TRANSDIFFERENTIATION OF SKIN EPITHELIAL CELLS INTO CORNEAL EPITHELIAL CELLS

A PROJECT REPORT

Submitted by

AKSHAYA RAVICHANDRAN

ANUSHA .R

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

ANNA UNIVERSITY: CHENNAI 600 025

APRIL 2010

ANNA UNIVERSITY : CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report **"Transdifferentiation of Skin Epithelial Cells into Corneal Epithelial Cells"** is a bonafide work of **Akshaya Ranvichandran and Anusha. R** who carried out the project work under my supervision.

SIGNATURE

Dr.S.SADASIVAM

PROJECT GUIDE

Dean (Biotechnology)

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore – 641 006

SIGNATURE

Dr.S.SADASIVAM

DEAN (Biotechnology)

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore – 641 006

CERTIFICATE OF EVALUATION

COLLEGE : **Kumaraguru College of Technology**

BRANCH : **Biotechnology**

SEMESTER : **Eighth semester**

NAME OF THE STUDENTS	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
AKSHAYA RAVICHANDRAN (71206214001) R.ANUSHA (71206214002)	TRANSDIFFERENTIATION OF SKIN EPITHELIAL CELLS INTO CORNEAL EPITHELIAL CELLS	DR.S.SADASIVAM Dean (Biotechnology)

The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed. It was submitted for viva voce exam on 20·4·2010.

(INTERNAL EXAMINER) (EXTERNAL EXAMINER)

30.10.2010
VIBS/Corres/2010/26

VIDYASAGAR INSTITUTE OF BIOMEDICAL TECHNOLOGY AND SCIENCE

(a Unit of Medical research Foundation)

SANKARA NETHRALAYA

BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"Transdifferentiation of skin epithelial cells into corneal epithelial cells"** is a bonafide work done by **Akshaya Ravichandran** and **Anusha R** (final year Bachelor of Technology in Biotechnology, Kumaraguru college of Technology, Affiliated to Anna University, Chennai) under my supervision in Vidyasagar Institute of Biomedical Technology and Science, Vision Research Foundation, Sankara Nethralaya, Chennai from January to March, 2010. I also certify that the best of my knowledge, this work has not been a part of any other thesis or dissertation.

Project Guide

Dr. S.Krishnakumar MD
Director, Nanobiotechnology Department
Incharge, Stem Cell Laboratory
L&T ocular pathology Department
Vision Research Foundation
Sankara Nethralaya, Chennai

Project Director

Dr. H.N. Madhavan. MD., PhD., FAMS., FIC
President, Vision Research Foundation
Director & Professor of Microbiology Departm
Director, Vidyasagar Institute of Biomedical
Technology and Science,
Sankara Nethralaya,Chennai.

ACKNOWLEDGEMENT

We wish to express our gratitude to **Dr.S.Sadasivam**, Dean of Biotechnology and our project guide, Kumaraguru College of Technology, for his unfailing support and guidance throughout the course of our project.

We sincerely thank **Dr.Krishnakumar**, Director of Nanobiotechnology and In charge of stem cell and ocular pathology laboratory, Sankara Nethralaya, for providing us the opportunity to carry out our project in his laboratory under his guidance.

We express our sincere gratitude to **Ms.Sasirekha Krishnan**, Senior research fellow and Ph.D student for providing us with all the required preparation and for guiding us through every stage of the project.

We also thank all the other members of the L&T ocular pathology laboratory and Nanobiotechnology division for their help and support.

We express our sincere gratitude to **Dr.N.Saraswathy**, Senior Lecturer, Kumaraguru College of Technology, our project co-ordinator for her help and guidance regarding all the processes involved in carrying out the project to completion.

We also thank all the teaching and non-teaching staff of the Department of Biotechnology, Kumaraguru College of Technology, for their assistance and support.

We finally thank the Almighty for enabling us at every step.

AKSHAYA RAVICHANDRAN

ANUSHA R

ABSTRACT

Bilateral limbal stem cell deficiency, one of the most common diseases causing loss of vision, can be treated effectively by allogenous transplantation which involves regeneration of cornea from a source other than the limbus, such as stroma, conjunctiva, oral mucosa, nasal mucosa and so on. This study explores the feasibility of the use of skin as an alternative source to produce corneal epithelium. Skin biopsy from seven random subjects was taken and cultured using explants culture technique. The skin explants were grown in corneal microenvironment for a period of 22 days until confluent growth was achieved. The cells were then harvested and trypsinised and the viability was estimated by trypan blue assay. Skin displayed confluent growth in a corneal microenvironment with high percentage of viability. Further, RNA was extracted from the cells and cDNA conversion was carried out using RT PCR technique. When the skin was analysed using markers such as ABCG2 (putative stem cell marker), p63 (keratinocyte marker) and K3/K12 (cornea phenotype marker), it did not express the markers responsible for stemness (ABCG2), indicating that the skin cells were completely differentiated after passing through a stage of de-differentiation. The differentiated cells expressed markers for keratinocytes (p63) and for the corneal phenotype (K3/K12) which are also expressed by the cornea. This is indicative of the fact that the differentiated cells are the corneal epithelial cells. The study concludes that skin may be used as a viable alternative for corneal growth since both skin epidermis and cornea are keratinocytes, with high optical clarity. Further research in the area of transplantation of the cultured cells using appropriate scaffolds would ensure that transdifferentiation of skin to obtain cornea is a definitive treatment of bilateral LSCD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ng	Nanogram
mg	Milligram
ml	Millilitre
µl	Microlitre
°C	Degree Celsius
bp	Base pairs
FP	Forward primer
RP	Reverse primer
MQ	MilliQ
rpm	Rotations per minute
DEPC	Diethyl Pyro Carbonate

INTRODUCTION

1. INTRODUCTION:

Of the five sensory organs, the eye holds a place of prime importance as the organ of vision. Vision is highly essential for smooth functioning of everyday life. In order to preserve effective vision maintenance of a healthy eye is mandatory.

The eye is seen to consist of three main layers namely, the external layer, formed by the sclera and cornea, the intermediate layer, divided into two parts: anterior (iris and ciliary body) and posterior (choroid) and the internal layer, or the sensory part of the eye, the retina. Of these the outermost layer, the cornea is the first and most powerful lens of the optical system of the eye and allows, together with the crystalline lens the production of a sharp image at the retinal photoreceptor level.

