 weeks of culture on the intact and denuded membrane the cultures showed stratification. The basal epithelium of the cells cultured over the intact epithelium showed the ABCG2 expression and there was negative expression for ABCG2 on the cells cultured over the denuded membrane. Positive expression for ABCG2 showed the staining of the cytoplasm only by the few basal cells on the cells cultured over the intact membrane. And for the p63 staining strong nuclear staining was seen on the cells cultured over both intact and denuded AM. **(Figure4.3)**

4.3.3 RT-PCR

Semiquantitative RT-PCR results show the expression of various markers such as p63, ABCG2, Connexin 43 and K3/K12 at the end of 21st day of cultivation on the cells grown over the intact and denuded AM. The expression of ΔNp63 is more on the cells

Figure 4.2:

Growth rate of limbal epithelial cells on the intact and denuded amniotic membrane



This figure show Outgrowth rate on the cells cultured over the intact and denuded AM: A significantly faster growth rate was observed on the denuded membrane (pink bar) than in intact membrane (blue bar ;) Limbal epithelial cells on the intact membrane started to grow only on the 3rd day compared to that of the denuded membrane which started to growth by the end of 2nd day and after approximately of 3 weeks they reached the confluence

Figure 4.3:

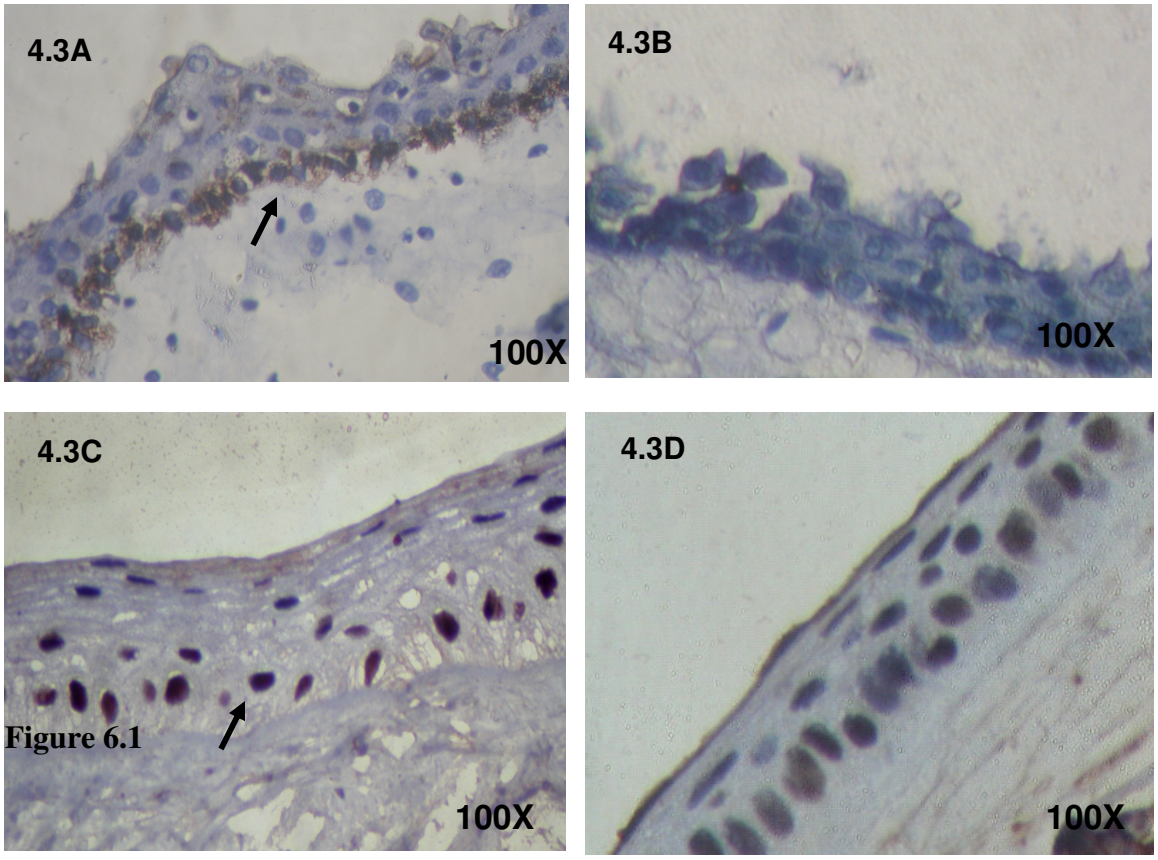


Figure 4.3 shows the immunohistochemistry picture of the limbal epithelial cells cultured on the intact and denuded membrane harvested at the end of the 3weeks. **4.3A** shows the ABCG2 expression on the cells cultured over the intact membrane basal layer of cells showing the positive expression. **4.3B** - cells on the denuded membrane there was no expression. **4.3C** p63 expression on the cells cultured over the intact. Positive cells are seen as the brown nuclei. **4.3D**- p63 staining on the denuded Amniotic

cultured over the intact membrane when compared to the cells cultured over the denuded membrane. ABCG2 expression was observed only on the cells cultured over the intact membrane and it was completely absent on the cells cultured over the cells cultured over the denuded membrane. Similarly, we compared the expression of the Connexin 43 and K3/K12 on the cells cultured over the intact and denuded membrane. The cells cultured over the intact amniotic membrane showed the negative expression of the Connexin 43 whereas cells cultured over the denuded AM showed high expression of the Connexin 43. Expression of Keratin 3 and 12 was high on the cells cultured over the denuded membrane whereas expression of K3/K12 was less on cells cultured on the intact membrane. (**Figure 4.4 A & B)**

4.3.4 Western Blot

Expression of the p63 and ABCG2 was confirmed with the Western blot. Proteins were extracted from the cells cultured over the intact and denuded membrane at the end of 21 days of incubation. The cells harvested from the intact membrane at the end of 3 weeks showed the expression for ABCG2 whereas cells on the denuded showed did not show. Expression of p63 (clone 4A4) was present in both. **Figure 4.5**

4.3.5 Brdu labeling index on the cells cultured over the intact and denuded membrane

Thymidine or BrdU labeling has been successfully used to identify 'label retaining' stem cells that are slow cycling or mitotically quiescent (Cotsarelis *et al*., 1989; Lauweryns *et*

Figure 4.4 a

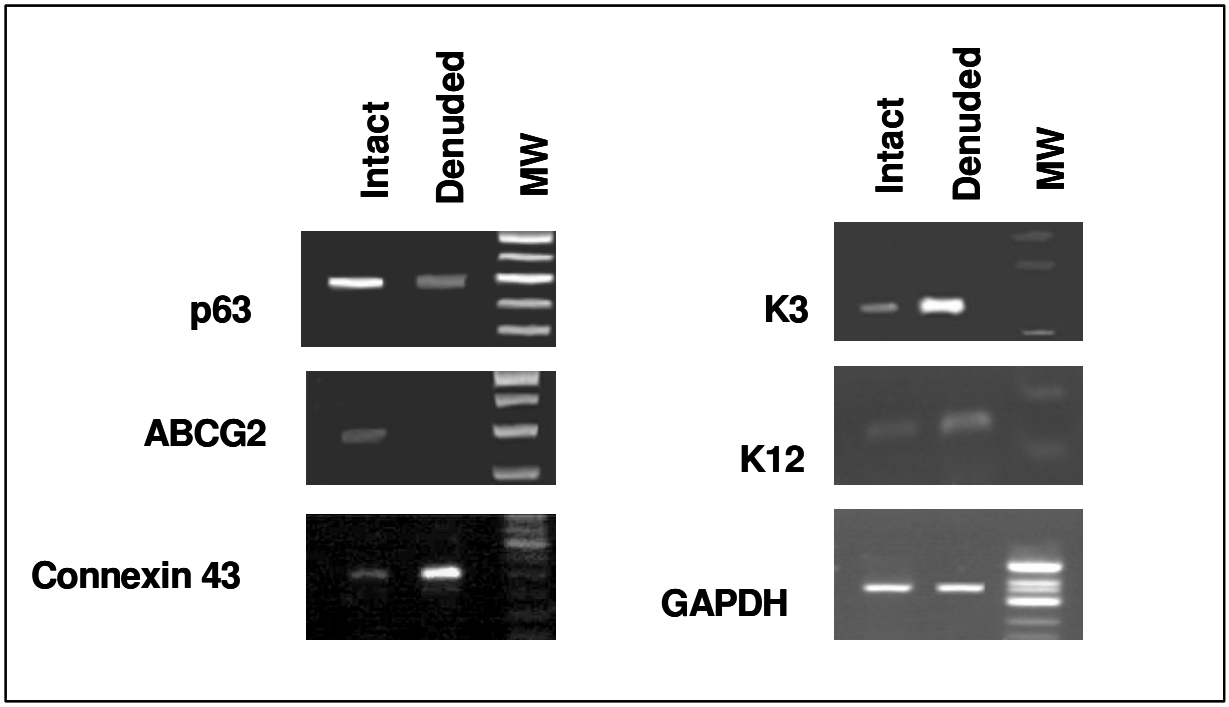


Figure 4.4A shows the electrophoretogram of the semiquantitative RT-PCR. The figure shows the expression p63, ABCG2, connexin-43, Keratin 3 and K12 on the cells cultured over the intact and denuded membrane. GAPDH is the internal control. MW- Molecular weight *ΦX Hinf I* digest

Figure 4.4 b

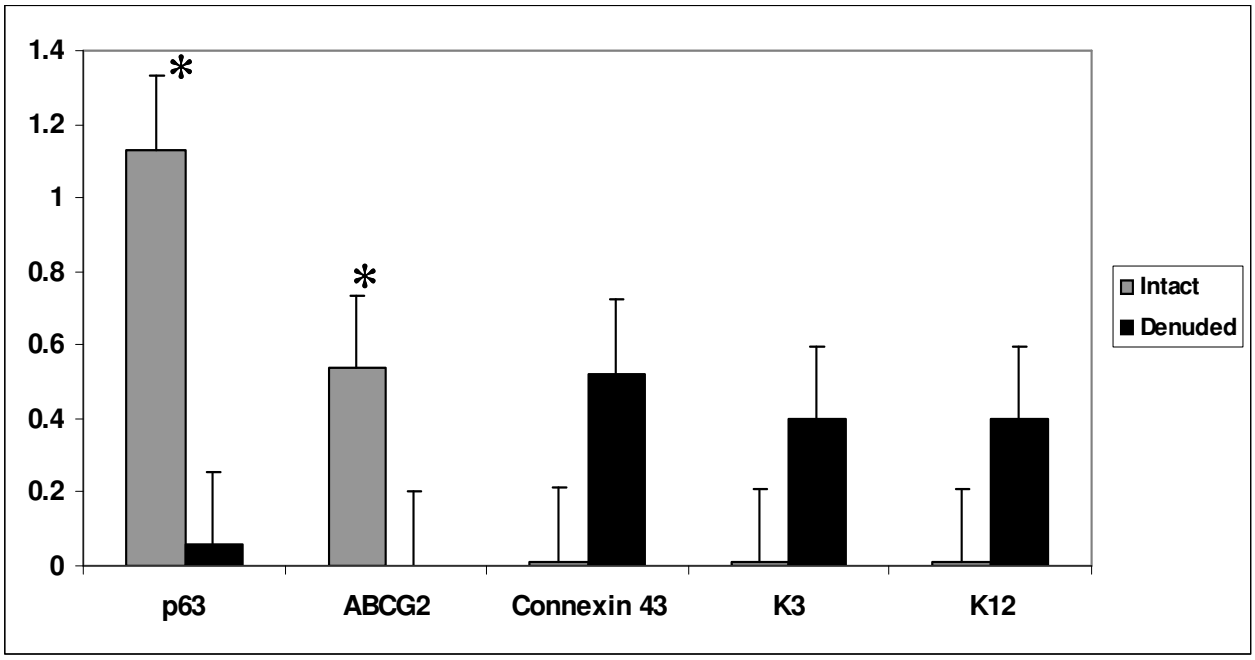


Figure 4.4 b shows the RT-PCR results on the cells harvested from the intact and denuded membrane. ΔNp63 and ABCG2 expression was more on the cells cultured over the intact amniotic membrane (∗p<0.001). Connexin -43, K3/K12 expression was more on the cells harvested from the denuded Amniotic membrane.

Figure 4.5 Western blot results

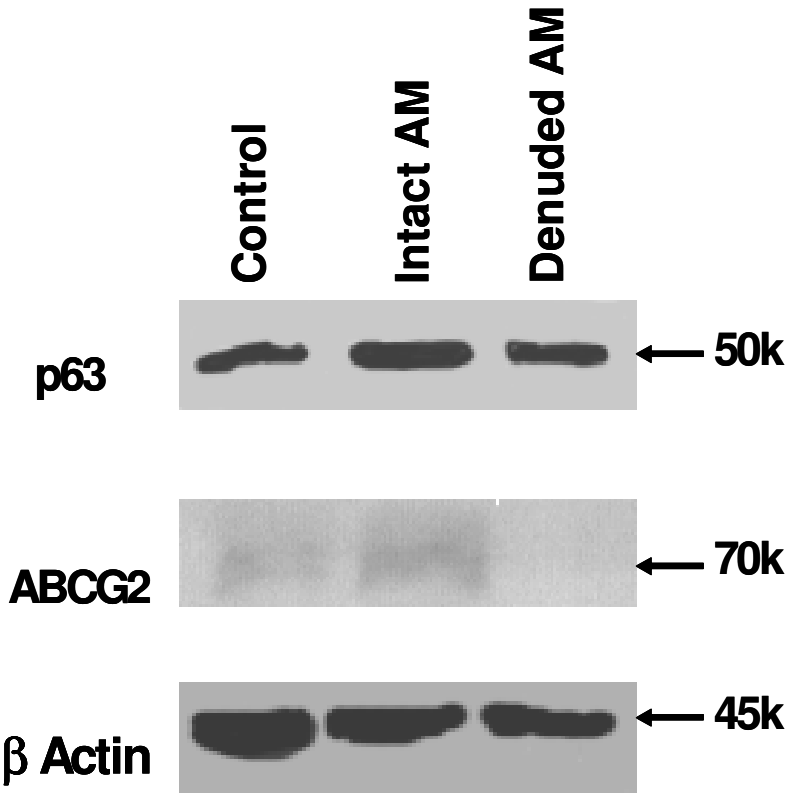


Figure 4.5 shows the western blot data on the cells harvested at the end of 3 weeks on incubation on the intact and denuded membrane. The positive expression of p63 was observed on both intact and denuded membrane. ABCG2 expression was completely absent on the cells harvested from the denuded membrane. Control lane – p63 – SiHa cell lysate; control lane – ABCG2 – MCF 7 cell lysate. β actin shows the loading control.

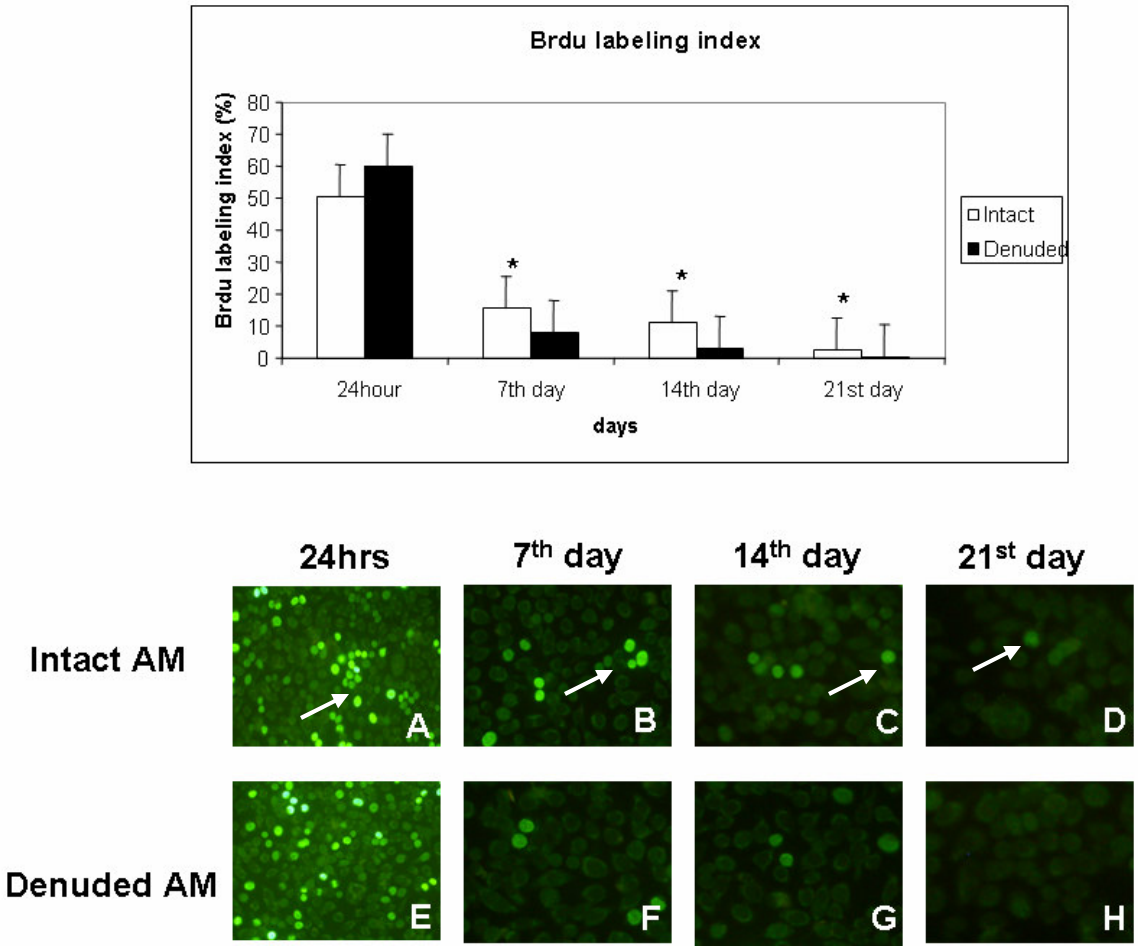
al., 1993). The substitution of an endogenous DNA base, thymidine, with the analogue BrdU allows specific labeling of only the dividing cells. Once the slow cycling cells have been labeled, they should retain this label for much longer period while other more mitotically active cells will loose the label through multiple mitosis.

In this study, we labeled the both cultures with BrdU containing medium for 24 hr at their early growth stage (about 1 week). The labeling index was high on denuded AM (64.0±7.76%) when compared to the intact AM on 1 day i.e. after 24 hours. After 24hours of labeling the cultures were chased continuously at the end of 7th day, 14th day and 21st day. On the 7th day it was 15.3.0±2.24% and on the 14th day it was 10.94±2.0% and it was 2.35±0.2% on the 21st day on the cells cultured over the intact membrane. Similarly on the denuded AM the labeling index was 7.85±3.2% on the 7th day, on 14th day it was 3.222±0.33% and on the 21st day it was 0.6±0.002%. **(Figure 4.6)**

4.4 Discussion:

The application of intact or denuded AM as a substrate for the *ex vivo* expansion of the Human Limbal epithelial cells (HLEC) has been considered as the effective method for the reconstruction of the ocular surface. However, the follow-up periods reported in the clinical studies are still too short to define the longevity of transplanted HLEC, a well known characteristic sign of stem cells. (Schawab *et al*, 2000; Tsai *et al*, 2000, Koizumi *et al*, 2001a, 2001b ;) Grueterich *et al* and Meller *et al* have demonstrated that the *ex vivo* expanded HLEC share the properties similar to the limbal epithelial cell progenitor in vivo. But the underlying mechanism which preserves the progenitor properties of the

Figure 4.6 Brdu Labeling index



Identification of label retaining cells in human corneal epithelial cultures. After 24 hr of Brdu labeling at one week of early growth stage, 64.0% on the cells cultured over the denuded membrane (E) and 51% on the cells cultured over the intact membrane (A) the cultured limbal epithelial cells from the explants have positively stained nucleus. In the cultures the labeling index decreased on the chasing. The index was 15.3%, 10.9, 2.3% after chasing for 7 (B), 14 (C), and 21 days (D) respectively. The cells on the denuded membrane showed 7.8%, 3.2%, 0.6% on the 7th day (F), 14th day (G) and 21st day (H). Arrows indicate the positive nucleus. The top figure there was significantly higher percentage of label retention on cells cultured over the intact membrane at the end of 7th, 14th and 21st day. (*$p<0.05$)

cultured limbal epithelial cells is not clearly understood. (Meller *et al*, 2002; Grueterich *et al*, 2002b) Grueterich *et al* has reported that the denudation of the devitalized AM promotes the corneal TAC differentiation. These important data suggested that during *ex vivo* expansion intact membrane seems to preserve the limbal progenitor cell properties. (Grueterich *et al*, 2002 ;)

Our data shows that cells cultured over the intact membrane have a slower growth rate when compared to that grown over the denuded AM. The reason might be due to the delayed onset of growth on the intact membrane. Our results are consistent with the results of the Grueterich *et al* (2000) and Koizumi *et al* (2000) where they studied using rabbit limbal and corneal explants.(Koizumi *et al*, 2000;)

Comparison of the Connexin and Keratin expression was done by many workers. Grueterich *et al* and Tseng *et al* has reported that the cells cultured over the intact membrane lack the Connexin 43 and they express low levels of keratin and they were able to retain the label retaining cells.(Grueterich *et al*, 2002;) ABCG2 transporters have been identified as a molecular determinant for bone marrow stem cells, and it has been proposed as a universal marker for stem cells from a variety of sources. (Goodell *et al*, 1996; Zhou *et al*, 2001;) Although ABCG2 expression occurs in a variety of normal tissues, its expression and functional role in corneal epithelial cells has been only recently reported. (Chen *et al*, 2004; Watanabe *et al*, 2004;) de Paiva *et al* (2004) has shown the exclusive expression pattern of ABCG2 transporter by the basal cells of human Limbal epithelium and its potential role in identifying the corneal epithelial stem cells.

We compared the ABCG2 expression and p63 expression on the cells cultured over the intact and denuded membrane by immnunohistochemistry. Basal cells on the intact AM shows the expression of the ABCG2, whereas it was absent on the cells cultured over the denuded AM. p63 gave similar pattern of expression on the cells cultured over the intact and denuded AM. We also compared the expression of ΔNp63, ABCG2 along with the Connexin 43 and Keratin 3 & 12 by semiquantitative Reverse Transcriptase PCR. Expression of ABCG2 was seen on the cells cultured over the intact membrane harvested at the end of 4^{th} week ($P < 0.001$), whereas it was completely absent on the cells cultured over the denuded membrane. ΔNp63 expression was seen on both, but the expression was comparatively lesser on the cells cultured over the denuded membrane when compared to the intact membrane. Similarly the expression of other markers like Connexin 43 and keratin 3 & 12 was expressed in low levels on the cells over the intact membrane. Expression of p63 and ABCG2 was confirmed by the Western blot. Thus our data is supportive to the earlier reported results by other workers. (Grueterich *et al*, 2002a; 2002b; Galindo *et al*, 2003; Koizumi *et al*, 2001a)

Although several stem cell markers have been proposed, their role in identifying keratinocyte stem cells is still very controversial. Therefore, the identification of stem cells relies on either evaluating their proliferative capacity *in vitro* (Barrandon *et al*, 1987;) or identifying slow-cycling cells ([3H]-thymidine- or BrdU-label retaining cells) in vivo. (Cotsarelis *et al*, 1989; Lavker *et al*, 2000 ;) Cells that retain these labels over a

long period (label retaining) are slow cycling, which is one known characteristic of epithelial stem cells in vivo.

The labeling index was high initially on the cells cultured over the denuded membrane which shows that the rate of proliferation was high on the denuded AM when compared to the intact membrane. But the chasing experiment revealed that the cells cultured over the intact membrane were able to retain the label till the end of 21st day when compared to that on the denuded membrane, which was observed only till day 14. ($p<0.05$). The high labeling index at 1 day indicated that BrdU was incorporated into the DNA during S phase in all mitotic cells, including the stem cells and transient amplifying cells. The decreased labeling index after chasing indicated that the Brdu-labeled transient amplifying cells (rapid cycling cells) were reduced in number or disappeared, while the Brdu-labeled stem cells still remained. Some of them had a cell cycle length of at least 21 days. The low labeling index noted after chasing for 7–21 days was indeed the result of slow cycling cells.

4.5 Conclusion:

Thus Limbal epithelial cultured on the intact AM, the basal layer of the cultured cells on expresses ABCG2 and there is low expression level of Connexin – 43 and keratin 3 & 12. The cultured cells were also able to retain the label till 21 days of incubation resembling the phenotype of stem cell containing human Limbal basal epithelial cells in vivo. Devitalized amniotic membrane with the epithelium mimics the in vivo stromal niche to maintain the stem cell characteristics.

CHAPTER 5 - USE OF NON POLYMERIC SCAFFOLDS IN CULTIVATION OF LIMBAL STEM CELLS

5.1 Introduction

In the recent years the treatment of severe ocular surface disorder is the transplantation of the cultured limbal stem cells on the human amniotic membrane (HAM) as the carrier for the cells. (Tan *et al*, 2004; Kim *et al*, 1995; Meller *et al*, 2002; Ti *et al*, 2002; Koizumi *et al*, 2001, 2000; Ang *et al*, 2004a; 2004b) Although the results are quite promising, this new procedure is still facing some challenges. However, the use of biological material is associated with the risks of disease transmission (e.g., HIV, hepatitis B and C, and bacterial and fungal infections) In addition; many of the substrates lack the mechanical properties that allow easy handling and suturing, as well as prolonged endurance after transplantation.

There are serious issues regarding the procuring and storing HAM. As a natural product HAM consistency cannot be controlled, and the physical structure of HAM does not provide significant mechanical strength to act as a suitable base for support of sclera or cornea and it is not optically clear. To overcome the disadvantages of HAM, it would be better to use synthetic and optically clear material that will replace HAM. The material used as an alternative and should be biocompatible and it should be durable, suturable and gas permeable and allow the free diffusion of glucose, ions and other growth factors.

In recent years one such functional biomaterial which has drawn attention in the tissue engineering field is Chitosan CS-based material. (Khor *et al*, 2003 ;) CS is a deacetylated derivative of chitin, a high molecular weight, second most abundant natural biopolymer commonly found in shells of marine crustaceans and cell walls of fungi. CS is a linear polysaccharide, composed of glucosamine and *N*-acetyl glucosamine linked in a β (1–4) manner; the glucosamine/*N*-acetyl glucosamine ratio being referred as the degree of deacetylation). It's chemically similar to cellulose, which is a plant fiber. Like plant fiber, chitosan accomplishes many of the same properties as fiber. However unlike plant fiber, it has the activity to significantly bind fat acting like a "fast sponge" in the digestive tract. It's also structurally identical to cellulose, but it has acetamide groups (NHCOCH3) at the c-2 positions Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000 KD with a degree of deacetylation from 30% to 95%. , CS is normally insoluble in aqueous solutions above pH 7; however, in dilute acids (pH<6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule. (Dornish *et al*, 2001; Athanasiou *et al*, 2001; Madihally *et al*, 1999 ;)

The cationic nature of CS is primarily responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to GAG (mostly with heparin and heparan sulphate), and a matrix incorporating a CS–GAG complex may retain and concentrate growth factors secreted by colonizing cells. (Madihally *et al*, 1999) The present study was undertaken to investigate the Chitosan as a matrix for culturing corneal limbal stem cells and to characterize the

cells cultured on the matrix and also to study the growth characteristics on the cells cultured over the chitosan and its derivatives.

5.2 Design of experiments:

5.2.1 Preparation of chitosan matrix:

Prawn shells were collected from local fish market of flesh and washed well with distilled water. The experimental procedure for isolation of chitin can now be divided in to three stages.

Deproteinization:

The chitin in the prawn shells is associated with proteins, inorganic materials that are mainly $CaCO_3$ pigments and liquids. The deproteinization was carried out by soaking the washed prawn shells, which were obtained after cleaning, in 5% NaOH solution and followed by refluxing for 2 to 3 hours. The NaOH solution was decanted and washed with distilled water and the pH was brought to 7.

Protein + chitin + NaOH $\rightarrow$ Amino acid + dipeptide + tripeptide + chitin

Demineralization:

The demineralization was carried out by soaking the shells obtained from deproteinization step, in 2N HCl for 4 hours. The HCl solution was decanted and washed with distilled water and the pH was brought to 7.

$$CaCO_3 + HCl \rightarrow CaCl_2 + H_2O + CO_2$$

Decolorization:

The exo-skeletons of crustacean contain coloring matter. Principally carotenoid, they do not appear to be complexes with inorganic materials or protein since treatment, which remove the carotenoids. Alternatively bleaching it with diluted $KMnO_4$ or Acetone may destroy the coloring matter; the pure chitin so obtained is oven dried and crushed to a thin powder, the structure of chitin is verified by IR-spectra. **Figure 5.1** shows the sequential steps involved in the preparation of the chitosan scaffold.

Preparation of Chitosan from pure Chitin:

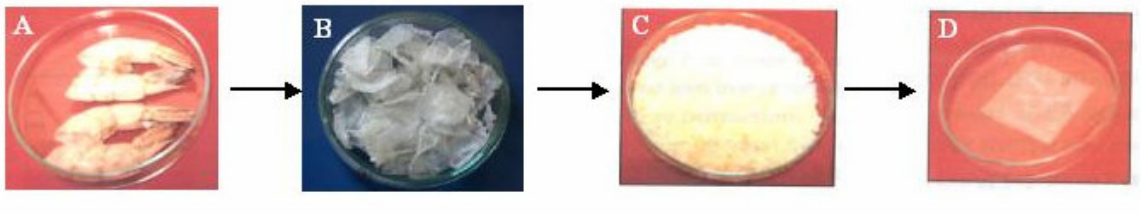
The pure chitin obtained was treated with 40 % NaOH and autoclaved at about 180^o C for 2 to 3 hours. The contents were routinely checked for their solubility in HCl (0.1N) solution or acetic acid (0.3N). Once the contents were dissolved in acetic acid or HCl, the same was removed from the autoclave and washed thoroughly with water to bring pH 7, and dried well.

Preparation of gold and silver nanocrystals:

All the chemicals were procured from Sigma Aldrich Chloroauric acid ($HAuCl_4$), Silver Sulphate ($AgSO_4$) and Sodium borohydride ($NaBH_4$) were obtained from Aldrich Chemicals and used as received. In a typical experiment 50 ml of chloroauric acid ($HAuCl_4$) (10^{-4}M) and 50 ml of silver sulphate ($AgSO_4$) (10^{-4}M) were taken in a beaker under stirring condition to this solution 0.1M of sodium borohydride was added. The color of chloroauric acid changes to ruby red color and the color of silver sulphate

Figure 5.1

Sequential steps involved in the preparation of the chitosan scaffold



This figure shows the sequential step involved in the preparation of chitosan scaffold. A is the collected prawns washed and B shows the shells after demineralization and Decolorization. C shows the preparation of chitosan and finally they are molded into chitosan sheets (D)

changes to yellow color, this shows the presence of colloidal gold and silver nanoparticles.

Incorporation of gold and silver nanoparticles in chitosan scaffold

0.1g of chitosan was dissolved in 100ml of 0.3N acetic acid. To this solution 25ml of gold solution and 25ml of silver were added separately and cast into a film and dried at RT over night. Thus the gold and silver nanoparticles were incorporated in the chitosan scaffold.

5.2.2 Characterization of the chitosan scaffold:

Tensile Test:

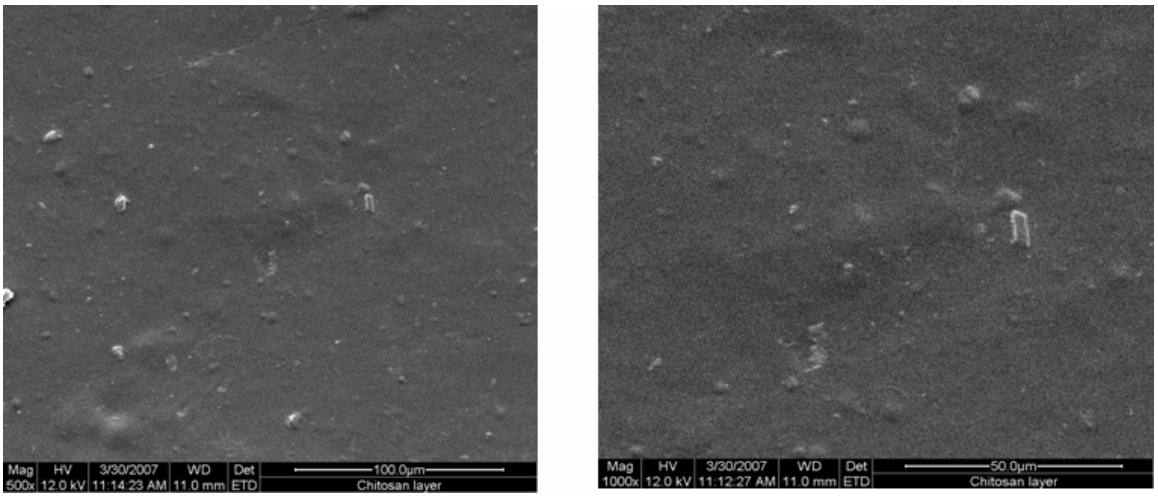
The tensile tests were carried out using an Instron universal material testing system (model 5567) at room temperature with gauge length of 10 mm and crosshead speed of 5 mm/min. Property values reported here represent averaged results for at least six specimens.

Scanning electron microscopy:

The samples in film form were coated with gold ions using an ion cater (Fisons sputter coater) under the following condition: 0.1 Torr pressure, 20mA current, and 70s coating time. Surface morphology was studies by scanning electron microscope (SEM model JSM 5300) **Figure 5.2** shows the scanning electron microscopic picture of the chitosan matrix.

Figure 5.2

Scanning Electron Microscopic picture of the chitosan sheet



This picture shows the scanning electron picture of the molded chitosan matrix. There surface is uniform and smooth. Left image magnification 500X and right image magnification 1000X

Infrared spectroscopy (FT-IR):

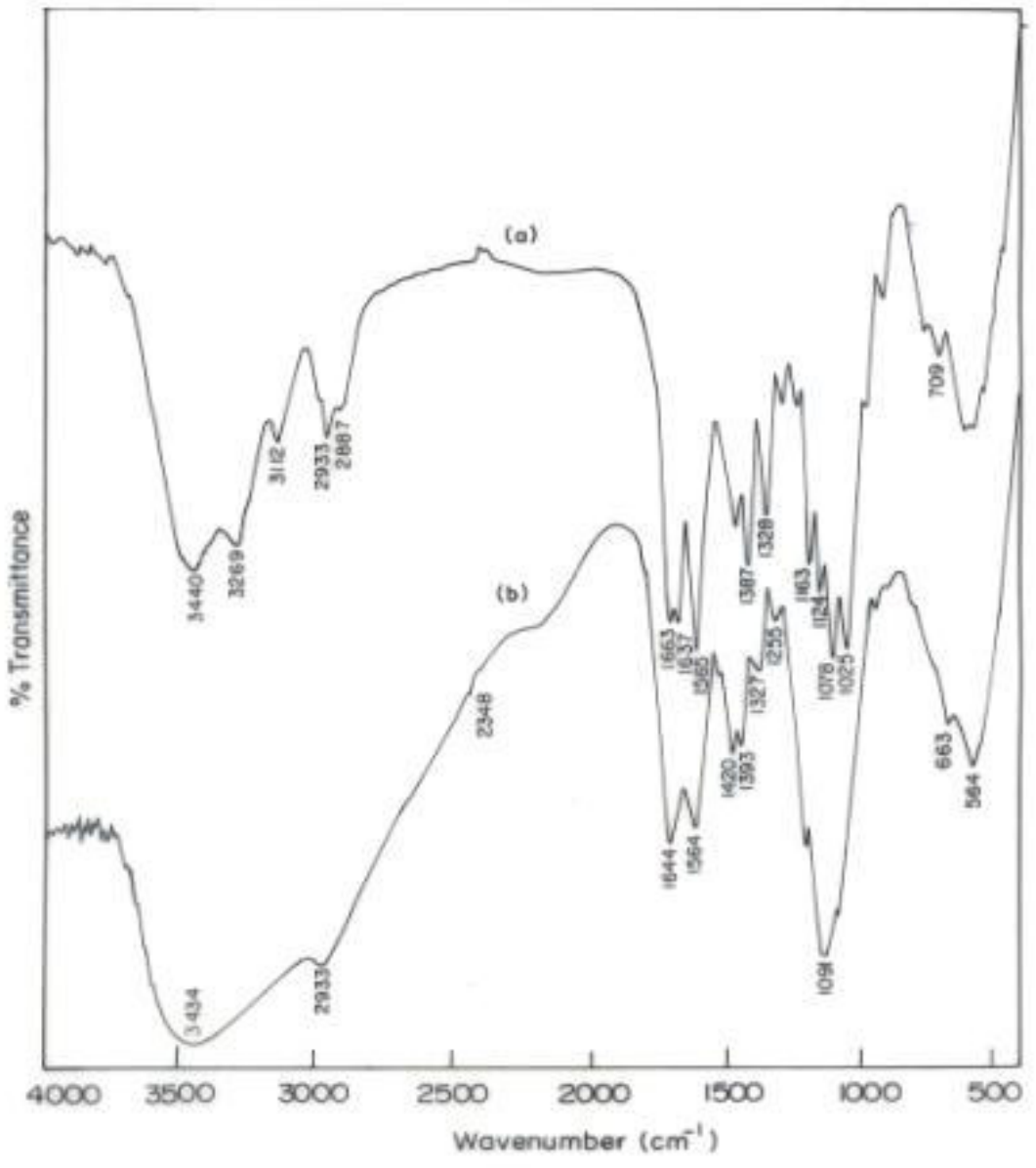
Infrared spectra of the samples were taken in a Nicolet Impact 400 Fourier Transform Infrared spectrometer, using 500 mg Kbr pellet containing 2 – 6 mg of the sample. The Chitosan film, were prepared for Fourier transform infrared (FTIR) spectroscopy measurements by drop-coating films on Si (111) wafers. FTIR measurements were carried out on a Perkin-Elmer Spectrum One FTIR spectrometer operated at a resolution of 4 cm^{-1} in the range of 450-4000 cm^{-1}. **Figure 5.3** shows the FI-TR spectrum of chitosan.

5.2.3 Cultivation of corneal epithelial cells on the chitosan scaffolds and its derivatives:

Human corneal limbal tissues:

Human corneal limbal tissues, which did not meet the criteria for clinical use from the donor aged 8 – 90 years, were obtained from the CU SHAH eye bank, Medical Research Foundation Sankara Nethralaya. About $2mm^2$ biopsy was taken from the cadaveric donor eye and stored in the 1ml of the transport medium at 4^0C. Human tissue was handled according to the tenets of Declaration of Helsinki. Cultivation of the limbal biopsies was done by the explant culture technique as per the procedure mentioned in the methodology section. The cultures were incubated for 21 days until they reach the confluence and the medium was changed every 3^{rd} day.