Being the first line of defense, by anatomy, the cornea is the layer that is most exposed to physical damage due to environmental factors and the like. The cornea is not only the layer that covers the underlying layers of the eye but is also the transparent film, which together with the lens, accounts for approximately two-thirds of the eye's total optical power (Goldstein and Bruce, 2007).

The cornea has unmyelinated nerve endings sensitive to touch, temperature and chemicals. Because transparency is of prime importance the cornea does not have blood vessels; it receives nutrients via diffusion from the tear fluid at the outside and the aqueous humour at the inside. The cornea has no blood supply; it gets oxygen directly through the air. Transparency, avascularity, the presence of immature resident immune cells, and immunologic privilege makes the cornea a very special tissue (http://en.wikipedia.org/wiki/Cornea).

The cornea borders with the sclera by the corneal limbus. The limbus is a common site for the occurrence of corneal epithelial neoplasm. Limbus contains radially oriented fibrovascular ridges known as the palisades of Vogt. The palisades of Vogt are distinctive normal features of the human corneoscleral limbus. The palisades of Vogt have a distinct vasculature with narrow, barely visible, arterial and venous components of radially oriented hairpin loops. Angiography reveals that these vessels leak fluorescein relatively late and only to a moderate extent. They respond to inflammation by dilatation and gross breakdown of their physiologic barrier properties. The functions of the palisades of Vogt are not known with certainty, but their interpalisadal epithelial rete ridges may serve as a repository for corneal epithelial progenitor cells (Goldberg and Bron, 1982).

In the case of depletion of the stem cells from the limbus, a condition known as limbal stem cell deficiency occurs. Limbal stem cell deficiency can be caused by a variety of hereditary or acquired disorders like Stevens-Johnson syndrome, chemical injuries, ocular cicatricial pemphigoid, contact lens induced keratopathy, multiple surgeries or cryotherapy to the limbal region, neurotrophic keratopathy and peripheral ulcerative keratitis. Acquired disorders form the majority of cases seen in the clinical setting.

The occurrence of bilateral limbal stem cell deficiency is treated commonly by allogenous transplantation. This involves the growth of corneal tissue from other sources such as nasal and oral mucosa, conjunctiva, stroma and, more recently, skin. Alternative sources of corneal growth are being studied in order to replace limbus as the only source. Limbus being a relatively small region hosting a limited number of stem cells cannot be used as the only source of stem cells for corneal growth.

As skin is the largest and the most accessible organ of the human body, it is naturally considered to be a promising source for cornea. By the process termed as transdifferentiation, which is the conversion of one differentiated tissue into another, it is possible to convert skin into cornea (Xueyi Yang et al, 2008). This process is being studied in order to provide alternative sources for corneal transplantation thereby treating the resultant effects of LSCD.

The study to examine alternative sources of corneal regeneration is of importance for the following reasons:

- Loss of limbus from both eyes caused by bilateral LSCD leads to depletion of the regular source.
- Allogenous transplantation involving the process of sourcing alternative tissue from the affected individual reduces chances of rejection
- Use of rapidly regenerating tissue such as skin aids in quick growth and does not cause irreplaceable loss to the patient.

Several studies have been conducted on various species such as rabbits and goats to determine the feasibility of using skin as a source of cornea. This project is an attempt to source skin from humans and to generate corneal tissue from it. The specific objectives of the project are:

- Standardization of growth of skin in skin growth medium
- Transdifferentiation of skin in corneal microenvironment
- Study of markers to detect differentiation, characteristic and ultimate conversion of skin into cornea

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE:

2.1 Importance of the human eye:

The eye is the organ that is responsible for vision in all organisms. The human eye is not properly a sphere; rather it is a fused two-piece unit. The smaller frontal unit, more curved, called the cornea is linked to the larger unit called the sclera. The cornea and sclera are connected by a ring called the limbus (http://en.wikipedia.org/wiki/Human_eye). The corneal layer is observed to be the transparent tissue that covers the front of the eye (http://www.medhelp.org/gov/cornea.htm). Its functions include providing a physical barrier preventing entry of foreign objects and a refractive medium aiding better focusing of light. The iris – the color of the eye – and its black center, the pupil, are seen instead of the cornea due to the cornea's transparency. When viewed through an ophthalmoscope, the fundus (area opposite the pupil) shows the characteristic pale optic disk (papilla), where vessels entering the eye pass across and optic nerve fibers depart the globe.

2.2 Cornea:

The human eye consists of several layers, the outermost being the cornea. It is the clear, dome-shaped surface that covers the front of the eye. The cornea provides the eye with protection and the refractive properties essential for visual acuity. The transparent epithelium is highly specialized with basal and stratified squamous cells that are renewed throughout life from a stem cell population (Daniels et al, 2001).

2.2.1 Structure:

The corneal tissue is arranged in five basic layers, each having an important function. These five layers are:

- Epithelium:

  The healthy ocular surface is composed of two different types of epithelial cells: corneal epithelial and conjunctival epithelial cells. Corneal epithelial cells are essential for a clear cornea and good vision (Noriko Koizumi et al, 2002). The epithelium functions primarily: (a) to block the passage of foreign material, such as dust, water, and bacteria, into the eye and other layers of the cornea thereby serving as a part of the ocular biodefense system (http://wuphysicians.wustl.edu/dept.aspx). Its cells are tightly arranged without intercellular spaces, creating a barrier that is relatively impermeable to water and external substances (Dua et al, 2000); (b) to provide a smooth surface that absorbs oxygen and cell nutrients from tears, then distributes these nutrients to the rest of the cornea.

The epithelium is filled with thousands of tiny nerve endings that make the cornea extremely sensitive to pain when rubbed or scratched. The part of the epithelium that serves as the foundation on which the epithelial cells anchor and organize themselves is called the basement membrane, aiding in support.

- Bowman's Layer:

  Lying directly below the basement membrane of the epithelium is a transparent sheet of tissue known as Bowman's layer. It is composed of strong layered protein fibers called collagen. Once injured, Bowman's layer can form a scar as it heals. If these scars are large and centrally located, some vision loss can occur.

- Stroma:

Beneath Bowman's layer is the stroma, which comprises about 90% of the cornea's thickness. It consists primarily of water (78%) and collagen (16 %), and does not contain any blood vessels. Collagen gives the cornea its strength, elasticity, and form. The collagen's unique shape, arrangement, and spacing are essential in producing the cornea's light-conducting transparency.