Figure 5.3 IF-TR spectrum of chitosan scaffold



5.2.4 Morphology and Viability of Cultivated Cells:

Cultures were monitored under an inverted phase-contrast microscope (Nikon, Japan). The viability of cultivated cells was determined by staining with Trypan Blue (Sigma Aldrich, USA). The cells were harvested at the end 4^{th} day, 12^{th} day and 21^{st} day, washed twice with PBS and to the cell pellet the 0.5% Trypan Blue solution in PBS was added and incubated at room temperature for ten minutes. About 10 µl of the sample was loaded on the heamocytometer chamber and number of viable cells and non-viable cells were counted under the microscope.

5.2.5 Proliferation study by Brdu labelling:

Brdu labeling was done on the cultured cells over chitosan matrix and chitosan matrix incorporated with the Chitosan with gold and silver nanoparticles. When the outgrowth reached 5–8 mm in diameter, explant cultures were incubated with a fresh medium containing 10 µM Brdu for 24 hour. After labeling with BrdU for 24 hr continually, the cultures were chased for 1–21 days by switching to BrdU free medium. All samples in triplicate were fixed in cold methanol at 4^0C for 10 min and processed for BrdU immunofluorescent staining according to the procedure given in the methodology section.

5.2.6 Marker study by RT-PCR analysis:

Total RNA was isolated from the cells cultured over the chitosan and derivatives using Qiagen RNA extraction mini kit according the procedure given in the methodology

section. First strand cDNA synthesis was done using the Qiagen sensiscript RT kit. Then amplification was done using the primers mentioned in the Table 2.1 of methodology.

5.2.7 Western Blotting analysis:

Western blot analysis was done to confirm the expression of ABCG2 and p63 at the end of 3 weeks on the cultured limbal epithelial cells on chitosan and its derivatives. After 21days of incubation the cultured cells over the membrane was scraped gently using cell scraper and protein extraction was carried out according to the procedure given in the methodology. Protein concentration was determined by Lowry method. 10 µg of each sample was used for running the gel. After the gel running and transfer the membranes were reacted with an anti-p63 (clone 4A4. Santacruz biotechnology dilution 1: 1000) and anti-ABCG2 (Clone BXP21 Chemicon. dilution 1: 500)

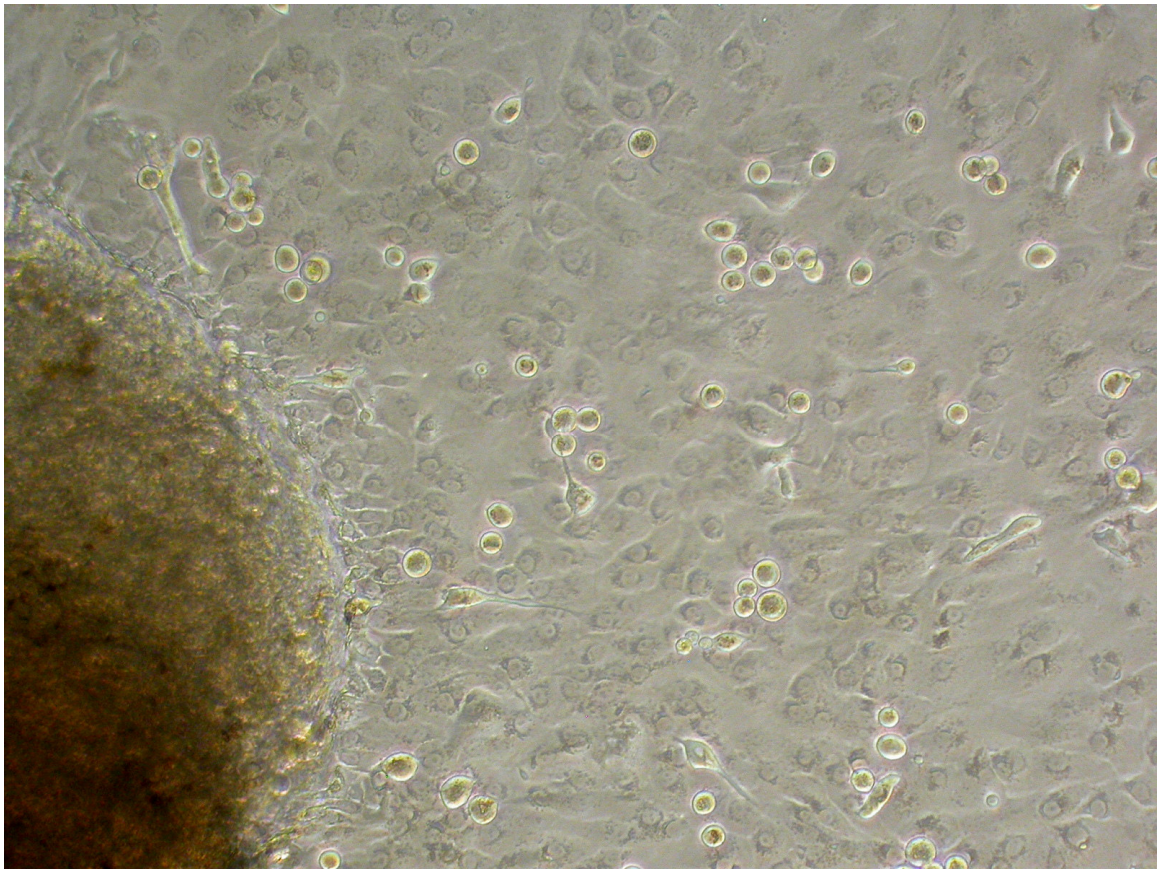
5.3 Results:

Cellular Morphology of the Chitosan and Chitosan Fabricated scaffolds:

Limbal explants started growing on the various scaffold from the explant by the end of 2day and formed a monolayer by the end of the 21st day. Morphology of the cells grown on the various scaffolds is shown in the **Figure 5.4** Outgrowth of the cells was observed under the inverted phase contrast microscope (Optiphot, Nikon, Japan), picture was documented using the Nikon cool pix digital camera. All the scaffolds supported the growth of the corneal limbal epithelial cells with ease and there is no observable cytotoxic effect on the cells cultured over these scaffold. The rate of growth on chitosan and chitosan + gold is almost similar with average reaching about 150mm^2 on chitosan

Figure 5.4

Growth of the cells on the chitosan matrix



This figure shows the growth of the limbal epithelial cells from the explant at the end 2 weeks of incubation they have formed a confluent growth and magnification is under 100X

and, whereas it is slightly lesser on the chitosan with silver. **Figure 5.5** shows rate of growth of cells in chitosan scaffold and its derivatives. **Figure 5.6** shows the H&E picture of the cells expanded over the chitosan scaffold.

Viability of cells by trypan blue dye exclusion test:

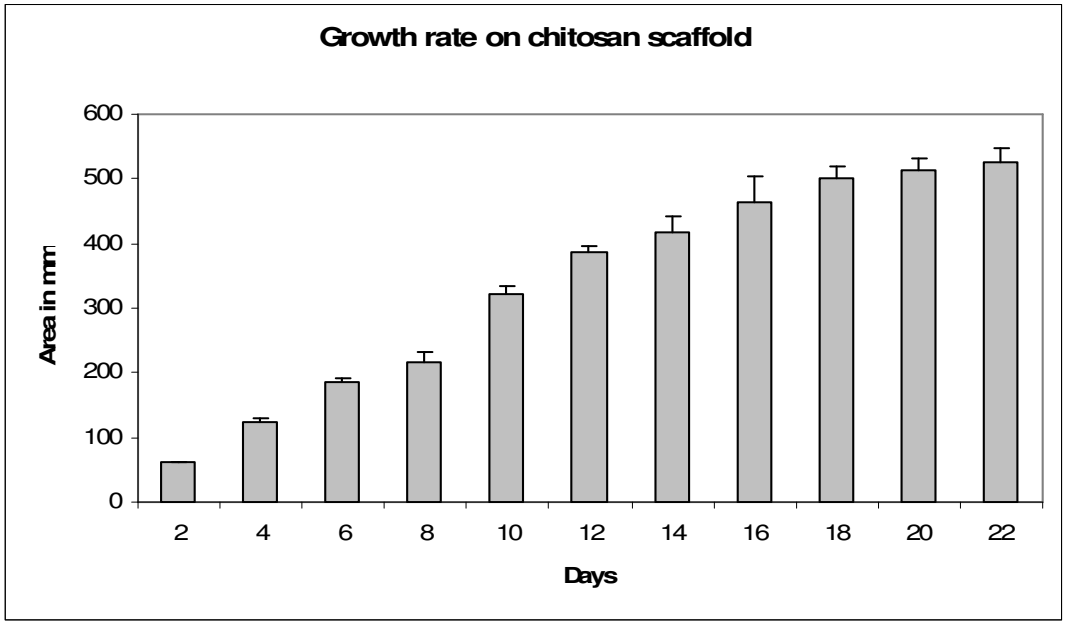
The viability of the cells checked by trypan blue dye exclusion test at the end of 4th day, 12th day and 21st day. All the scaffolds supported the growth of the cells and viability of the cells was well preserved till 21st day of incubation and results of which is shown in the **Figure 5.7** the percentage of viability ranged from 95 – 60%

Expression of corneal stem cell associated markers and differentiation markers on the various scaffolds using semiquantitative RT-PCR:

Semiquantitative RT-PCR was performed on the cells harvested from the various scaffolds at the end of 21st day. There was faint expression of the p63 (proposed keratinocyte stem cell marker) and ABCG2 (putative stem cell marker) connexin 43 and K3 / K12 on the cells expanded on the Chitosan, Chitosan-gold, whereas ABCG2 expression was completely absent on the cells expanded on chitosan-silver whereas the expression of all the other markers was seen on the chitosan silver scaffold. **Figure 5.8** shows the expression of the markers on the cells harvested at the end of 2 weeks from the chitosan matrix and its derivatives.

Figure 5.5

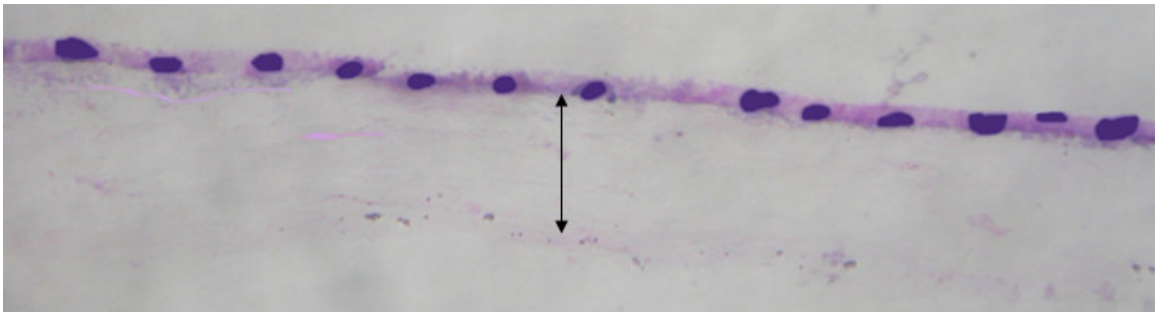
Rate of growth on the chitosan scaffold



This figure shows the rate of the growth of limbal epithelial cells on the chitosan scaffold. The growth commenced by the end of day 2 and almost reached confluence by the end of day 21 on the six well tissue culture plate.

Figure 5.6

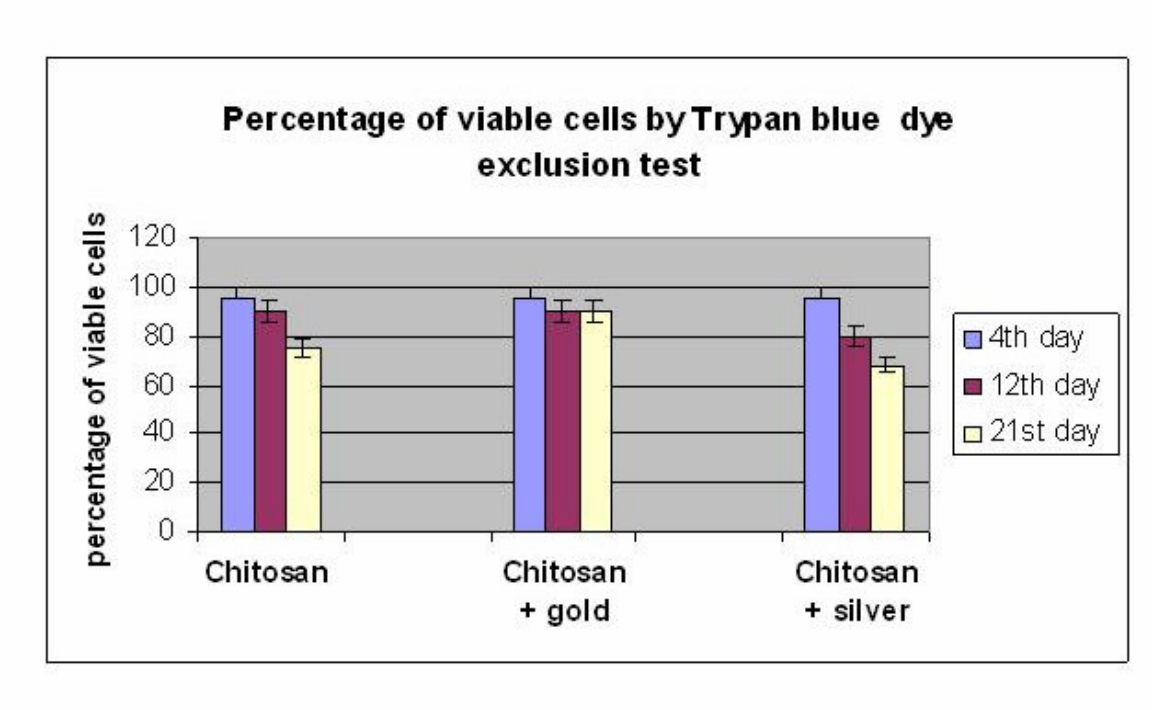
H & E shows the growth of the limbal epithelium on the chitosan scaffold



This figure shows the monolayer of limbal epithelial cells grown on the chitosan matrix at the end of 2 weeks of incubation. The chitosan matrix as such was removed and frozen section of 5m thickness was taken and stained by Haematoxylin and Eosin stain (Magnification 100X)

Figure 5.7

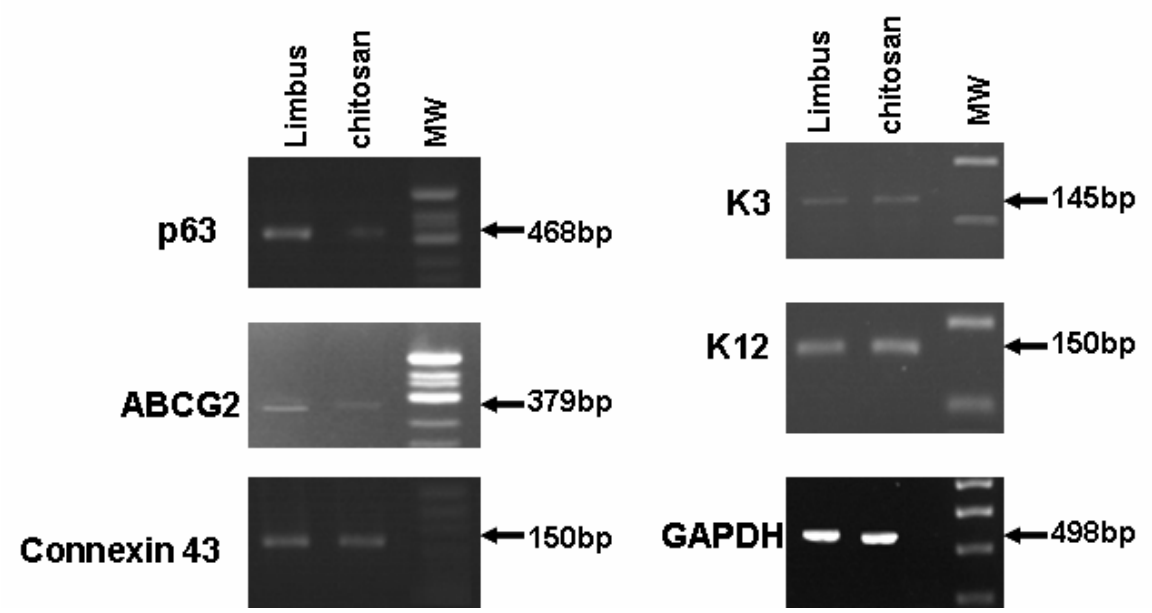
Percentage of viable cells by trypan dye exclusion method on the cells cultured over the chitosan matrix and its derivatives



This figure shows the percentage of viable cells present at the end of the 4th, 12th and 21st day. On day 4 the viability of cells was 100% on the cells cultured over all the scaffolds. On the 21st day the viability was maximum on the cells cultured over the chitosan and chitosan +gold.

Figure 5.8

Electrophoretogram showing the expression of the stem cell associated and differentiation markers on the cells cultured over the chitosan matrix



Electrophoretogram shows the expression of the stem cell associated markers (p63 and ABCG2) and connexin 43 and Keratin expression on the cells cultured over the chitosan scaffold at the end of 2 weeks cells harvested from limbus is used as a control. GAPDH is used as an internal control. Expression of p63 and ABCG2 was retained till 21 days on the cells cultured over the chitosan matrix.

Western blot results:

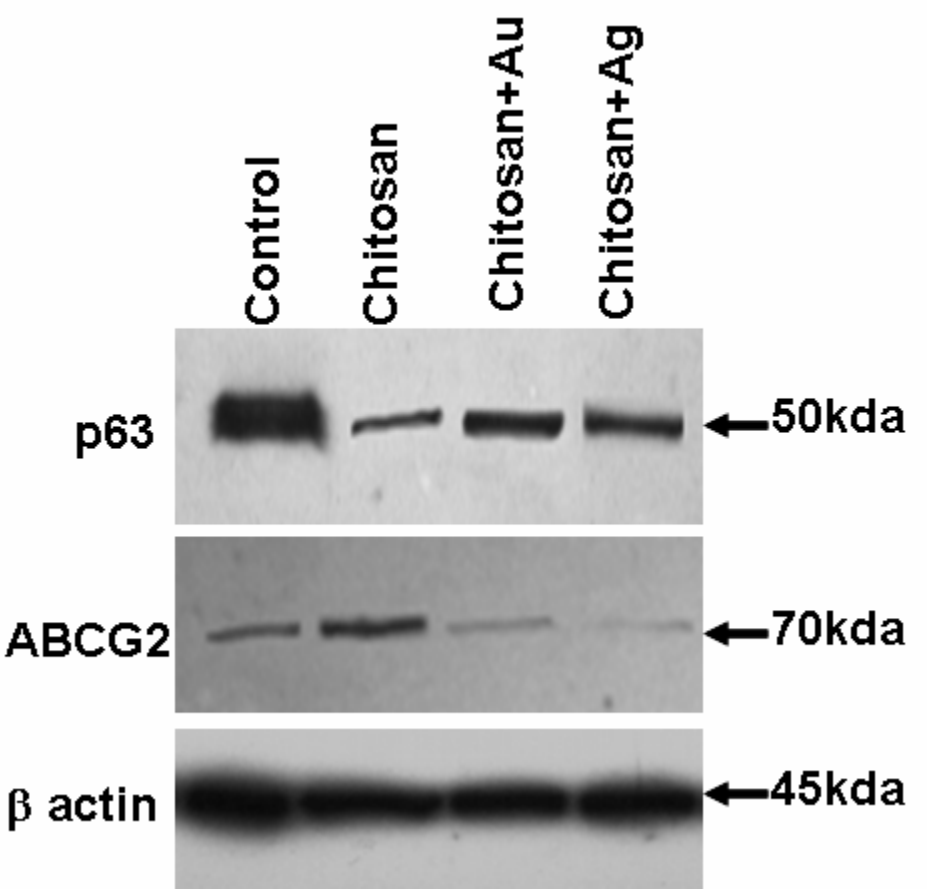
The expression of p63 and ABCG2 was confirmed by western blot. There was expression of p63 on the cells harvested from all the three chitosan matrices. ABCG2 expression was completely absent on the cells harvested from the chitosan + Silver matrix. But there was a faint expression of the ABCG2 on the cells harvested from the other two matrixes. **Figure 5.9** shows the expression of the markers on the cells harvested from the chitosan matrices.

Brdu labelling:

Brdu labelling showed that the cells cultured over the chitosan and chitosan with gold were able to retain the label, whereas on the cells over the chitosan + silver matrix retaining cells were seen only till the day 14 of chasing. **Figure 5.10** shows the Brdu labelling on the cells cultured over chitosan matrix. The labeling index at the end of 24 hours for the cells cultured over the chitosan is 64.0 ± 2.0%, for Chitosan with Au it is 55.0 ± 3.5% and chitosan + Ag it is 45 ± 2.0% . At the end of 7^{th} day it was 15±6.0% for chitosan and 8.0±2.0% on chitosan + Au, 6.8±2.2% on chitosan +Ag. On 14^{th} day it was 6.0±.28%, 3.0±0.18% and 2.0±0.28% respectively. At the end of 21^{st} day it was 2.3±0.02% on the chitosan and 1.0±0.01% on the chitosan+Au whereas it was completely absent on the cells cultured over the chitosan + Ag.

Figure 5.9

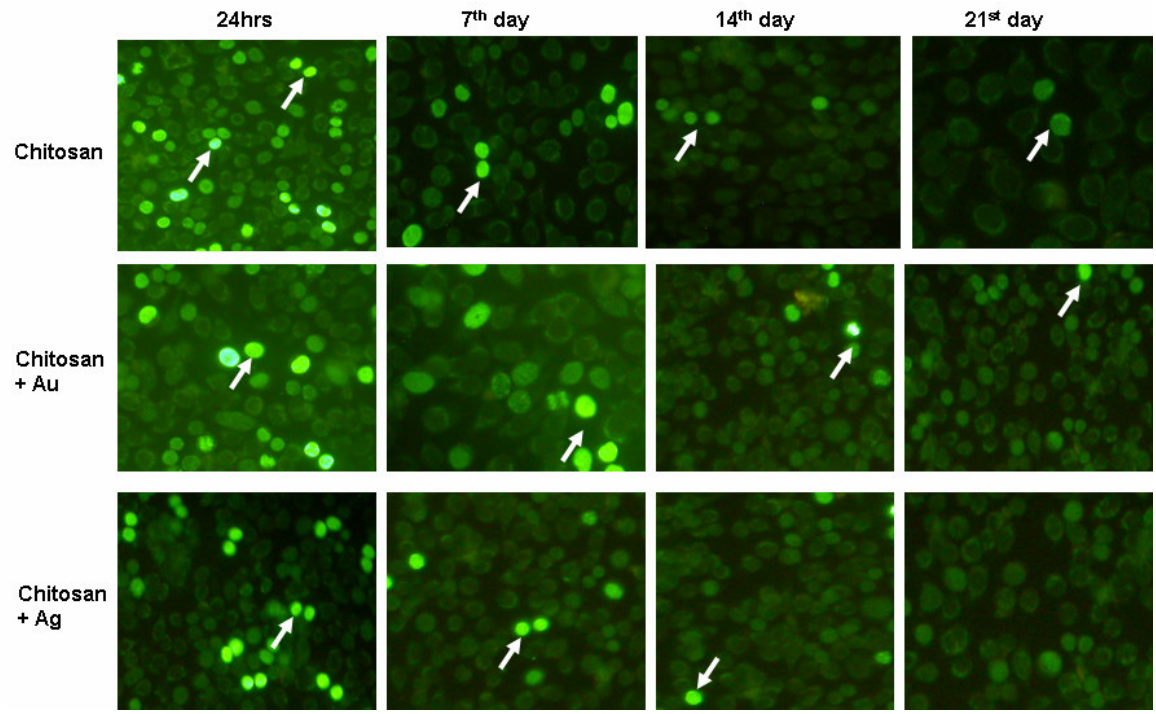
Western blot results showing the expression of p63 and ABCG2 on the cells cultured over the chitosan matrix and its derivatives



This figure shows the expression of the p63 and ABCG2 on the cells cultured over the chitosan scaffold at the end of 21 days of incubation. HeLa cells were used for p63 control and MCF cell line was used for ABCG2 control. P63 and ABCG2 expression was seen on the cells harvested from the chitosan and its derivatives.

Figure 5.10

Brdu labelling on the cells cultured over the chitosan matrix



This figure shows the labeling index of the cells cultured over the chitosan matrix and its derivatives. All the experiments were done in triplicates and Standard mean ± SD was taken. At the end of 24hours the labeling index of the chitosan is more when compared to the other two. The cells were able to retain the label on those cultured over the chitosan and chitosan + Au. Whereas it was less on those cultured over the chitosan + Au.

5.4 Discussion:

The main objective of this chapter is to compare the growth characteristics of the cells cultured over the chitosan matrix and its derivatives. We cultured limbal epithelial cells from the explants over the chitosan and its derivative (Chitosan +gold, Chitosan +silver). The cultured cells were looked for the viability, and characterization was done using molecular markers by semiquantitative RT-PCR, proliferation index was studied by Brdu labeling. The expression of p63 and ABCG2 markers was confirmed by Western Blot.

Chitosan and its derivatives possess some special properties for use in regenerative medicine. Several studies have examined the host tissue response to chitosan based implants. In general, these materials are non-toxic and biodegradable with living tissues, evoke a minimal foreign body reaction with little or no fibrous encapsulation. Lysozyme is the primary enzyme responsible for *in vivo* degradation of chitosan through hydrolysis of acetylated residues. (Ren *et al*, 2005) Chitosan have cationic nature that is primarily responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. This property allows chitosan to retain and concentrate biomolecules since a large number of cytokines/growth factors are linked to GAG. (Madihally *et al*, 1999 ;) Chitosan also have intrinsic antimicrobial activity against bacteria and fungi. (Hu *et al*, 2003) The exact mechanism of the antimicrobial action of chitosan and its derivatives is not fully known, but different mechanisms have been proposed. Its cationic amino group associates with anions on the bacterial cell wall, suppressing biosynthesis; moreover, chitosan disrupts the mass transport across the cell wall accelerating the death of bacteria. (Rabea *et al*, 2003 ;) This

chitosan also play a role in inhibiting the fibroblast growth. (Mori *et al*, 2005) With all these favorable properties we had used the chitosan and its derivatives for culturing limbal epithelial stem cells. Zhu *et al* has already reported the use of chitosan-gelatin biomatrix for the cultivation of conjunctival epithelial cells. (Zhu *et al*, 2006)

In our study we have cultured the cells on the chitosan and chitosan + gold and chitosan + silver. The cells grew well in all the three matrices but the rate of proliferation was more on the chitosan matrix comparatively. Vialbility of the cells were also measured at the end of 3 weeks of incubation. The viability of the cells was maintained almost equally in all the three matrices. The percentage of viability ranged from 95-65%.

The marker study revealed that the cells cultured over the chitosan and chitosan with gold were able to retain expression of p63 and ABCG2 even after 3 weeks of incubation. Expression of differentiation marker connexin 43 and K3/K12 was expressed on the cells over all the three matrices. The expression of the p63 and ABCG2 was confirmed by western blot, where it showed the faint expression of the protein on the cells cultured over the chitosan and chitosan with gold, while ABCG2 was completely absent on the cells cultured over the chitosan + silver. The cells over the chitosan and chitosan with gold were able to retain the label till 3 weeks of incubation but the cells over the chitosan with silver were able to retain the label only till 7^{th} day. Thus; we were able to culture the limbal epithelial cells on the chitosan matrix and its derivatives and cells over the chitosan matrix and chitosan with gold were able to retain the limbal phenotype cells even after 3 weeks of incubation. Thus chitosan would be better biomatrix for supporting the cell growth without any cytotoxic effect and which can also be used for the ocular surface reconstruction.

5.5 Conclusion:

Thus the novel properties of chitosan make it one of the most promising biopolymers for cell therapy, tissue engineering and gene therapy. It is hoped that these diverse approaches for regenerative medicine will translate from 'bench to bedside' in the future. However, efforts to improve the mechanical properties of chitosan-based composite biomaterials are essential for this type of application, especially for the ocular surface reconstruction. Further issues on the transparency and tensile strength have to be studied in detail before we go in for the clinical application.

CHAPTER 6 - USE THERMOREVERSIBLE GELATION POLYMER IN CULTIVATION OF CORNEAL STEM CELL

6.1 Introduction:

Ocular surface diseases, such as Stevens-Johnson syndrome, chemical and thermal burns, and inflammatory lesions like bacterial, fungal and parasitic infections, allergies, dry eye, keratoconus etc., result in damage to the corneal, limbal, and conjunctival epithelia, often resulting in significant visual morbidity. Cultivated limbal stem cell transplantation has been used in the treatment of limbal stem cell deficiency. (Koizumi *et al*, 2001; Tsai *et al*, 2000; Schwab *et al*, 2000) The substrates that have been used for the development of ocular surface tissue-equivalents include human amniotic membrane (HAM), collagen gel and fibrin glue, of which HAM is currently the most commonly used substrate for ocular surface transplantation. (Koizumi *et al*, 2001; Tsai *et al*, 2000, 1988, 1994; Schwab *et al*, 2000; Ang *et al*, 2005; Tan *et al*, 2004; Pellegrini *et al*, 1997;)

However, the use of allogeneic biological material is associated with the risks of disease transmission (e.g., HIV, hepatitis B and C, and bacterial and fungal infections) and there are reports showing the persistence of the human amniotic membrane after the transplantation. (Connon *et al*, 2006)

In addition, many of the substrates lack the mechanical properties that allow easy handling and suturing, as well as prolonged endurance after transplantation. As such, there is a perceived need to develop new methods of ocular surface epithelial cell replacement.

The use of biosynthetic materials as stromal substitutes to support epithelial cell growth would overcome some of the problems related to the use of allogeneic tissue and biological substrates. These materials may be custom fabricated to suit each condition and could provide a ready supply of material for clinical use and avoid the shortcomings, such as poor mechanical strength and risk of immunologic rejection. In this context developing synthetic polymers, which are non-toxic and biodegradable with adequate tensile strength is important.

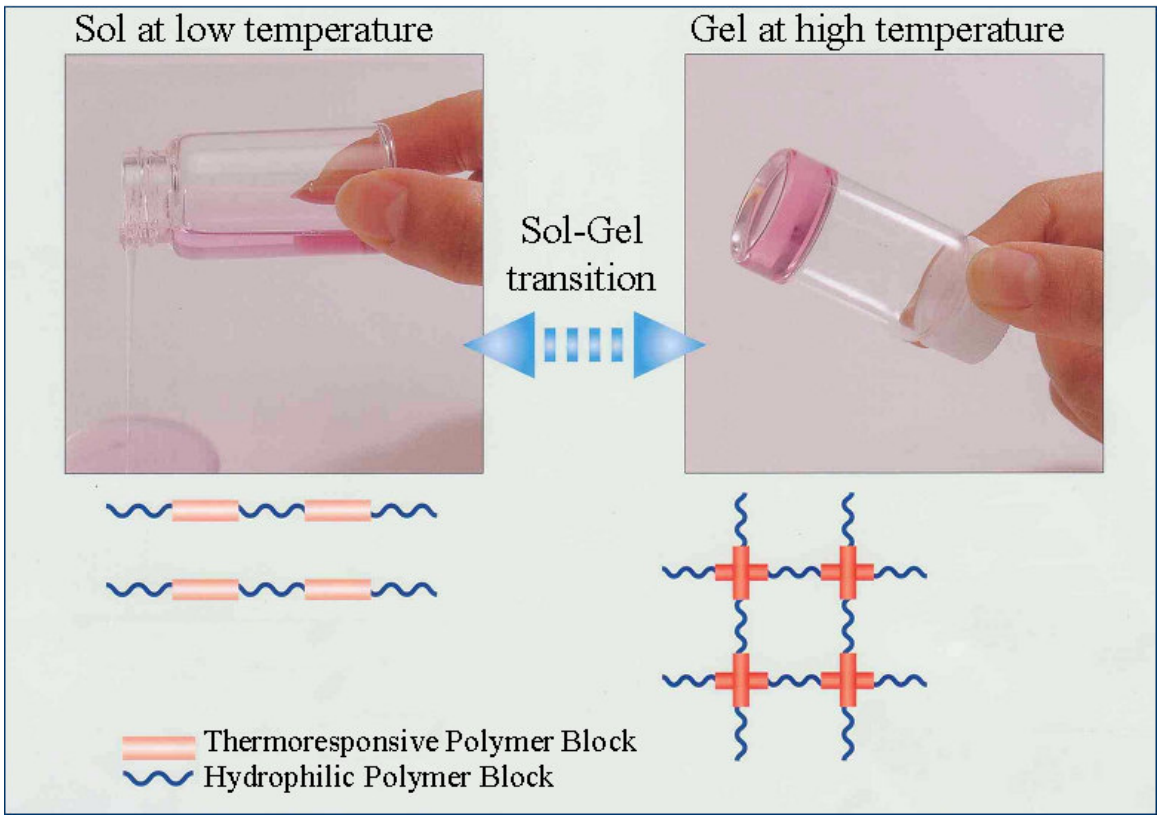
Mebiol Gel is a copolymer composed of the thermo responsive polymer block [poly (N-isopropylacrylamide-co-n-butyl methacrylate) (poly NIPAAm-co-BMA)] and the hydrophilic polymer block [polyethylene glycol (PEG)]. This polymer block is hydrophilic at temperatures below 20^0C and hydrophobic at temperatures above 20^0C forming cross-linking points and homogenous three-dimensional (3-D) network of Mebiol Gel in water. **Figure 6.1** Shows the transition state of the mebiol gel at the low and high temperatures.

Cells or tissues can be embedded in a liquid Mebiol Gel solution at lower than 20^0C and cultured three-dimensionally in a hydrogel state at 37^0C. The sol-gel transition temperature can be controlled by chemical composition of Thermo Reversible Gelation polymer (TGP). (Yoshioka 1999b, 2003 ;) The present study was undertaken to investigate the growth characteristics and the phenotype of the human cadaveric limbal explants embedded in Mebiol Gel.

Figure 6.1

(Adapted from the www.mebiol.co.jp)

Mebiol gel transition state



Finally, we investigated reconstruction of the rabbit corneal ocular surface with rabbit limbal cells cultured on Mebiol gel. This model provides a novel ability to determine the source of cells repopulating the reconstructed epithelium.

6.2 Materials and Methods

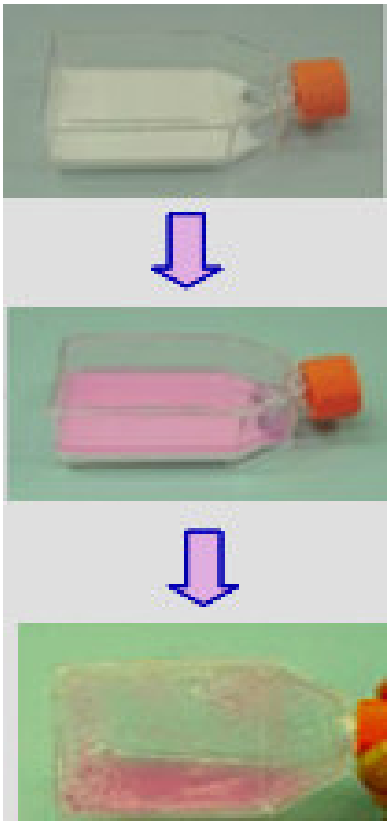
6.2.1 Collection of Human limbal tissues

Twenty-four human limbal biopsies of approximately 2-mm^3 sizes were obtained from the superior and the inferior portion of the limbus from the human donor eyes using sterile Bard-Parker blade within 3-6 hours of death from the C.U Shah eye bank of Sankara Nethralaya, Chennai, India. Each human limbal biopsy was placed in one mL of tissue culture (TC) medium of DMEM containing 3% FCS and antibiotics (penicillin, gentamicin and amphotericin B) and transported to cell biology laboratory immediately. All the tissue samples were handled according to the declaration of Helsinki.

6.2.2 Preparation of Mebiol Gel-tissue culture growth medium mixture

Mebiol gel provided in 10 mL amount as lyophilized and sterilized form in a flask was purchased from Mebiol Inc. (Tokyo, Japan, through Nichi-In Bio Sciences Pvt. Ltd, Chennai, India). The gel in the flask was dissolved in 10ml of 2X strength of TC growth medium of DMEM + Ham F-12 containing 10% FCS at pH 7.0 and placed in a refrigerator at $4^{\circ}C$ – $8^{\circ}C$ overnight, yielding a viscous transparent Mebiol Gel-TC growth medium mixture (gel-TC medium mixture) **(Figure 6.2)** of uniform liquid without any air bubbles for use in the experiments.

Figure 6.2



The figure shows the lyophilized powder becomes gel on incubation at 4^0C with the tissue culture growth medium after 48 hours.

6.2.3 Cultivation of Corneal limbal tissue embedded within the Mebiol Gel:

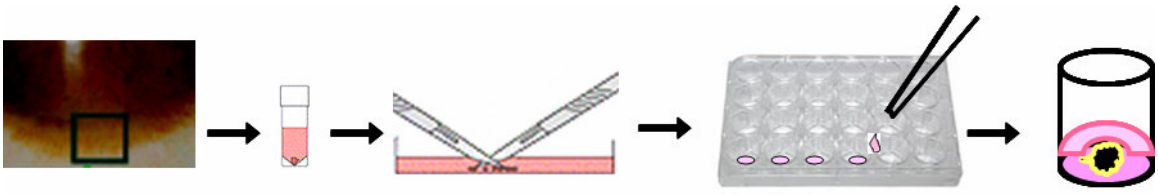
Limbal biopsy was washed thrice using TC growth medium and the limbal epithelium was carefully dissected from the underlying stroma, cut into 0.5- to 1-mm pieces, and cultivated as explants within the Mebiol gel. A drop of the liquefied (4 - 8°C) gel-TC medium mixture was placed in the center of the 24-well TC plate and solidified at 37°C for about 20 – 30 minutes. The explant piece of tissue was placed on the surface of the solid gel and another cold liquefied drop of the gel-Tissue culture medium mixture was added to cover the gel with the tissue bit inside. **Figure 6.3** shows the sequential steps involved in the cultivation of limbal biopsy within the mebiol gel. The tissue culture plates were incubated in 10% CO_2 atmosphere at 37°C. The growth pattern of limbal explants within the gel was observed and documented under the phase contrast microscope.

6.2.4 Evaluation of Morphology and Viability of Cultivated Cells:

Cultures were monitored under an inverted phase-contrast microscope (Nikon, Japan). The viability of cultivated cells was determined by staining with Trypan Blue (Hi Media, India). The cells were harvested and washed twice with PBS and to the cell pellet the 0.5% Trypan Blue solution in PBS was added and incubated at room temperature. About 10µl of the sample was loaded on the heamocytometer chamber and number of viable cells and non-viable cells were counted.

Figure 6.3

Sequential steps involved in the cultivation of corneal limbal biopsy in Mebiol gel



This picture shows the sequential steps involved in the cultivation of limbal biopsy in mebiol gel. About 2mm^2 biopsy was taken from the limbal region and washed thrice and removed of from excessive sclera, cut into multiple bits and placed within the mebiol gel.

6.2.5 Evaluation of Proliferative Capacity of Cultivated Cells:

The proliferative capacity of cultivated cells was evaluated by using the following method.

^{3}H thymidine assay.

Growth rate of human corneal limbal tissue (HCLT) cells within Mebiol Gel was determined by the rate of uptake of ^{3}H thymidine by growing cells as described by (Mc Ateer *et al*, 2002;) and modified according to Beckmann instruction manual along with control wells with and without gel. (*Beckmann Instruction LS 6500 scintillation system operating manual, Beckmann Instruments Inc, U.S.A)*

The HCLT biopsy bits were placed over the 12-well tissue culture plate. One µCi of ^{3}H thymidine was incorporated into each well. Fifty µl of medium from each well was sampled every day for ten days and relative consumption percentage was measured and calculated using the disintegrations per minute of test and reference standard using LSS (Beckmann Instruments Inc, LS6500, CA, USA*)* scintillation counter. Reduction of ^{3}H thymidine indicated its incorporation by multiplying cells for DNA synthesis. The cultivated HCLTs along with the outgrowth of cells were harvested by liquefying Mebiol Gel by placing the 12-well TC plates in the refrigerator for two hours to release tissue and cells. Their viable count was done by trypan blue dye exclusion test. (Schmidt *et al*, 1988).

6.2.6 Characterization of cultured cells using immunohistochemistry and RT-PCR

Immunohistochemistry and Immunofluorescence

The cultured cells were washed in cold phosphate buffered saline at pH 7.0, cytospinned (Cytospin 2, Shandon, UK) on the microscopic slides and stained for the various stem cell association markers using monoclonal antibodies against p63 (clone 4A4), ABCG2 and differentiation markers Connexin 43 and Integrin β1 (Santa Cruz Biologicals, Santa Cruz, CA, USA) by Immunoperoxidase and immunofluorescent methods.

Immunoperoxidase staining was performed on cytospinned smear preparation. The smears were fixed in methanol and then dried and treated with primary antibody for 2 hours at room temperature, further washed with Tris HCl buffer at pH 8.0 and then was incubated for one hour with horseradish peroxidase conjugated Anti Mouse Secondary antibody at 1:100 dilution. Substrate Diamino Benzidine (1:100) (DAKO cytomation corp, Glostrup, Denmark) was added and incubated for 5 minutes washed and counter stained with Harris Haematoxylin for 30 seconds.

Immunofluorescence staining was performed on smears fixed in cold acetone. Staining was done with primary antibodies at 1:50 dilution with 1 hour incubation and after washing with PBS-Tween 20 (PBST) was treated with anti mouse conjugated with FITC (DAKOcytomation corp, Glostrup, Denmark) at 1:5 dilutions for an hour and counterstained with 0.5% Evans blue. The slides were mounted with glycerol and observed under the fluorescent microscope (Optiphot, Nikon, Japan).

RT–PCR:

Total RNA was isolated from tissue and cells on days 2, 4, 6, 8 and 10 of incubation to determine the pattern of expression of different presumed corneal limbal stem cell association markers and cornea differentiation markers on the cultured cells by RT-PCR. Cells were collected, treated with Tri-Reagent (Sigma Aldrich St Louis, USA) according to the manufacturer's recommended protocol and total RNA was extracted and stored at -80^0C until use. Reverse Transcription was performed using sensiscript reverse transcripatase (Qiagen), which is a recombinant heterodimeric enzyme. PCR amplification of the first – strand cDNAs were performed using specific primer pairs, designed from published human gene sequences along with house keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as internal control, expression of following markers viz. ΔNp63, ABCG2, Integrin α9 Connexin 43, K3 and K12 were performed on the cultured cells (**Table 3.1**) in the Eppendorf PCR systems. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide with molecular marker *Hinf I* Φ digest to confirm the size of the resultant product.

6. 3 Animal model of limbal stem cell deficiency:

6.3.1 Selection of animals for the study:

The entire animal investigation was approved and supervised by the Society for prevention of cruelty to animals. (SPCA) All experiments satisfied the ARVO recommendations for the humane treatment of animals. Fourteen Newzeland white male rabbits weighing 2 – 2.5 kg were used for the study. The study protocol was done according to the schedule given in the **Table 6.1** below.

All animals were handled according to the SPCA guidelines and an alphabet was assigned each animal, the alphabet was marked on the left ear with the permanent marker. The animals were anaesthetized 20mins before the surgical procedure and they were restrained and taken to theater where the further experimental procedures are carried out.

Table: 6.1

Experimental schedule

Stage	**Procedure**	**Day Count**	**Gr.1 (A,B,C)***	**Gr.2 (D,E,F)**	**Gr.3 (G,H,I)**	**Gr.4 (J,K,L)**
Stage:1	**Deficiency Creation**	**1**	1	4	8	11
Stage:2	**Imp. Cyt for Conjtvzn.**	**11**	11	22	25	29
Stage: 3	Limb. Culture Start	**1**	1	5	8	11
Stage:4	Limb. Culture harvest	**22**	22	25	29	32
Stage:5	**Transplant**	**22**	22	25	29	33
Stage:6	1 week follow up	**29**	29	32	36	39
Stage:7	3 week follow up	**43**	43	46	50	53
Stage:8	6 week follow up	**64**	64	67	71	74
Stage:9	12 week follow up	**99**	99	102	113	116
Stage:10	24 week follow up	**176**	176	186	197	200
Stage:11	**Biopsy/PK (182 days after Tx)**					

6.3.2 Creation of total Limbal stem Cell Deficiency:

Total limbal stem cell deficiency was created by the following method. The animal were anesthetized by giving systemic injection of Ketamine Xylazine at the dose of 35 – 50mg/Kg of body weight 20 mins prior to the experimental procedure, If the redosing is required only ketamine is administered. The corneal epithelium was removed by the topical application of n –heptanol and by the mechanical debridement, followed by the 360^0C surgical removal of a lamellar limbal ring (1.5mm segment on either side of anatomic junction of cornea and conjunctiva)

Postoperatively the rabbits received Ciplox eye ointment and Ciplox eye drops. They were followed up at the first postoperative day and weekly there after till the clinical signs of total LSCD (conjunctivalization, neovascularisation, haze etc) were evident.

Impression cytology was done by placing cellulose acetate paper onto the cornea at the end 2^{nd} week and sampling was done in all the four quadrants of the eye to establish the total Limbal stem cell deficiency (LSCD). The paper was fixed in 95% alcohol and stained by modified PAS staining according to the protocol mentioned in the methodology section. The grading was done mainly on the presence conjunctival goblet cell.

6.3.3 Biopsy collection and cultivation:

Limbal biopsy was done on the right eye of the rabbits before making it limbal deficient. After anesthetizing the animal and sterilizing the eye with povidone iodine, 2x2mm^3 limbal tissue containing the limbal epithelial cells and part of the corneal stroma will be separated from the limbal margin and excised from corneal stroma by lamellar keratectomy. The tissue will then be placed in a vial containing Dulbecco's Minimum Essential Medium (DMEM) containing suitable penicillin, streptomycin and amphotericin.

Corneal limbal biopsy of 2mm^3 was collected in the transport medium with suitable antibiotics. The tissue bit will be cut in multiple bits of 0.5 – 1.0 mm^3. A drop of the Mebiol gel TC medium mixture (4 - 8^{0}C) was placed in the center of the 6 well TC Plate and so liquefied at 37^{0}C before placing the tissue. The cut tissue bit will be placed on the solid gel and drop of the gel – Medium mixture was added to cover the tissue bit. Thus the tissue bit was embedded in the Mebiol gel. The growth of cells from the tissue bit was observed everyday under the phase contrast microscope. **Figure 6.4a** shows the sequential procedure involved in the cultivation of the rabbit limbal tissue within the Mebiol gel.

6.3.4 Harvesting of cultured cells for transplantation:

Once the cells reach confluence the gel the culture plate with the cells were incubated at 4^{0}C for half an hour. The cells with the gel were collected in sterile vial and transported in the isotherm cooler racks. (BD Biosciences)

Figure 6.4a

Sequential steps followed for the expansion and transplantation of limbal epithelial cells in Mebiol gel

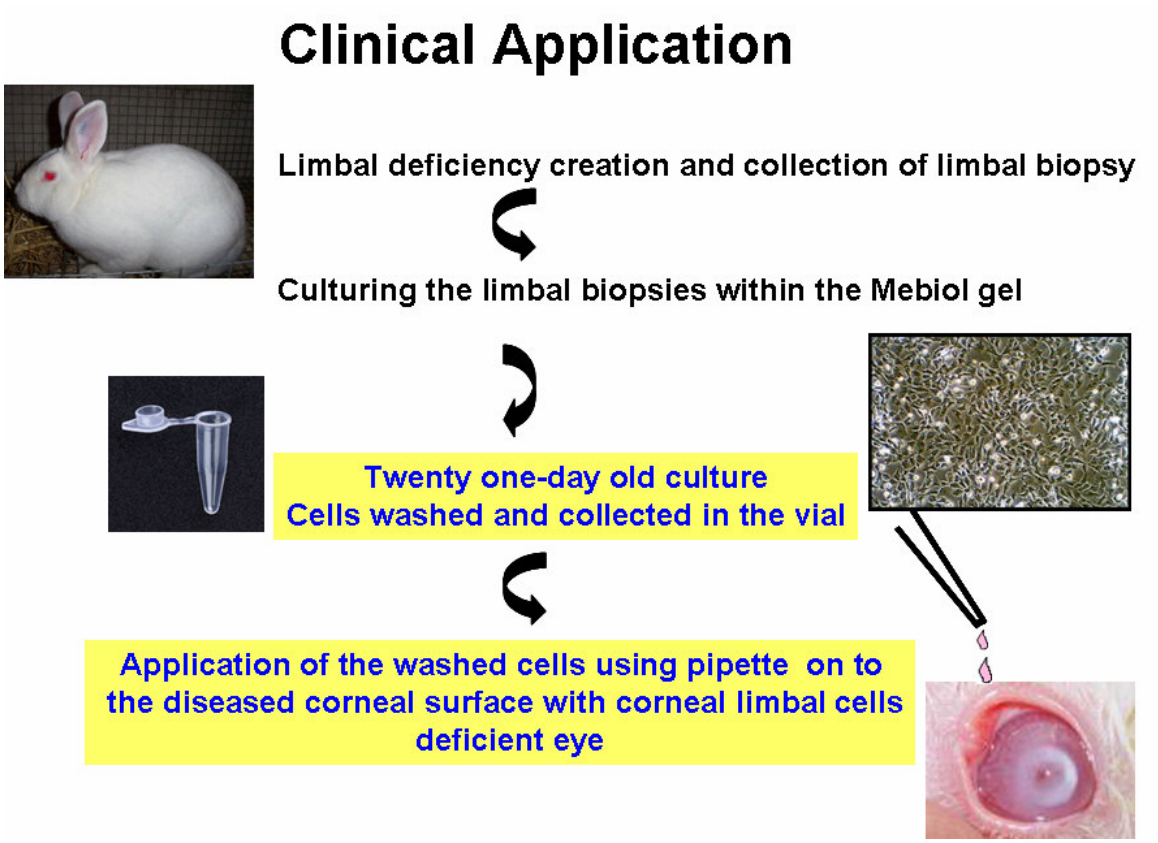
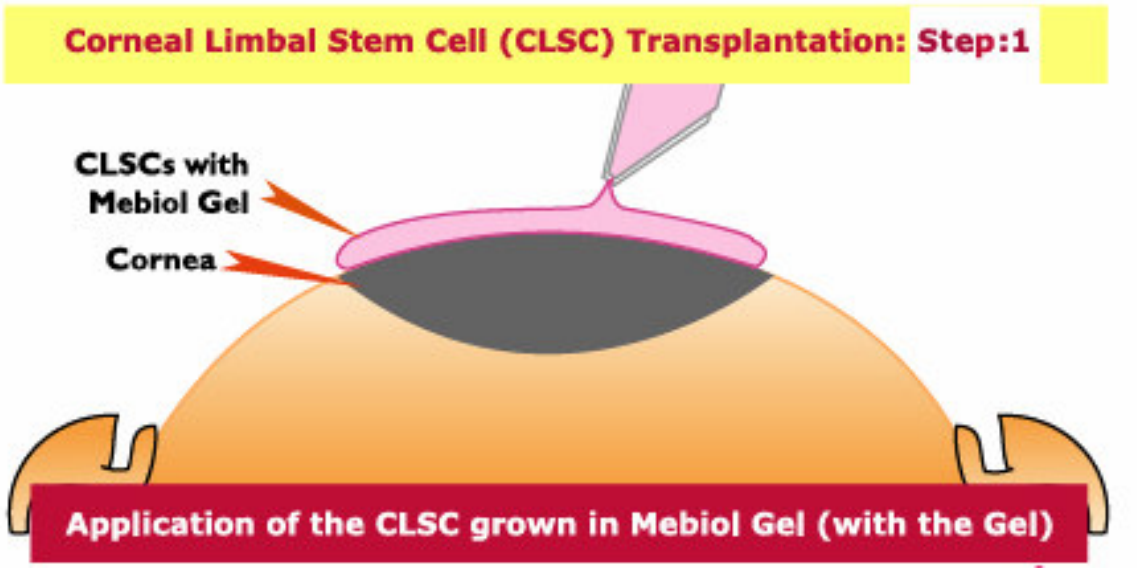
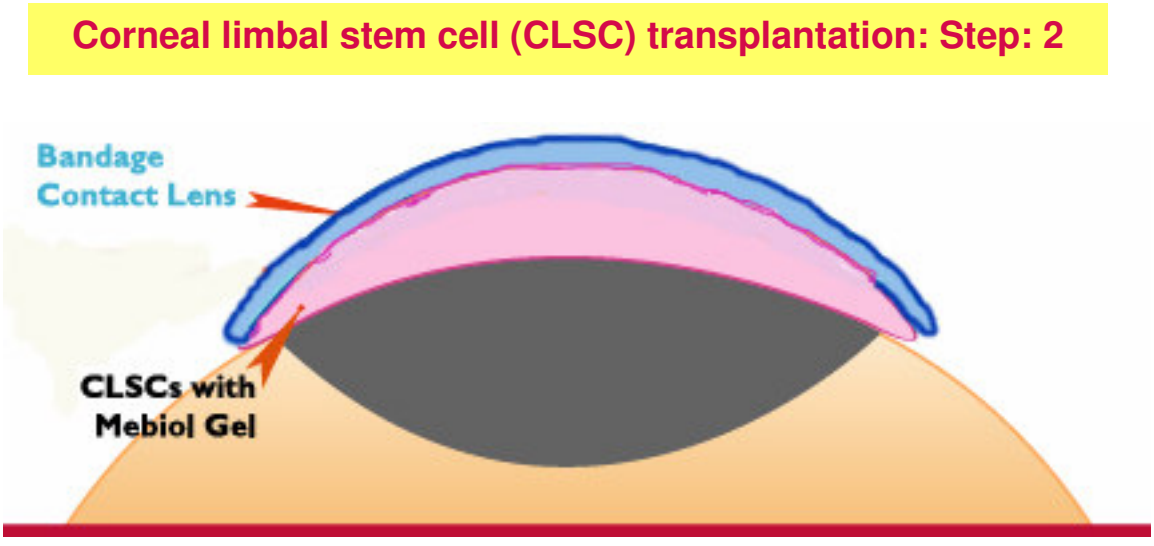


Figure 6.4b

Transplantation procedure of the cultivated limbal epithelial cells onto the rabbit Cornea





6.3.5 Transplant procedure:

Two weeks after the initial removal of the limbal epithelial stem cells and unilateral ocular surface injury, a second procedure was performed on these 14 rabbits. The animals were successfully anesthetized according to the ARVO recommendations for the ethical treatment of animals using ketamine, xylazine, and buprenorplline. Once successful anesthesia had been obtained, all rabbits were examined.

A 360 degrees conjunctival peritomy followed by removal of fibrovascular pannus will be done initially. The free end of conjunctiva will be anchored to the episclera 3-4mm away from the limbus using 7.0 vicryl sutures. This will be followed by transplant of cultured cells on to the ocular surface by one of the two methods below. A drop of Mebiol gel containing the cultured LSC was placed on the surface of cornea using Pasteur pipette and it was allowed to spread over the entire corneal surface by gravity. A bandage contact lens was then placed over the cornea for 3 months. Two rabbits were used as control. During transplantation only the gel was transplanted without the cells. **Figure 6.4b** shows the transplantation procedure of the cultivated cells

6.3.6 Postoperative follow-up:

Postoperatively the rabbits received topical antibiotics for a week and topical steroids for 3 months (prednisolone acetate 1%, 4 times daily for one month and then tapered over two months).

The rabbits were examined on slit lamp the first postoperative day, at 1 week, 1 month and monthly thereafter to document epithelization. Slit lamp examination, fluorescein

staining, impression cytology as mentioned in the methodology section and photography was performed at each visit.

6.3.7 Outcome measures:

Outcome will be defined as successful if the cornea regains smoothness, avascularity and clarity. Partial success is the return of vascularized irregular corneal surface in two or less quadrants. More than two quadrants of vascularization will be taken as failure

6.3.8 Marker study:

After transplantation follow up was carried out the end of the 1st week, 3rd week 6th week, 12th week and 24th week and the follow up was done till 9th month. Impression cytology was done on the At the end 9 months the animals were sacrificed and the eyeballs were enucleated and it was immediately snap frozen, after half an hour the eyeballs were cut into two equal halves and one half was processed for the histopathology and embedded in paraffin wax other half was used for the molecular work, total RNA was extracted from the limbus portion and the central corneal portion, cDNA was converted using sensiscript reverse transcriptase and amplification for the p63, ABCG2 and connexin -43 and Keratin was carried out using the specific primer sequence.

6.4 Results:

Morphology and Viability of Cultivated Cells:

The growth of cells from the limbal biopsy tissue embedded within the Mebiol gel commenced on day 4. The cells further multiplied with outgrowth migrating out of the margin of Mebiol gel forming monolayer outside the gel margin. **(Fig 6.5)** Viable count, as estimated by trypan blue exclusion test, ranged from 95 to 98 % with approximate yield of $4X10^4$ cells per mm^3 of liquefied gel.

Proliferation Assay:

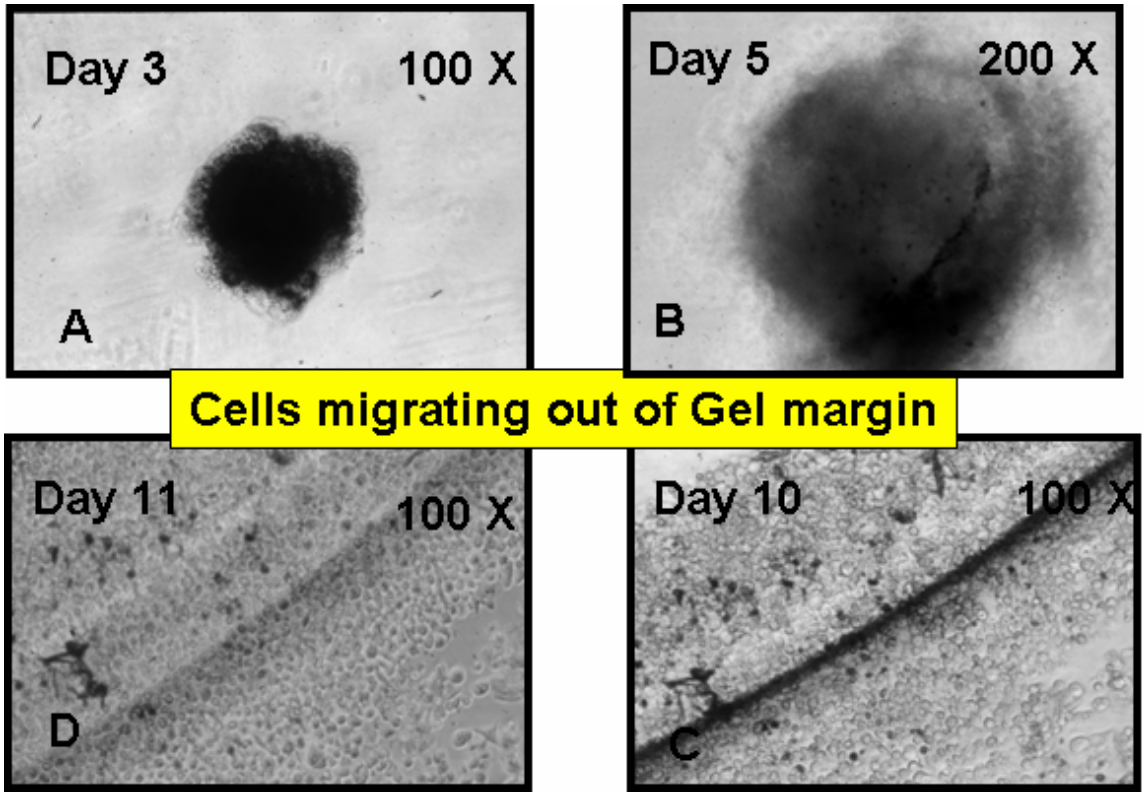
Thymidine incorporation studies:

Thymidine incorporation studies show that there was rapid incorporation of thymidine in limbal explants embedded within the Mebiol gel. Rapid 3H thymidine incorporation occurred from day-1 by cells in Mebiol Gel and control cells did so only on the initial day. **Figure 6.6** shows the results of 3H thymidine incorporation studies performed on one of the limbal explants cultivated in Mebiol Gel for 10 days.

Immunohistochemistry and Immunofluorescence:

The cultured limbal epithelial cells demonstrated a positive immunoreactivity for antibodies for presumed limbal stem cell associated markers: ABCG2 and p63 and cornea differentiation markers Connexin 43 and Integrin α9 by both immunoperoxidase and immunofluorescence methods. **(Figure 6.7 and 6.8)** The **table 6.2** summarizes the results of the marker study on the cells cultured within the mebiol gel.

Figure 6.5



Figs. A, B, C & D show the sequential three dimensional (3-D) increase in size of HCLT biopsy tissue embedded within the Mebiol Gel. Fig A shows the growth as on day 3 with 3-D increase in size of the tissue & cells proliferation as seen in Fig. B. The Fig. C shows the migration of proliferating cells breaking through the margin of Mebiol Gel on day 10. Fig. D shows the proliferating cells outside Mebiol Gel margin forming a monolayer on day 11. Photomicrography of all figures done at Mag. 100X

Figure 6.6

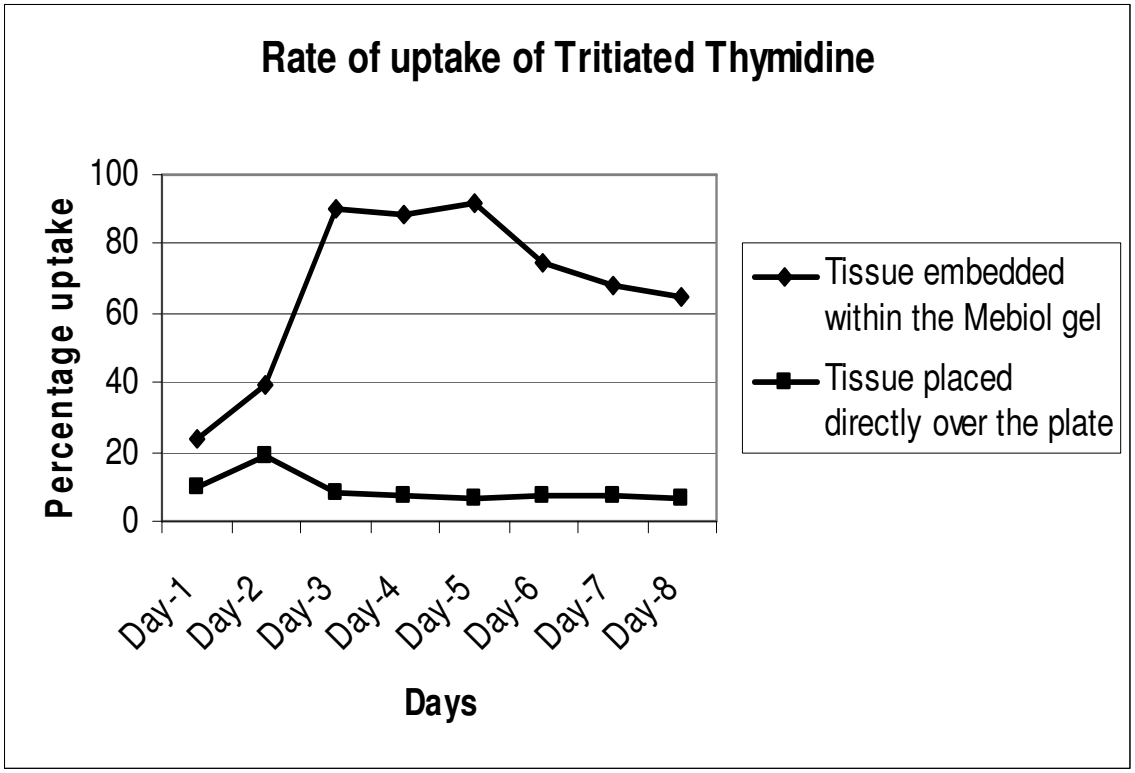
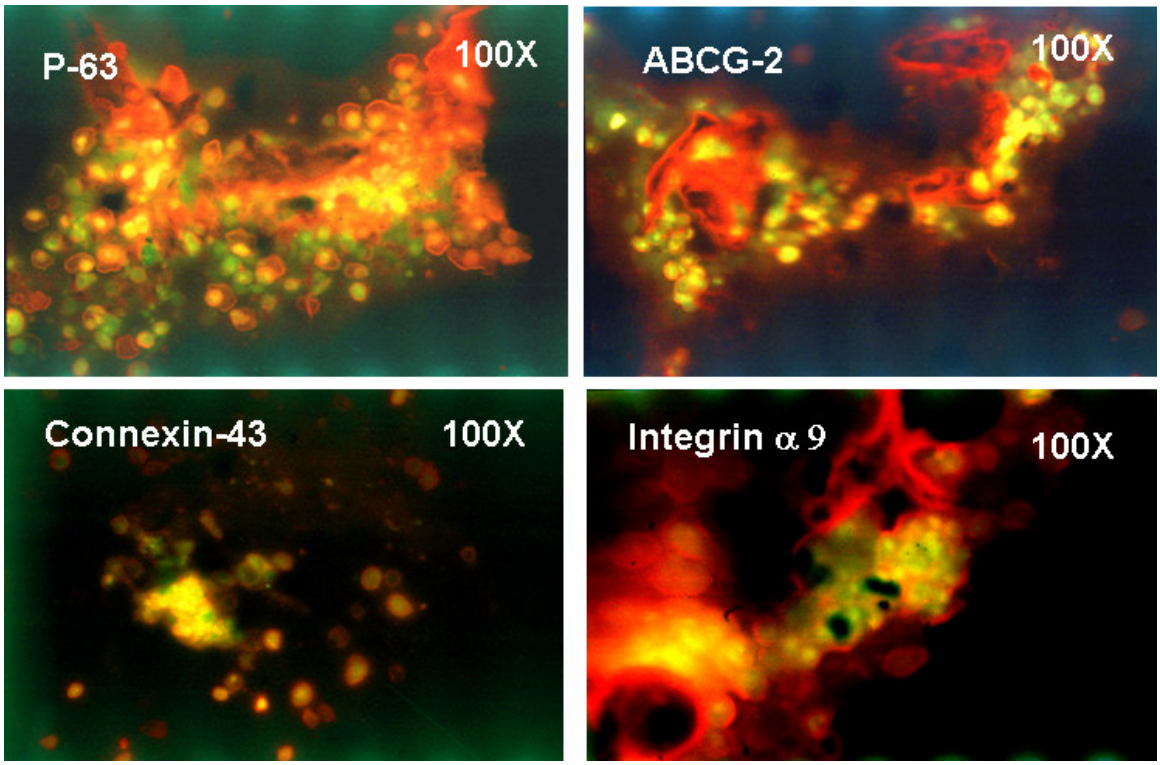

Rate of uptake of Tritiated Thymidine
Percentage uptake
100
80
60
40
20
0
Day-1
Day-2
Day-3
Day-4
Day-5
Day-6
Day-7
Day-8
Days
Tissue embedded within the Mebiol gel
Tissue placed directly over the plate

Figure 6.7:

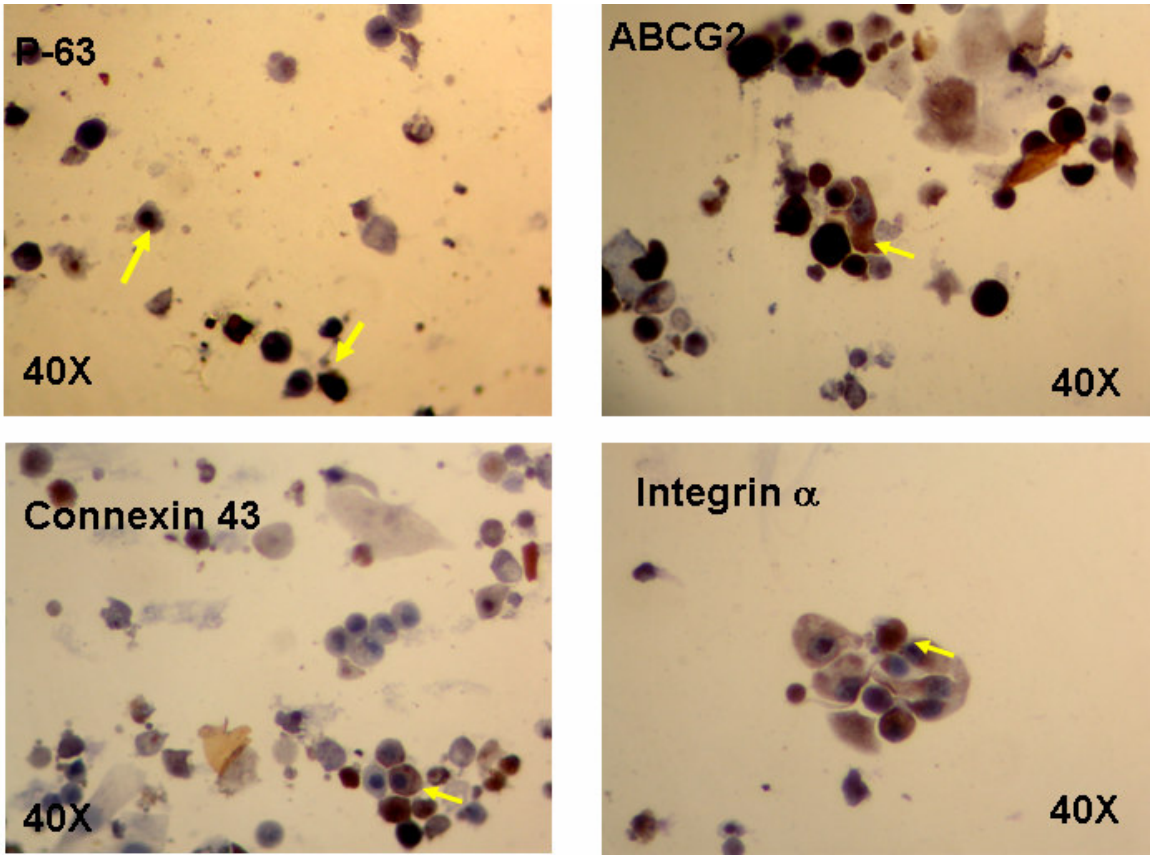
Immunofluorescence staining



This picture shows the expression of the p63, ABCG2 (stem cell associated marker) and connexin 43 and integrin. Green fluorescence shows the positive expression by immunofluorescence method.

Figure 6.8

Immunocytochemistry



This picture shows the immunocytochemistry on the cytospinned smear preparation of the cells harvested from the gel. The cells harvested were washed and cytospinned at 1000rpm for 5 minutes, and stained with the specific antibodies. Positive cells are shown with yellow arrow marked.

RT-PCR Results of the various stem cells associated and differentiation markers on the limbal explants cultured within the Mebiol Gel

Results of RT-PCR on the RNA extracted from the HCLT cultivated cells harvested from the Mebiol on days 4th, 8th, 12th, 16th and 20th days of incubation are shown in **Figure 6.9**

Table: 6.2. Shows the results of the immunomarker studies of each of the human corneal limbal explants cultivated within Thermoreversible polymer

S.No.	Marker study				Day of Harvesting
	P-63	ABCG-2	Connexin-43	Integrin α9	
1	+	+	+	+	13th
2	-	+	+	-	12th
3	+	+	-	-	10th
4	Cultivated tissues & cells were lost during processing				10th
5	+	+	+	+	10th
6	+	+	+	+	11th
7	+	+	+	+	12th
8	+	+	-	+	10th
9	+	+	-	-	11th
10	+	-	-	-	12th
11	+	+	-	-	11th
12	+	+	+	+	13th
13	+	+	+	-	13th
14	+	+	-	-	13th
15	+	+	-	-	10th
16	+	+	-	+	11th
17	+	+	-	-	11th
18	+	+	-	+	12th
19	+	+	+	+	11th

20	+	+	+	+	10^{th}
21	+	+	+	+	10^{th}
22	+	+	-	-	10^{th}

· Immunofluorescence staining was performed

 Tritiated thymidine uptake study was performed

+ Positive expression of the marker

- Negative for the marker

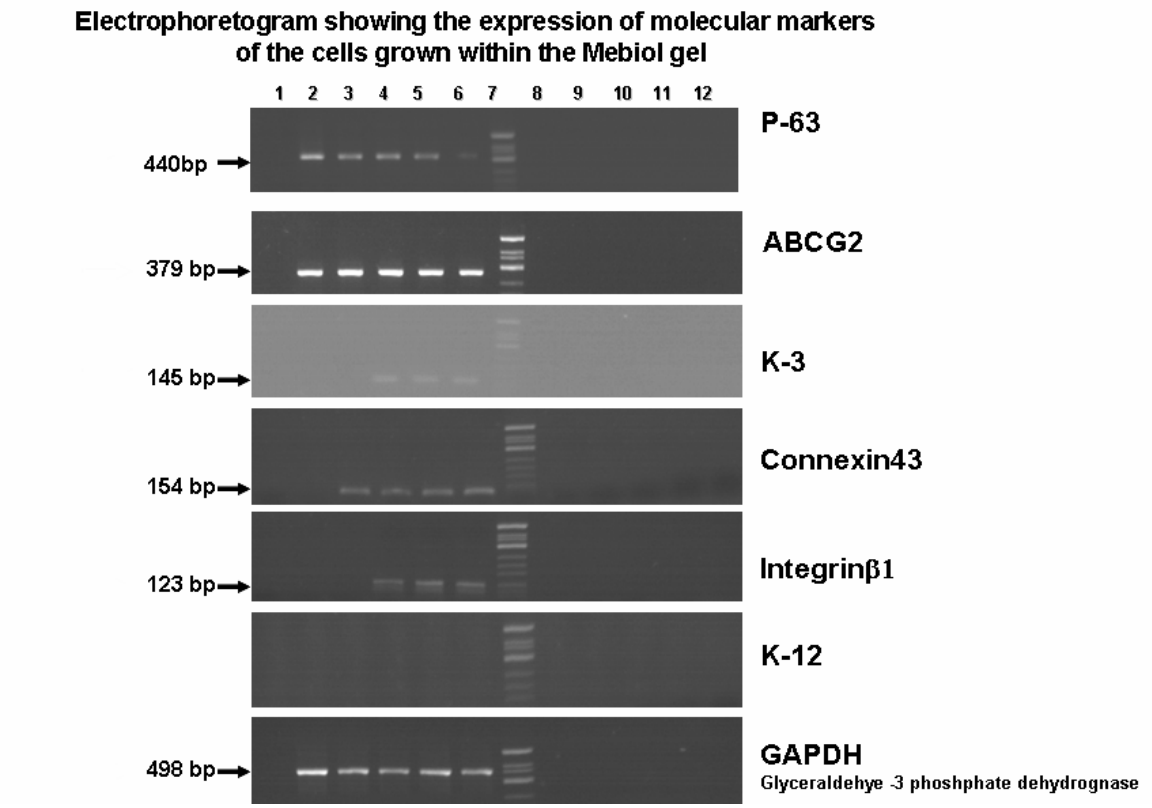
p63

P63, a transcription factor, has a role in morphogenesis of cells and is expressed in the nuclei of keratinocytes with proliferative potential and as keratinocyte stem cell marker of corneal epithelium and epidermis on the basis that it preferentially stained cells with greatest clonogenic capacity was shown to be marker of transient amplifying progenitor cells. Cells harvested from all the days expressed P63 and ABCG2 on all days.

ABCG2

ABCG2 (ATP-binding cassette super family G member 2), found in a variety of stem cells, is expressed on the plasma membrane with a functional role in developmental stem cell biology. Ocular surface epithelia contain ABCG2 dependent side population cells exhibiting features associated with stem cells representing putative corneal epithelial stem cells. (Budak *et al*, 2005; Chen *et al*, 2004) Expression of ABCG2 by immunophenotyping and RT-PCR on days all days shows that proliferating pluripotent stem cells in the cells cultivated within Mebiol Gel.

Figure 6.9:



This figure shows the results of reverse transcriptase polymerase chain reaction (RT-PCR) for m-RNA expression of ΔNp63, ABCG2, and Integrin β 1 Connexin43, K3 and K12 markers on the cells grown within the Mebiol gel. Tissues along with cells cultivated were harvested on days 4^{th}, 8^{th}, 12^{th}, 16^{th} and 20^{th}. Lane 1 is the Negative control and Lanes 2 – 6 is the m-RNA expression of the cells harvested on days 4^{th}, 8^{th}, 12^{th}, 16^{th} and 20^{th} days respectively. Lane 7 is the Molecular weight marker *ϕ X Hinf I* Digest, Lanes 8 – 12 are controls viz. DNA controls. P-63 and ABCG2 expression was seen on all days. Connexin 43 expression was seen from day 8 and K3 and Integrin β 1 expression from 12^{th} – 20^{th} day. K12 expression was absent.

Connexin 43:

Connexin 43, belonging to the family of 21 transmembrane proteins, is widely expressed in all layers of the corneal epithelium with the exception of the most superficial cells. This was not found on the expanded limbal epithelial cells on amniotic membrane. (Meller *et al*, 2002) Expression of this marker from 8^{th} day on the cells cultivated within Mebiol Gel indicating multiplication of transient amplifying cells.