- Descemet's Membrane:

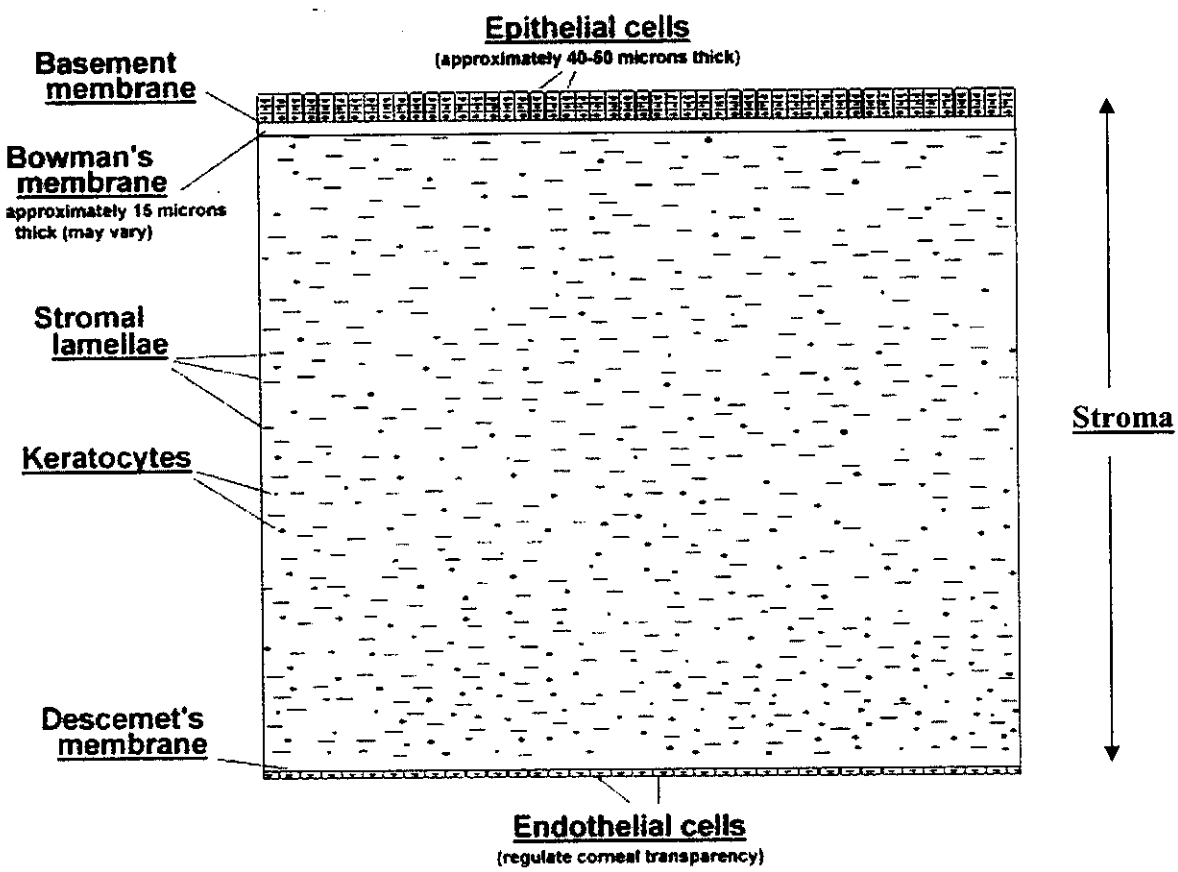
Under the stroma is Descemet's membrane, a thin but strong sheet of tissue that serves as a protective barrier against infection and injuries. Descemet's membrane is composed of collagen fibers (different from those of the stroma) and is made by the endothelial cells that lie below it. Descemet's membrane is readily regenerated after injury.

- Endothelium

The endothelium is the extremely thin, innermost layer of the cornea. Endothelial cells are essential in keeping the cornea clear. Normally, fluid leaks slowly from inside the eye into the middle corneal layer (stroma). The endothelium's primary task is to pump this excess fluid out of the stroma. Without this pumping action, the stroma would swell with water, become hazy, and ultimately opaque. In a healthy eye, a perfect balance is maintained between the fluid moving into the cornea and fluid being pumped out of the cornea. Once endothelium cells are destroyed by disease or trauma, they are lost forever. If too many endothelial cells are destroyed, corneal edema and blindness ensue, with corneal transplantation the only available therapy (Dua et al, 2000).

The different layers of the cornea are represented in Figure 2.1.

Figure 2.1: Layers of the cornea



The figure depicts the structural composition of the cornea delineating the several layers that constitute the cornea. As depicted, the stromal region comprises of a major portion of the corneal structure, protected by the external epithelium.

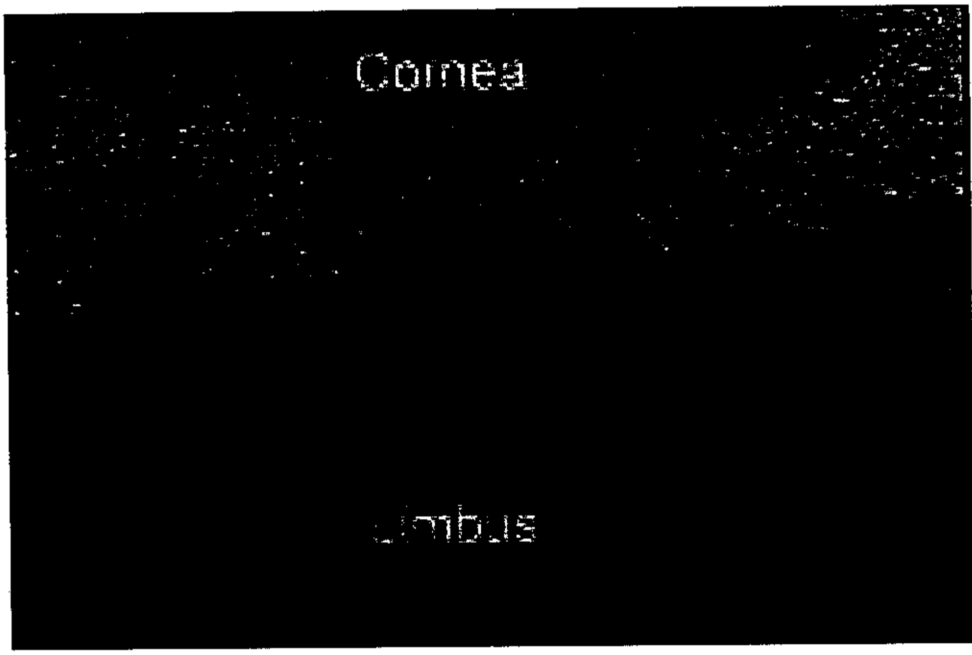
2.2.2 Features of the corneal epithelium:

The corneal epithelium is characterized by several unusual features. (a) To maintain its transparency, corneal epithelium is extremely fiat with no papillary structures. Hence, the ratio between the basal and superficial surface areas approaches one; this corresponds to an exceptionally high demand on the regenerative capacity of the basal cells (Davanger and Evensen, 1971). (b) Because of the avascular nature of the underlying stroma, the bulk of corneal epithelium is remote from a capillary network, the nearest being located in the limbus which is the transitional zone between the cornea and conjunctiva (Hogan et al., 1971; Cogan and Kuwabara, 1974). (c) Corneal epithelium can be regenerated efficiently from the limbal epithelium and to a lesser extent from the conjunctival epithelium (Maumenee, 1964). (d) The mitotic index of corneal epithelium tends to be higher toward the edge of the cornea (Friedenwald and Buschke, 1944). (e) Corneal epithelial neoplasms are in general quite rare, with most of the reported cases involving the limbus (Pizzarello and Jakobiec,1978).

2.3 Corneal Regeneration:

Renewal and regeneration of the cornea is effected by the stem cell storehouse of the eye, the limbus. The limbus is a transition zone where the cornea meets with the sclera. The transition zone begins at a region designated by the termination of Bowman's layer and Descemet's membrane. The transition ends at a region designated by the scleral spur drawn perpendicular to the ocular surface. The transition zone with these limits is then a donut that is about 1.5 mm in width (Schermer et al, 1986).

Figure 2.2: Limbal undulations

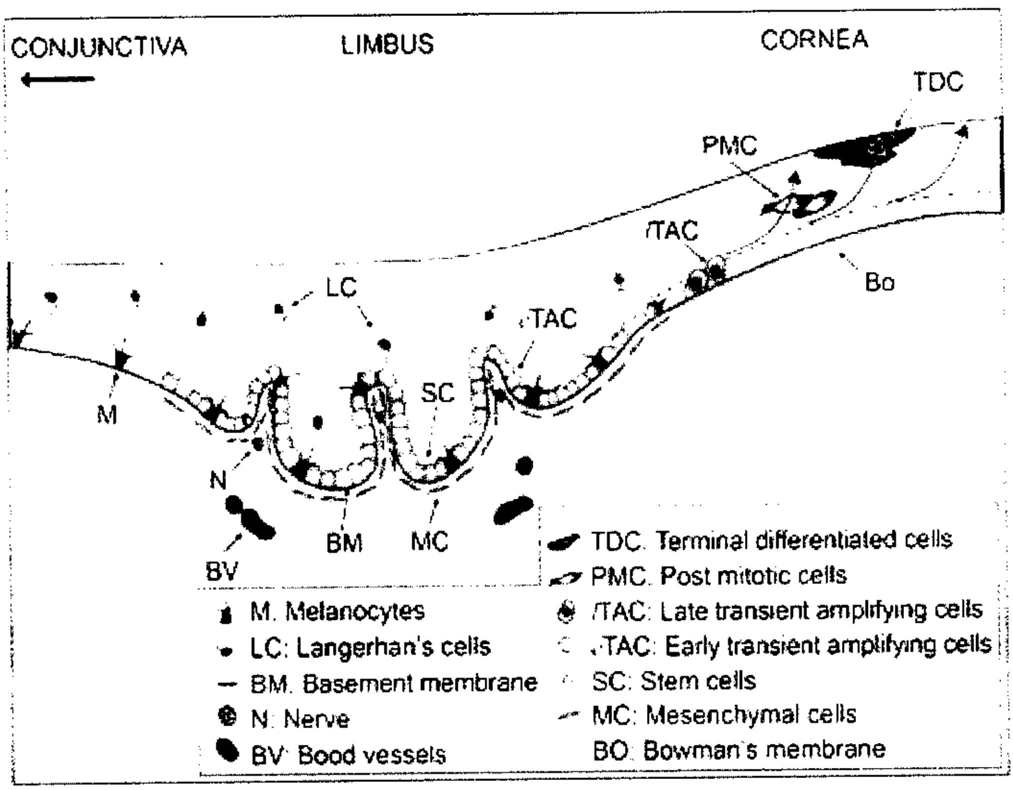


The architecture of the limbus demonstrates a palisade (of Vogt) arrangement indicated in the Figure 2.2 (Goldberg and Bron, 1982 ; Townsend, 1991). The limbal stem cells probably reside in the basal layer of the palisades of Vogt (Davanger and Evensen, 1971). This region is seen to be a physically protective area sheltering the stem cells and also providing them with the necessary nutrients due to its vascularization.

2.3.1 Differentiation of limbal epithelial cells into cornea:

Stem cells that are housed in the palisades of Vogt gradually mature to form differentiated corneal epithelial cells. It has been hypothesized that this storehouse of stem cells is responsible for the regeneration of the conjunctiva as well. A schematic diagram of the stages of differentiation of the stem cells is given in Figure 2.3.

Figure 2.3: Stages of differentiation of cornea



2.4 Limbal stem cell deficiency (LSCD):

The maintenance of a healthy functional corneal epithelium is provided by the subpopulation of stem cells located in the limbal region (Cotsarelis et al, 1989). When limbal epithelial stem cells are destroyed or become dysfunctional, a pathological state known as limbal stem cell deficiency (LSCD) manifests. The hallmark of LSCD is the conjunctivalization of the cornea (Puangsricharern and Tseng, 1995), and is frequently associated with superficial vascularization and compromised corneal surface (Dua et al, 2000). LSCD can be found with a number of corneal diseases such as chemical burns, Stevens Johnson syndrome, aniridia, peripheral keratitis, severe limbitis.