Integrin alpha 9

Expression of integrin [alpha] α9 in cornea, limbus and limbal explant cultured on amniotic membrane showing its role in cell binding to laminin-1/5 of basement membrane. (Meller *et al*, 2002 ;) These integrins mediate adhesion of basal cells to the underlying basement membrane play a role in lateral cell migration and stratification to regulate onset of terminal differentiation. (Jones *et al*, 1993 ;) Keratinocytes possessing highest proliferative potential (stem cells) are distinguished from other proliferative cells (transient amplifying cells) because of higher expression levels of alpha 9. integrins, which are putative stem cell markers for epidermal keratinocytes. In our study, Integrin β1. positivity in cells cultured in Mebiol gel demonstrated multiplying transient amplifying cells further confirmed by results of RT-PCR experiments. Integrin α9 expression was seen from the 12^{th} day.

Keratins K3 & K12:

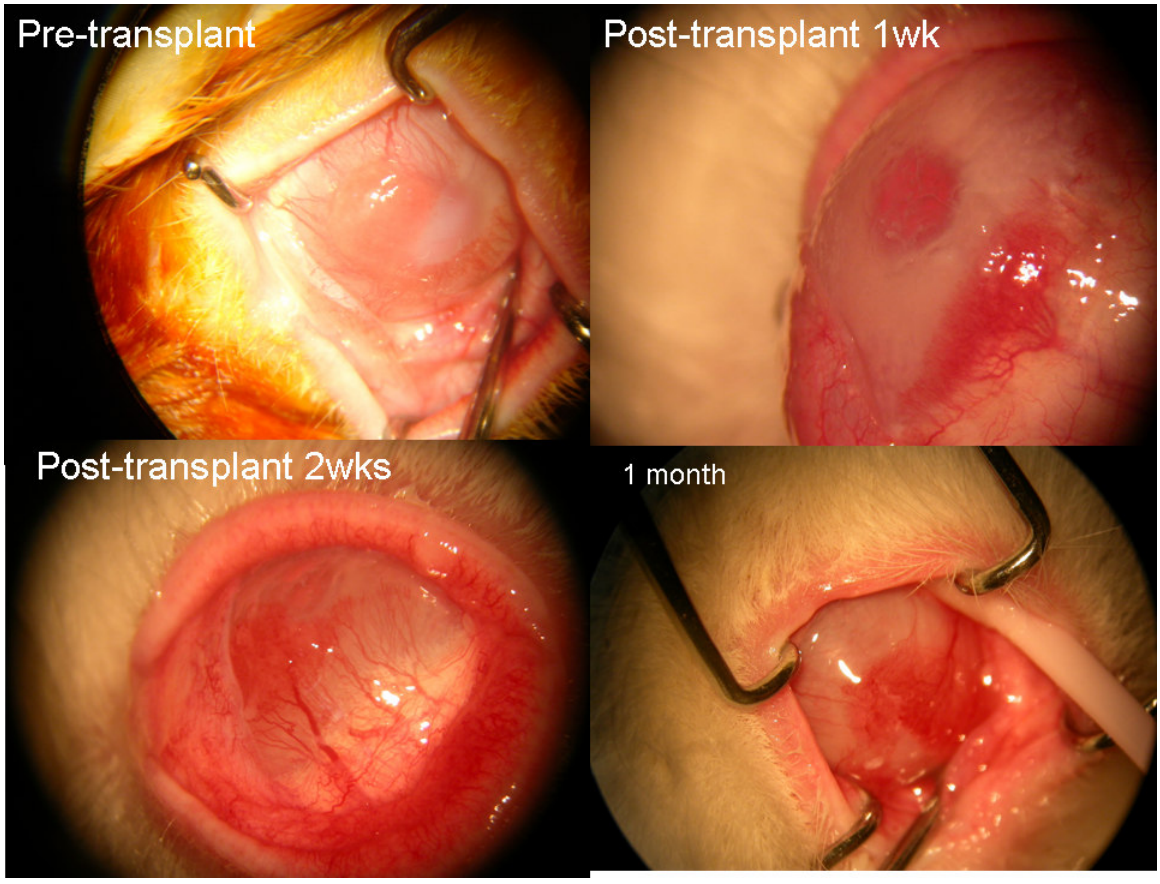
K3 and K12 are cornea specific keratins expressed by the mature corneal epithelium. RT-PCR results show the expression of the K3 from the 12^{th} day. Whereas K12 expression was absent.

Results of the rabbit model experiment:

We were able to grow the rabbit corneal epithelium in the Mebiol gel. The cells were cultured within the Mebiol gel for two weeks. After two weeks all the rabbits underwent similar surgery with the removal of the limbal tissues and application of ethanol as mentioned above. Clinically 12 rabbits had similar injuries, with evidence of moderate to severe damage to the ocular surface of the right eye, including superficial and deep neovascularisation and an irregular but intact epithelium with subepithelial haze. Based on the criteria as mentioned below the success rates was graded as mentioned in the **Table. 6.3**

Figure- 6.10a, b and c shows the results of one of the rabbits which gave total success. **Figure 6.11** shows the overall results of the rabbit model experiment. **Table 6.4** shows the complete results of the rabbit model experiment after the clinical and histopathological evaluation

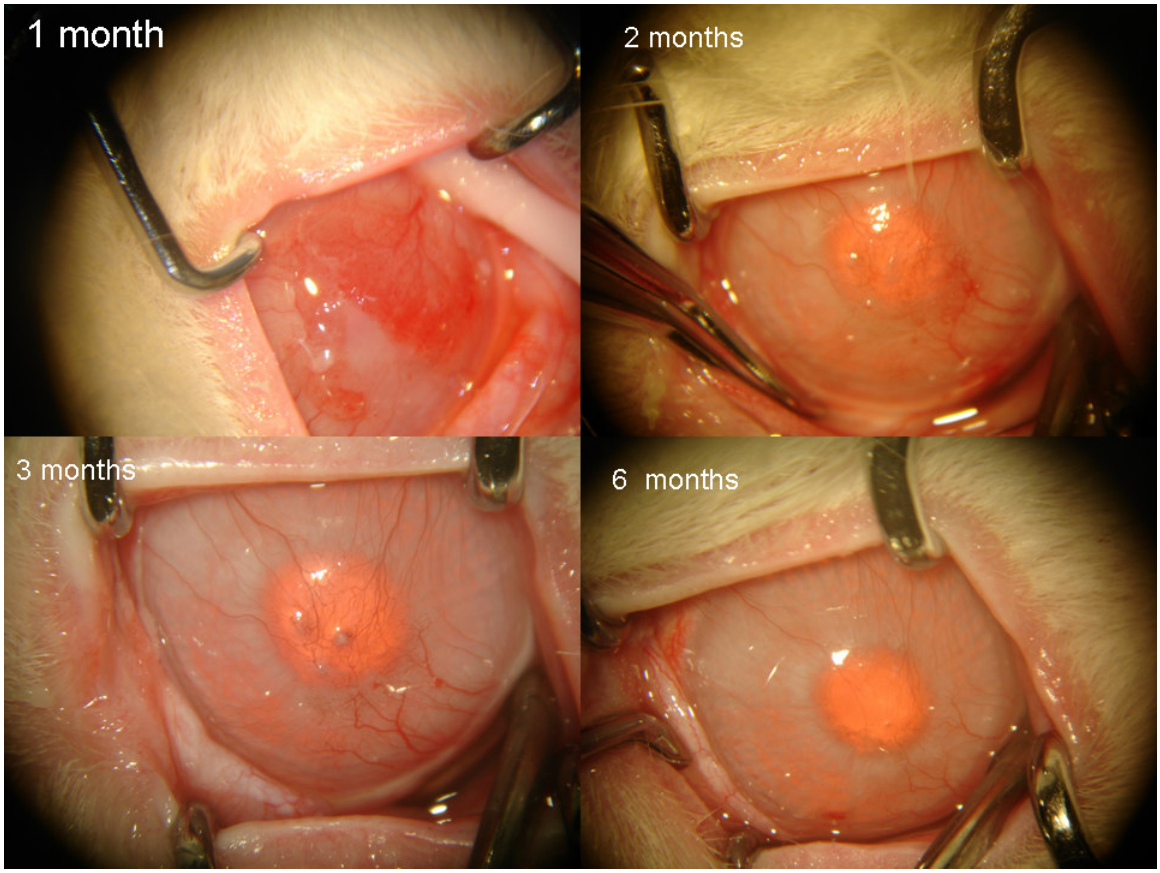
Figure 6.10 a



This figure shows the pre transplant and the post transplant picture of the Rabbit J

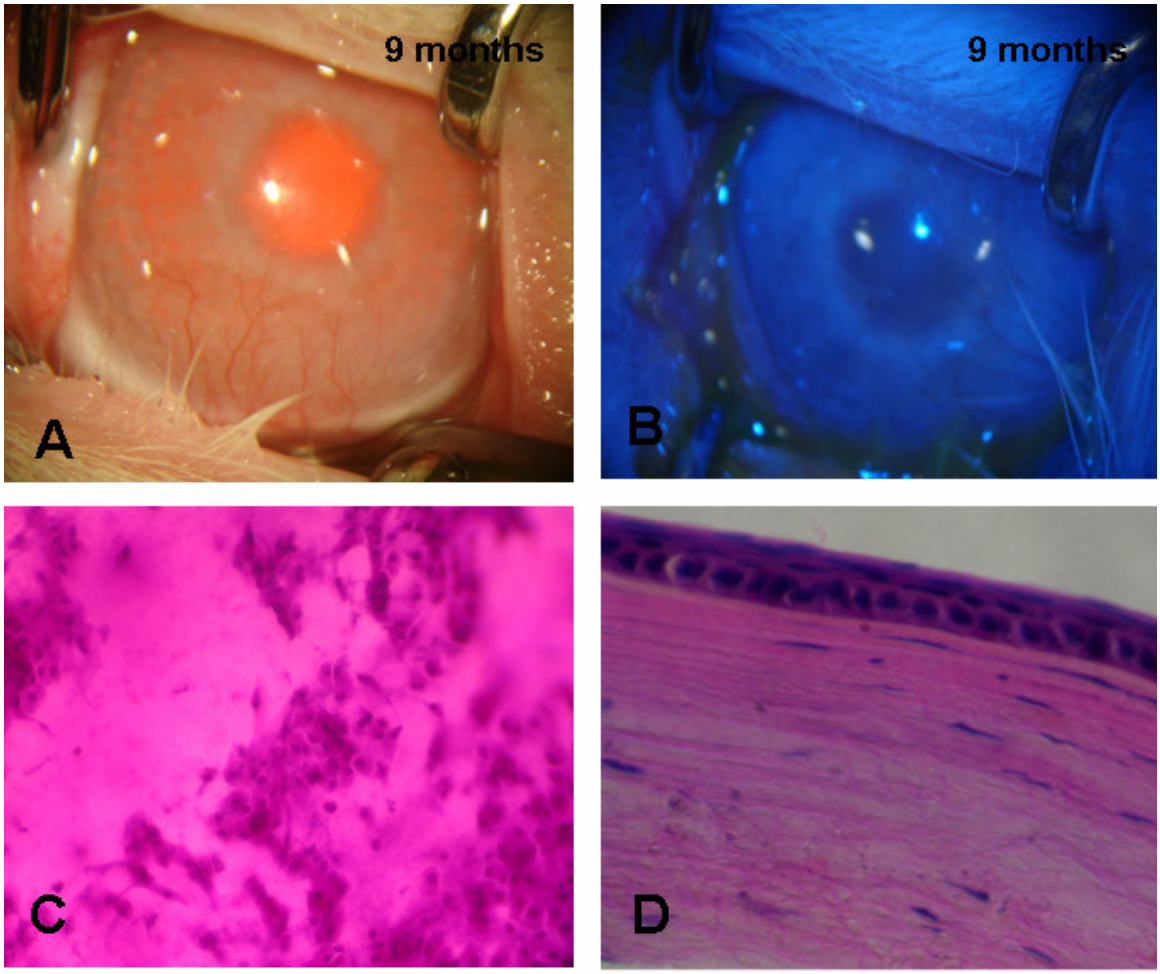
Severe vascularization was seen in both pre and post transplant pictures.

Figure 6.10b



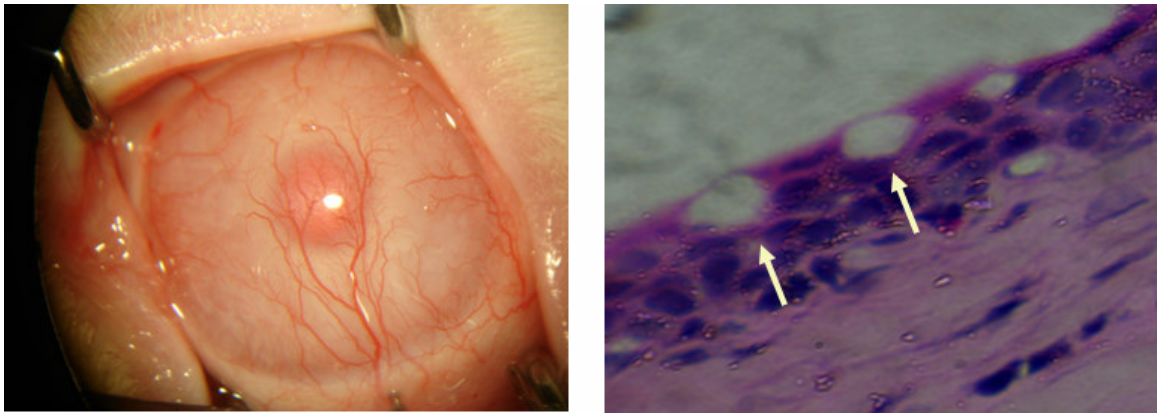
This figure shows the post transplantation picture at the end of 1, 2, 3 and 6 months. There is a gradual increase in the clarity; The vascularization has reduced. The cornea looks clear and smooth at the end of 6 months.

Figure 6.10c



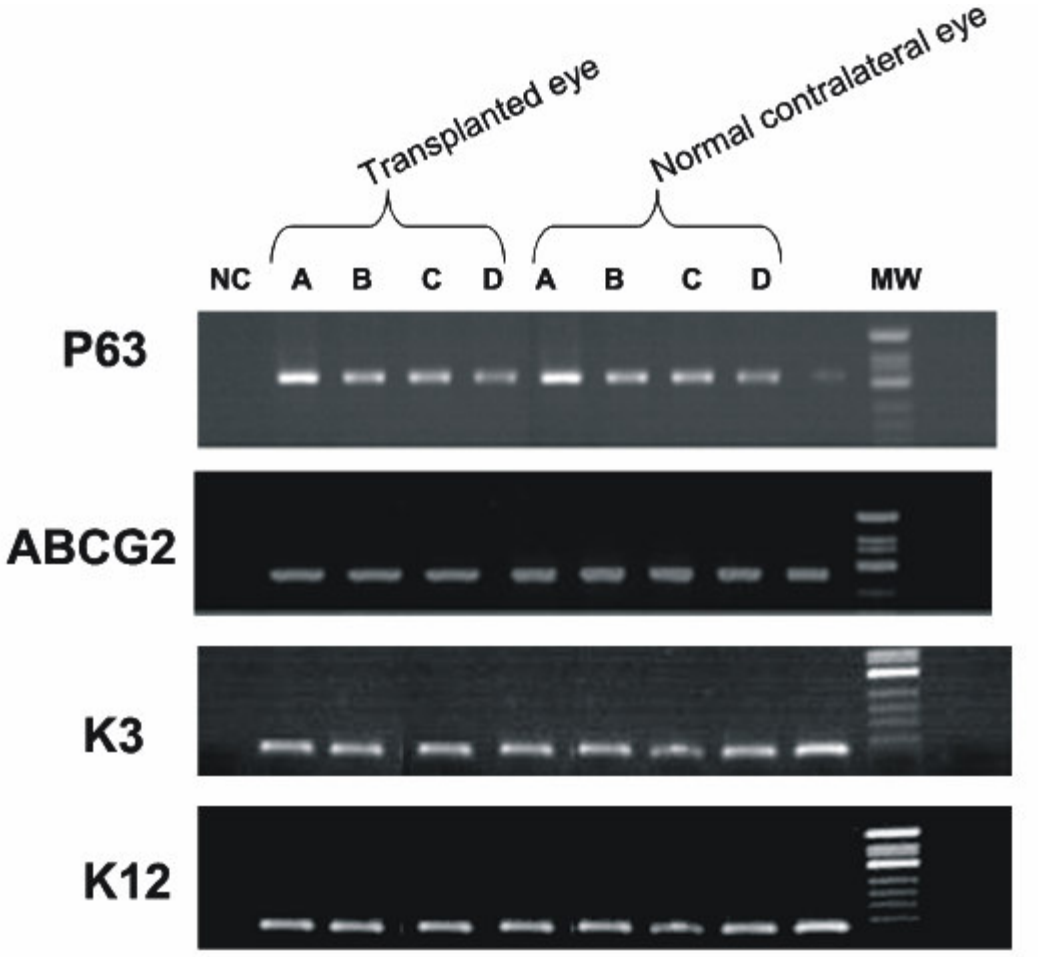
This figure shows the follow up of the rabbit J at the end of 9months. The vascularization has completely regressed and the corneal surface is totally clear and smooth. The figure B is negative fluorescein stain of the cornea, which shows that there is no epithelial defect of the cornea. Figure C is the impression cytology done at the end of nice months. Impression cytology shows no goblet cells and sheets of epithelial cells are seen. Figure D shows the histology of the sections made from the rabbit eye at the end of 9 months. The section shows 3 – 4 layered epithelium and there are no blood vessels and the epithelial cells looks healthy.

Figure 6.11:



This figure shows the picture of the failure case (score, <5) in 3 (25%). At the end of 9months there is no regression of the conjunctivalization and picture in the right shows the histology of the same rabbit, where there are numerous goblet cells seen in between.

Figure 6.12:



This figure shows the electrophoretogram of the markers done on the cells harvested from the rabbits at 9 months. The transplanted eye and the other normal contralateral eye showed similar pattern of expression of all the markers. Lane Nc is the negative control. Lane A-D on the transplanted eye and lane A-D on the contralateral eye showed the expression. Lane MW is the molecular weight marker (*Hinf I* ϕX digest)

Table 6.3

The following are the criteria used for the evaluation.

	Evaluation of vascularization	**Score**
A	No vascularization beyond 3 mm in any area	4
B	Neovascularisation of 4mm or more in $\leq$ 3 clock hours	3
C	Neovascularisation of 4mm or more in > 3 clock hours to $\leq$ 6 clock hours	2
D	Neovascularisation of 4mm or more in > 6 clock hours to $\leq$ 9 clock hours	1
E	Neovascularisation of 4mm or more in > 9 clock hours	0

	Evaluation of corneal haze	**score**
1	No haze: clear cornea, iris details seen clearly	2
2	Mild haze: visible but iris details visible	1
3	Severe haze: iris details obscured	0

Evaluation of fluorescein staining		**score**
I	Negative	1
II	Positive	0

Grading scale for corneal epithelial architecture based on hematoxylin and eosin stain seen with histologic examination		**score**
I	Nearly normal with few, if any, goblet cells; basal cells with maturation seen	3
II	Goblet cells (< 10%); abnormal epithelium but basal cells with some maturation seen	2
III	Goblet cells (> 25%); abnormal epithelium cells with flattened cells; some layering of epithelial cells; some normal appearing basal cells	1
IV	Disorganized epithelium with flattened abnormal appearing cells; minimal, if any maturation or layering	0

Maximum score that can be given is 10.

Success - **8 or more**

Partial success – **6, 7**

Failure - **0 to 5**

Table 6.4

Results after clinical and histopathological evaluation:

Rabbit	**Fluorescent stain**	**Vascularisation**	**Corneal haze**	**Histopathology**	**Results**
1	1	4	2	3	**S**
2	1	4	2	3	**S**
3	1	2	1	3	**PS**
4	1	4	1	3	**S**
5	1	4	0	3	**S**
6	1	0	0	2	**F**
7	1	0	0	1	**F**
8	1	3	2	3	**S**
9	1	2	2	2	**PS**
10	1	4	2	3	**S**
11	1	4	2	3	**S**
12	1	2	1	1	**F**

Success – S – 7

Partial Success – PS – 2

Failure – F – 3

Evaluation of the molecular markers on the RNA extracted from the limbal region and central cornea after the enucleation:

From the extracted RNA expression of ΔNp63, ABCG2, Connexin 43, and Keratin3 &12 was examined. The RNA extracted from the limbus shows the expression of all the markers, whereas the central cornea expresses only p63, connexin 43, and Keratin 3 and 12 but there is complete absence of the ABCG2 marker. In control rabbits, where only gel was transplanted without the cells, expression of ΔNp63 and ABCG2 was negative whereas there was faint expression of connexin43 and keratin 3 and 12.

6.5 Discussion:

The objective of our study was to observe the following: 1) whether the polymer Mebiol gel supported the growth of the cells from the limbal explants,2) whether the cultured cells were able to multiply, were viable and 3) most importantly, whether the cultured cells were able to retain the limbal phenotype and to show cornea-specific differentiation.

We observed that the Mebiol gel facilitates the growth of cells from the limbal explants. This was also supported by the 3H-thymidine incorporation studies. The cultured cells were shown to be viable by the trypan blue exclusion test. The results from the immunochemistry and the RT-PCR studies established that the cultured cells were limbal phenotype.

The cells cultured in Mebiol gel, were positive for presumed limbal stem cell association markers ABCG2, and p63 cells from the day 4. ABCG2, found in a variety of stem cells,

is expressed on the plasma membrane with a functional role in developmental stem cell biology. Ocular surface epithelia contain ABCG2 dependent side population cells exhibiting features associated with stem cells representing putative corneal epithelial stem cells. (Budak *et al*, 2005; Chen *et al*, 2004 ;)

Transcription factor p63 initially was considered as a new marker for limbal stem cells. p63 is expressed in committed progenitor cells of both corneal and conjunctival epithelium as well as in basal/parabasal cells in other squamous epithelial tissues (Meller *et al*, 2002 ;) Using the monoclonal antibody clone 4A4, which reacts with all p63 isoforms, our immunocytochemical investigation showed p63 staining on cells harvested on all the days. Since p63 was expressed on the cells harvested initially and on day 20, it suggests that p63 may represent both presumed limbal stem cells and corneal epithelial cells in a proliferative state, such as transient amplifying cells (TAC) (Budak *et al*, 2005; Koizumi *et al*, 2001;)

The differentiation markers connexin 43, belonging to the family of 21 transmembrane proteins, is widely expressed in all layers of the corneal epithelium with the exception of the most superficial cells. This was not found on the expanded limbal epithelial cells on amniotic membrane. (Meller *et al*, 2002 ;)

Integrin $\alpha 9$, a marker for transient amplifying cells and K3 corneal phenotype markers were positive from day 12. K3 and K12 are specifically expressed in corneal epithelial cells and are regarded as markers of corneal epithelial differentiation. The appearance of

the K3/K12 keratin pair during migration from the limbal to the corneal stroma has been interpreted as differentiation of SC into TAC. (Grueterich *et al*, 2003 ;)

Thus, our study demonstrated that Mebiol gel supported the growth of cultured cells from limbal explants. Since we were able to observe the expression of presumed limbal stem cell association markers in limbal explants cultured in Mebiol gel, there was no need for using 3T3 feeder layer when culturing cells for human clinical use.

Mebiol gel has been used as wound dressing, microcapsule for islets, (Shimizu *et al*, 1996 ;) electrophoretic gels for DNA separation, (Tsukikawa *et al*, 2003) and three dimensional culture matrix for various cells (Hishikawa *et al*, 2004;) Cancer cells rapidly grew in Mebiol gel while fibroblasts did not grow (Madhavan *et al*, 2004 ;) Our earlier investigations on cultivation of standard animal cell cultures embedded in Mebiol gel indicated their rapid multiplication with formation of monolayers without signs of cytotoxicity.

In summary, we demonstrated the use of a synthetic polymer for the growth of corneal limbal epithelial cells. The Mebiol gel supported the proliferation of epithelial cells in culture and the cells were viable. The cells expressed presumed limbal stem cell association markers and cornea phenotype suggesting that Mebiol gel retains a mixed population both corneal stem cells and differentiated cells. The use of biosynthetic, biocompatible membranes offers several advantages in terms of eliminating the risk of disease transmission, reducing the inconsistency in tissue composition associated with

biological substrates, being able to be custom fabricated to suit specific requirements, and possibly providing a readily available alternative tissue source for clinical use.

The Mebiol gel is also far more transparent than HAM. This promising material may have the potential to be used in tissue engineering ocular surface equivalents in the future. These findings have important clinical implications and are an important step toward the development of a safe and effective bioengineered tissue equivalent for clinical use.

Animal model experiment gave the promising result. Using the Mebiol gel we were able to successfully reconstruct the damaged corneal epithelium. Out of the 12 rabbits 7 had successful epithelization and was complete success and 2 had the partial success. Only 3 had failure results. The method used for the reconstruction is sutureless technique. Thus transplantation of cultured cells becomes very easy by this carrier free method.

12 rabbits were rendered limbal deficient surgically after a limbal biopsy was taken to grow the limbal epithelial cells. The limbal stem cells were then cultured in a TGP (Mebiol Gel). 3 weeks later, transplantation was done on the rabbits after removal of the corneal pannus. The rabbits were evaluated at 1month, 3 months, & 6 months.

Outcome measures (modified from Schwab *et al*, were

- Vascularisation of cornea >4mm from the limbus
- Corneal haze
- Fluorescein staining
- Histopathology

Based on these outcome measures a comprehensive scoring system was devised.

At last follow up we had 58% success and 17% partial success. 25% of rabbits failed. Histopathologically there was evidence of three to four layer epithelium formations with basement membrane. Immunomarker study documented presence of p63 and the ABCG2 marker stem cell associated marker on the cells harvested from the limbal region. Cornea specific markers K3/K12, Connexin 43 on the cells harvested on the central portion of the cornea.

The results of this rabbit model suggest that autologous corneal limbal epithelial cells grown in TGP (Mebiol Gel) may restore a nearly normal ocular epithelial surface in eyes with unilateral LSCD.

Conclusion:

Limbal epithelial cells grow with ease in the thermoreversible gelation polymer. The cultured cells were able to retain the limbal phenotype. The results of the rabbit model suggest that autologous limbal epithelial cells grown in thermo-reversible gel polymer may restore a nearly normal ocular epithelial surface in eyes with unilateral LSCD.

CHAPTER 7 - EFFECT OF 3T3 IN CULTURING OF CORNEAL LIMBAL STEM CELLS

7.1 Introduction:

Ex vivo expansion of the limbal epithelium using amniotic membrane (AM) as a biological substrate is a well known technique for ocular surface reconstruction in patients with partial or total limbal stem cell deficiency. (Tsai *et al*, 2000; Schwab *et al*, 2000, Koizumi *et al*, 2001a; 2001b; Grueterich *et al*, 2002;) There are many studies which have shown favorable clinical outcomes, they adopted different culturing techniques for *ex vivo* expansion, especially regarding the preparation of AM and the inclusion of 3T3 fibroblast feeder layers.

The use of feeder cells in cell culture was first reported by Puck *et al*. (Puck *et al*, 1956 ;) Feeder cells are especially effective for the support of growth of cells that are difficult to culture. Feeder cells provide a suitable environment in the co-culture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interaction (Ehmann *et al*, 1998;), production of soluble growth factors and removal of toxicants from the culture medium. Fibroblasts are commonly used as feeder layer for the culturing of the corneal limbal stem cells; Fibroblast culture produces a variety of necessary growth factors and ECM components (Rajabalian *et al*, 2003 ;). These components are dependent on or mediated by the expression of the other. Studies on the growth of the limbal stem cells showed that they have a greater growth potential in explant cultures and higher clonogenicity when co-cultured on 3T3 fibroblasts feeder

layers and their proliferative potential is resistant to tumor promoting phorbol esters (Meller *et al*, 2002; Grueterich *et al*, 2003;).

There are several studies which has shown favorable clinical outcome on using 3T3 fibroblast feeder layer. Koizumi *et al*. (Koizumi *et al*, 2000) seeded limbal explants on epithelially denuded AM with an additional feeder layer of mouse 3T3 fibroblasts on the plastic dish underneath the AM. Schwab *et al*. first expanded epithelial cells by a 3T3 fibroblast feeder layer and then seeded them on denuded AM. Tsai *et al*., however, seeded limbal explants on intact AM without the use of a 3T3 fibroblast feeder layer.

Grueterich *et al* has reported the beneficial role of the 3T3 feeder layer. In their study, they have examined the beneficial role of 3T3 fibroblast feeder layers in human limbal explant cultures on denuded AM in comparison with those on intact AM alone by studying the modulation of connexin 43 and keratin expression. (Grueterich *et al*, 2003)

Efforts have been made for establishing a limbal cell culture procedure; however, the state of differentiation of the cells as defined by the cytokeratin profile and Pluripotent stem cell marker expression has not been examined. (Kiritoshi *et al*, 1991; Li *et al*, 1996; Joseph *et al*, 2004;)

In this study we compared the expression of the stem cell associated marker (p63 and ABCG2) and differentiation marker (Connexin 43 and K3/K12) on the cells cultured in the presence of mitomycin C treated 3T3 feeder layer on various culture conditions.

7.2 Design of experiments:

7.2.1Preparation of Human Amniotic Membrane:

Human Amniotic membrane was prepared according to protocol mentioned in the methodology section.

7.2.2 Preparation of 3T3 fibroblasts:

Confluent murine 3T3 fibroblasts obtained from National Centre for Cell sciences (NCCS, Pune) were incubated with 4 µg/mL mitomycin C (MMC) for 2 hours at 37°C under 5% CO2, trypsinised and plated onto cell culture dishes at a density of 2.2 x 10^4 cells/cm^2. These feeder cells were used 4 h to 24 h after plating.

7.2.3 Limbal Biopsy preparation:

Corneal limbal biopsy of 2 mm^3 from the cadaveric donor eye was collected in DMEM with 3% FCS and antibiotics as the transport medium and was transported to the cell biology laboratory for further processing was done according to the protocol given in the methodology section.

7.2.4 Culture of limbal explants on the denuded AM in the presence and absence of 3T3 feeder layer

Human Limbal Explants culture on the deepithelialized amniotic membrane with and without the feeder layer:

AM with the basement membrane facing upward was fastened on the culture insert as described previously. (Meller *et al*, 1990 ;). Two different culture conditions for the

limbal epithelium were analyzed; denuded AM and denuded AM with 3T3 fibroblast feeder layer. On the centre of the EDTA treated AM an explant was placed and cultured in the medium containing equal volume of DMEM and F12 mixture and the medium was changed once in three days and cell growth was monitored daily for 3 weeks with an inverted phase contrast microscope (Nikon, Tokyo, Japan). Ten of the 20 cultures with the denuded AM were co cultured on a 3T3 fibroblast feeder layer that was prepared as mentioned on the tissue cultured plate with a method that has been reported. (Meller *et al*, 1999 ;) For the culture with the 3T3 the culture inserts with the AM and limbal explants were placed on top of the 3T3 layer. The medium was changed every 2 to 3 days. The cultures were incubated for about 3 to 4 weeks until they reach the confluence. After 3 weeks the cultures were embedded in optimal cutting temperature (OCT) compound and snap frozen for immunohistochemistry.

7.2.5 Immunostaining:

Immunostaining was done on the frozen section with the antibody dilution of 1: 100 and 1: 75 for p63 and ABCG2 respectively.

7.2.6 Western Blot analysis:

Western blot was used to confirm the expression of ABCG2 and p63 by cultured limbal limbal epithelial cells on denuded membrane and denuded membrane with 3T3 feeder layer. Two cultures from each donor were initiated and sampled to achieve sufficient cells for protein extraction. After limbal epithelium cultures on denuded (n=3) and denuded AM with 3T3 (n=3) reached plateau growth, the cultured cells over the

membrane were scraped using cell scraper and collected in lysis buffer (Proprep protein extraction Kit, CA, USA), protein was extracted according to protocol mentioned in the methodology section.

7.2.7 RNA isolation and RT- PCR analysis:

Once there was a confluent growth the cells were harvested and RNA was extracted and cDNA was converted according to the procedure given in the methodology section. (Li *et al*, 1995; 2001). Using specific primer pairs, designed from published human gene sequences (Chen *et al* 2004 ;) (**Table 1**) for different markers in Eppendorf PCR systems. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control, the mRNA expression of different molecular markers was analyzed by RT-PCR. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% ethidium bromide with molecular marker *Hinf I* φ digest to confirm the size of the resultant product of the amplification curve. The fidelity of the RT-PCR products was verified by comparing their size with the expected cDNA bands and by sequencing the PCR products.

Statistical analysis:

All the experiments were performed in triplicates. For proliferation index and the expression study the two tailed t test was performed.

7.3 Results:

Cultivation of corneal limbal explants on denuded AM in the presence of the 3T3 feeder layer:

A total of 20 limbal biopsies were obtained the over a period of 6 months between December 2005 – May 2006. The tissues from the donor aged 8 7.–90 years were collected within 2 hours after death. **Figure 7.1** shows the growth of the cells on the denuded AM and with 3T3 feeder layer. The growth of cells from the explants were started by the end of day 3 and almost reached the confluence by the end of day 21.

Cultivation of corneal limbal explants on denuded AM in the absence of the 3T3 feeder layer:

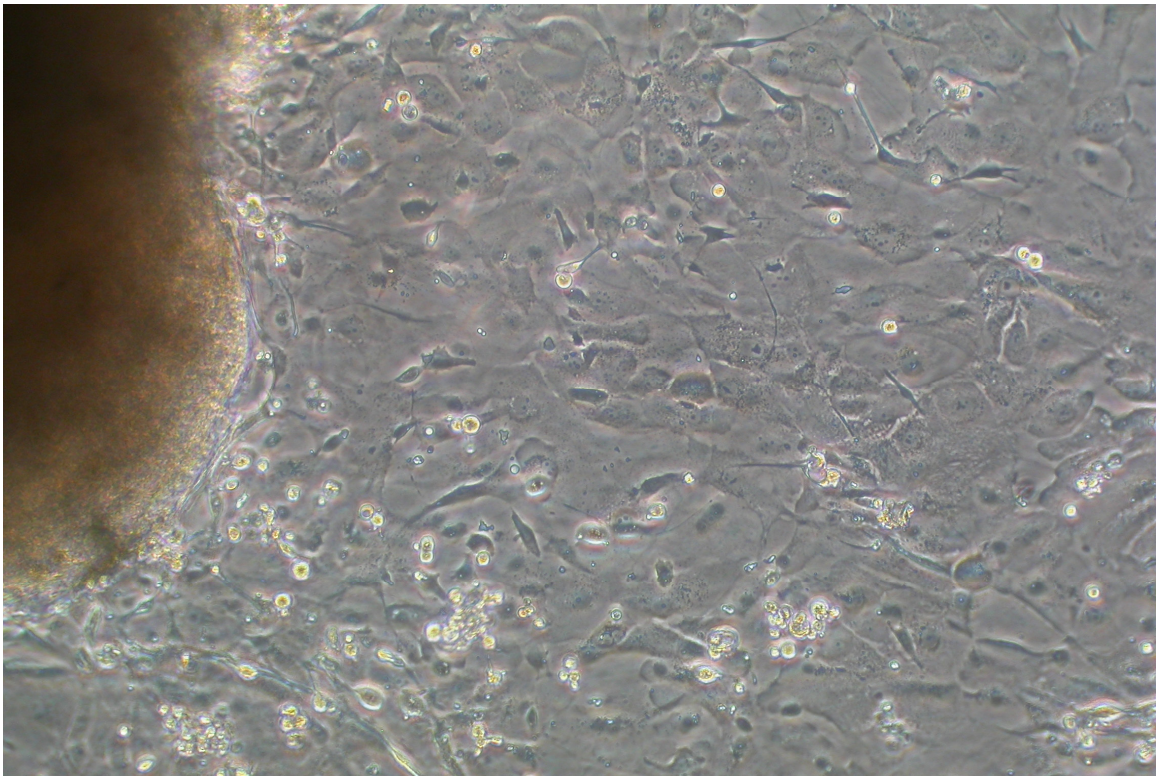
The outgrowth rate of the 10 cultures was photographed and measured each time the culture medium was changed until the cultures reached the confluence till 3 – 4 weeks time.(data not shown) The cells cultured over the denuded AM and denuded AM with 3T3 had similar growth rate and reached almost a confluent growth by the end of 21st day.

ABCG2 and p63 expression by immunohistochemistry and western blot on the cultured corneal limbal explants on denuded AM in the presence of the 3T3 feeder layer:

Immunohistochemistry was done on the cultured cells at the end of 3 weeks. The cells cultured over the denuded AM in the presence of 3T3 shows the expression of both p63 and ABCG2. A few basal cells showed the expression of both the markers. **(Figure 7.2)**

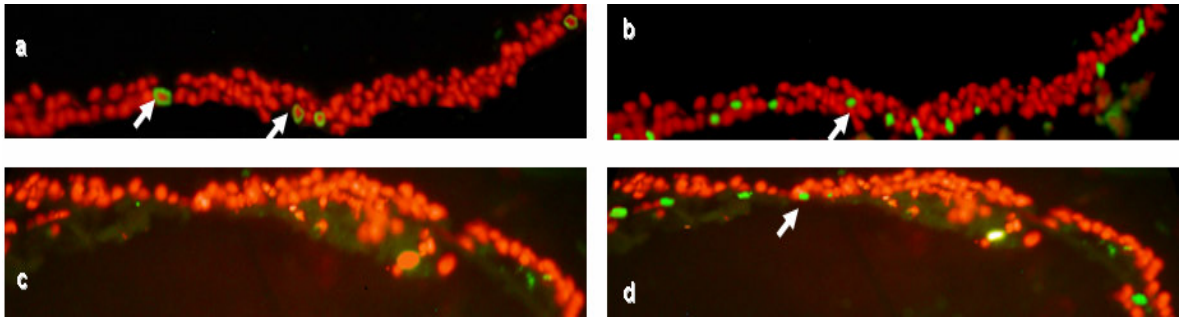
Figure 7.1

Growth of the cells in the presence of 3T3 feeder layer



This figure shows the growth of the limbal epithelial cells from the limbal explants cultured in the presence of the 3T3 feeder layer. This is the culture photograph taken at the end of 2 weeks of incubation. 100X Mag

Figure 7.2



This figure shows the immunofluorescence picture of the p63 and ABCG2 on the cultured over the denuded AM with and without 3T3 feeder layer at the end of 3 weeks. Figure 2a and 2c shows the expression of ABCG2. a & b cells grown on the denuded AM in the presence of 3T3 feeder layer, c & d cells cultured over the denuded AM without 3T3 feeder layer. Few basal cells show the expression of ABCG2 on the cells cultured in the presence of 3T3 feeder layer. C - cells cultured over the denuded without 3T3 feeder layer shows negative expression. b –shows the positive p63 expression on the cells cultured over the denuded AM +3T3 feeder layer. d – Positive expression on denuded AM without 3T3 feeder layer. (Mag – 20X)

The expression of this marker was confirmed by western blot. Western blot results showed the faint positive expression of both p63 and ABCG2 on the cells harvested at the end of 3 weeks. **(Figure 7.3)**

ABCG2 and p63 expression by immunohistochemistry and western blot on the cultured corneal limbal explants on denuded AM in the absence of the 3T3 feeder layer:

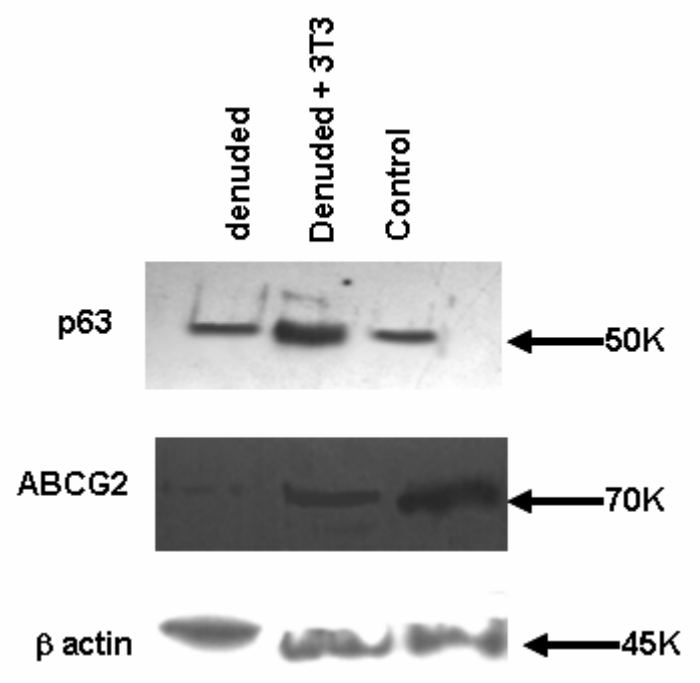
ABCG2 expression was completely absent on the cells cultured over the denuded membrane but the p63 expression was seen on the few basal cells at the end of 3 weeks**.(Figure 7.2c and 7.2d)** This was confirmed by the western blot on the cells harvested at the end of 3 weeks. There was complete absence of the ABCG2 but there was faint expression of the p63 protein**. (Figure 7.3)**

RT-PCR Results:

RT-PCR data on the expression of ΔNp63, ABCG2, Connexin 43 and K3/K12 on the cells cultured over the denuded AM without 3T3 feeder layer:

Faint expression of p63 and ABCG2 was seen only on the cells harvested at 8^{th} day but it was completely absent on cells harvested till 21^{st} day. Similarly the expression of Connexin – 43 and Keratin 3 &12 was comparatively more on the cells cultured over the denuded AM without 3T3 feeder layer**. (Figure 7.4)**

Figure 7.3 Western blot results



This figure shows the western blot data on the cells harvested at the end of 21days, cultured over the denuded AM and denuded AM with 3T3. The positive expression of p63 was observed on both denuded AM and denuded AM with 3T3 feeder layer. ABCG2 expression was completely absent on the cells harvested from the denuded membrane, whereas it is positive on the cells cultured with 3T3 feeder layer. Control lane – p63 – SiHa cell lysate (Positive control); control lane – ABCG2 – MCF 7 cell lysate (positive control). βactin shows the loading control.

RT-PCR data on the expression of ΔNp63, ABCG2, Connexin 43 and K3/K12 on the cells cultured over the denuded AM with the 3T3 feeder layer:

The cultured cells showed the expression of p63 and ABCG2 till 21 days of incubation. There was a gradual decrease in the expression of these stem cells associated markers; it was able to retain the expression till 21 days of incubation. Similarly the expression of connexin43 and keratin 3&12 was seen but it was comparatively less than those cultured only on the denuded AM. **(Figure 7.4)**

7.4 Discussion:

The limbal corneal epithelial cells cultured over denuded AM under the influence of feeder layer were able to retain the expression of the putative stem cell makers, ABCG2 and p63 in contrast to cells cultured over denuded AM minus feeder layer.

Immunohistochemical expression of p63 and ABCG2 expression was observed at the end 21 days and the expression was confirmed by the western blot. Western blot results show that the expression of p63 is seen both the culture conditions whereas the ABCG2 expression was seen only the cells cultured over the denuded AM with 3T3 feeder layer. RT-PCR results shows that expression of p63 and ABCG2 showed a gradual decrease by the end of day 21 on the cells cultured in presence of 3T3, but they were able to retain the expression of the p63 and ABCG2, but cells cultured on the denuded AM minus 3T3 were not able to retain the ABCG2 marker. Similarly, the expression of gap junction connexin 43 and corneal keratins keratin 3 and 12 was observed in cells cultured over denuded AM, in the absence of feeder layer. There was gradual increase in the

Figure 7.4 a

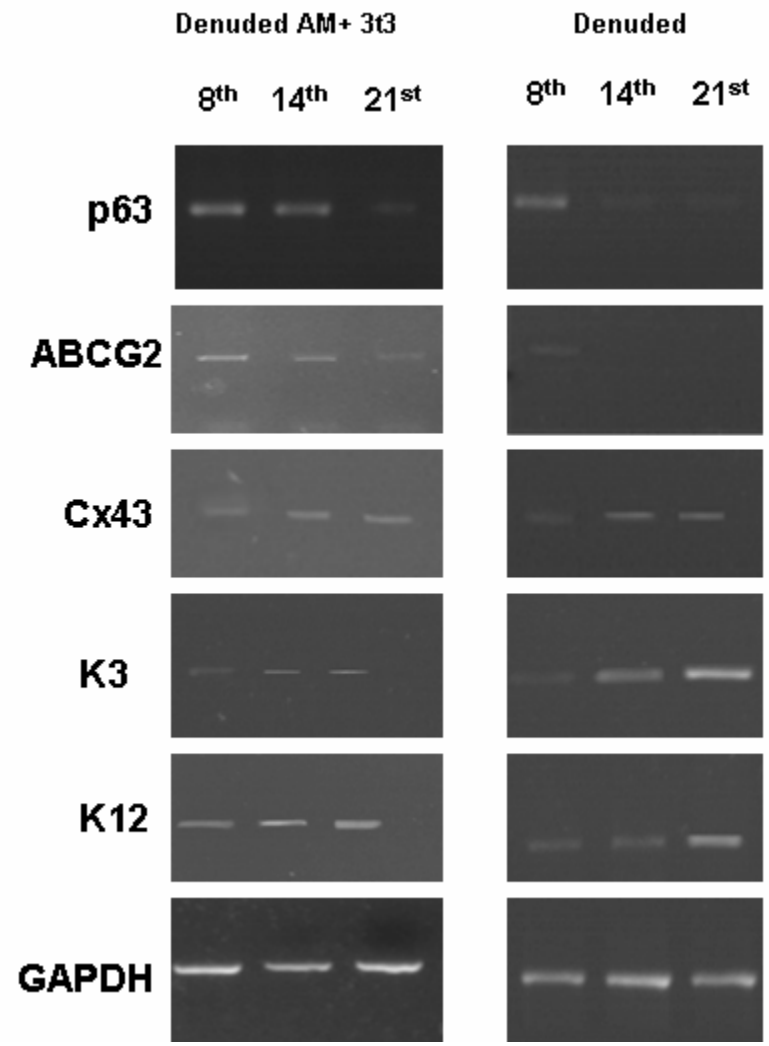
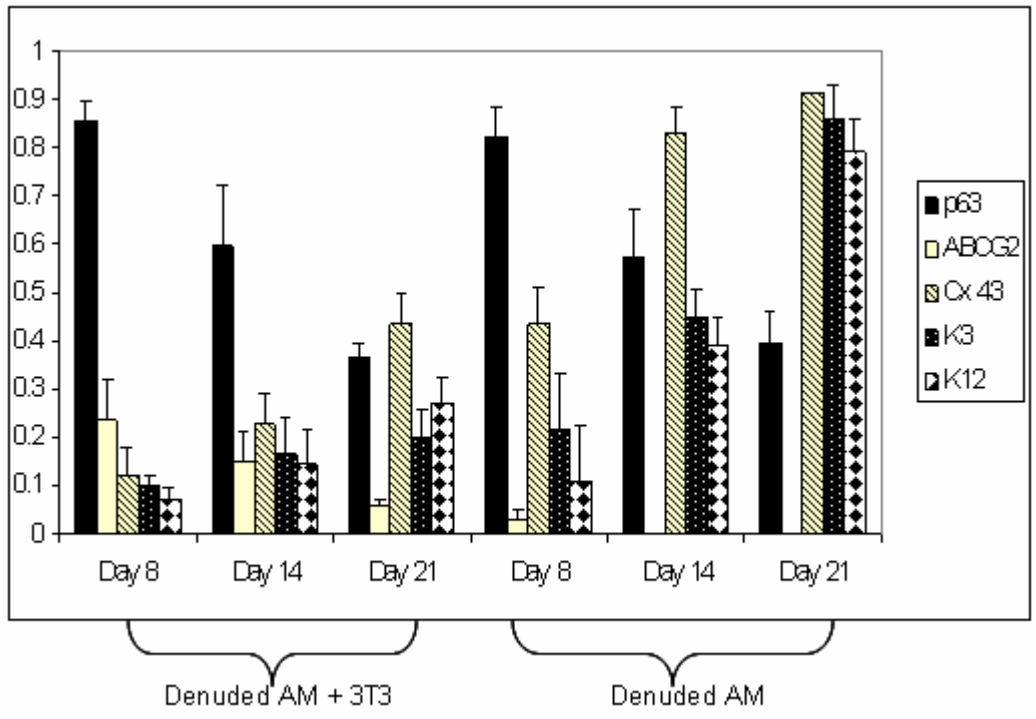


Figure 7.4a &b shows the RT-PCR results on the cells harvested from the denuded AM and denuded AM with 3T3 feeder layer. The gel photograph shows the expression of the p63, ABCG2, connexin43, K3 and K12 on the cells harvested at the end of 8th day, 14th and 21st day. p63 expression was seen till 21days on the cells cultured over both the conditions.ABCG2 expression was expressed till 21days only on the cells cultured in the presence of 3T3 feeder layer. Connexin 43 and keratin 3 and 12 was seen in both the culture conditions, comparatively the expression is more on the cells cultured over the denuded AM without 3T3 feeder layer

Figure 7.4b Expression of stem cell associated and differentiation markers on the denuded AM in the presence and absence of 3T3 feeder layer



expression Connexin 43 and Keratins markers from day 8 to day 21on the cells cultured over the denuded AM.

Our results are in concurrence with previous investigators. (Liu *et al*, 2006) Liu *et al* observed that the cells cocultured with 3T3 feeder layer shows the expression of p63 and faint expression of K3 and 12. On the continuous passage the expression of p63 was lost. (Liu *et al*, 2006) They also showed that the human limbal cells from the cadaveric donor eyes when cocultured with the mitomycin treated 3T3 feeder layer were able to maintain the features of limbal epithelial cells. (Liu *et al*, 2006) Grueterich *et al* and Tseng *et al* reported the low level expression of the keratin on the cells cultured in the presence of 3T3. (Grueterich *et al*, 2003; Tseng *et al*, 1996 ;) They also provided experimental evidence supporting the idea that a more differentiated epithelial phenotype of limbal explant cultures on the denuded AM is down regulated by 3T3 fibroblast feeder layers. Thus inclusion of 3T3 feeder layers appears to prevent epithelial differentiation when denuded AM is used for *ex vivo* expansion of limbal epithelial progenitor cells

The reason for the maintenance of the stem cell markers in the cultured cells under the influence of feeder layer is not clearly understood, but could be multifactorial such as the possible release of diffusible factors or cytokines released by the 3T3 fibroblast system , presence of antiapoptotic survival factor in 3T3 fibroblast conditioned medium. (Tseng *et al.,* 1996;) and the similarity of the 3T3 feeder layer in mimicking the numerous signal transduction pathways between the limbus and the limbal epithelial cells in maintaining

the stemness of the cells. (Li *et al*, 1996,) Further studies are needed to be studied on the growth factors released by the feeder layer.

However, there are limitations in the use of feeder layer for culturing stem cells [either embryonic or mesenchymal for human clinical applications. Recently, human embryonic stem cell lines cultured on mouse feeder cells were reported to be contaminated by the xeno-carbohydrate N-glycolylneuraminic acid (Neu5Gc) and considered potentially unfit for human therapy. (Heiskanen *et al*, 2007) We do not know whether this holds true for adult limbal stem cells cultured under the influence of 3T3 feeder layer. However, further *in-vitro* studies using feeder layer will enable us to understand the factors which play a role in maintaining the limbal stem cell niche.

7.5 Conclusion:

The limbal epithelial cells cultured in the presence of 3T3 feeder layer on the denuded AM were able to maintain the limbal phenotype cells. Further studies are needed to find out the factors released by these cells which help to maintain the limbal phenotype of the cells.

CHAPTER 8 - EXPRESSION OF P63 ISOFORMS IN CULTURED CORNEAL STEM CELLS

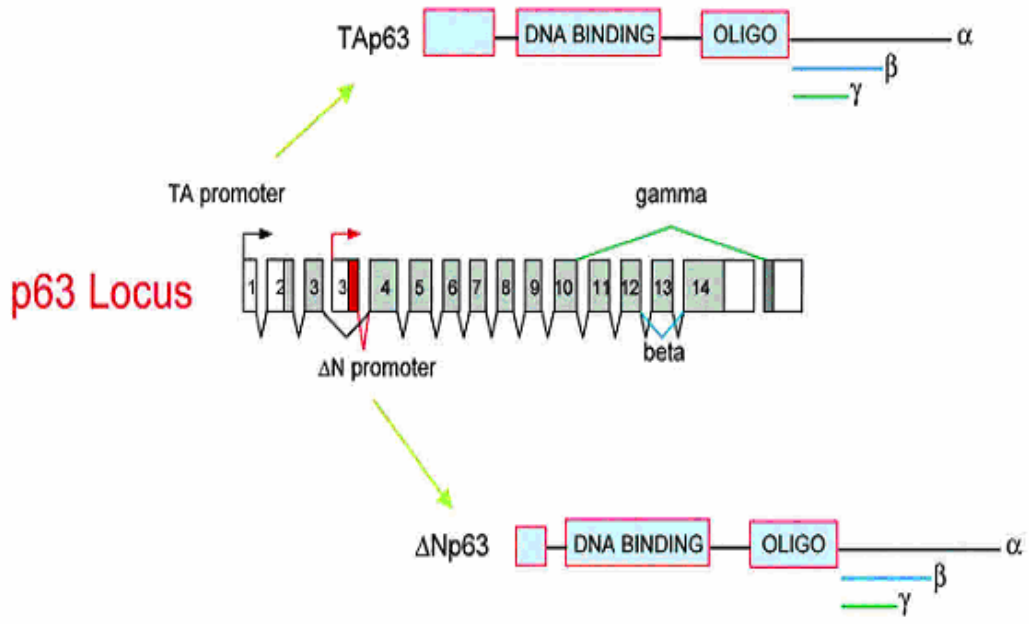
8.1 Introduction:

p63 gene has an important role on the development of stratified epithelia (Yang *et al*, 1998; 1999; Mills *et al*, 1999; Koster *et al*, 2003 ;). Ablation of this *p63* gene in mice results in the absence of these epithelia. In humans, mutations of the *p63* gene cause disorders of the epithelia and of nonepithelial structures whose development depends on the epithelial functions (Celli *et al*, 1999 ;). The function of p63 was elegantly shown using knockout (KO) mice, in which the absence of p63 results in severe anomalies in limb and craniofacial morphogenesis, as well as severe hypoplasia of squamous epithelia (Mills *et al*., 1999; Yang *et al*., 1999).

p63 gene is located at chromosome 3q27-29 and contains 15 exons. Its messenger RNA (mRNA) has been detected in skin, cervix, urothelium, prostate, thymus, placenta, testis, and skeletal muscle, and the protein is mainly expressed in proliferating basal cells of several types of multilayered epithelia, including epidermis, cervix, urothelium, prostate, and other tissues (Yang *et al*, 1998; Benard *et al*, 2003). p63 is also a member of the p53 protein family and is expressed as six distinct isoforms due to alternative transcription start sites and splicing at the C terminus resulting in α, β, and γ variants. (Yang *et al*. 1998). **(Figure 8.1)**

The TA isoforms β and χ of p63 bind to p53 and induce p53 target genes, and c isoform is able to induce cell cycle arrest and apoptosis, whereas TAp63α isoform lacks this

Figure 8.1



Adapted from http://www.genesdev.org

This figure shows the Generation of TA and ΔN isoforms from the p63 gene. Schematic of the exon structure and promoter positions of the p63 gene as well as the splicing events that give rise to the TA and ΔN isoforms and their respective β and γ spicing variants. The TA isoforms have an acidic N-terminal domain common to many transcriptional activators whereas the ΔN isoforms lack this acidic N-terminal domain. The p63 gene is distributed over < 200 kb of mouse chromosome 16 and human chromosome 3q27.

ability (Yang *et al*, 1998). However, three ΔN isoforms lack the transactivational domain but have a DNA-binding domain that can bind to p53 or the TA isoforms, rendering them inactive, thus have an antiapoptotic activity (Bernard *et al*, 2003; Irwin *et al*, 2001;) . The ΔNp63α isoform is highly expressed in stem cells of epithelial tissue, which is required to proliferate and maintain a stem cell epithelial population (Reis *et al*, 2002; Signoretti *et al*, 2000). The TA and ΔN isoforms of p63 have opposing functions, the former having potential tumor suppressor activity, whereas the latter may have oncogenic activity. Consistent with having both positive and negative gene regulatory roles, p63 can induce expression of some target proteins. (Sasaki *et al*. 2002; Ihrie *et al*. 2005). The functions of the TA and ΔN p63 isoforms are described in the **table 8.1.**

Table 8.1 Functions of p63 isoforms:

S.No.	Isoform name	Function	Reference
1	ΔN isoform	Necessary for the maintenance of proliferative state and differentiation, they also have oncogenic activity	Dohn *et al*, 2002, King *et al*, 2003
	Alpha	Predominant isoform expressed in basal layer of the epidermis and maintain the stem cell population	Yang *et al* 1998
2.	TA (α,β γ)	Initiation of epithelial stratification , tumor suppressing activity	Koster *et al*, 2004;

Among the various p63 isoforms as mentioned above this ΔNp63α has also been reported to be strongly expressed in the epidermis (Yang *et al*, 1998; 1999; Parsa *et al* 1999;) and is selectively expressed in the basal cell compartment of a variety of epithelial tissues (e.g., cervix, urogenital tract, prostate, breast, (Yang *et al*, 1998, 1999; Parsa *et al*, 1999; Bamberger *et al*, 2002; Noszezyk *et al*, 2001; Di Como *et al*, 2002;) and cornea).11 (Pelligrini *et al*, 2001;) Therefore, it has been suggested that ΔNp63α may be essential in the maintenance of a stem cell (SC) population in various epithelial tissues. Furthermore, ΔNp63 has been also implicated in the control of epithelial cell proliferation and migration during wound healing. Yet, the expression of p63 by the majority of basal cells and by suprabasal cells, as assessed by the 4A4 antibody recognizing all p63 isoforms, has been considered too broad to be stem cell specific (Sun *et al*, 2004; Kaur *et al*, 2004;).

For understanding the role of the p63 isoforms in the ocular surface epithelia there are studies showing the spatial distribution of these isoforms in the ocular surface epithelia. Is has been clearly stated that the single antibody cannot detect the specific type of the p63 isoform Kawasaki *et al* has used the primers to determine the specific p63 isoform, which are located in the N terminus and C-terminus. (Kawasaki *et al*, 2006 ;).

Expression of this p63 isoform has been studied in detail in the only on the ocular surface epithelium, but there are no data available on the *in vitro* cultured cells. It is therefore essential to identify the proliferative cells cultured *in vitro*. So in this study, we have evaluated the expression of the p63 isoforms in the cultured corneal limbal stem cells.

And as intact AM preserves the limbal phenotypic cell population of the cultured cells when compared to denuded AM we wanted to study the expression of the p63 isoforms on the cells cultured over both intact and denuded AM and compare

8.2 Design of Experiments:

8.2.1 Cultivation of limbal epithelial cells on the intact and denuded AM:

Limbal biopsies from the cadaveric donor eyes were collected and cultured on the intact and denuded AM according to the protocol given in the methodology section. DMEM and F12 mixture was used for culturing and medium was changed every other day and the plates were incubated for 3 weeks until they reach the confluence.

8.2.2 Study of expression of p63 isoforms:

Total RNA was extracted once the cells over the intact and denuded AM reach the confluence and after RNA extraction and cDNA conversion amplification was done using the specific primer sequences for p63 isoforms as given in the **Table – 3.2 methodology section** (Kawasaki *et al*, 2006 ;) Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control, the mRNA expression of different molecular markers was analyzed by RT-PCR. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% ethidium bromide with molecular marker *Hinf I* φ digest to confirm the size of the resultant product. The fidelity of the RT-PCR products was verified by comparing their size with the expected cDNA bands and by sequencing the PCR products.

8.3 Results:

The results of evaluation of p63 isoforms are summarized in the **Table 8.2** .Of the samples that were obtained for the isoform analysis, all were successfully amplified with Qiagen Sensiscript Reverse Transcription cDNA conversion kit, exhibiting moderately strong smearing, (Data not shown). The RTase-omitted sample did not exhibit such smearing; indicating that contaminated genomic DNA was not amplified by the above procedure. All samples exhibited positive PCR bands for the GAPDH gene, demonstrating successful reverse transcription and cDNA amplification.

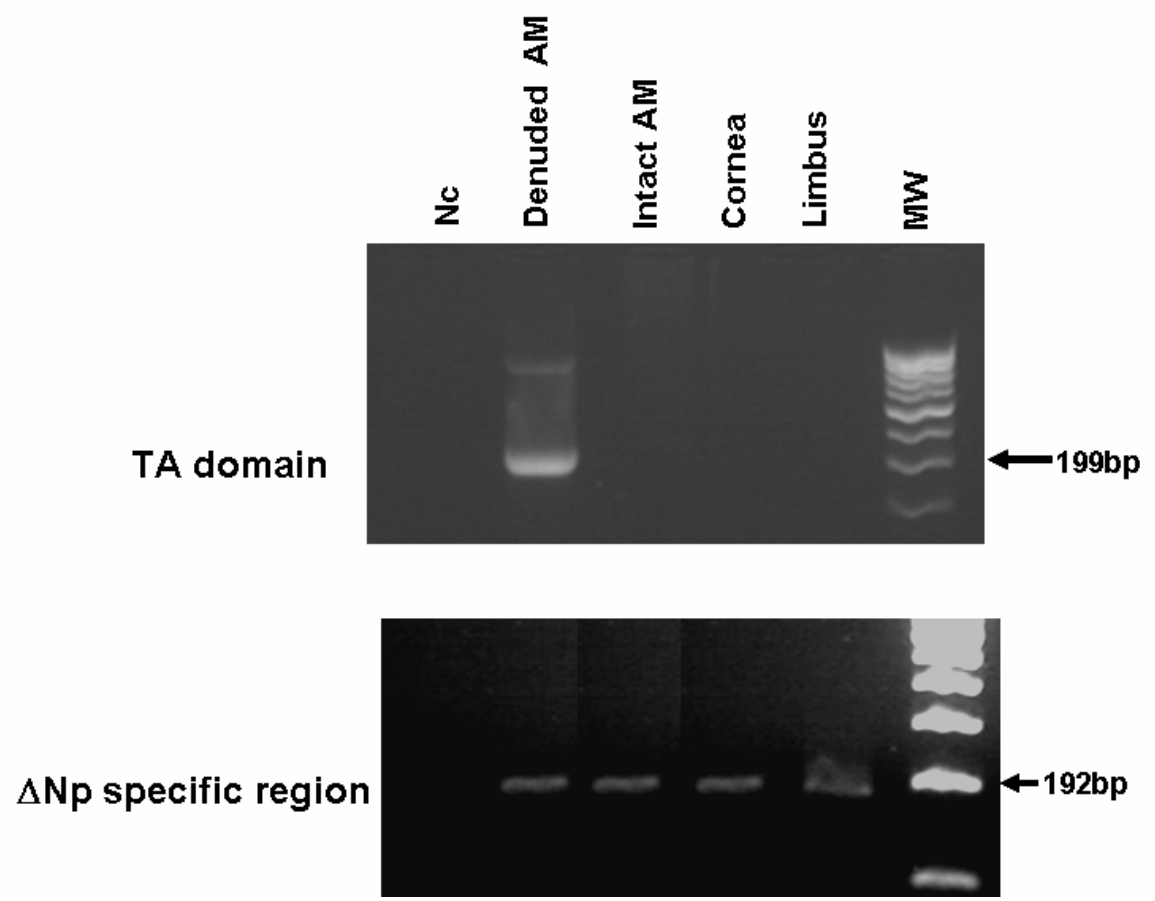
ΔN specific region and TA region:

In our study the TA domain, which is not detected in any layers of any epithelia, was completely absent on the cells cultured over the intact AM, corneal epithelial cells and RNA extracted from the limbus. But it was positive for the cells cultured over the denuded AM. **(Figure 8.2)** The ΔNp specific region which is usually detected in the basal to intermediate layers of all types of epithelia and in the superficial layer of the limbus and the cornea was positive for the cells cultured over the intact AM, denuded AM and the cells harvested from the cornea and the limbus. **(Figure 8.2)** HeLa gave bright positive expression for both. But ΔN expression was comparatively less when compared to TA. **(Figure 8.7)**

Isoform determining region:

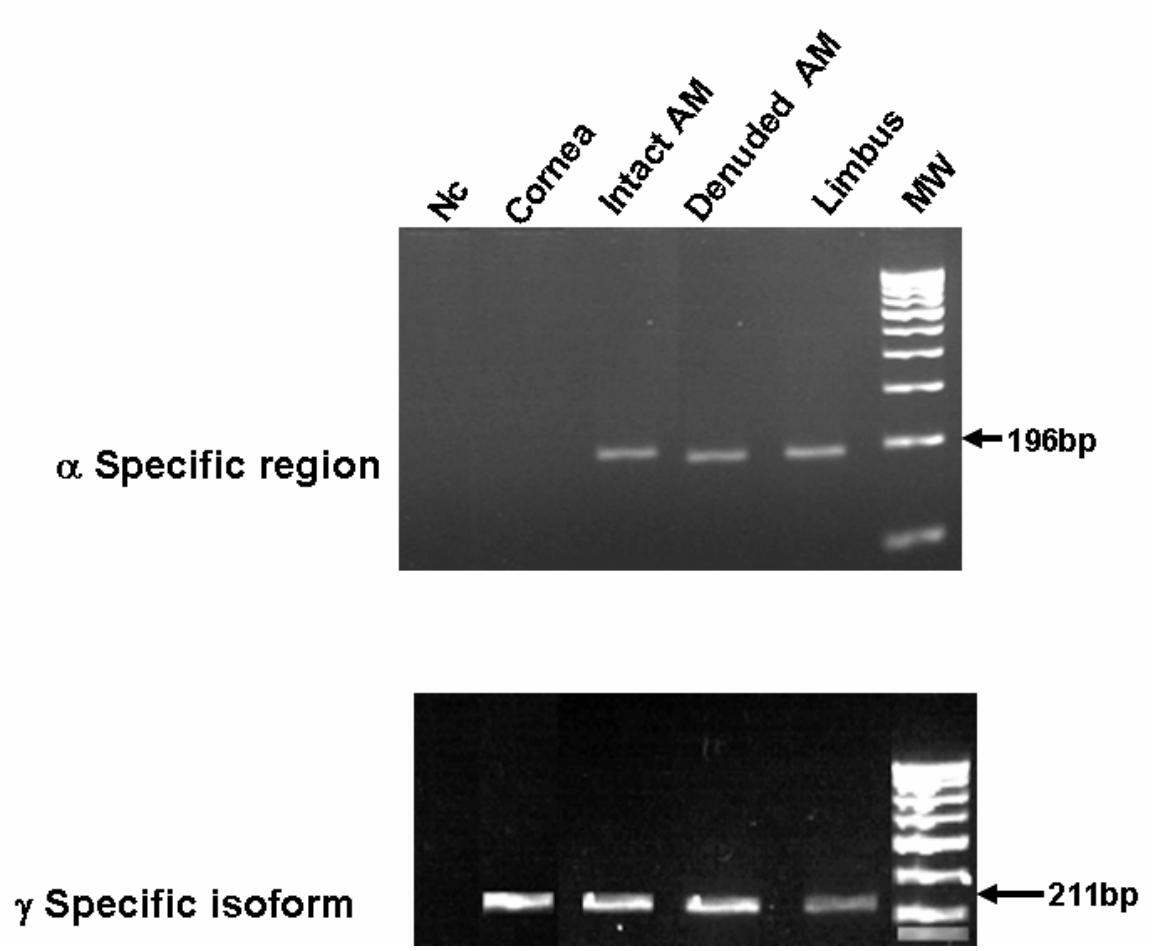
The α-isoform specific region, which is also detected in all layers of the conjunctiva and the limbus and was detected in the cells cultured over the intact and denuded AM and

Figure 8.2:



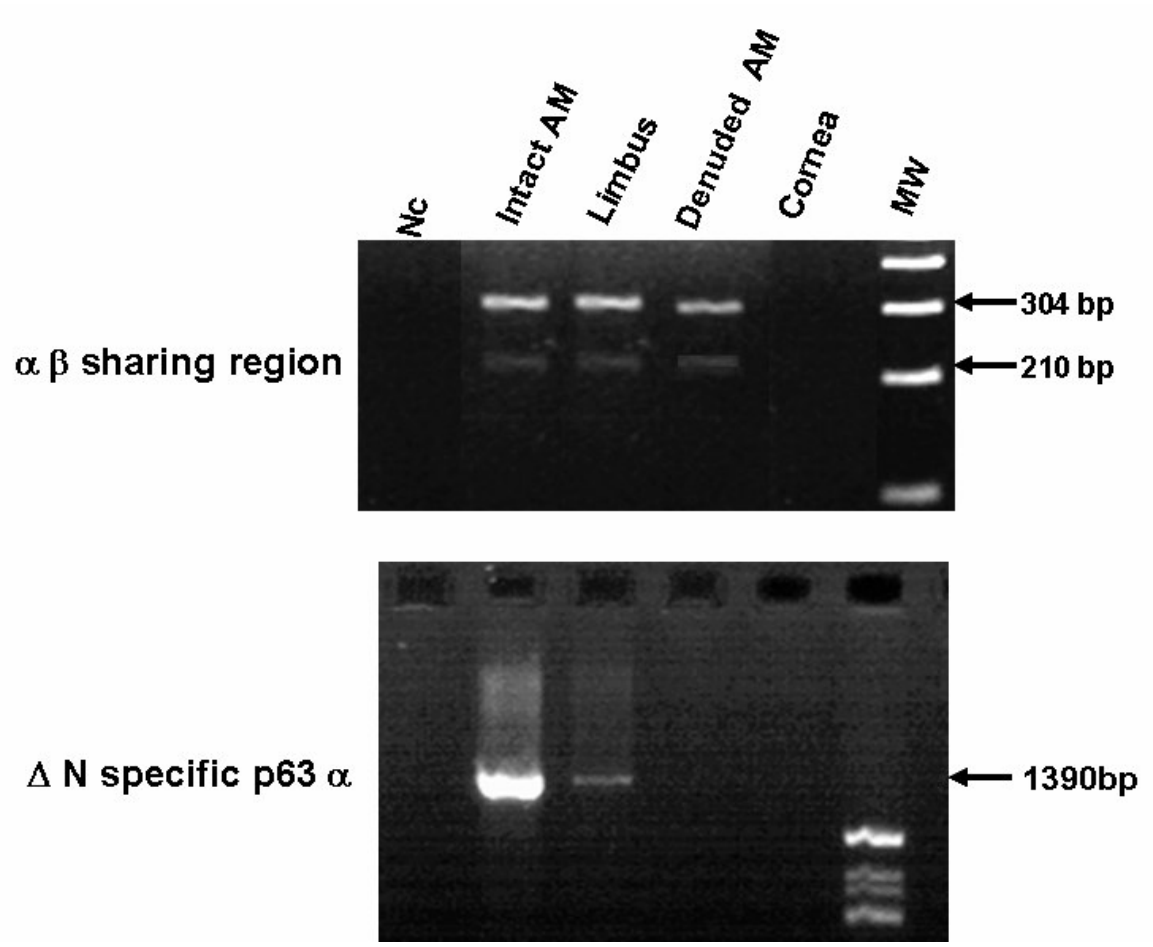
This figure shows the electrophoretogram of the RT-PCR for TA domain and □N specific region of the p63 isoform. Lane NC – Negative RT reaction. Lane denuded AM – Cultured cells grown on the denuded AM, Lane Intact AM cells grown on the Intact membrane, Lane Limbus – Cells harvested from the limbal region of the donor cornea, Lane Cornea – cells harvested from the corneal epithelial cells of donor eye ball. Lane Mw is Molecular weight marker – 100bp ladder. TA domain gives the amplified product size of 199 bp and ΔN domain gives the amplified product size of 192 bp. TA domain was positive only for the cells cultured over the denuded AM and D N domain was positive for all.

Figure 8.3



This figure shows the electrophoretogram of the RT-PCR for α and γ isoform of p63 Lane NC – Negative RT reaction. Lane denuded AM – Cultured cells grown on the denuded AM, Lane Intact AM cells grown on the Intact membrane, Lane Limbus – Cells harvested from the limbal region of the donor cornea, Lane Cornea – cells harvested from the corneal epithelial cells of donor eye ball. Lane Mw is Molecular weight marker – 100bp ladder α isoform specific primer gives the amplified product size of 196 bp and γ isoform specific primer gives the amplified product size of 211 bp. α specific isoform was positive for the cells cultured over the intact AM only whereas γ specific isoform was positive for both intact and denuded AM.

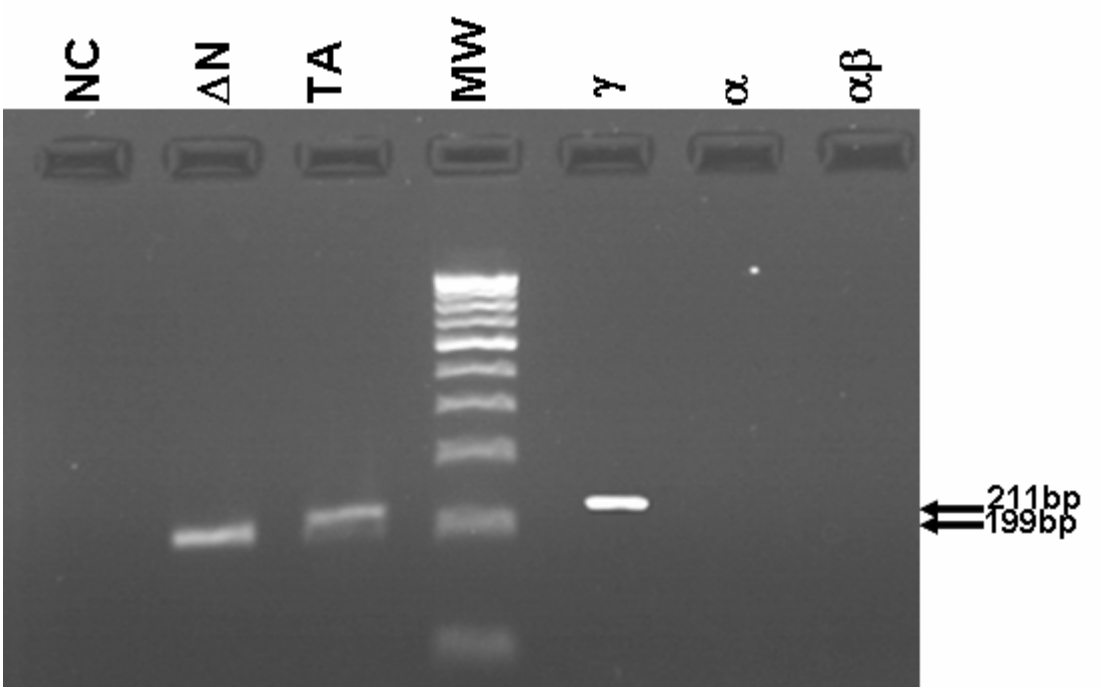
Figure 8.4



This figure shows the electrophoretogram of the RT-PCR for αβ and ΔN specific isoform of p63 α Lane NC – Negative RT reaction. Lane denuded AM – Cultured cells grown on the denuded AM, Lane Intact AM cells grown on the Intact membrane, Lane Limbus – Cells harvested from the limbal region of the donor cornea, Lane Cornea – cells harvested from the corneal epithelial cells of donor eye ball. Lane Mw is Molecular weight marker – 100bp ladder. αβ sharing region primer gives the amplified product size of about 304 bp for α and 210 bp for β. ΔNp63α gives the amplified product size of about 1390bp

Figure 8.5

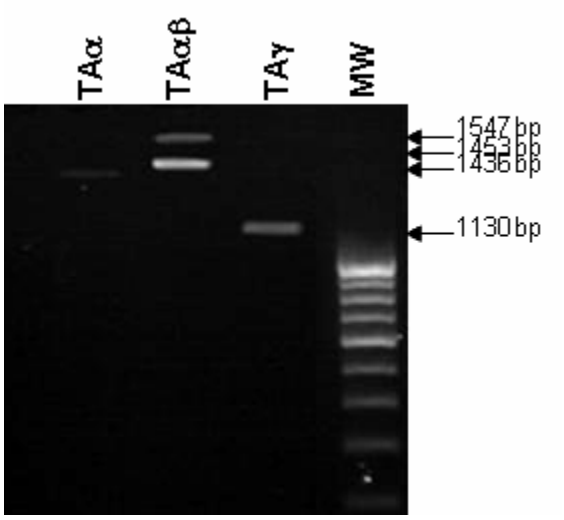
Expression of isoform specific region on the HeLa cell line



This figure shows the electrophoretogram of the isoform specific region on the HeLa cell line. Lane NC –Negative control. HeLa cell line showed the positive expression of the DN specific region, TA, and gamma specific region whereas it is negative for the alpha and alpha beta sharing region. DN specific gives the amplified product 192bp, TA region gives the amplified product of 199bp and gamma specific region gives the product size of 211bp. Lane MW is the molecular weight marker (100bp Ladder).