2.5 Treatment of LSCD:

In patients with total limbal stem cell deficiency, limbal auto- or allo-transplantation are indicated for corneal surface reconstruction. Donor tissue can be obtained from the fellow eye (limbal autograft) in cases of unilateral disease, or from a living related donor (usually gives a better tissue match), or from a cadaver donor (limbal allograft) when both eyes are affected.

Damaged corneas have been successfully reconstructed by transplanting autologous limbal epithelial cells (Schwab *et al*, 2000) but not in severe ocular surface diseases such as Stevens-Johnson syndrome (SJS) and ocular cicatricial pemphigoid (Ti *et al*, 2004). In the case of severe ocular diseases, the bilateral damage to LSCs and the immune response to heterologous corneal epithelial transplantation are the most challenging clinical entities (Xueyi Yang *et al*, 2008).

2.5.1 Treatment by transdifferentation:

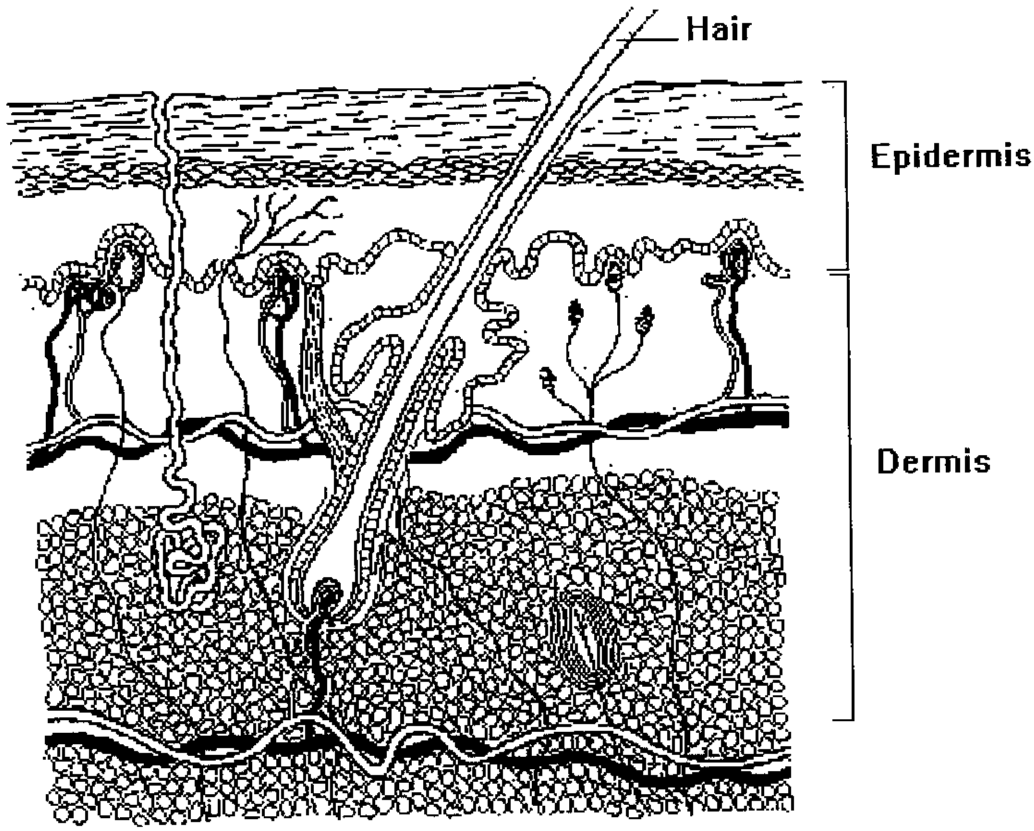
Transdifferentiation of differentiated tissue such as skin and oral mucosa has been employed as a method to generate corneal epithelium that is required for transplantation in bilateral LSCD. It has been observed that epidermal adult stem cells can be converted into corneal epithelium by corneal stroma. The use of human skin for transdifferentiation has also been studied by Huang *et al* (2004) and they concluded that corneal epithelium can be reconstructed from skin stem cell, which may be an alternative for constructing autogeneous bioengineered cornea.

Some of the most commonly studied sources are hair follicle stem cells, oral mucosa, conjunctiva and more recently, the epidermis of the skin. Skin has the advantage of being the largest and the most accessible organ of the human body and has been the obvious choice to attempt trandifferentiation for transplantation purposes. Yang *et al* (2008) reported considerable success in performing autologous transplantation to correct limbal deficiency by the use of epidermal adult stem cells. Human skin has also previously been employed to grow cornea with sufficient success having been reported.

2.6 Skin epithelial cells:

Skin is known to be the first line of defense to the body, much like the cornea acting as a physical barrier protecting the eye. The skin basically consists of a covering epithelium and an underlying connective tissue separated by a basal membrane (Arnold *et al*, 1990). The epithelium acts as a barrier against exogenous substances and pathogens and, mainly in skin, dehydration. The main cells are keratinocytes that are tightly attached to each other by desmosomes, and arranged in a number of distinct layers. The connective tissue supports and nourishes the epithelium, and connects it to the underlying structures (Liu *et al*, 2010). The layers of the skin are shown in Figure 2.4. Skin being a keratinocyte is therefore a good prospective for use as an alternative source of corneal epithelium.

Figure 2.4: Layers of the skin



2.7 Corneal epithelial markers and Stem cell markers:

2.7.1 ATP-binding cassette subfamily G, member 2 (ABCG2):

ATP-binding cassette subfamily G, member 2 (ABCG2), otherwise known as breast cancer resistance protein 1 (BCRP1), is a member of the Adenosine Tri Phosphate (ATP)-binding cassette (ABC) transporters. It has been identified as a molecular determinant for hematopoietic stem cells (HSCs), and has been proposed as a universal marker of stem cells. Chen et al 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. Other investigators have made similar observations. 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 (Chen *et al*, 2004).

2.7.2 p63:

p63 has recently been proposed as a keratinocyte stem cell marker. p63 is a transcription factor belonging to the p53 gene family. However, unlike p53, it is not a tumour suppressor gene.

The p63 gene shows remarkable structural similarity to the p53 and p73 genes. Because of two promoters, the p63 gene generates two types of protein isoforms, one with N terminal trans-activating domain TAp63 and one without Np63. Each type yields three isotypes because of differential splicing of the p63 COOH terminus. The isotypes of both p63 contain an extended COOH terminus with a conserved SAM domain implicated in protein-protein interactions (Di Iorio *et al*, 2005). In general, the TAp63 isotypes might behave like p53 because they transactivate various p53 downstream targets, induce apoptosis, and mediate cell cycle control. However, the Np63 isotypes have been shown to display opposing functions vis-a`-vis the TAp63 isotypes, including acting as oncoproteins . It has been found that p63 can regulate genes with diverse roles in cellular function and possesses opposing regulatory effects based on the expression of two main p63 isotypes.

p63 is normally expressed in the nuclei of keratinocytes with proliferative potential, including skin, cervix, prostate and cornea. It plays a key role in morphogenesis by regulating epithelial development and differentiation. 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. Pellegrini *et al* 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. 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. The authors concluded 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. Dua et al found that in addition to limbal basal cells, most of the basal cells of central cornea in adult humans also expressed p63. Dua *et al* (2000) concluded that p63 was too ubiquitous to be a stem cell marker in their work on human corneal epithelial cells expanded *ex vivo* (Davengar. M and Evansen A, 1971).

The expressions of TAp63 and Np63 transcripts appeared to be site specific. TAp63 was expressed at the highest level in limbus, decreased by approximately 10-fold in peripheral cornea and was undetectable in the central cornea. Np63 was also expressed at the highest level in limbus, decreased by approximately 35% in peripheral cornea, and was undetectable in the central cornea. Suppression of TAp63 expression inhibited limbal keratinocyte proliferation but promoted differentiation. Suppression of Np63 expression also inhibited cell proliferation but had no obvious effect on cell differentiation (Der-Yuan Wang *et al*, 2005).

2.7.3 Keratins:

K3 and K12 form a 'keratin pair' and are considered markers of corneal epithelial differentiation.

2.7.4 Connexin:

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. Connexin 43 is expressed by the corneal basal epithelium but not by limbal basal epithelium. This implies that Connexin 43 expression is acquired during the process of TAC differentiation. It has been suggested that the absence of intracellular communication helps to maintain a unique intracellular environment. The intracellular communication helps to maintain the stem cell niche, reflecting the need for LSCs to maintain a unique intracellular environment. The absence of gap junction proteins could also be a protective mechanism as it makes an individual stem cell less vulnerable to insults affecting its neighbor. 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. In contrast connexin50 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. Both proteins are considered useful markers of advanced corneal differentiation (Sheng Zhou *et al*, 2001).

2.7.5 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. A further study of the mouse cornea has shown that expression of integrin α9 is upregulated during wound healing. As the numbers of TACs are known to increase in response to stress, it seems clear that integrin α9 should be expressed on TACs. Furthermore, whole-mount preparations of murine cornea showed that the number of integrin α9-positive cells in the limbus exceed those generally accepted as indicative of a stem cell population. Together, these studies imply that the majority of integrin α9-positive cells in adult mice are TACs, although it does not rule out the possibility that murine LSCs also express the protein. In human corneas, Integrin α9 has been localized to a small subset of cells in the limbal basal epithelium. 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 (De Paiva *et al*, 2004).

2.8 Alternative sources for corneal growth:

The principle of transdifferentiation, which is the conversion of one differentiated cell type to another (Tosh and Slack, 2003), is currently employed as a means to combat the consequences of limbal stem cell deficiency. Reconstruction of the stratified ocular surface epithelium in patients with bilateral limbal stem cell deficiency is one of the most challenging problems in clinical ophthalmology. In order to replenish the stem cell pool, it is clearly desirable to use autologous cells for ex vivo culture, tissue engineering, and transplantation, as this avoids the risk of allogenic immune rejection and the need for immunosuppression. A major strategy is based on autologous stem cells taken from stratified epithelia of other areas of the body (Blazejewska *et al*, 2008). In the case of absence of limbus to grow cornea from, sources such as oral and nasal mucosa, conjunctiva, stroma and, more recently, skin have been studied. Schrader *et al* concluded that the advantages of autologous tissues such as conjunctiva and oral or nasal mucosa are evident, as they contain an appropriate matrix with epithelium and do not stimulate rejection. Blazejewska, Schlotzer-Schrehardt, Zenkel *et al* isolated adult epithelial stem cells from bulge region of hair follicle and cultured them in different conditioned media derived from central and peripheral corneal fibroblasts, limbal stromal fibroblasts, and 3T3 fibroblasts. They concluded that the HF may be an easily accessible alternative therapeutic source of autologous adult stem cells for replacement of the corneal epithelium and restoration of visual function in patients with ocular surface disorders. It has been suggested that subjecting hair follicle stem cells to a limbal microenvironment can induce differentiation to corneal epithelial tissues. Recent progress in this field suggests that oral mucosal epithelium (Nakamura *et al*, 2003; Nishida *et al*, 2004; Inatomi *et al*,2006), conjunctival epithelium (Tanioka *et al*, 2006; Ono *et al*,2007), and epidermis (Gao *et al*, 2007; Yang *et al*, 2007) may serve as alternative sources of autologous adult stem cells, which can be used to reconstruct the ocular surface in animal models and patients with limbal stem cell deficiency.

Hayashida *et al* demonstrated that autologous tissue-engineered cell sheets fabricated from oral mucosal epithelium are effective substitutes for allogeneic limbal tissues in ocular surface reconstruction. They also provide a compelling argument extolling the advantages of oral mucosa over other sources. Further, skin has also been widely studied as a source by several investigators. Yang *et al* concluded that that EpiASC repaired the damaged cornea of goats with total LSCD and demonstrated that EpiASC can be induced to differentiate into corneal epithelial cell types in vivo. Huang *et al* conducted similar studies using human and rabbit skin to rectify limbal deficiency in rabbits and achieved results that corroborate the fact that skin may be used as a viable source of corneal growth.

MATERIALS AND METHODS

3. MATERIALS AND METHODS:

3.1 Collection of samples:

The source to be used in order to generate corneal tissue was the epidermal layer of the skin. Biopsy of the skin tissue was done after obtaining informed consent of each of the donors. The biopsy was performed by the surgical staff of Sankara Nethralaya, Chennai and transferred to the research wing for further use.

Several skin samples were obtained from the forearm region from donors of diverse age groups. The samples and the corresponding donor description are provided (Table 3.1).