Figure 8.6:

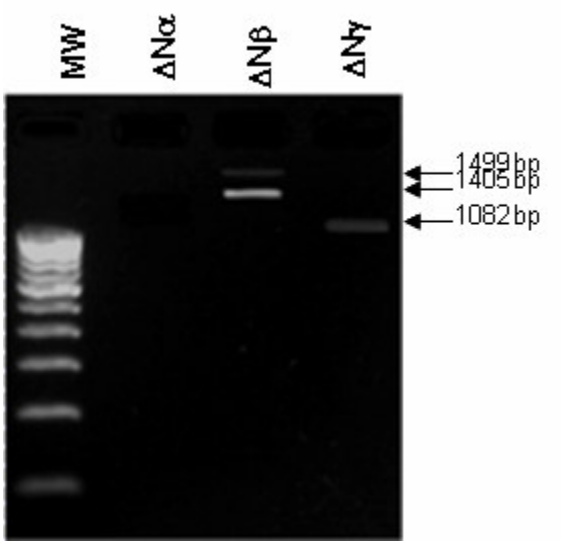
Electrophoretogram showing the TA specific isoforms on the HeLa cell line



This figure shows the electrophoretogram of the TA specific region on the HeLa cell line. HeLa gave the positive amplification for the all the TA specific isoforms. TAα gave the product size of 1436bp and TAαβ gave the 1547bp and 1453bp respectively. Taγ gave a product size of 1130bp. Lane MW molecular weight marker 100bp ladder.

Figure 8.7

Electrophoretogram showing the ΔN specific isoforms on the HeLa cell line



This figure shows the electrophoretogram of the ΔN specific region on the HeLa cell line. HeLa gave the positive amplification for the all the ΔN specific isoforms. ΔNα is negative on the HeLa cell line. ΔNαβ gave faint expression of 1499bp of α isoform and 1405bp respectively. ΔNγ gave a product size of 1082bp. Lane MW molecular weight marker 100bp ladder.

also in the cells harvested from the limbus but it is negative on the cells harvested from the cornea. **(Figure 8.3)** As there is no specific region for the β-isoform, a primer pair flanking the α-isoform specific region (exon 14) was designed to detect both the α- and β-isoforms with a different product length of 304 or 210, respectively. The αβ sharing region was detected on the cells cultured on the Intact AM, denuded AM, cells harvested from the limbus. **(Figure 8.4)** The β-isoform, which was detected in the basal to intermediate layers of the limbus, was detected on the cells harvested from the Intact, denuded and limbus but negative on the corneal epithelial cells. The γ-isoform specific region, which was detected in almost all layers of all epithelia, is expressed in cells cultured over the intact and denuded and also on the cells harvested from the limbus and the corneal epithelial cells. **(Figure 8.5)** HeLa gave a positive expression only for the γ isoform whereas other two were negative. **(Figure 8.7)**

TA and DN isoforms:

The TAp63 isoforms, including TAp63α, TAp63β, and TAp63γ, which is not detected in any layer of any epithelia is negative in both the cells cultured over the intact and denuded and also on the cells from the limbus and the cornea. (data not shown) Similarly ΔNp63α, which is detected in the basal to intermediate layers of the limbus and conjunctiva and no layer of the cornea, exhibited this isoform in the previous study of Kawasaki *et al*. The results of the ΔNp63α shows the expression only on the cells cultured over the intact AM and the limbus. **(Figure 8.5)** The ΔNp63β and ΔNp63γ isoforms, which is negative in all the layers of any epithelia, is negative in all. (Data not

shown) RT-PCR for the TAp63 isoforms and ΔNp63 isoforms on the HeLa cDNA gave positive result for all the isoforms except the ΔNp63α. **(Figure 8.6, 8.7)**

Table 8.2

Results of the RT-PCR on the p63 isoforms:

Isoforms	Intact AM	Denuded AM	Limbus	Cornea	HeLa
TA	Negative	Positive	Negative	Negative	Positive
ΔNp	Positive	Positive	Positive	Positive	Positive
α	Positive	Positive	Positive	Negative	Negative
αβ	Positive	Positive	Positive	Negative	Negative
γ	Positive	Positive	Positive	Positive	Positive
TAP63α	Negative	Negative	Negative	Negative	Positive
TAP63 β	Negative	Negative	Negative	Negative	Positive
TAP63γ	Negative	Negative	Negative	Negative	Positive
ΔNp63α	Positive	Negative	Positive	Negative	Negative
ΔNp63 β	Negative	Negative	Negative	Negative	Positive
ΔNp63γ	Negative	Negative	Negative	Negative	Positive

8.4 Discussion:

Human amniotic membrane is a widely used surgical material, (Trelford *et al*, 1979) and in recent years there has been a renewed interest of culturing limbal phenotype cells *in vitro* using this AM with or without epithelium. Grueterich *et al* has shown the downregulation of Connexin 43 expression and the keratin expression on the cells cultured over the intact membrane, which shows that cells cultured over the intact membrane preserves the stemness of the cultured cells. There are several studies going which has shown the usefulness of the intact and denuded AM for culturing corneal limbal stem cells. There are also many reports available (Koizumi *et al*, 2007) that the intact AM supports the limbal phenotype and they do preserve the stemness of the cultured cells. (Grueterich *et al*, 2002; Koizumi *et al*, 2007, 2000; Galindo *et al*, 2000 ;) Whereas, there is not much data available on the p63 isoform status on the cultured limbal stem cells. Since the common clone used for identification of the keratinocyte stem cell is 4A4 of p63 which is not much useful in identifying the proliferative cells.

In this present study we investigated the expression of the p63 isoforms on the cells cultured over the intact and denuded AM and also we saw the expression of the isoform status on the HeLa adenocaricnoma cell line. Since HeLa expresses all the p63 isoforms except we few we used it as a control for the PCR and also we wanted to know the p63 isoform status on the carcinoma cell line. The studies on the p63 isoforms on the different layers of the cornea were reported by the Pellegrini *et al,* Di Iorio *et al,* Kawasaki *et al*. (Pellegrini *et al*, 1999; Kawasaki *et al*, 2006 ;) From their studies that ΔNp63α isoform seems to be the major transcript for determining the corneal stem cells.

Di Iorio *et al* has shown the evidence that the ΔNp63α is likely to identify the stem cell population in the limbus supports the concept that the α isoform of ΔNp63 is essential for regenerative proliferation in ocular surface. We studied the ΔNp63α expression on the cells cultured over the intact and denuded AM and also on the cells harvested from the cornea and the limbus. Similarly cells harvested from the limbus also showed the positive expression for ΔNp63α. And the results were consistent with the previous reports. (Di Iorio *et al*, 2005; Kawasaki *et al*, 2006 ;) Kawasaki *et al* and Pellegrini *et al* has identified that ΔNp63α isoform of p63 can be used to identify the proliferative cells. The presence of the ΔNp63α isoform shows that the intact AM supports the amplification of the proliferative cells when compared to the denuded AM. In HeLa adenocarcinoma cell line, weak expression of ΔN form and no expression of β isoform may explain the fact that the Uterine cervical adenocarcinoma is consistently negative for p63 protein on immunohistochemical study (Wang *et al*, 2001)

Little is known about the functional difference and biological significance of the individual p63 isoforms. The biological relationship of the TAp63 and ΔNp63α is that, the TAp63 group is required for or elicits several p53-dependent cellular processes, including cell cycle arrest or apoptosis (Flores *et al*, 2002) via its N-terminal TA domain. On the other hand, the ΔNp63 group lacks this domain, thus yielding a dominant-negative effect against such cellular processes and ultimately achieving the maintenance of cell proliferation. (Yang *et al*, 1998 ;) In our study there was the complete absence of the TAp63 isoforms, but TA specific domain is present only on the cells cultured over the denuded membrane.

Thus, the predominantly expressed ΔNp63α within ocular surface epithelia suggests several complicated but as yet unknown interactions via these functional domains. How, why and when ocular surface epithelial cells use a specific ΔNp isoform is the next matter to be resolved. Similarly why the cells cultured on the intact AM express this marker and functional role of the AM epithelial cells has to be studied in detail.

Conclusion:

In conclusion, ΔNp63α appears to be the most dominant isoform within the ocular surface epithelia but also to detect the high proliferative cells *in vitro* also. The cells cultured over the intact AM were able to maintain the high proliferative potential of the cells when compared to the cells cultured over the denuded membrane. The biological function of this isoform is to retain the proliferative capacity of these cells via a dominant negative effect against the p53-dependent cell cycle arrest or apoptosis. Identification of the upstream regulators and downstream effectors of this protein should be the next goal to expand our understanding of how cell proliferation and differentiation are regulated within the ocular surface epithelia.

CHAPTER 9 - SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE *EX-VIVO* EXPANSION OF THE CORNEAL STEM CELLS

9.1 Introduction:

In the recent years, one of the major advances in treating the patients with total limbal stem cell deficiency is transplantation of the epithelial progenitor cells expanded from the small biopsy tissue bit cultured on the amniotic membrane with or without the feeder layer. (Tsai *et al*, 2000; Koizumi *et al*, 2001a, 2001b; Grueterich *et al*, 2002;)

Ti *et al* in their rabbit model of unilateral total limbal stem cell deficiency have demonstrated that the long-term efficacy of this new surgical procedure (Ti *et al*, 2002) and that clinical success correlates with the recovery of a normal corneal epithelial phenotype, and clinical failure correlates with the maintenance of a conjunctival epithelial phenotype. (Ti *et al*, 2003) Furthermore, restoration of a clear and transparent cornea is associated with a normal corneal epithelium and complete wound remodeling. (Espana *et al*, 2003)

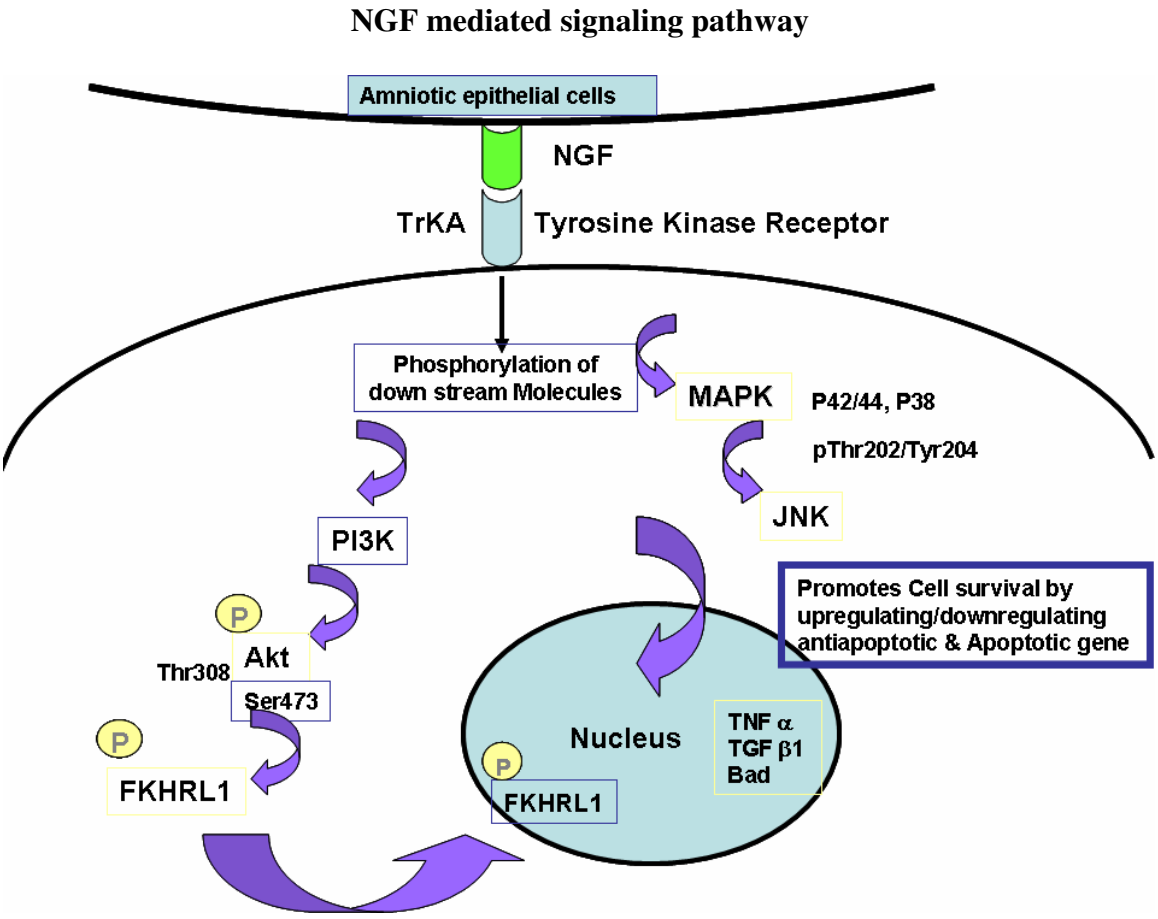
There are several reports that the *ex vivo* expanded limbal epithelial cells on the intact amniotic membrane with the feeder layer were able to preserve the characteristics of limbal epithelial cells. (Gruterich *et al*, 2002; Meller *et al*, 2002; Galindo *et al*, 2003 ;)

Since Amniotic membrane have a number of cytokines, growth factors and protease inhibitors such as IL-4, 6 and EGF, FGF,TGF, NGF and HGF have been found in cryopreserved amniotic membranes. The presence, concentration and action of these substances may account for most of the observed clinical effects and its mechanisms of

action (Tseng *et al*, 2001 ;) As a result of exploring the molecular mechanism governed by the amniotic membrane, Tseng *et al*, has noted that the nerve growth factor (NGF) is abundantly present in AM stromal matrix and that TrkA (The NGF high affinity receptor) is expressed by basal cells of the limbal epithelium. Through this the basal epithelial cells sustains the antiapoptotic survival.

It has been reported that NGF/TrkA signaling leads to the PI3K-Akt-FKHRL1 pathway, (Zheng *et al*, 2002 ;) which is know to govern cell survival (Datta *et al*, 1999) and to the mitogen activated protein kinase (MAPK) pathway which is know to control cell mitosis. (Johnson *et al*, 2002) In the MAPK pathway, extracellular signals such as growth factors, mitogens, cytokines, and various forms of environmental stresses activate phosphorylation of one or several of the three members: extracellular signal-regulated kinases (ERKs), also referred to as p44/42 MAP kinase; p38 MAP kinases; and c-Jun N-terminal kinases (JNKs, also termed stress-activated protein kinases). (Graves *et al*, 1996; Xia *et al*, 1995 ;) He *et al* (He *et al*, 2006 ;) has demonstrated the role of this signaling pathways on the cells expanded over the intact membrane. **Figure 9.1** shows the schematic picture of activation of PI3K signaling path way mediated through NGF. In this study we studied the signaling pathways on the cells expanded over the denuded, Intact and chitosan matrix.

Figure 9.1:


NGF mediated signaling pathway
Amniotic epithelial cells
NGF
TrKA
Tyrosine Kinase Receptor
Phosphorylation of down stream Molecules
MAPK
P42/44, P38
pThr202/Tyr204
JNK
PI3K
Promotes Cell survival by upregulating/downregulating antiapoptotic & Apoptotic gene
P
Akt
Thr308
Ser473
P
FKHRL1
Nucleus
TNF α
TGF β1
Bad
P
FKHRL1

9.2 Design of experiments:

9.2.1 Cultivation of the limbal explants on the intact, denuded Amniotic membrane (AM) & chitosan matrix:

Limbal biopsies were collected from the cadaveric donor eyes were cultured on the intact, denuded AM and the chitosan matrix by explant culture technique. Total of 5 biopsies were processed. The cultures were maintained in DMEM & F12 mix and were incubated at 37^0C with 5% CO_2. The cultures were incubated for 3 weeks until they reach the confluence. The cultures were also tested for the phosphorylation status of the signaling molecules after incubation with the specific inhibitors. We also used one of the PI3K inhibitor LY294002 and their effect on the epithelial outgrowth and the phosphorylation status. For the addition of inhibitor the specific concentration of the inhibitor dissolved in DMSO.

9.2.2 Harvesting cells for protein extraction and western blot:

Once the cell cultured over the intact, denuded and chitosan reach confluence the cells were treated with mild trypsin treatment. The collected cells were washed thrice and protein was extracted according to the procedure give in the methodology section. Western blot was carried out using the following antibodies given in the table 9.1.

Table 9.1 List of antibodies and dilutions

S.No.	Antibody	Company	Dilution used
1	Akt	Cell signaling Technology	1:1000
2	PAkt (Thr 308)	Cell signaling Technology	1:1000
3.	FKHRL1	Cell signaling Technology	1:500
4	MAPKs P44/42 P38	Cell signaling Technology	 1:1000 1:1500
5	JNK	Cell signaling Technology	1:2000
6	Inhibitor LY294002 inhibitor for PI3K pathway	Cell signaling technology	25mM and 50mM concentration was used

All the procedures were followed according to the protocol given in the methodology section.

9.3 Results:

Total of 5 limbal explants from 5 donors ranging from 48 to 57 years of age. Under microscopic observation we noted epithelial migration from all the 5 limbal biopsies by the end of 48 hours on the denuded and chitosan matrix. But the growth on the intact AM commenced only by the end of 3rd day. By the end of the day 15 good confluent growth was formed and cells were harvested for the western blot.

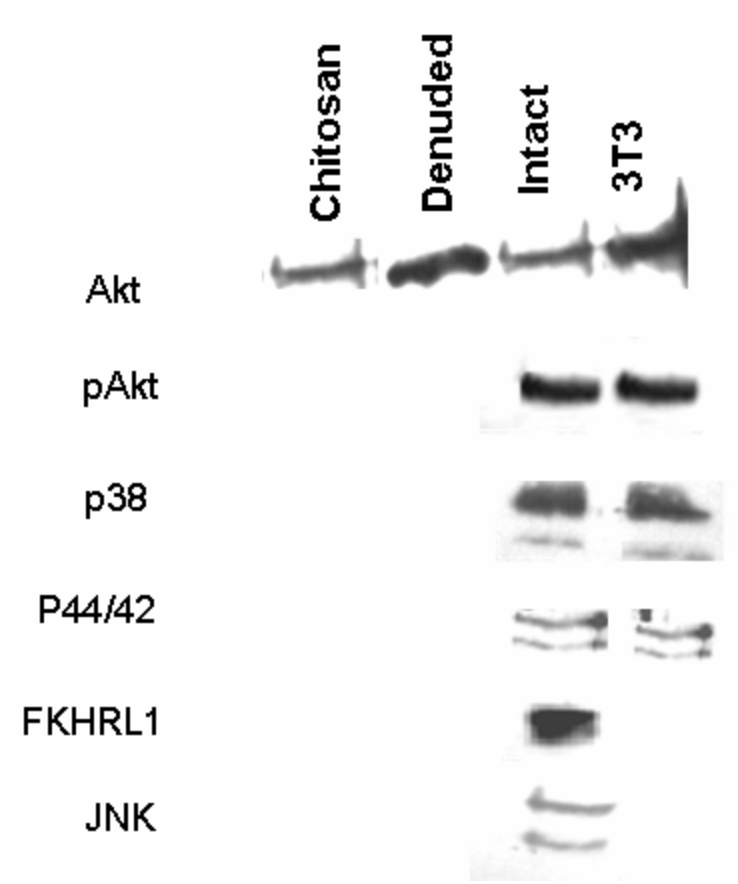
PI3K-Akt pathway:

The PI3K –Akt pathway controls the cell survival and inhibition of this pathway frequently leads to apoptosis (Datta *et al*, 1999; Franke *et al*, 2003) one of the down stream targets of PI3K is phosphorylation and activation of Akt kinase. We could demonstrate the phosphorylation of Akt at the Thr 308 position and Ser 143 position on the cells cultured over the intact membrane but it was absent on the cells cultured over the denuded and chitosan matrix, **(Figure 9.2)** our result is consistent with the results of He *et al*, 2006.

P44/42 MAPK, p38 MAPK, FKHRL1, JNK MAPK pathway:

The MAPK pathway controls the cellular proliferation, migration and differentiation (Johnson *et al*, 2002 ;) The cells cultured over the intact membrane shows the phosphorylation of Thr 202/Tyr204 (p42/44) and p38MAPK, whereas it was absent on the cells cultured over the denuded and chitosan matrix. Similarly Phosphorylation of FKHRL1 and JNK MAPK was observed only on the cells cultured over the intact

Figure 9.2: Western blot results



This figure shows the western blot of the Akt and other downstream pathways on the cells cultured over the various matrices. Intact membrane shows the positive expression of all the molecules. 3T3 is used as the control.

amniotic membrane. It was completely absent on the cells cultured over the denuded and chitosan. **(Figure 9.3)**

Inhibition of the PI3k –Akt pathway:

The PI3K-Akt pathway controls cell survival, and inhibiton of this pathway frequently leads to apoptosis. LY294002 is a specific inhibitor of PI3K, and one of the downstream targets of PI3K is phosphorylation and activation of Akt kinase. We used the concentration of 25mM and 50mM of the inhibitor, which seems to have inhibited the epithelial outgrowth significantly. This result suggests that inhibition of either PI3K or Akt could completely abolish epithelial outgrowth from a limbal explant cultured on AM.

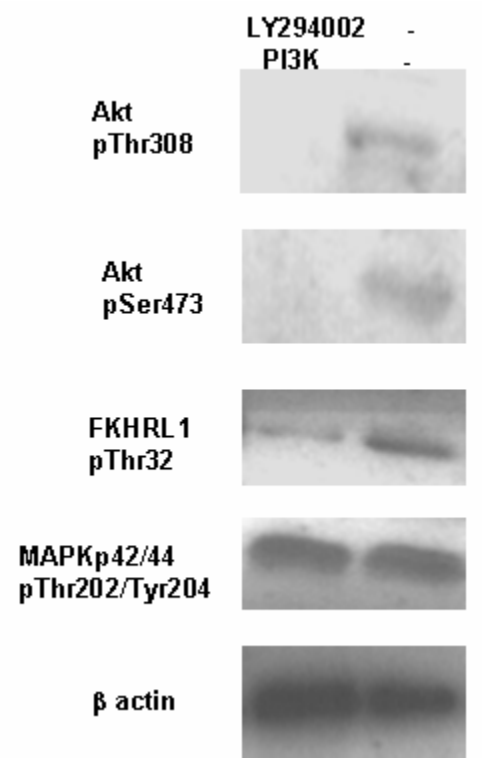
Western blot analysis was performed to check the phosphorylation status of the kinases, which was inhibited after the treatment with the inhibitor. The results showed that addition of 50 μM of LY294002 abolished phosphorylation of Akt at Thr308 and Ser473. 50 μM LY294002 decreased, Thr32 phosphorylation of FKHRL1, a downstream target of Akt (Kops *et al*, 2000; Burgering *et al*, 2002; Carlson *et al*, 2002 ;) 50 μM LY294002 did not change the p44/42 MAPK phosphorylation. **(Figure 9.4)**

9.4 Discussion:

Grueterich *et al*, 2002, Meller *et al*, 2002, Galindo *et al*, 2003a, 2003b have demonstrated that human limbal epithelial progenitor cells can be expanded and maintained on intact, but not denuded, AM or plastic in the absence of 3T3 fibroblast feeder layers. Touhami *et*

Figure 9.3

Western Blot results on the cells cultured over the intact AM after treatment with LY294002 inhibitor



This figure shows the WB picture on the cells harvested from the Intact AM after treatment with the inhibitor LY204002, inhibitor of PI3K. After treatment with the inhibitor there is complete absence of phosphorylation of the AKT at Ser473 and Thr 308 position. Whereas there is no change in the expression of other downstream pathway molecules.

al, 2002 and He *et al*, 2006 have reported that the signaling mediated by NGF is involved in the exvivo expansion. He *et al* have shown that the specific inhibitors of PI3K, Akt and MAPKs (p44/42, p38, and JNK) showed that both PI3K-Akt-FKHRL1 and P44/42 pathways provide the downstream signaling. In this study we studied the phosphorylation of the above mentioned signaling molecules on the cells cultured over the intact, denuded and chitosan matrix.

Any differentiated mammalian cells require the presence of mitogenic signals to initiate cell cycle re-entry and exit quiescence (G0) and to progress through the restriction point in late G1. Serum and combinations of growth factors are known to induce the activation of cyclin dependent kinases (cdks) that drive cell cycle progression. Thus PI3K activation is triggered by binding of such survival factors such as insulin like growth factors (IGF)-1 and neruotrophins (e.g., NGF) to their cell surface receptors. (Furman *et al*, 1998;) Activated PI3K in turn leads to the activation of serine/threonine kinase, termed as Akt or PKB, by phosphorylation at Thr 308 and Ser473 (Datta *et al*, 1999) which then phosphorylates its downstream target FKHRL1 to be translocated into the nucleus to transactivate proapoptotic genes such as tumor necrosis factor α, TGF-β1 and Bad. (Franke *et al*, 2003 ;)

In this study we studied the phosphorylation of the NGF mediated signaling pathway on the cells cultured over the intact, denuded membrane and chitosan scaffold. NGF mediated signaling pathway is activated only on the cells cultured over the intact membrane. It was completely absent on the cell cultured over the denuded and chitosan

scaffold. We also used one of the specific the specific inhibitor of the PI3K-Akt pathway. We observed that the phosphorylation of the Akt Thr 308 and Ser 243 was completely inhibited whereas it reduced the phosphorylation of the pThr32 FKHRL1, but the expression of p42/44 remains unaltered. Our result is consistent with the earlier reports of He *et al*, 2006.

Since our results and previous reports speculate that the antiapoptotic survival signaling mediated by the PI3K-Akt-FKHRL1 pathway is necessary to sustain *ex vivo* expansion of limbal epithelial progenitor cell including the stem cells. But further studies are needed to determine whether PI3K-Akt-FKHRL1 is involved in the self-renewal of limbal epithelial stem cells, and whether antiapoptosis is the key mechanism governing stem cell renewal.

Since these signaling pathways are mediated by the neruotrophins like (NGF) in the substrate like chitosan we can incorporate them and look for the activation of the signaling pathway. So other than intact membrane we can also try maintain the progenitor cell properties of the cultured limbal cells *in vitro*.

9.5Conclusion:

Thus cellular survival and continuous self renewal is dependent on the NGF mediated PI3K-Akt-FKHRL1 pathway. Since Amniotic membrane releases all these cytokines and neruotrophins which can be utilized for creating the substrates which mimics the Amniotic membrane. But still further studies are needed to elucidate the exact mechanism by which intact AM were able to maintain the progenitor cell like properties of the cultured limbal epithelial cells.

CHAPTER 10 - EMBRYONIC STEM CELL MARKER EXPRESSION IN CULTURED CORNEAL LIMBAL STEM CELLS

10.1 Introduction:

Recent development of therapeutic strategy for the management of total limbal deficient cases are the transplantation of the *ex vivo* expanded human limbal epithelial cells on the amniotic membrane or other substrates. (Koizumi *et al*. 2001; Grueterich *et al*. 2002a; Tseng *et al*. 2002). Expansion of cells from the small biopsies reduces the risk of damage for the healthy eye or donor eye. (Tsai *et al*, 2000)

Although these techniques are currently introduced into clinical practice, their biological background is still poorly understood, primarily due to a lack of knowledge about the nature of limbal SC. The maintenance of undifferentiated stated of the cultured limbal epithelial cells *in vitro* conditions is still questionable. A major challenge in corneal SC biology is the ability to identify SC *in vitro* and *in situ*, and one of the major controversies in this field relates to the issue of reliable SC markers. To date, limbal SC can be only identified by indirect methods. Although a number of molecular markers for the limbal SC compartment have been proposed (Grueterich *et al*. 2003; Chen *et al*. 2004), their role in specifically identifying limbal SC is still under debate and further study is needed to identify novel marker(s). Moreover, the identification of the new stemness marker(s) is critical for maintenance of limbal epithelial SC properties during culture.

Generally, stem cells exist in most adult organs, being best characterized in the bone marrow and gut. They are defined as cells that undergo symmetric and asymmetric division to give rise to daughter cells needed for self-renewal and amplification or to a daughter cell that acts as a progenitor cell for the purpose of producing specific differentiated lineages, respectively. Oct3/4 or Oct4 (also referred to as Pou5f1), a transcription factor, is a specific gene marker for totipotency. (Pesce *et al*, 1998; 2001; Deyev *et al*, 2004 ;) Nanog, a second homeodomain protein with functional relevance to embryonic stem cells (ES) cells has been identified (Chambers *et al*, 2003; Mitsui *et al*, 2003 ;) and maintains self renewal in the absence of STAT3 activation, conditions under which ES cells would normally differentiate. Nanog is capable of sustaining ES cell pluripotency (Vernallis *et al*, 1997 ;) Nanog function is critical at a later time during development than Oct 4. Similarly another marker Pax6, which is a homeobox gene and know to play an important role in the eye development. They have also identified that mutations in this gene is responsible for human aniridia. (Ton *et al*, 1991; Glaser *et al*, 1992 ;) In this study we looked for the expression of the Oct 4, Nanog and Pax6 in the cultured limbal epithelial cells.

10.2 Design of experiments:

The study protocol for the cultivation of Human cadaveric corneal limbal biopsy tissue was approved by the ethics sub-committee (Internal Review Board) of Vision Research Foundation. Human tissues were handled according to the Declaration of Helsinki

10.2.1 Expansion of corneal limbal stem cells on the intact and denuded AM:

Corneal limbal biopsies of approximately 2 mm^3 size obtained from human donor eyes collected within 3-6 hours of death was provided by the C.U Shah eye bank

of Sankara Nethralaya ophthalmic hospital, Chennai, India. (n= 5) Limbal biopsies were collected in the transport medium and they were processed according to the protocol mentioned in the methodology section. The biopsy bits were cultured by explant culture technique on the intact and denuded AM.

10.2.2 To study the expression of OCT4, Nanog and Pax6

Once the cultures over the AMs (intact and denuded) reach the confluence the cells were harvested by mild trypsin and EDTA treatment and the RNA was extracted according to the kit protocol mentioned in the methodology section. First strand cDNA synthesis was done and amplification of using specific primer sequence was as given in the table 10.1

Table 10.1 Primer sequence:

S.No	Primer 5'-3'	Annealing temp	Product size
1	**OCT 4 FP**: GAC AAC AAT GAG AAC CTT CAG GAG A **OCT 4 RP:** CTG GCG CCG GTT ACA GAA CCA	55^0C	220bp
2.	**Nanog FP:** GCA GAA GGC CTC AGC ACC TA **Nanog RP:** AGG TTC CCA GTC GGG TTC A	55^0C	120bp
3	**Pax6 FP:** CCG GCA GAA GAT TGT AGA GC **Pax6 RP:** CTA GCC AGG TTG CGA AGA AC	62^0C	326bp

10.3 Results:

Expression of Oct 4, Nanog and Pax 6:

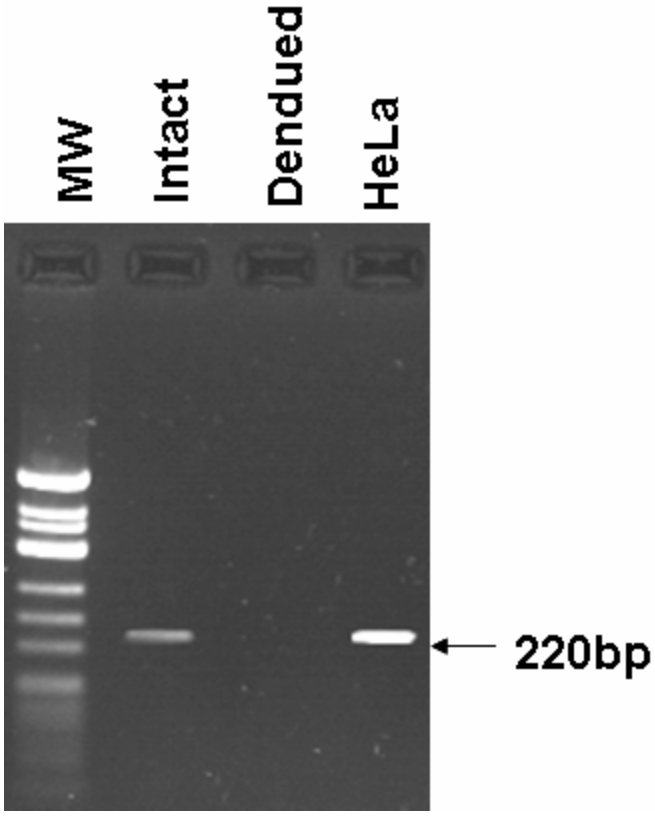
Total RNA was isolated from HeLa was used as a positive control for the presence of the three early stem cell markers OCT and Nanog. Single amplification products of expected sizes were observed using HeLa RNA. The expression of OCT4 and Nanog was present only on the cells cultured over the intact AM which was harvested after two weeks of culture, whereas it was completely absent on the cells cultured over the denuded AM. Similarly Pax6 expression was more on the cells cultured over the intact membrane whereas faint expression was seen on the cells cultured over the denuded AM. **(Figure 10.1)** Reactions omitting the reverse transcriptase (-RT) using *Nanog* and Oct-4 and Pax 6 produced no product. Primers for GAPDH were included as an internal control.

10.4 Discussion:

Given acceptance of the expression of Oct4 & Nanog as a marker of embryonic stem cells, our study was to see the presence of Oct 4 and Nanog expression. The expression of *Nanog*, *Oct-4 and Pax 6* was detected by RT-PCR on the cells cultured over the intact membrane whereas it was completely absent on the cells cultured over the denuded AM. The amplification of these transcription factors using RT-PCR techniques produced bands of expected sizes for each PCR product. These data suggest that cultured corneal limbal stem cells cultured on intact were able to harbor a population of stem cells that share some of the phenotypic characteristics of ESCs and also of non-embryonic stem cell populations. Oct-4 and Nanog transcription factors are considered key regulators of gene transcription in primitive stem cells. These factors have been reported to occupy the

Figure 10.1a:

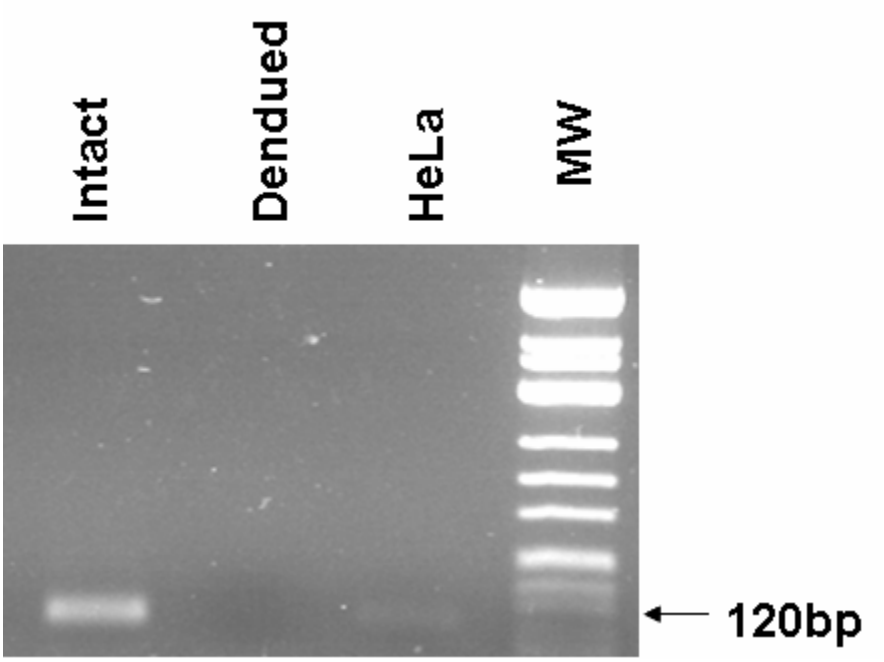
Electrophoretogram showing the amplification of OCT4



This electrophoretogram shows the expression of OCT4 on the cells cultured over the intact and denuded AM. The positive expresssion gives the specific amplification product size of 220bp. The cells harvested from the intact AM shows the expression of OCT4 whereas cells on the denuded show negative expression for OCT4. MW is molecular weight ϕX Hinf I digest. HeLa is used as the positive control

Figure 10.1b

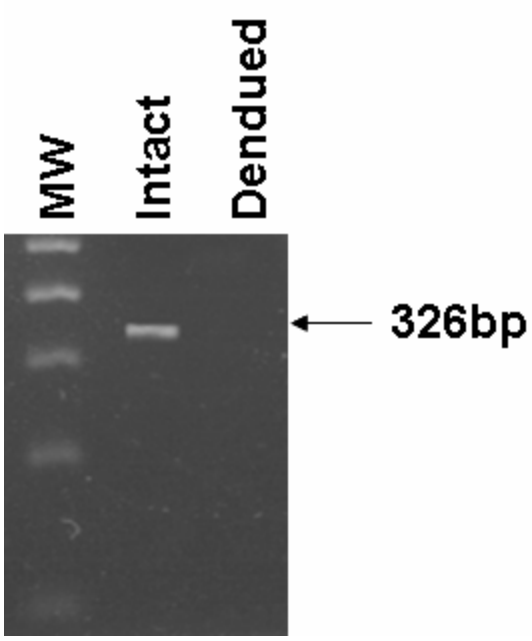
Electrophoretogram showing the amplification of Nanog



This electrophoretogram shows the expression of Nanog on the cells cultured over the intact and denuded AM. The positive expresssion gives the specific amplification product size of 120p. The cells harvested from the intact AM shows the expression of Nanog whereas cells on the denuded show negative expression for Nanog. MW is molecular weight ϕX Hinf I digest. HeLa is used as the positive control.

Figure 10.1c

Electrophoretogram showing the amplification of Pax6



This electrophoretogram shows the expression of Pax6 on the cells cultured over the intact and denuded AM. The positive expresssion gives the specific amplification product size of 326bp. The cells harvested from the intact AM shows the expression of pax6 whereas cells on the denuded show negative expression for Nanog. MW is molecular weight ϕX Hinf I digest.

promoters of at least 2,260 human genes and these transcription factors co occupy 353 gene promoters. Thus, these transcription factors regulate pluripotency and self-renewal both jointly and individually in human ESCs. A recent paper described colony formation and Oct-4 expression in fetal murine and porcine fibroblasts, but failed to replicate these findings in adult-derived porcine fibroblasts. (Kues *et al*, 2005) Expression of Oct-4 in fetal fibroblasts was only detected when the cells were maintained in 30% calf serum, suggesting that some constituent of the serum must be present in sufficient quantities for expression to be seen. The growth medium used in the present study contains only 10% FBS, although it is supplemented with several other factors, including epidermal growth factor (EGF), and there are reports of the existence of these transcription factors in the adult stem cell. (Tai *et al*, 2005) Recently Bharvand *et al*, has also reported the existence of the stem cell marker such as Oct4, Nodal, Ac133 and Pax6 on the corneal and conjunctival epithelial cells. (Bharvand *et al*, 2007 ;) Pax-6- which is known to play an essential role in the establishment and growth of the vertebrate lens. (Glaser *et al*, 1992; Hogan *et al*, 1986; hill *et al*, 1991 ;) We have found that the gene is expressed prominently in the early cellular precursors of the cornea and conjunctiva, (Walther *et al*, 1991; Grindley *et al*, 1995; Li *et al*, 1994 ;) which are located in the periphery of the lens placode. (Schook *et al*, 1994 ;) Since Pax6 is generally associated with the corneal abnormalities, there was question raised whether human Pax6 has a functional role later in life and continues to be expressed at significant levels in the ocular surface. The corneal epithelium is highly specialized tissue designed to maintain a smooth and transparent surface for the eye, Pax6 might be involved in regulating the expression of genes that determine its cellular structure and composition. Because there is evidence that

Pax-6 directly regulates the expression of crystallin genes in the lens (Richardson *et al*, 1995; Cvekl *et al*, 1995) and it is known that the corneal epithelium contains crystallin-like proteins, (Cuthberson *et al*, 1992;) Pax-6 might regulate the expression of such crystallin-like proteins in the in the adult cornea.