Table 3.1: The administration number, age, gender and medical details of the tissue biopsy donors

Sample	MRD No.	Age	Gender	Admitted for
I	999526	47	Male	Occuloplasty
II	767216	37	Male	Lymphangioma of the eyelid
III	1323-03	46	Male	Occuloplasty
IV	1068130	21	Male	Occuloplasty
V	1311011	26	Female	Occuloplasty
VI	1311004	17	Male	Occuloplasty
VII	2147890	20	Female	Occuloplasty

3.2 Transfer of skin samples:

The skin biopsy obtained from the donor was transferred to the lab in Dulbecco's Modified Eagle's Medium (DMEM) with 3% antibiotics and maintained at 4°C. The tissue is usually processed within 6 hours of excision in order to ensure maximum viability and growth.

3.3 Preparation of growth supplements and media for culture of epidermal cells:

The skin cells were cultured by two different procedures involving different media. The first process involved use of the skin growth medium whose composition is as follows: DMEM, 20 % FBS, 2ng/ml of EGF, 1 % Antibiotic – antimycotic solution. The second process used conditioned media as that was used for limbal epithelial culture which contained equal volume of Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 5μg/ml of transferrin, 5ng/ml of selenium, 5μg/ml of insulin, 2.5ng/ml EGF, 0.5mg/ml hydrocortisone, 2.5 μg/ml keratinocyte growth supplement, 10% FCS (fetal calf serum) antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin (Hi-media).

3.4 Processing of skin:

The processing of skin was done under sterile conditions. The biopsied tissue was transferred to a 35mm Petri plate and washed thoroughly with Phosphate buffered saline (PBS) twice. An antibiotic-antimycotic solution was also used for washing. The tissue was then washed with DMEM once and the solution was drained out. The epidermal layer of the skin was carefully separated from the other layers of the tissue and it was cut into multiple small pieces using sterile sharp curved scissors/Bard-Parker blade.

3.4.1 Explant culture in skin growth medium:

The explants were transferred to another Petri plate using sterile needles. The plate was then placed in a 5% CO_2 incubator at 37°C for five minutes to enable attachment. On removal from the incubator, the explants were flooded with 5ml of DMEM supplemented with 20% FBS. 100µl of antibiotic solution was also added and incubated in CO_2 incubator till confluent growth was achieved. The growth of the explants was checked using an inverted phase contrast microscope (Nikon, Tokyo, Japan) and the medium was changed every three days. Photographs of the stages of growth of the cells were taken and recorded.

3.4.2 Explant culture in corneal growth medium:

The procedure used for growth in skin medium was repeated. The medium added to the explants contained equal volume of DMEM and F12, 10% FBS, 50ng/mL of streptomycin, 1.25ng/mL of Amphotericin B, 2ng/ml of mouse epidermal growth factor (EGF), 5ng/mL of insulin, 5ng/mL of Transferrin, 5ng/mL of selenium, 5mg of Keratinocyte growth supplement and 0.5mg/mL of Hydrocortisone. Growth until confluency was observed and photographed with a regular change in medium every three days.

3.5 Trypsinisation of cells:

The medium was removed from the cultures at the end of 22 days and 1ml of 0.1% EDTA-Trypsin solution was added and incubated for 3 minutes at room temperature. The solution was then drained and the plates were tapped. Now the medium containing FBS was added. With the help of a cell scraper the cells were scraped, collected and centrifuged.

3.6 Viability assay:

Viability assays measure the percentage of a cell suspension that is viable. This is generally accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the coloring agent. The dye used for exclusion stain is usually trypan blue but erythrosin and naphthalene black have also been used. The trypan blue dye exclusion is commonly used and a protocol for this procedure is included here:

- Cell pellet obtained following trypsinisation was resuspended and 10µl of the same was used for the assay.

- A 1:1 dilution of the suspension using a 0.4% trypan blue solution was prepared.

- The counting chambers of a hemocytometer were loaded with 10 µl of the pellet suspension and 10 µl of trypan blue.

- The solution was allowed to sit for 1-2 minutes.

- The number of stained cells and total number of cells were counted manually.

- The calculated percentage of unstained cells represents the percentage of viable cells (Freshney, R.I., 1994).

3.7 RT- PCR analysis:

Total RNA was isolated from tissue and cells at the end of 22days of incubation to see the expression of different stem cell markers specific for corneal limbal stem cells by Reverse Transcriptase PCR (RT-PCR). At the end of 22 days of incubation the cells with the tissue bit was collected, and total RNA was isolated using Qiagen-kit according to the manufacturer's recommended protocol and the total RNA was stored at-80^0C before use. Along with housekeeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as internal control, mRNA expression of stem cell and corneal epithelial cell molecular markers viz. ABCG2, p63 and K3/K12 was studied.

RT-PCR was performed using sensiscript reverse transcriptases, which are recombinant heterodimeric enzymes. PCR amplification of the first – strand cDNAs were performed using specific primer pairs, designed from published human gene sequences for different markers in Eppendorf PCR systems. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide.

3.7.1 RNA isolation by Qiagen-Kit method

Protocol

- The cells were harvested from the cultures by trypsinisation and collected in a vial.
- The harvested cells were centrifuged at 10000rpm for 5-10 minutes and the supernatant discarded using a pipette.
- To the pellet 350μl of the RLT buffer reconstituted with β-mercaptoethanol and 350μl of 70% ethanol was added.

- It was mixed well with pipette and then added to the RNase mini column placed in 2ml collection tube
- Centrifuged for 30 sec at 10000rpm
- To the column 350µl of RW1 buffer (given in the kit) was added and centrifuged at 10,000rpm for 15 seconds.
- The flow through was discarded
- The spin column was transferred to a new collection tube. Then 500µl of RPE buffer (given in the kit) was added and centrifuged at 10,000rpm for 15 seconds.
- The flow through was discarded and the empty column was centrifuged at 14000rpm for 1 minute.
- The column was placed in a new DEPC treated Eppendorf and 10µl of RNase free water was added directly on to the membrane and centrifuged at 10,000rpm for1minute.
- 5µl of the total RNA extracted was run in 1% agarose gel to see the quality of RNA extracted and the rest was stored at -80^{0}C before use.

3.7.2 cDNA conversion:

cDNA conversion was performed on the RNA isolated from the cells using c-DNA conversion kit.