10.5 Conclusion:

We conclude that cultured corneal limbal stem cells contain unique and primitive cells whose potential is as yet undefined. The presence of these transcriptions factors and stem cell marker suggests that corneal limbal stem cells have properties of primitive pluripotent stem cells. Thus further studies are needed to understand the role of these transcription factors in the self-renewal of limbal stem cells cultured on these substrates.

CHAPTER 11 - SUMMARY AND CONCLUSIONS

The ocular surface is uniquely suited to study epithelial cell biology since both differentiated cells and their precursors reside therein. There is convincing clinical as well as laboratory evidence that corneal epithelial stem cells are located within the precincts of the limbal palisades. In this study **we tried to understand the biology of the corneal limbal stem cells.**

Since corneal epithelium is a nonkeratinized epithelial multilayer that covers the anterior surface of cornea. The integrity of this self-renewing epithelium relies on the existence of limbal stem cells (LSCs). Severe ocular surface injuries such as alkali burn, thermal injury, Stevens-Johnson syndrome, contact lens related epitheliopathy and ocular cicatricial pemphigoid might cause limbal stem cell deficiency (LSCD). Severe LSCD can finally result in reduced visual acuity, even blindness. Various surgical techniques have been developed to treat LSCD. Treatment is mainly through the transplantation of ex – vivo expanded corneal limbal stem cells on Human amniotic membrane matrix. **The diseases and conditions as mentioned above affect the vision of approximately 3000 patients every year in India.** Usually during such damages, the corneal limbal stem cells spontaneously multiply and repairs. But when the damage due to disease is very large or when the stem cell themselves are damaged, such repair is not feasible. At present the treatment available consists of either full thickness corneal transplant *(only one among every 10 person waiting for a transplant gets the corneal transplant done, due to shortage of available cadaver corneas)* or corneal epithelial transplant which are grown

on homologous biological materials like Human amniotic membrane. The success rate with such methods has been less than 30% due to haziness as well as rejection.

In this study, we tried to find out the usefulness of the intact (AM with devitalized epithelium) and denuded (AM removed of its epithelium layer) Intact AM was able to retain the stemness of the cultivated cells when compared to the denuded membrane.

We standardized culturing corneal limbal epithelial cells on the polymeric and nonpolymeric matrices which can be used as an alternative matrix for culturing these cells. As polymeric scaffold we used a Mebiol gel (which is NIPPAM polymer) and it has thermoreversible property. We were successfully able to culture and study the characteristics of the cultured cells. Autologous rabbit limbal corneal epithelial cells could be cultured successfully on the thermo-reversible polymer (Mebiol gel). These cultured cells on transplantation were able to adhere to the denuded corneal surface, replicate and regenerate the corneal epithelium. Reparative surgery was a total success in 7 (58.3%), partial success in 2 (16.7%) and failure in 3 (25%). The transplantation technique was very simple where cells with the gel could be applied as drops. The procedure is **sutureless technique**.

We also studied the effect of 3T3 feeder layer in cultivation of corneal limbal epithelial cells. The inactivated mouse feeder layer is commonly used for the expansion of the corneal epithelial cells. We studied the expression of the various stem cells associated markers and differentiation makers on cultivation of corneal epithelial cells with and without the 3T3 feeder layer. The cells cultured in the presence of 3T3 feeder layer were

able to maintain the limbal phenotype cells. Further studies are needed to know the cytokines and growth factors released by these feeder cells.

P63, which is one of the important marker for the identification of the keratinocyte stem cells. We studied the different isoforms of the p63 on the corneal limbal epithelial cells cultured over the intact and denuded AM. ΔNp63α which is mainly expressed by the basal limbal and corneal epithelial cells seems to be expressed only on the cells cultured over the intact AM. This shows that cells cultured over the intact membrane were able to maintain the progenitor cell properties. Which we also proved by the label retaining properties of the cells cultured over the intact AM. And we were also able to demonstrate the expression of the embryonic stem cell marker like OCT4, Nanog and Pax6 on the cells cultured over the intact AM.

Since AM releases lot of growth factors and other cytokines we studied NGF mediated signaling on the cells cultured over the intact, denuded and chitosan matrix. The cells cultured over the intact AM were able to activate the downstream signaling pathway like Akt –FKHRL1-MAPK pathway, which are involved in the regulation of cellular proliferation and mitosis.

Further studies are needed to elucidate the actual role of these matrixes and signaling molecules which mediate the proliferation of the progenitor cell population *in vitro*.

REFERENCES

- Adds PJ, Hunt C, Hartley S. Bacterial contamination of amniotic membrane. *Br J Ophthalmol* 2001; 85:228-30.
- Akpek and Foster, Limbal stem cell transplantation, *Opthalmol. Clin* 1999;29: 71 – 82
- Alison MR, Poulsom R, Forbes S, Wright NA. An introduction to stem cells. *J Pathol* 2002; 197: 419–23.
- Amino S, Itakura M, Ohnishi H, *et al*. Nerve growth factor enhances neurotransmitter release from PC12 cells by increasing Ca(2+)-responsible secretory vesicles through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *J Biochem* .2002; 131:887–894.
- Ang LP, Tan DT, Beuerman RW, Lavker RM. Development of a conjunctival epithelial equivalent with improved proliferative properties using a multistep serum-free culture system. *Invest Ophthalmol Vis Sci* 2004a; 45:1789-95.
- Ang LP, Tan DT, Phan TT, Li J, Beuerman R, Lavker RM. The in vitro and in vivo proliferative capacity of serum-free cultivated human conjunctival epithelial cells. *Curr Eye Res*. 2004b, 28(5):307-17.
- Ang LPK, Tan DTH, Cajucom-Uy H, Phan TT, Beurman R, Lavker R. Autologous cultivated conjunctival transplantation for pterygium surgery. *Am J Ophthalmol.* 2005; 139:611–619.
- Armulik, A., T. Velling, and S. Johansson, The integrin beta1 subunit transmembrane domain regulates phosphatidylinositol 3–kinase-dependent tyrosine phosphorylation of Crk-associated substrate. *Mol. Biol. Cell*, 2004; *15*(*6*): 2558–2567.
- Arpitha P, Prajna NV, Srinivasan M, Muthukkaruppan V. High expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: implications on epithelial stem cells. *Invest Ophthalmol Vis Sci*. 2005; 46(10):3631-6.

- Athanasiou K.A., Shah A.R., Hernandez R.J, LeBaron R.G., Basic science of articular cartilage repair, *Clin Sports Med,* 2001; 20: 223–247
- Azuara-Blanco A, Pillai CT, Dua HS. Amniotic membrane transplantation for ocular surface reconstruction. *Br J Ophthalmol.* 1999; 83:399-402.
- Baharvand H, Ebrahimi M and Javadi MA Comparison of characteristics of cultured limbal cells on denuded amniotic membrane and fresh conjunctival limbal and corneal tissues *Develop. Growth Differ* 2007; 49, 241–251
- Bamberger C, Pollet D, Schmale H. Retinoic acid inhibits downregulation of DeltaNp63alpha expression during terminal differentiation of human primary keratinocytes. *J Invest Dermatol.* 2002; 118:133–138.
- Barishak, R. Y. Embryology of the Eye and Its Adnexa. 2nd, revised edition ed. Basel, Switzerland: Karger; 2001.
- Barnabe-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. *J Neurosci*. 2003; 23:5149–5160.
- Baum JL. Melanocytes and Langerhans cell population of the cornea and limbus in the albino animal. *Am J Ophthalmol* 1970; 69:669-676.
- Beebe D.C and Masters D.R Cell lineage and differentiation of corneal epithelial cells *Invest. Opthalmol. Vis. Science*1996; 37, 1815 – 1825
- Bell E, Ehrlich HP, Buttle DJ, *et al.* Living tissue formed *in vitro* and accepted as skin-equivalent tissue of full thickness. *Science* 1981;211:1052–4
- Benard J, Douc-Rasy S., Ahomadegbe JC. TP53 family members and human cancers. *Hum Mutat* 2003; 21:182–91.
- Bickenbach, J.R., Identification and behaviour of label-retaining cells in oral mucosa and skin. *J. Dent. Res.* 1981; 60, 1611–1620.
- Boiani, M., Eckardt,S., Scholer,H.R. and McLaughlin,K.J. Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.*, 2002; 16, 1209–1219.
- Bosman FT, Stamenkovic I. Functional structure and composition of extracellular matrix. *J Pathol,* 2003; 200:423-428.

- Bourne GL. The microscopic anatomy of the human amnion and chorion. *Am J Obstet Gynecol.* 1960; 79:1070-1073.
- Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature*1987; 326: 515–17.
- Brun P, Cortivo R, Zavan B, Vecchiato N, Abatangelo G. *In vitro* reconstructed tissues on hyaluronan-based temporary scaffolding. *J Mater Sci Mater Med* 1999; 10:683-688.
- Buck RC: Cell migration in repair of mouse corneal epithelium. *Invest Opthalmol Vis Sci* 1979;18:767–784.
- Buck, R.C., Measurement of centripetal migration of corneal epithelial cells in the mouse. *Invest. Ophthalmol. Vis. Sci.* 1985; 26, 1296– 1299.
- Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, De Paiva JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci.* 2005; 118:1715-1724.
- Burgering BM, Kops GJ. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci*. 2002; 27:352–360.
- Cai, L., N.L. Hayes, and R.S. Nowakowski, Synchrony of clonal cell proliferation and contiguity of clonally related cells: production of mosaicism in the ventricular zone of developing mouse neocortex. *J Neurosci*., 1997; *17*(*6*): 2088–2100
- Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003 Oct 23;425(6960):841-6.
- Cao Y, Vacanti JP, Paige KT, *et al.* Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue engineered cartilage in the shape of a human ear. *Plast Reconstruct Surg* 1997; 100:297–302.
- Carlin, R Duane Davis, Mark Weiss, Bruce Schultz, and Deryl Troyer Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells *Reprod Biol. Endocrinol*., 2006, 4: 8 -14
- Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. *Dev Biol*. 2002; 250:1–23.

- Celli, J., Duijf, P., Hamel, B. C. J., Bamshad, M., Kramer, B., Smits, A. P. T., Newbury-Ecob, R., Hennekam, R. C. M., Van Buggenhout, G., van Haeringen, A., *et al. Cell* 1999: 99, 143–153.
- Chaignaud B, Langer RS, Vacanti JP. The history of tissue engineering using synthetic biodegradable scaffolds and cells. Atala A, Langer RS, Mooney DJ, acanti JP, editors. Synthetic Biodegradable Polymer Scaffolds. Boston: irk Hauser; 1997:1-14.
- Chaloin-Dufau, C., Sun, T. T., & Dhouailly, D. Appearance of the keratin pair K3/K12 during embryonic and adult corneal epithelial differentiation in the chick and rabbit. *Cell Differentiation and Development*, 1990 ;*32*, 97–108
- Chambers, I., Colby, D., Robertson, M., *et al*. Functional expression cloning of Nanog, a pluripotency-sustaining factor in embryonic stem cells. *Cell* 2003; 113: 643–655.
- Chandy T, Sharma CP. Chitosan – as a biomaterial. *Biomater Artif Cells Artif Organs* 1990; 18:1-24.
- Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells*. 2004; 22(3):355-66
- Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 2004; 22: 355–66.
- Chen, J.J., Tseng, S.C, Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Invest. Ophthalmol. Vis. Sci* 1991;32, 2219–2233.
- Chen, J.J., Tseng, S.C., Corneal epithelial wound healing in partial limbal deficiency. *Invest. Ophthalmol. Vis. Sci*. 1990; 31, 1301–1314.
- Cintia S. de Paiva, Zhuo Chen, Rosa M. Corrales, Stephen C. Pflugfelder, De-Quan Li ABCG2 Transporter Identifies a Population of Clonogenic Human Limbal Epithelial Cells. *Stem Cells* 2005;23:63–73
- Cintron, C., Covington, H. And kublin, C. L. Morphogenesis of rabbit corneal stroma. *Invest Ophthalmol Vis Sci* 1983; 24:543-556.

- Connon CJ, Nakamura T, Quantock AJ, Kinoshita S. The persistence of transplanted amniotic membrane in corneal stroma. *Am J Ophthalmol.* 2006 Jan; 141(1):190-2.
- Coroneo MT, Muller-Stolzenburg NW, Ho A. Peripheral light focusing by the anterior eye and the Ophthalmohelioses. *Ophthalmic Surg* 1991; 22:705-711.
- Cotsarelis, G., Cheng, S.Z., Dong, G., Sun, T.T., Lavker, R.M. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989;57, 201–209.
- Cuthberson RA, Tomarev SI, Piatigorsky J. Taxon-specific recruitment of enzymes as major soluble proteins in the corneal epithelium of three mammals, chicken, and squid. *Proc Natl Acad Sci USA.* 1992; 89:4004-4008.
- Cvekl A, Sax CM, Li X, McDermott JB, Piatigorsky J. Pax-6 and lens-specific transcription of the chicken delta 1-crystallin gene. *Proc Natl Acad Sci USA.* 1995;92:4681-4685.
- Daniels, J.T., Dart, J.K.G., Tuft, S.J., Khaw, P.T., 2001. Corneal stem cells in review. *Wound Rep. Reg*. 9, 483–494.
- Danjo, Y. and Gipson, I.K. (2002) Specific transduction of the leading edge cells of migrating epithelia demonstrates that they are replaced during healing. *Exp. Eye Res.* 74, 199–204
- Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev*. 1999; 13:2905–2927.
- Davanger M, Evensen A: Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971; 229:560–561
- Davis JW. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. *Johns Hopkins Med J*. 1910; 15:307.
- Davis SS, Illum L. Polymeric microspheres as drug carriers. *Biomaterials* 1988; 9:111-115.
- De Paiva CS, Chen Z, Corrales RM, Pflugfelder S, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005; 23:63-73.

- De Rotth A. Plastic repair of conjunctival defects with fetal membrane. *Arch Ophthalmol.* 1940; 23:522-525.
- Deyev, I.E. and Polanovsky,O.L. The OCT genes and Oct proteins. *Mol. Biol.*, 2004; 38, 48–55
- Deyev, I.E., Polanovsky, O.L. The Oct genes and Oct proteins. *Mol. Biol.* 2004; 38, 48--55.
- Di Como CJ, Urist MJ, Babayan I, *et al*. p63 expression profiles in human normal and tumor tissues. *Clin Cancer Res.* 2002; 8:494– 501.
- Di Iorio E., Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De LM. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci* U S A 2005; 102:9523-9528.
- Dilly PN, Mackie IA: surface changes in the anaesthetic conjunctiva in man, with special reference to the production of mucus from a non-goblet cell source, *Br. J. Ophtalmol.* 1981; 65:833-842.
- Dogru M, Tsubota K. Current concepts in ocular surface reconstruction. *Semin Ophthalmol*. 2005; 20:75-93
- Dong Y, Roos M, Gruijters T *et al.* Differential expression of two gap junction proteins in corneal epithelium. *Eur J Cell Biol* 1994; 64: 95–100.
- Dornish M, Kaplan D, Skaugrud O. Standards and guidelines for biopolymers in tissue-engineered medical products, *Ann N Y Acad Sci,* 2001*;* 944: 388–397.
- Dua HS, Azuara–Blanco A. Amniotic membrane transplantation. *Br J Ophthalmol.* 1999; 83:748–752.
- Dua HS, Jagjit SS, Azuara-Blanco A, Gupta P. Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. *Indian J Ophthalmol* 2000; 48:83-92.
- Dua HS, Shanmuganathan VA, Powell-Richards A, Tiqhe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005; 89:529-532.
- Dua, H.S., Azuara-Blanco, A., Limbal stem cells of the corneal epithelium. *Surv. Ophthalmol.* 2000; 44, 415–425.

- Dua, H.S., Joseph, A., Shanmuganathan, V.A., Jones, R.E., Stem cell differentiation and the effects of deficiency. *Eye* 2003; 17, 877–885.
- Duke-Elder WS: Normal and abnormal development. In system of ophthalmology, Vol III, Pt I, London, 1963, Kimpton.
- Dutton, R. and P.F. Bartlett, Precursor cells in the subventricular zone of the adult mouse are actively inhibited from differentiating into neurons. *Dev. Neurosci*., 2000; 22(1–2): 96–105.
- Ebato, B., Friend, J., & Thoft, R. A. Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Investigative Ophthalmology & Visual Science*, 1988; 29, 1533–1537.
- Ebato, B., Friend, J., Thoft, R.A., Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest. Ophthalmol. Vis. Sci.* 1988; 29, 1533–1537.
- Ehmann, U.K., Stevenson, M.A., Calderwood, S.K. and DeVries, J.T. Physical connection between feeder cells and recipient normal mammary epithelial cells. *Exp. Cell Re*s. 1998: 243: 76-86
- Espana EM, Grueterich M, Touhami A, Tseng SCG. Corneal stromal changes following reconstruction by *ex vivo* expanded limbal epithelial cells in rabbits with total limbal stem cell deficiency. *Br J Ophthalmol.* 2003; 87:1509–1514.
- Espana EM, Kawakita T, Romano A, Di Pascuale M, Smiddy R, Liu CY, Tseng SC. Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. *Invest Ophthalmol Vis Sci* 2003; 44:5130- 5135.
- Espana EM, Romano AC, Kawakita T, Di Pascuale M, Smiddy R, Tseng SC. Novel enzymatic isolation of an entire viable human limbal epithelial sheet. *Invest Ophthalmol Vis Sci* 2003; 44:4275-4281.
- Farjo, A.A. and Soong, K.H. Corneal epithelium. In Ophthalmology (Yanoof, M. and Duker, J.S., eds.), pp. 413–421, Mosby, MO, U.S.A. conjunctival limbal autograft for total limbal stem cell deficiency. *Ophthalmol.* 2004;110, 1585–1592
- Flaumenhaft R, Rifkin DB. The extracellular regulation of growth factor action. *Mol Biol Cell* 1992; 3:1057–1065.

- Flores, E.R., Tsai, K.Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., Jacks, T., p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* 2002; 416, 560–564.
- Foster CS, Sainz de la Maza M: Structural considerations of the sclera. In The Sclera, New york, 1994, Springer-Verlag.
- Franke TF, Hornik CP, Segev L, *et al*. PI3K/Akt and apoptosis: size matters. *Oncogene*. 2003; 22:8983–8998.
- Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. *Int J Pharm* 2004; 282:1-18.
- Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Annu Rev Biochem*. 1998; 67:481–507.
- Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell* 2004; 116:769-778.
- Galindo H, EE, Theiss C, Steuhl KP, Meller D. Gap junctional communication in microinjected human limbal and peripheral corneal epithelial cells cultured on intact amniotic membrane. *Exp Eye Res.* 2003 Mar; 76(3):303-14.
- Germain L, Auger FA, Grandbois E, Guignard R, Giasson M, Boisjoly H, Guerin SL. Reconstructed human cornea produced *in vitro Tissue engineering. Pathobiology* 1999; 67:140-7.
- Ghioni, P., Bolognese, F., Duijf, P.H., Van Bokhoven, H., Mantovani, R., and Guerrini, L. Complex transcriptional effects of p63 isoforms: Identification of novel activation and repression domains. *Mol. Cell. Biol.* 2002; 22: 8659–8668.
- Gipson, I.K. The epithelial basement membrane zone of the limbus. *Eye,* 1989; 3, 132–140.
- Glaser T, Walton DS, Maas RL. Genomic structure, evolutionary conservation and aniridia mutations in the human Pax-6 gene. *Nature Genet.* 1992;2:232-239.
- Goldberg MF, Bron AJ. Limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1982; 80:155-171.
- Gomes JA, Romano A, Santos MS, Dua HS. Amniotic membrane use in ophthalmology. *Curr Opin Ophthalmol*. 2005; 16:233-40.

- Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* 1996; 183:1797-1806.
- Goodell MA, Rosenzweig M, Kim H, *et al*. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997; 3:1337-1345.
- Goodson, W.H. III, Moore, D.H., Ljung, B.M., Chew, K., Florendo, C., Mayall, B., Smith, H.S., Waldman, F.M., The functional relationship between in vivo bromodeoxyuridine labeling index and Ki-67 proliferation index in human breast cancer. *Breast Cancer Res. Treat*. 1998;49, 155–164
- Graves JD, Draves KE, Craxton A, *et al*. Involvement of stressactivated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. *Proc Natl Acad Sci USA*. 1996; 93:13814–13818.
- GrindleyJC, Davidson DR, Hill RE. The role of Pax-6 in eye and nasal development. *Development.*1995; 121:1433-1442.
- Grueterich M, Edgar M. Espana, Scheffer C. G. Tseng. Modulation of Keratin and Connexin Expression in Limbal Epithelium Expanded on Denuded Amniotic Membrane with and without a 3T3 Fibroblast Feeder Layer *Invest Ophthalmol Vis Sci.* 2003a;44:4230–4236
- Grueterich M, Espana E, Tseng SC. Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. *Invest Ophthalmol Vis Sci.* 2002a; 43:63–71.
- Grueterich M, Espana EM, Tseng SC. *Ex vivo* expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. *Surv Ophthalmol* 2003b; 48: 631–46.
- Grueterich M, Tseng SC. Human limbal progenitor cells expanded on intact amniotic membrane *ex vivo*. *Arch Ophthalmol.* 2002b; 120:783–790.
- Grueterich, M., Espana, E., Touhami, A., Ti, S. & Tseng, S.. Phenotypic study of a case with successful transplantation of *ex vivo* expanded human limbal epithelium for unilateral total limbal stem cell deficiency. *Ophthalmology* 2002c; 109, 1547–1552.

- Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996; 84:345-577.
- Hay E.D. Development of the vertebrate cornea. *Int Rev Cytol* 1979; 63:263-322.
- Hayashi R, Yamato M, Sugiyama H, *et al*. N-cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells* 2006; Feb; 25(2):289-96.
- He Hua, Hee-Tae cho, Wei Li, Tetsuya Kawakita, Ling Jong and Scheffer Tseng Signal transduction pathways required for *ex vivo* expansion of human limbal explants on intact amniotic membrane. *Invest. Opthalmol. Vis. Science*; 2006: 47: 151 -157
- Heiligenhaus A, Li H, Hernandez Galindo EE, *et al*. Management of acute ulcerative and necrotizing herpes simplex and zoster keratitis with amniotic membrane transplantation. *Br J Ophthalmol*. 2003;87:1215-1219
- Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M, Olonen A, Salo H, Lehenkari P, Tuuri T, Otonkoski T, Natunen J, Saarinen J, Laine J. N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. *Stem Cells*. 2007 Jan; 25(1):197-202.
- Helton, E.S., Zhu, J., and Chen, X. The unique NH2-terminally deleted (_N) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the ΔN variant of p63. *J. Biol. Chem.* 2006; 281: 2533–2542.
- Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D. Expression of Delta Np63 in response to phorbol ester in human limbal epithelial cells expanded on intact human amniotic membrane. *Invest Ophthalmol Vis Sci* 2003a; 44: 2959–65.
- Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D. Gap junctional communication in microinjected human limbal and peripheral corneal epithelial cells cultured on intact amniotic membrane. *Exp Eye Res*. 2003b; 76:303–314.
- Higa K, Shimmura S, Miyashida H, Shimazaki J, Tsubota K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. *Exp Eye Res* 2005; 81:218-223.

- Hill RE, Favor J, Hogan BLM, *et al*. Mouse *Small eye* results from mutations in a paired-like homeobox-containing gene. *Nature.* 1991; 354:522-525.
- Hishikawa K, Miura S, Marumo T, Yoshioka H, Mori Y, Takato T, *et al*. Gene expression profile of human mesenchymal stem cells during osteogenesis in three dimensional thermoreversible gelation polymer. *Biochem Biophys Res Commun* 2004; 317: 1103-7.
- Hogan BLM, Horsburgh G, Cohen J, Hetherington CM, Fisher G, Lyon MF. Small eyes *(Sey):* A homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. */Embryol Exp Moiphol.* 1986; 97:95-110.
- Holland, E.J., Schwartz, G.S., The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. *Cornea* 1996;15, 549–556.
- Houlihan JM, Biro PA, Harper HM, *et al*. The human amniotic membrane is a site of MHC class 1b expression: evidence for the expression of HLA-E and HLA-G. *J Immunol.* 1995;154:565-574.
- Hsueh, Y.-J., Wang, D.-Y., Cheng, C.-C., Chen, J.-K., Age-related expressions of p63 and other keratinocyte stem cell markers in rat cornea. *J. Biomed. Sci.* 2004;11, 641–651.
- Huang, A.J., Tseng, S.C., Corneal epithelial wound healing in the absence of limbal epithelium. *Invest. Ophthalmol. Vis. Sci*1991;32, 96–105.
- Ihrie, R.A., Marques, M.R., Nguyen, B.T., Horner, J.S., Papazoglu, C., Bronson, R.T., Mills, A.A., and Attardi, L.D.. Perp is a p63-regulated gene essential for epithelial integrity. *Cell* 2005;120: 843–856.
- Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U. Stem Cells Derived from Human Fetal Membranes Display Multi-Lineage Differentiation Potential.Biol Reprod. 2007 May 9
- Irwin MS, Kaelin WG. Role of the newer p53 family proteins in malignancy. *Apoptosis* 2001;6:17–29.

- Jester, J.V., Moller-Pedersen, T., Huang, J., Sax, C.M., Kays, W.T., Cavangh, H.D., Petroll, W.M. and Piatigorsky, J. The cellular basis of corneal transparency: evidence for 'corneal crystallins'. *J. Cell Sci*. 1999;112, 613–622
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002; 298:1911–1912.
- Johnson MD, Okedli E, Woodard A, *et al*. Evidence for phosphatidylinositol 3-kinase-Akt-p7S6K pathway activation and transduction of mitogenic signals by platelet-derived growth factor in meningioma cells. *J Neurosurg*. 2002; 97:668–675.
- Jones J, Sugiyama M, Watt FM, Speight PM, Integrin expression in normal, hyperplastic, dysplastic and malignant oral epithelium, *J. Pathol* 1993:169: 235 – 243
- Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. *Cell* 1995; 80: 83–93.
- Kaur, P., Li, A., Redvers, R. & Bertoncello, I. *J. Invest. Dermatol. Symp. Proc.* 2004; 9, 238–247.
- Kawakita T, Espana EM, He H, Li W, Tseng SCG. Intrastromal invasion by limbal epithelial progenitor cells is mediated by epithelial-mesenchymal transition activated by air exposure. *Am J Pathol* 2005; 167:381-393.
- Kawakita T, Espana EM, He H, Yeh LK, Liu CY, Tseng SCG. Epidermal differentiation promoted by high calcium serum free condition in mouse corneal/limbal epithelial culture. *Invest Ophthalmol Vis Sci* 2005; 45:3507-3512.
- Kawasaki S, Tanioka H, Yamasaki K, Connon CJ, Kinoshita S. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. *Exp. Eye Res* 2006; 82:293–299
- Kedinger M, Lefebvre O, Duluc I, Freund JN, Simon-Assmann P. Cellular and molecular partners involved in gut morphogenesis and differentiation. *Philos Trans R Soc Lond B Biol Sci* 1998; 353:847-856.
- Kenyon, K.R., Tseng, S.C., Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;96, 709–722.

- Khor E, Lim LY Implantable applications of chitin and chitosan, *Biomaterials*, 2003, 13: 2339–2349
- Kim JC, Tseng SC. The effects on inhibition of corneal neo vascularization after human amniotic membrane transplantation in severely damaged rabbit corneas. *Korean J Ophthalmol*. 1995; 9:32-46.
- Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit conjunctival epithelial cells. *Curr Eye Res* 2004b; 28:307-17.
- Kim JC, Tseng SCG. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea*. 1995; 14:473–484.
- King, K.E., Ponnamperuma, R.M., Yamashita, T., Tokino, T., Lee, L.A., Young, M.F., and Weinberg, W.C. ΔNp63 functions as both a positive and a negative transcriptional regulator and blocks *in vitro* differentiation of murine keratinocytes. *Oncogene* 2003; 22: 3635–3644.
- Kinoshita S, Friend J, Thoft RA. Sex chromatin of donor corneal epithelium in rabbits.*Invest Ophthalmol Vis Sci.* 1981 Sep; 21(3):434-41.
- Kiritoshi, A., SundarRaj, N., Thoft, R.A., Differentiation in cultured limbal epithelium as defined by keratin expression. *Invest. Ophthalmol. Vis. Sci* 1991;32, 3073–3077
- Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R. *et al*. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*; 2003; 425, 841–846
- Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ. Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. *Invest Ophthalmol Vis Sci.*2000; 41:2506–2513.
- Koizumi N, Inatomi T, Quantock AJ, Fullwood NJ, Dota A, Kinoshita S. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits.*Cornea.* 2000;19:65–71.

- Koizumi N, Inatomi T, Suzuki T, *et al*. Cultivated corneal epithelial transplantation for ocular surface reconstruction in the acute phase of Stevens-Johnson syndrome. *Arch Ophthalmol*. 2001a; 119: 298–300.
- Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 2001b; 108:1569-74.
- Koizumi N, Rigby H, Fullwood NJ, Kawasak S, Tanioka H, Kinoshita S Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells. *Graefe's Arch Clin Exp Ophthalmol.* 2007; 245: 123–134
- Kops GJ, Burgering BM. Forkhead transcription factors are targets of signalling by the proto-oncogene PKB (C-AKT). *J Anat*. 2000; 197:571–574.
- Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR. p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev.* 2004; 18:126–131.
- Kruse, F.E. Stem cells and corneal epithelial regeneration. *Ey*e, 1994; 8, 170–183.
- Kruse, F.E., Reinhard, T., Limbal transplantation for ocular surface reconstruction. *Ophthalmologe* 2001;98, 818–831.
- Kruse, F.E., Tseng, S.C., Retinoic acid regulates clonal growth and differentiation of cultured limbal and peripheral corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 1994;35, 2405–2420.
- Kruse, F.E., Tseng, S.C.G., A tumor promoter-resistant subpopulation of progenitor cells is larger in limbal epithelium than in corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 1993a;34, 2501–2511.
- Kubo M, Sonoda Y, Muramatsu R, Usui M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Invest Ophthalmol Vis Sci*. 2001 Jun; 42(7):1539-46.
- Kues WA, Petersen B, Mysegades W, Carnwath JW, Niemann H. Isolation of murine and porcine fetal stem cells from somatic tissue. *Biol Reprod.* 2005; 72:1020–1028

- Kurpakus, M.A., Stock, E.L., Jones, J.C., Expression of the 55- kD/64-kD corneal keratins in ocular surface epithelium. *Invest. Ophthalmol. Vis. Sci.* 1990;31, 448–456.
- Kuwabara T, Perkins DG, Cogan DG: Sliding of the epithelium in experimental corneal wounds. *Invest Opthalmol* 1976;15:4–14.
- Lambiase A, Bonini S, Micera A, Rama P, Bonini S, Aloe L. Expression of nerve growth factor receptors on the ocular surface in healthy subjects and during manifestation of inflammatory diseases. *Invest Ophthalmol Vis Sci* 1998; 39: 1272–5.
- Langer RS, Vacanti JP. Tissue engineering. *Science* 1993;260:920–6
- Langer RS, Vacanti JP. Tissue engineering: the challenges ahead. *Sci Am* 1999; 280:62–5.
- Lauweryns, B., van den Oord, J.J., De Vos, R., Missotten, L., A new epithelial cell type in the human cornea. *Invest. Ophthalmol. Vis. Sci.* 1993a. ;34, 1983–1990.
- Lauweryns, B., van den Oord, J.J., Missotten, L.,.The transitional zone between limbus and peripheral cornea. An immunohistochemical study. *Invest. Ophthalmol. Vis. Sci* 1993. 34, 1991–1999.
- Lavker RM, Sun T-T: Epidermal stem cells: Properties, markers, and location. *Proc Nat Acad Sci USA* 2000;97:13473–13475,
- Lavker RM, Tseng SC, Sun TT. Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle. *Exp Eye Res* 2004; 78:433-446.
- Lavker, R.M., Dong, G., Cheng, S.Z., Kudoh, K., Cotsarelis, G., Sun, T.T., Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. *Invest. Ophthalmol. Vis. Sci*. 1991;32, 1864–1875.
- Lavker, R.M., Wei, Z.-G., Sun, T.-T., Phorbol ester preferentially stimulates mouse fornical conjunctival and limbal epithelial cells to proliferate in vivo. *Invest. Ophthalmol. Vis. Sci.* 1998;39, 301–307.
- Lawrenson JG, Ruskell GL. The structure of corpuscular nerve endings in the limbal conjunctiva of the human eye. *J Anat* 1991; 177:75-84.

- Lee SH, Tseng SC. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol*. 1997; 123:303-312.
- Lehrer, M.S., Sun, T.-T., Lavker, R.M., Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J. Cell Sci*. 1998;111, 2867–2875.
- Li DQ and Scheffer C. G. Tseng. Differential Regulation of Cytokine and Receptor Transcript Expression in Human Corneal and limbal Fibroblasts by Epidermal Growth Factor, Transforming Growth Factor-α, Platelet-Derived Growth Factor B, and Interleukin-l β. *Invest Ophthalmol Vis Sci*. 1996; 37:2068-2080.
- Li DQ, Lokeshwar BL, Solomon A *et al*. Regulation of MMP-9 production by human corneal epithelial cells. *Exp Eye Res* 2001; 73:449–459.
- Li DQ, Tseng SC. Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. *J Cell Physiol* 1995; 163:61–79.
- Li HS, Yang Jacobson RD, Pasko D, Sundin O. Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: Implications for stepwise determination of the lens. *Dev Biol.* 1994; 162:181-194.
- Li L, Xie T. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol* 2005; 21:605-631.
- Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. *In vitro* propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci.* 1993; 34:2672–2679.
- Liu S, Li J, Wang C, Tan D, Beuerman R. Human limbal progenitor cell characteristics are maintained in tissue culture. *Ann Acad Med Singapore*. 2006; 35(2):80-6.
- Liu, C.Y., Zhu, G., Westerhausen-Larson, A., Converse, R., Kao, C.W., Sun, T.T., Kao, W.W., Cornea-specific expression of K12 keratin during mouse development. *Curr. Eye Res* 1993. 12, 963–974.
- Ljubimov AV, Burgeson RE, Butkowski RJ, Michael AF, Sun T-T, Kenney MC. Human corneal basement membrane heterogeneitiy: topographical differences in

the expression of type IV collagen and laminin isoforms. *Lab Invest* 1995; 72:461-473.

- Lohrum MA, Vousden KH. Regulation and function of the p53-related proteins: same family, different rules. *Trends Cell Biol* 2000; 10: 197–202.
- Lu, B., L. Jan, and Y.N. Jan, Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu. Rev. Neurosci*., 2000; *23*: 531–556.
- Madhavan HN, Malathi J, Joseph RP, Mori Y, Abraham SJK, Yoshioka H. A study on the growth of continuous culture cell lines embedded in Mebiol Gel. *Curr Sci* 2004; *87:* 1275-7.
- Madihally S.V, Matthew H.W.T., Porous chitosan scaffolds for tissue engineering, *Biomaterials*, 1999; 20:1133–1142.
- Matic, M., Petrov, I.N., Chen, S., Wang, C., Dimitrijevich, S.D., Wolosin, J.M., Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation,* 1997; 61, 251–260.
- McAteer JA & Davis, J.M. Basic cell culture technique and the maintenance of cell lines. In Davis, J.M., eds. Basic cell culture – A practical approach. 2nd edition. Oxford university press, London, 2002; 93 – 148
- Meller D, Dabul V, Tseng SC. Expansion of conjunctival epithelial progenitor cells on amniotic membrane. *Exp Eye Res* 2002; 74:537-45.
- Meller D, Pires RT, Mack RJ, Figueiredo F, Heiligenhaus A, Park WC, Prabhasawat P, John T, McLeod SD, Steuhl KP, Tseng SC. Amniotic membrane transplantation for acute chemical or thermal burns. *Ophthalmology.* 2000 May;107(5):980-9;
- Meller D, Pires RT, Tseng SC. *Ex vivo* preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. *Br J Ophthalmol*. 2002; 86: 463-71.
- Meller D, Tseng SCG. Conjunctival epithelial cell differentiation on the amniotic membrane *Invest. Opthalmol. Vis. Sci*.1990:40; 878- 886
- Meredith JE, Fateli B, Schwartz MA. The extracellular matrix as a cell survival factor, *Molecular Cellular Biology* 1993; 9:953-961.

- Miller SJ, Lavker RM, Sun T-T: Keratinocyte stem cells of cornea, skin and hair follicle: Common and distinguishing features. *Semin Dev Biol* 1993; 4:217–240.
- Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., Bradley, A., p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 1999; 398, 708–713.
- Mishima S Corneal thickness, *Surv Ophthalmol* 1968;13, 57 – 96.
- Mitsui, K., Tokuzawa, Y., Itoh, H., *et al*. The homeoprotein Nanog is required for maintenance of Pluripotency in mouse epiblast and ES cells. *Cell* 2003; 113: 631–642.
- Moore KA, Lemischka IR. Stem cells and their niches. *Science* 2006; 311:1880-1885.
- Moore, J.E., McMullen, C.B.T., Mahon, G., Adamis, A.P., The corneal epithelial stem cell. DNA *Cell Biol.* 2002; 21, 443–451.
- Mori T. M. Murakami, M. Okumura, T. Kadosawa, T. Uede and T. Fujinaga, Mechanism of macrophage activation by chitin derivatives, *J Vet Med Sci;* 2005: 67, 51.
- Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S. Characterization and distribution of bone marrow-derived cells in mouse cornea. *Invest Ophthalmol Vis Sci* 2005; 46:497-503.
- Nakamura T, Koizumi N, Tsuzuki M, Inoki K, Sano Y, Sotozono C, Kinoshita S. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. *Molecular Vision* 2007; 13:524-533
- Narisawa Y, Kohda H, Tanaka T. Three-dimensional demonstration of melanocyte distribution of human hair follicles:special nreference to the bulge area. *Acta Derm Venereol* 1997; 77:97-101.
- Noszczyk BH, Majewski ST. p63 expression during normal cutaneous wound healing in humans. *Plast Reconstr Surg.* 2001;108: 1242–1247.
- Okada H, Toguchi H. Biodegradable microspheres in drug delivery. Crit Rev Ther *Drug Carrier Syst* 1995; 12:1-99.

- Okamoto,K., Okazawa,H., Okuda,A., Sakai,M., Muramatsu,M. and Hamada,H. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell*, 1990; 60, 461–472.
- Pachence JM. Collagen-based devices for soft tissue repair. *J Biomed Mater Res* 1996; 33:35-40.
- Pancholi, V., Multifunctional a-enolase: its role in diseases. *Cell. Mol. Life Sci.* 2001;58, 902–920.
- Park KS, Lim CH, Min BM, *et al*. The side population cells in the rabbit limbus sensitively increased in response to the central cornea wounding. *Invest Ophthalmol Vis Sci* 2006; 47:892-900.
- Parsa, R., Yang, A., McKeon, F. & Green, H. *J. Invest. Dermatol.* 1999; 113, 1099–1105.
- Peifer M. Signal transduction. Neither straight nor narrow. *Nature* 1999; 400:213-215.
- Pellegrini G, Dellambra E, Golisano O, *et al*. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA.* 2001; 98:3156–3161.
- Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1997; 349:990–993.
- Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P. & De Luca, M. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface *J. Cell Biol* 1999*;* 145, 769–782
- Pesce, M. and Scholer, H.R. Oct-4: gatekeeper in the beginnings mammalian development. *Stem Cells,* 2001; 19: 271-278.
- Pesce, M., Gross, M.K. and Scholer, H.R. In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays* 1998; 20: 722--732.
- Piatigorsky, J. A case for corneal crystallins. *J. Ocul. Pharmacol. Ther*. 2000;16, 173–180
- Potten CS, Booth C: Keratinocyte stem cells: A commentary. *J Invest Dermatol* 119:888–899, 2002

- Potten CS, Loeffler M: Stem cells: Attributes, cycles, spirals, pitfalls and uncertainties.Lessons for and from the crypt. *Development* 110:1001–1020, 1990
- Prabhasawat P, Barton K, Burkett G, Tseng SCG. Comparison of conjunctival autografts, amniotic membrane grafts, and primary closure for pterygium excision. *Ophthalmology.* 1997; 104:974–985.
- Prusa, A.R., Marton,E., Rosner,M., Bernaschek,G. and Hengstschlager,M. Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research *Hum. Reprod.*, 2003; 18, 1489–1493
- Prydal JI, Artal P, Woon H, Campbell FW. Study of human precorneal tear film thickness and structure using laser interferometry. *Invest Ophthalmol Vis Sci.* 1992 May;33(6):2006-11.
- Puangsricharern V, Tseng SCG. Cytologic evidence of corneal diseases with limbal stem cell deficiency. *Ophthalmology* 1995; 102:1476-1485.
- Puck, T.T., Marcus, P.I. and Cieciura, S.J. Clonal growth of mammalian cells *in vitro*. Growth characteristics of colonies from single HeLa cells with and without a feeder layer. *J. Exp. Med*. 1956: 103: 273-283.
- Quesenberry PJ, Becker PS. Stem cell homing: rolling, crawling, and nesting. *Proc Natl Acad Sci U S A* 1998; 95:15155-15157
- Rabea E.I., Badawy, M.E. Stevens, C.V. Smagghe G. and Steurbaut W., Chitosan as antimicrobial agent: applications and mode of action, *Biomacromolecules* 2003; 4: 1457.
- Rajabalian S, Zahra Samadi Bahrami1, Vahideh Farahat, Fazel Shokri Supportive Effects of Human Embryonic Fibroblast Cell Lines on Growth and Proliferation of EBV-Transformed Lymphoblastoid Cells. *Iranian Biomedical Journal,* 2003; 7 (4): 147-153.
- Rama P, Bonini S, Lambiase A, Golisano O, Paterna P, De Luca M, Pellegrini G. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation* 2001; 72:1478-85

- Ramaesh K, Ramaesh T, Dutton GN, Dhillon B. Evolving concepts on the pathogenic mechanisms of aniridia related keratopathy. *Int J Biochem Cell Biol* 2005; 37:547-557.
- Ramaesh T, Ramaesh K, Martin CJ, Chanas SA, Dhillon B, West JD. Developmental and cellular factors underlying corneal epithelial dysgenesis in the Pax6+/- mouse model of aniridia. *Exp Eye Res* 2005; 81:224-235.
- Raoul, C., B. Pettmann, and C.E. Henderson, Active killing of neurons during development and following stress: a role for p75(NTR) and Fas *Curr. Opin. Neurobiol*., 2000; *10*(*1*): 111–117.
- Records RE: The conjunctiva and lacrimal system, In Tasman W, Jaeger EA, editors: Duane's foundations of clinical Ophthalmol- Jaeger EA, editors: Duane's Foundations of clinical ophthalmology, Philadelphia, 1994, JB Lippincott.
- Redvers RP, Li A, Kaur P. Side population in adult murine epidermis exhibits phenotypic and functional characteristics of keratinocyte stem cells. *Proc Natl Acad Sci U S A* 2006; 103:13168-13173.
- Reisenberger K, Egarter C, Knofler M. Cytokine and prostaglandin roduction by amnion cells in response to the addition of different bacteria. *Am J Obstet Gynecol*. 1998; 178:50-53.
- Reis-Filho JS, Schmitt FC. Taking advantage of basic research: p63 is a reliable myoepithelial and stem cell marker. *Adv Anat Pathol* 2002; 9:280–9.
- Ren D, Yi H, Wang W and Ma X., The enzymatic degradation and swelling properties of chitosan matrices with different degrees of N-acetylation, *Carbohydr Res* 2005; 340: 2403. Abstract
- Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; 6:331-337.
- Rice RH, Green H. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 1979; 18: 681–94.
- Richardson J, Cvekl A, Wistow G. Pax-6 is essential for lens-specific expression of zeta-crystallin. *Proc Natl AcadSci USA*. 1995;92:4676-4680.

- Romano, A.C., Espana, E.M., Yoo, S.H., Budak, M.T., Wolosin, J.M., Tseng, S.C.G., Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest. Ophthalmol. Vis. Sci.* 2003; 44, 5125–5129.
- Rosner,M.H., Vigano,M.A., Ozato,K., Timmons,P.M., Poirier,F., Rigby,P.W. and Staudt, L.M. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature*, 1990;345, 686–692
- S.G. Hu, C.H. Jou and M.C. Yang, Protein adsorption, fibroblast activity and antibacterial properties of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) grafted with chitosan and chitooligosaccharide after immobilized with hyaluronic acid, *Biomaterials*; 2003: 24; 2685.
- S.V. Madihally and H.W.T. Matthew, Porous chitosan scaffolds for tissue engineering, *Biomaterials* 1999;20:1133.
- Sangwan VS, Vemuganti GK, Iftekhar G, *et al*. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. *Cornea*. 2003; 22:478-481.
- Sangwan, V.S., Limbal stem cells in health and disease. *Biosci. Rep.* 2002;21, 385–405.
- Sasaki, Y., Ishida, S., Morimoto, I., Yamashita, T., Kojima, T., Kihara, C., Tanaka, T., Imai, K., Nakamura, Y., and Tokino, T. The p53 family member genes are involved in the Notch signal pathway. *J. Biol. Chem.* 2002; 277: 719–724.
- Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; 103: 49–62.
- Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005; 81: 247–64.
- Schmale, H., Bamberger, C.A novel protein with strong homology to the tumor suppressor p53. *Oncogene;* 1997:15, 1363–1367.
- Schmidt, N.J. Cell culture procedures for diagnostic virology. In Schmidt N.J., and Emmons R.W., eds. Diagnostic procedures for viral, Rickettsial and Chlamydial

infections. 6th Edition, American Public Health Association Inc, Washington DC, 1988; 51-100.

- Scholer, H.R., Ruppert,S., Suzuki,N., Chowdhury,K. and Gruss,P. New type of POU domain in germ line-specific protein Oct-4. *Nature*, 1990; 344, 435–439.
- Schook P. Cell action and cell interaction during ocular morphogenesis. In: Tasman W, Jaeger E, eds. Duane's Foundations of Clinical Ophthalmology. Philadelphia: JB Lippincott; 1994.
- Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea*. 2000; 19:421-426.
- Schwab IR. Cultured corneal epithelia for ocular surface disease. *Trans Am Ophthalmol Soc.* 1999; 97:891–986.
- Schwab, IR. Cultured corneal epithelia for ocular surface disease. *Tr. Am. Ophth. Soc*, 1999, Vol XCVII
- Sechriest VF, Miao YJ, Niyibizi C, Westerhausen-Larson A, Matthew HW, Evans CH, *et al*. GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. *J Biomed Mater Res* 2000; 49:534-41.
- Seigel GM, Sun W, Salvi R, Campbell LM, Sullivan S, Reidy JJ. Human corneal stem cells display functional neuronal properties. *Mol Vis* 2003; 9: 159–63.
- Sevel, D. and Isaacs, R. A re-evaluation of corneal development. *Trans Am Ophthalmol Soc* 1988;86:178-207.
- Shanmuganathan VA, Foster T, Kulkarni BB, Hopkinson A, Gray T, Powe DG, Lowe J, Dua HS. Morphological Characteristics of the Limbal Epithelial Crypt. *Br J Ophthalmol* 2007 Apr;91(4):514-9.
- Shimazaki J, Yang HY, Tsubota K. Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. *Ophthalmology*. 1997;104:2068-2076
- Shimizu S, Yamazaki M, Kubota S, Ozasa T, Moriya H, Kobayashi K, *et al*. *In vitro* studies on a new method for islet microencapsulation using a thermoreversible

gelation polymer, N-isopropylacrylamide-based copolymer. *Artif Organs* 1996; *20:* 1232-7.

- Signoretti S, Waltregny D, Dilks J *et al*. p63 is a prostate basal cell marker and is required for prostate development. *Am J Pathol* 2000; 157:1764–75.
- Silvestri, F., Banavali, S., Baccarani, M., Preisler, H.D., The CD34 hemopoietic progenitor cell associated antigen: biology and clinical applications. *Haematologica* 1992; 77, 265–273
- Solter, D. Mammalian cloning: advances and limitations. *Nature Rev. Genet.*, 2000; 1, 199–207
- Sommer, L. and M. Rao, Neural stem cells and regulation of cell number. *Prog. Neurobiol*., 2002; *66*(*1*): 1–18.
- Srinivasan BD, Eakins KE: The reepithelialization of rabbit cornea following single and multiple denudation. *Exp Eye Res* 1979; 29:595–600
- Stemple DL, Anderson DJ. Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* 1992; 71: 973– 85.
- Stepp MA, Zhu L, Sheppard D, Cranfill RL. Localized distribution of alpha 9 integrin in the cornea and changes in expression during corneal epithelial cell differentiation. *J Histochem Cytochem* 1995; 43: 353–62.
- Stepp MA, Zhu L. Upregulation of alpha 9 integrin and tenascin during epithelial regeneration after debridement in the cornea. *J Histochem Cytochem* 1997; 45: 189–201.
- Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. *Biomaterials;* 2000; 21:2589-98.
- Sun, L., Sun, T.-T., Lavker, R.M., 2000. CLED: a calcium-linked protein associated with early epithelial differentiation. *Exp. Eye Res*. 259, 96–106.
- Sun, T.-T. & Lavker, R. M. *J. Invest. Dermatol. Symp. Proc.* 2004; 9, 202–207.
- Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. *Carcinogenesis*. 2005; 26: 495—502.
- Taipale J, Keski-Oja J. Growth factors in the extracellular matrix. *FASEB*; 1997; 11:51-59.

- Talmi YP, Sigler L, Inge E. Antibacterial properties of human amniotic membranes. *Placenta.* 1991; 12:285-288.
- Tan DT, Ang LP, Beuerman RW. Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent. *Transplantation.* 2004 Jun 15; 77(11):1729-34.
- Tan DTH, Ang LPK, Beuerman RW. Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent. *Transplantation.* 2004; 77:1729–1734
- Teruo Nishida: Cornea In Fundamentals of cornea and external disease. Editors: H. Krachmer, MD, Mark J. Mannis, MD and Edward J. Holland, MD Mosby, 1997; Elsevier.
- Ti S-E, Anderson DF, Touhami A, *et al*. Factors affecting outcome\following transplantation of *ex vivo* expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci*. 2002; 43:2584–2592.
- Ti S-E, Grueterich M, Espana EM, *et al*. Correlation of long-term phenotypic and clinical outcomes following limbal epithelial transplantation cultivated on amniotic membrane in rabbits. *Br J Ophthalmol* 2003; 88:422–427.
- Ton CC, Hirvonen H, Miwa H, *et al*. Positional cloning and characterization of a paired-box and homeobox containing gene from the aniridia region. *Cell.* 1991; 67:1059-1074.
- Touhami A, Grueterich M, Tseng SC. The role of NGF signaling in human limbal epithelium expanded by amniotic membrane culture *Invest Ophthalmol Vis Sci*. 2002; 43:987–994.
- Trelford JD, Trelford–Sauder M. The amnion in surgery, past and present. *Am J Obstet Gynecol.* 1979; 134:833–845.
- Tsai RJ and Tseng SC Human allograft limbal transplantation for ocular surface reconstruction. *Cornea* 1994; 13; 389 -400
- Tsai RJ, Ho YS, Chen JK. The effects of fibroblasts on the growth and differentiation of human bulbar conjunctival epithelial cells in an *in vitro* conjunctival equivalent. *Invest Ophthalmol Vis Sci.* 1994; 35:2865–2875.

- Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med.* 2000; 343:86–93.
- Tsai RJ, Tseng SC. Substrate modulation of cultured rabbit conjunctival epithelial cell differentiation and morphology. *Invest Ophthalmol Vis Sci.* 1988; 29:1565–1576.
- Tsai, R.J., Sun, T.-T., Tseng, S.C., Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology* 1990; 97, 446–455.
- Tsai, R.J.F., Li, L.-M., Chen, J.-K., Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N. Eng. J. Med*. 2000;343, 86–93.
- Tseng SC, Farazdagi, M and Roider AA, Conjunctival transdifferentiation induced by systemic vitamin A deficiency in conjunctivalized rabbit corneas. *Invest. Opthal. Vis. Science* 1987: 28,1497 – 504.
- Tseng SC, Kruse FE, Merritt J, Li DQ. Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media. *Curr Eye Res*. 1996 Sep; 15(9):973-84.
- Tseng SC., Conjunctival grafting for corneal diseases In: E.A. Jeager, Tasman, W. Ed Duane's clinical ophthalmology. Lippincott, Philadelphia; 1994; p1 -11
- Tseng SCG, Li DQ, Ma X. Down-regulation of TGF-ß1, ß2, ß3, and TGF-ß receptor II expression in human cornealfibroblasts by amniotic membrane. *Invest Ophthalmol Vis Sci.* 1998; 39 :(suppl)428.
- Tseng SCG, Tsubota K. Amniotic Membrane Transplantation for Ocular Surface Reconstruction. In: *Ocular Surface Diseases: Medical and Surgical Management* Ed. Holland EJ and Mannis MJ, Sringer, 2001
- Tseng, S., Meller, D., Anderson, D. *et al.. Ex vivo* preservation and expansion of human limbal epithelial stem cells on amniotic membrane for treating corneal diseases with total limbal stem cell deficiency. *Adv. Exp. Med. Biol.* 2002; 506, 1323–1334.

- Tseng, S.C., Zhang, S.H., Limbal epithelium is more resistant to 5- fluorouracil toxicity than corneal epithelium. *Cornea* 1995;14, 394–401.
- Tseng, S.C.G., Regulation and clinical implications of corneal epithelial stem cells. *Mol. Biol. Rep*1996;23, 47–58.
- Tseng, S.C.G., Li, D.Q., Comparison of protein kinase C subtypes expression between normal and aniridia human ocular surfaces: implications for limbal stem cell dysfunction in aniridia. *Cornea* 1996; 15, 168–178.
- Tseng, SCG. Concept and application of limbal stem cells. *Eye* 1989; 3,141–157.
- Tsubota K, Satake Y, Ohyama M, *et al*. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. *Am J Ophthalmol.* 1996;122: 38 –52.
- Tsubota k, Satake Y., Kaido M., Shinozaki N., Shimmura S, Bissen Miyajama H., and Shimazaki J Treatment of severe ocular surface disorder with corneal epithelial stem cell transplantation. *N Engl J Med* 1999;340; 1649 – 703.
- Tsukikawa S, Matsuoka H, Kurahashi Y, Konno Y, Satoh K, Satoh R, *et al*. A new method to prepare multicellular spheroids in cancer cell lines using a thermo-reversible gelation polymer. *Artif Organs* 2003; 27: 598-603.
- Tuori A, Uusitalo H, Burgeson RE, Terttunen J, Virtanen I. The immunohistochemical composition of the human corneal basement membrane. *Cornea* 1996; 15:286-294.
- Umemoto T, Yamato M, Nishida K, *et al*. Rat limbal epithelial side population cells exhibit a distinct expression of stem cell markers that are lacking in side population cells from the central cornea. *FEBS Lett* 2005; 579:6569-6574
- Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, Okano T. Limbal epithelial side-population cells have stem cell-like properties, including quiescent state. *Stem Cells* 2006; 24:86-94
- Ungaro F.,. Biondi M, Indolfi L., De Rosa G.,. La Rotonda M. I, Quaglia F.and. Netti P Bioactivated Polymer Scaffolds for Tissue Engineering EXPERTISSUES e-book Topics in Tissue Engineering 2004, volume 2. Eds. N. Ashammakhi & R.L. Reis © 2004

- Ursula Schlo¨tzer-Schrehardt, Friedrich E. Kruse Identification and characterization of limbal stem cells *Experimental Eye Research* 2005; 81:247–264
- Vantrappen L, Geboes K, Missotten L, Maudgal PC, Desmet V. Lymphocytes and Langerhans cells in the normal human cornea. *Invest Ophthalmol Vis Sci* 1985; 26:220-225.
- Varde NK, Pack DW. Microspheres for controlled release drug delivery. *Expert Opin Biol Ther* 2004; 4:35-51.
- Vernallis, A.B., Hudson, K.R., and Heath, J.K. An antagonist for the leukemia inhibitory factor receptor inhibits leukemia inhibitory factor, cardiotrophin-1, ciliary neurotrophic factor, and oncostatin M. *J. Biol.Chem.* 1997; 272, 26947–26952.
- Walther C, Gruss P. Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development.*1991;113:1435-1449.
- Wang TY, Chen BF, Yang YC *et al*. Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases. *Hum Pathol* 2001; 32:479–86.
- Wantanabe H *et al*: Human corneal and conjunctival epithelium produce a mucin-like glycoprotein for their apical surface, *Invest. Ophthalmol Vis sci* 1945;36; 337 – 34,
- Waring GO, Roth AM, Ekins MB: Clinical and pathologic description of 17 cases of corneal intraepithelial neoplasia. *Am J Ophthalmol* 1984 ; 97:547–559
- Watanabe K, Nishida K, Yamato M, *et al*. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett* 2004; 565:6-10.
- Westfall, M.D., Mays, D.J., Sniezek, J.C., and Pietenpol, J.A. The ΔNp63 phosphoprotein binds the p21 and 14–3–3σ promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol. Cell. Biol.* 2003;23: 2264–2276.
- Wolosin, J.M., Xiong, X., Schutte, M., Stegman, Z., Tieng, A., Stem cells and differentiation stages in the limbo-corneal epithelium. *Prog. Ret. Eye Res*. 2000;19, 223–255.

- Xia Z, Dickens M, Raingeaud J, *et al*. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*. 1995; 270: 1326 –1331.
- Xin X, Borzacchiello A, Netti PA, Ambrosio L, Nicolais L. Hyaluronic-acid-based semi-interpenetrating materials. *J Biomater Sci Polym Ed* 2004; 15:1223-1236.
- Yang A, Kaghad M, Wang Y *et al*. p63, a p53 homolog at 3q27- 29, encodes multiple products with transactivating, death inducing, and dominant-negative activities. *Mol Cell* 1998; 2: 305–16.
- Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., McKeon, F. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 1999; 398, 714–718.
- Yannas IV, Burke JF, Orgill DP, *et al.* Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. *Science* 1981;215:174– 6
- Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J., Buck, D.W., AC133, a novel marker for human haematopoietic stem and progenitor cells. *Blood* 1997: 90: 5002–5012.
- Yoshioka H, Cushman J.A & Mori Y, Synthetic hydrogel with thermoreversible gelation 3: an NMR study of the Sol-Gel transition. *Polym. Adv. Tech.*, 1994; 5: 122-26
- Yoshioka H, Mikami M., MoriY. & Tsuchida E. A synthetic hydrogel with thermoreversible gelation: A synthetic hydrogel with thermoreversible gelation 1: preparation and theological properties. *J. Macromol. Sci.* 1994a; A31: 113 – 21
- Yoshioka, H., Mori, Y & Shimizu, M. Separation and recovery of DNA fragments by electrophoresis through a thermoreversible hydrogel composed of poly (ethylene oxide) and poly (propylene oxide). *Anal Biochem*. 2003; 323, 218-22
- You L, Kruse FE, Volcker HE. Neurotrophic factors in the human cornea. *Invest Ophthalmol Vis Sci* 2000; 41: 692–702.
- Zhang Q, Shimoya K, Moriyama A. Production of secretory leukocyte protease inhibitor by human amniotic membranes and regulation of its concentration in amniotic fluid. Mol Hum Reprod. 2001; 7:573-579.

- Zheng WH, Kar S, Quirion R. FKHRL1 and its homologs are new targets of nerve growth factor Trk receptor signaling. *J Neurochem*. 2002; 80:1049–1061.
- Zhou S, Schuetz JD, Bunting KD, *et al*. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001; 7:1028-10
- Zhu X, Beuerman RW, Chan-Park MB, Cheng Z, Ang LP, Tan DT. Enhancement the ocular surface. *Ann Acad Med Singapore.* 2006 Mar; 35(3):210-4.
- Zhu X, Chian KS, Chan-Park MB, Lee ST. Effect of argonplasma treatment on proliferation of human-skin-derived fibroblast on chitosan membrane *in vitro*. *J Biomed Mater Res A* 2005;73:264-74.
- Zieske JD, Bukusoglu G, Yankauckas MA, Wasson ME, Keutmann HT. Alpha-enolase is restricted to basal cells of stratified squamous epithelium. *Dev Biol* 1992; 151: 18–26.
- Zieske JD, Bukusoglu G, Yankauckas MA. Characterization of a potential marker of corneal epithelial stem cells. *Invest Ophthalmol Vis Sci* 1992; 33: 143–52.
- Zieske JD. Perpetuation of stem cells in the eye. *Eye* 1994; 8:163–9.

APPENDICES

Appendix I

Cell culture Reagents
Preparation of Media

Dulbecco's Minimum Essential Medium:

1 vial of DMEM powder dissolved in 900 ml of double sterilized milli Q water and filter sterilized under vacuum (sterility check-up – Incubation at room temperature, overnight)

HAM'S F-12 MEDIUM:

1 vial F-12 medium powder is dissolved in 1 L of double sterilized milli Q water and filter sterilized under vacuum (sterility check-up – Incubation at room temperature, overnight)

DMEM-F 12 MIX: 50 ml of sterile DMEM is mixed with 50 ml of F-12 medium in a 100ml flask. The following are added to the mix:

- **L- glutamine** (0.3gm/10 ml of distilled water) - 2 ml/ 100 ml of the media mix.
- **Sodium Bicarbonate** (0.75 gm/10 ml of distilled water)
- **Antibiotics:** Penicillin (1 vial in 10 ml of distilled water), Streptomycin (0.75 gms/10 ml of distilled water), ciprofloxacin (10 mg/10 ml of distilled water)
- **Fetal Bovine Serum (FBS):** Gamma irradiated-commercially available.
  Heat inactivated at 56 deg C for 30 minutes.
  - 10 ml of FBS/ 100 ml of the DMEM / F-12 mix.
- **Growth Factors:**
  Epidermal Growth Factors- 50 ng/ ml
  Hydrocortisone- 0.5 µg/ ml
  Transferrin – 5 µg/ ml
  Sodium Selenite – 5 µg/ ml
  Keratinocyte growth supplement- 5 µg/ ml

After the addition of the above components to the DMEM- F12 mix, the medium is checked for its sterility (Incubation at room temperature, overnight).

TRANSPORT MEDIUM:

DMEM with 3 % FBS- aliquoted in 1.5 ml cryovials and stored at 4º C and is used for collecting limbal biopsies.

Gel Electrophoresis

Electrophoresis is a method by which the amplification of the gene sequence is confirmed after PCR. DNA, negatively charged moves to the cathode on subjected to electric field and moves according to the molecular weight in a matrix like agarose or polyacrylamide gels. The gels are suitably stained to visualize the product (Fluorescent dyes for agarose gels and silver staining of poly acryl amide gels).

Agarose gel electrophoresis

Preparation of TBE buffer: 54.1 g of Tris, 27.8g of boric acid and 3.65 g EDTA were added in 500 ml water and pH adjusted to 8.0.The stock solution is diluted

1: 10 for further use.

Tracking dye- Bromophenol blue : 0.1gm BPB+100ml 1X TBE buffer in equal volumes of 40%sucrose solution.

Requirements

Molecular weight marker, Agarose,10 X TBE buffer,Ethidium bromide (2mg/ml), BPB

Preparation of agarose gel

The gel trough was cleaned with ethanol and the ends were sealed with cellophane tape with the combs placed in the respective positions to form wells. Two percentage of agarose gel was prepared by dissolving agarose in 1X TBE buffer and 8µl of ethidium bromide being finally added and mixed thoroughly and poured on to the trough followed by electrophoresis of the amplified prodcuts at 100 V for 30 to 45 minutes.The gel was then captured in the Vilber Lourmat and analysed using Imagemaster gel documentation system.

Western blotting Reagents

1. **Acrylamide (30%):**

   Acrylamide-14.6gm

   Bis acrylamide-0.4gm

   Dissolved in 30ml of water made up to 50ml.

2. **Tris-HCl Buffer (PH 8.8):**

   Tris-9.0gm

   Water-25ml

   Adjust the PH to 8.8 with1N HCl and made up to 50ml with Distilled Water.

3. **Tris-HCl Buffer (PH6.8):**

   Tris-3.0gm

   Water-25ml

   Adjust the PH to 6.8 with 1N HCl and made up to 50ml with Distilled Water.

4. **10% APS: Polymerization catalyst required for gel formation. 10% solution used** – always prepared fresh. Increasing APS concentration will make the gel set quicker.

   Ammonium per sulphate-10gm

   Distilled water-100ml.

5. **TEMED** (readily available): Polymerization catalyst. Catalyses the formation of persulphate free radicals from the APS, which in turn initiates polymerization. Always the last reagent added to the gel.

6. **10% SDS**: Binds proteins so they all become negatively charged, therefore separation is on the basis of size alone and not the intrinsic protein charges.

   SDS-10gm

   Distilled water-100ml

   Stored at room temperature.

7. **Separating gel preparation:** Sieves and separates the proteins by size. Percentage gel depends upon the size of target proteins.

Acryl amide %	Range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

10%gel:

30%Acrylamide- 4ml

TrisHCl PH 8.8-2.5ml

10%APS-50µl

TEMED-5µl

10%SDS-100µl

Distilled water-3.344ml

8. **Stacking gel:** Large pore size gel with little or no molecular sieving on the sample. Standard 4% Acrylamide – large enough pore size for most samples.

   30% Acrylamide-1.33ml

   TrisHCl- (PH 6.8)-2.5ml

   10%APS-50µl

   TEMED-10µl

   10%SDS-100µl

   Distilled water-6.01ml.

9. **Western blotting transfer buffer:**

   Tris-3.3gm

   Glycine-14.4gm

   SDS-1.0 gm

The contents were dissolved in 800ml of distilled water. The final volume was then made up to 1000ml with methanol.

10. Ponseau stain:

Ponseau S-0.5gm

Glacial acetic acid-1ml

Made up to 100ml with distilled water. Prepared just before use.

11. Electrophoretic buffer (5x):

Tris base-15.1gm

Glycine-72.0gm

SDS-5.0gm

Distilled water-made up to 1000ml.Donot adjust the PH of the stock, as the PH will be 8.3 when diluted. Stored at 4^0C until use (up to 1 month).

12. Sample loading Buffer (3x):

1M Tris HCl (PH 6.8)-2.4ml

20%SDS-3ml

Glycerol-3ml

β-Mercaptoethanol-1.6ml

Bromophenol Blue-0.006gm

13. Tris buffered saline (pH:7.6) (TTBS):

Sodium chloride-8gm

1M tris HCl-20ml

Diluted to 1000ml with distilled water.

Tween- 20-10ml.

Appendix II

LIST OF CONSUMABLES

CONSUMABLES	COMPANY
Agarose	SRL, Mumbai, India
ABCG2 antibody	Chemicon, CA, USA
Acrylamide	SRL, Mumbai, India
Ammonium Persulphate	SRL, Mumbai, India
Anti Mouse FITC conjugate	Sigma Aldrich Pvt. Ltd. USA
Antibiotic Mixture	Invitrogen, USA
Boric acid, Molecular grade	SRL, Mumbai, India
Bromophenol blue	HiMedia, Mumbai, India
Chloroform	Merck Limited, Mumbai, India
100 bp DNA ladder, ϕ X bacteriophage DNA	Bangalore Genei Pvt. Ltd., India
Dulbeccos minimum essential medium	Invitrogne, USA
Hinf – I digest,	Bangalore Genei Pvt. Ltd
HAM's F12	Invitrogen, USA
Hydrocortisone	Sigma Aldrich Pvt. Ltd, USA
dNTPs	Bangalore Genei Pvt. Ltd
ECL kit , Femto kit	Pierce, USA
EDTA, Molecular grade	SRL, Mumbai, India
Ethanol, Absolute	S D fine chemicals Pvt Ltd, Mumbai,
Ethidium bromide	HiMedia, Mumbai, India
Epidermal Growth Factor	Sigma Aldrich Pvt. Ltd. USA
Fetal Bovine Serum	Gibco.
Glycine	SRL, Mumbai, India
Isoamyl alcohol	Merck Limited, Mumbai, India
Keratinocyte growth supplement	Sigma Aldrich Pvt. Ltd. USA
L-glutamine	Invitrogen, USA
Parafilm	Axygen Inc, USA: Dialab Pvt Ltd, India
P63 antibody	Santa Cruz, CA USA
Phenol	SRL, Mumbai, India
Primers	Sigma Aldrich Pvt Ltd., USA
Ponseau	HiMedia, Mumbai, India
Qiagen RNA extraction Mini kit	QIAGEN, Germany: Genetix Pvt Ltd,
Qiagen cDNA conversion Kit	QIAGEN, Germany: Genetix Pvt Ltd,
Sodium dodecyl sulphate	Merck Limited, Mumbai, India
Sodium bi carbonate	HiMedia, Mumbai, India
Sodium Selenite	Sigma Aldrich Pvt. Ltd, USA
Taq DNA Polymerase	Bangalore Genei Pvt. Ltd., India
Tris, Molecular grade	SRL, Mumbai, India
Transferrin	Sigma Aldrich Pvt. Ltd, USA
TEMED	SRL, Mumbai, India

List of Publications

Articles published:

1. **B. Sudha,** H.N. Madhavan, G. Sitalakshmi, J. Malathi, S.Krishnakumar, Y. Mori, H. Yoshioka, S. Abraham, "Cultivation of Corneal Limbal Stem cells in Mebiol gel – A Thermo-Reversible gelation Polymer Indian. J. Med. Res; 2006 Dec;124(6):655-64

Articles under revision:

1. **Sudha B**, Srilatha Jasty, Sithalakshmi G, Madhavan HN Subramanian Krishnakumar Influence of Feeder Layer on the Expression of Stem Cell Markers in the cultured Limbal Corneal Epithelial Cells *Indian. Med. Res*
2. **Sudha B**, Sithalakshmi G, Madhavan HN Subramanian Krishnakumar Comparison of stem cell markers on the limbal epithelial cells cultured on the intact and denuded amniotic membrane. *Indian. Med. Res*
3. **B. Sudha.,** Ms. Sujatha K Arumugam, Dr.Sanjeeb K Sahoo, Dr. T.P. Sastry, Dr.G.Sitalakshmi,.Dr. S. Krishnakumar Phenotypic characterization of limbal epithelial cells expanded on chitosan scaffold *Cornea*

Manuscripts under preparation:

1. **p63 isoform status on the cultured limbal epithelial cells on the cells expanded over the intact and denuded amniotic membrane.**

2. **A Rabbit model of Ex Vivo cultivation and transplantation of autologous limbal epithelial cells grown on a thermo reversible polymer (Mebiol gel)**

3. **Signal transduction pathway involved in the ex-vivo expansion of limbal epithelial cells on the cells cultured over the intact and denuded amniotic membrane**

List of presentations and awards

1. **B. Sudha,** Dr. H.N. Madhavan, Dr. G. Sitalakshmi, Dr. J. Malathi, Dr. S. Krishnakumar, Dr. Y. Mori, Dr. H. Yoshioka,Dr. S. Abraham Cultivation of Corneal Limbal Stem cells in Mebiol gel – A Thermo-Reversible gelation polymer presented in 2nd International conference on "Tissue Engineering and Regenerative Medicine" held at Bangalore on the 1st and 2nd April 2005 **(Oral presentation)**

2. **B. Sudha**, Dr. H.N. Madhavan, Dr. G. Sitalakshmi, Dr. J. Malathi, Dr. S. Krishnakumar, Dr. Y. Mori, Dr. H. Yoshioka, Dr. S. Abraham Cultivation of Corneal Limbal Stem cells in Mebiol gel – A Thermo-Reversible gelation polymer presented in **IERG conference held at L.V. Prasad eye institute on 30 & 31st of July 2005 (Oral presentation)**

3. H.N. Madhavan, **B.Sudha**, G.Sitalakshmi, S.Krishnakumar, Y.Mori, H.Yoshioka, S.Abraham. Comparative study on Growth Characteristics of Cadaveric Human Corneal Limbal Stem Cells in Mebiol Gel (a synthetic polymer) and on Human Amniotic Membrane was presented in **ARVO 2006**. **3033/B186 (poster presentation)**

4. **Sudha B,** Jasty S, Madhavan HN, Iyer GK, Sitalakshmi G, Krishnakumar S. Comparison of ABCG2, p63, Connexin 43, and k3/k12 in cultured corneal epithelial cells on intact and denuded human amniotic membrane presented **in IERG 2006 held on 29th and 30th July 2006 (Oral presentation)**

5. **Sudha B,** Labhasetwar V, Krishnakumar S. To study the uptake of PLGA nanoparticles by in vitro cultivated limbal progenitor cells presented **in IERG 2006 held on 29th and 30th July 2006 (Oral presentation)**

6. **Sudha B,** Labhasetwar V, Krishnakumar S. To study the uptake of PLGA nanoparticles by in vitro cultivated limbal progenitor cells presented in **International conference for Nanobioscience held on 6th to 8th August 2006 (Poster presentation)**

7. **Sudha B,** S. K. Arumugam, Srilatha. J , Nalini V. Sastry T P , Geetha K Iyer, Sitalakshmi G, Madhavan HN , Krishnakumar S Phenotypic characterization and signal transduction pathway involved in the exvivo expansion of corneal stem cells over various scaffolds presented in **First international Meeting Stem Cell Research Forum of India on Jan 29th – Feb 1st 2007. (Poster presentation)**

8. **Sudha B**, Krishnakumar S, Srilatha. J Expression of p63 isoforms on corneal limbal stem cells cultured on the intact and denuded human amniotic membrane presented in **First international Meeting Stem Cell Research Forum of India on Jan 29th – Feb 1st 2007 (Poster presentation)**

9. **Sudha B,** S. K. Arumugam, Srilatha. J, Nalini V. Sastry T P, Geetha K Iyer, Sitalakshmi G, Madhavan HN , Krishnakumar S Phenotypic Characterization And Signal Transduction Pathway Involved In The *Ex-vivo* Expansion Of Corneal Stem Cells Over Various Scaffolds **has been presented in ASIA ARVO 2007 at Singapore. March 2-5th (poster presentation)**

10. Dr. G. Sitalakshmi, **B. Sudha**, Dr. S. Vinay, Dr. H.N. Madhavan, Dr. J. Malathi, Dr. S. Krishnakumar, Dr. Y. Mori, Dr. H. Yoshioka, Dr. S. Abraham A Rabbit Model of Ex Vivo Cultivation and Transplantation of Autologous Limbal Epithelial Cells grown on a Thermo-reversible **Polymer (Mebiol Gel)** has been presented in **ASIA ARVO 2007 at Singapore. March 2 – 5th (poster presentation)**

Awards:

1. Received the **Dr. Amjad Rahi Prize for best paper presentation** for the abstract entitled, "**To study the uptake of PLGA nanoparticles by in vitro cultivated limbal progenitor cells" presented in IERG meeting at Hyderabad on 29th and 30th July 2006**

2. **Received Travel fellowship award in IERG 2006**

Brief biography of the candidate

Ms. B. Sudha obtained her B.Sc Microbiology degree from Valliammal College, University of Madras, Chennai in 2000. She obtained her M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 2003 with course work at Medical Research Foundation, Chennai. Then she joined the Larsen & Toubro Microbiology Centre, Sankara Nethralaya as Junior Scientist and worked in NMITLI (New millennium Indian Technology Leadership Initiative) project funded by Council of Scientific and Industrial Research. She registered for Ph. D in August 2003 in Birla Institute of technology & Science, Pilani. She has made presentations in 10 national level and international conferences, comprising 7 oral presentations and 3 poster presentations. She has 2 publications out of which 1 are on her thesis work and published 15 abstracts presented in various national and international conferences. 2 more publications are under review. 3 papers are under preparation. She has standardized the cultivation of limbal stem cells in vitro and she has also tried to characterize them by immunophenotypic methods and molecular methods. She has received "Best Paper Award" for paper "To study the uptake of PLGA nanoparticles by in vitro cultivated limbal progenitor cells" presented in IERG (Indian Eye Research Group" 2006. Her research interests are in the area of ocular stem cells.

Brief biography of the supervisor

Dr S Krishnakumar was selected as Best Student in MD pathology at Post-graduate Institute of Basic Medical Sciences, Taramani. He has been trained in Hematology, Hemato-Oncology, Dermato-pathology and Clinical Dermatology. He has undergone 6 months research fellowship in ophthalmic pathology at Doheny Eye Institute, University of Southern California, USA under Dr Narsing Rao. He has undergone training for both transmission and scanning electron microscopy and Zeis confocal microscopy which can be developed in future. He has been selected as DBT Associate and awarded short-term Overseas Fellowship Award in nanotechnology at University of Missouri-Columbia and ICMR Fellowship for training in nanotechnology and drug delivery at University of Nebraska Medical Center, Omaha. Selected for "Sir Sriram Travel Fellowship Award" for training in proteomics for the year 2006-2007 by National Academy of Medical Sciences. He has got 70 publications in the peer reviewed national and international journals. He has successfully completed 3 projects funded by the Indian Council of Medical research. To his credit he has 9 ongoing projects which are funded by DBT, DST and ICMR. Out of which two are stem cell grant one on studying the biology of the corneal stem cells funded by ICMR the other project is on the characterization of retinal stem / progenitor cells funded by DBT. He has good collaborations with many institutes all over India and also with the other foreign universities. The key areas of his research are tumor biology, stem cells and tissue engineering and Nanotechnology.