Reaction mixture:

dNTP	:	2.0µl
Oligo dT	:	2.0µl
RNase out	:	1.0µl
Sensiscript RT	:	1.0µl

MQ water	:	10.0μl
Template RNA	:	2.0μl

The above mixture was subjected to reverse transcriptase PCR and the product obtained was used in further analysis such as marker gene expression.

3.7.3 Polymerase Chain Reaction (PCR):

PCR reagents were prepared according to manufacturer's instruction.

Reaction mixture:

dNTP	:	2.5μl
FP	:	0.5μl
RP	:	0.5μl
Taq	:	0.3μl
10x buffer	:	2.5μl
MQ water	:	15.7μl
cDNA	:	3.0μl

The procedure was carried out to detect the expression of marker genes encoding for proteins such as ABCG2, p63, K3/K12 and GAPDH as internal control. The details are given below in Table 3.2.

Table 3.2: Primer sequence, annealing temperature and base pair size of the marker genes

Gene Name	Primer Sequence - 3'-5'	Annealing Temperature (^{0}C)	Base Pair Size (bp)
ABCG2	FP: AGTTCCATGGCACTGGCCATA RP: TCAGGTAGGCAATTGTGAAGG	62	379
Keratin 3	FP: GGCAGAGATCGAGGGTCTC RP: GTCATCCTTCGCCTGCTGTAG	64	145
Keratin 12	FP: CATGAAGAAGAACCACGAGGATG RP: TCTGCTCAGCGATGGTTTCA	63	150
p63	FP: CAGACTCAATTTAGTGAG RP: AGCTCATGGTTGGGGCAC	54	440
GAPDH	FP: GCCAAGGTCATCCATGACAAC RP: GTCCACCACCCTGTTGCTGTA	63	498

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION:

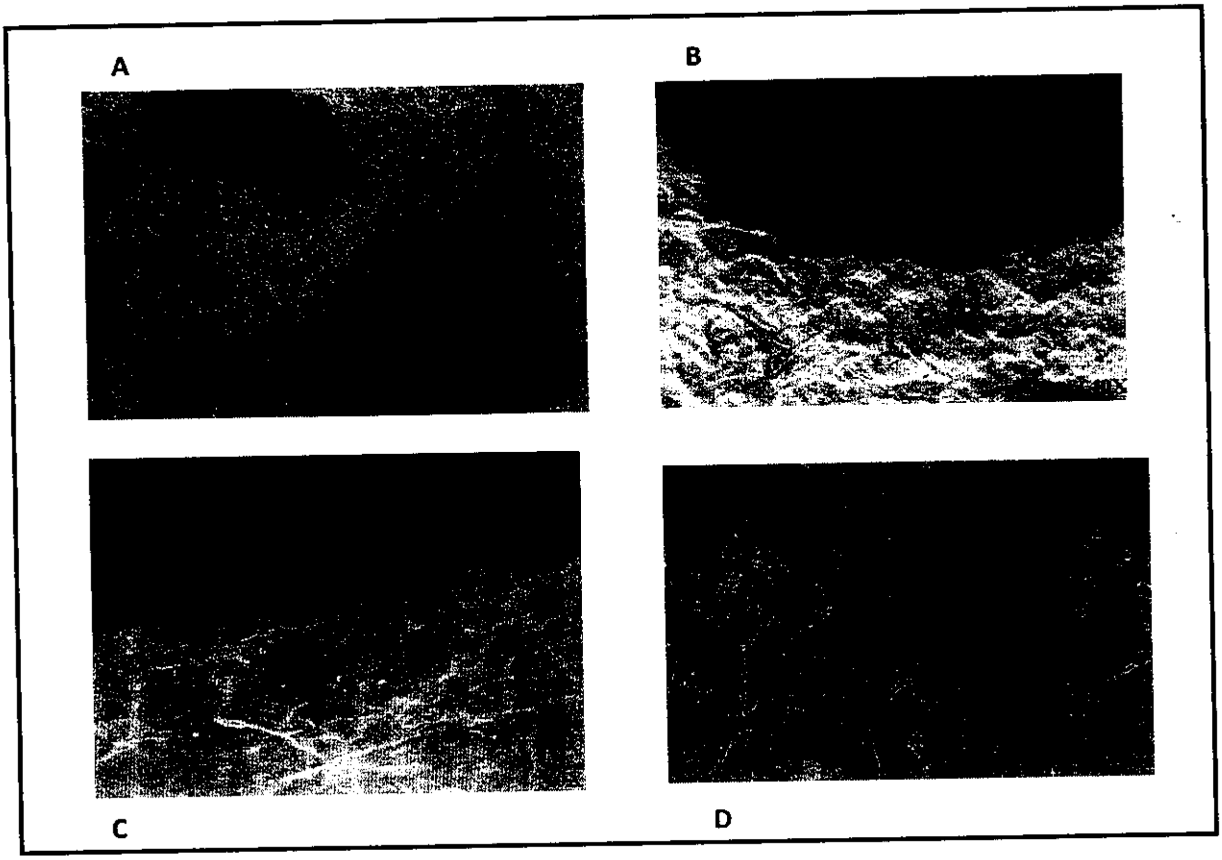
4.1 Tissue growth using skin medium:

Skin growth was standardized using DMEM medium supplemented with fetal bovine serum and EGF. No further supplements or growth factors were added and the growth was observed at regular intervals using phase contrast microscope. Pictures showing the growth of cells at different stages were taken and are presented in Figure 4.1.

It was observed that cell proliferation began at the outermost regions of the explants at the end of 24 hours. The cells increased in number and began extending in all directions as see by the picture taken after 48 hrs. Significant growth of cells was observed and the explants were seen to decolourise at the end of 72 hours. Confluent growth was achieved at the end of 15 days by which time, complete decolourisation was observed and the explants were no longer visible as separate entities.

Other media such as NCTC 168 have been employed (Price *et al*, 1979) and confluent growth has been obtained in 28 days. The use of DMEM is advantageous in comparison since the duration to achieve confluency is less. On the other hand, Yang *et al* (2008) employed Medium 199 for the culture of adult epidermal stem cells derived from skin and reported significantly better results.

Figure 4.1: The stages of growth of skin in DMEM medium



The figure depicts extent of growth of the cells after 24 hours (A), 48 hours (B), 72 hours (C) and 15 days (D)

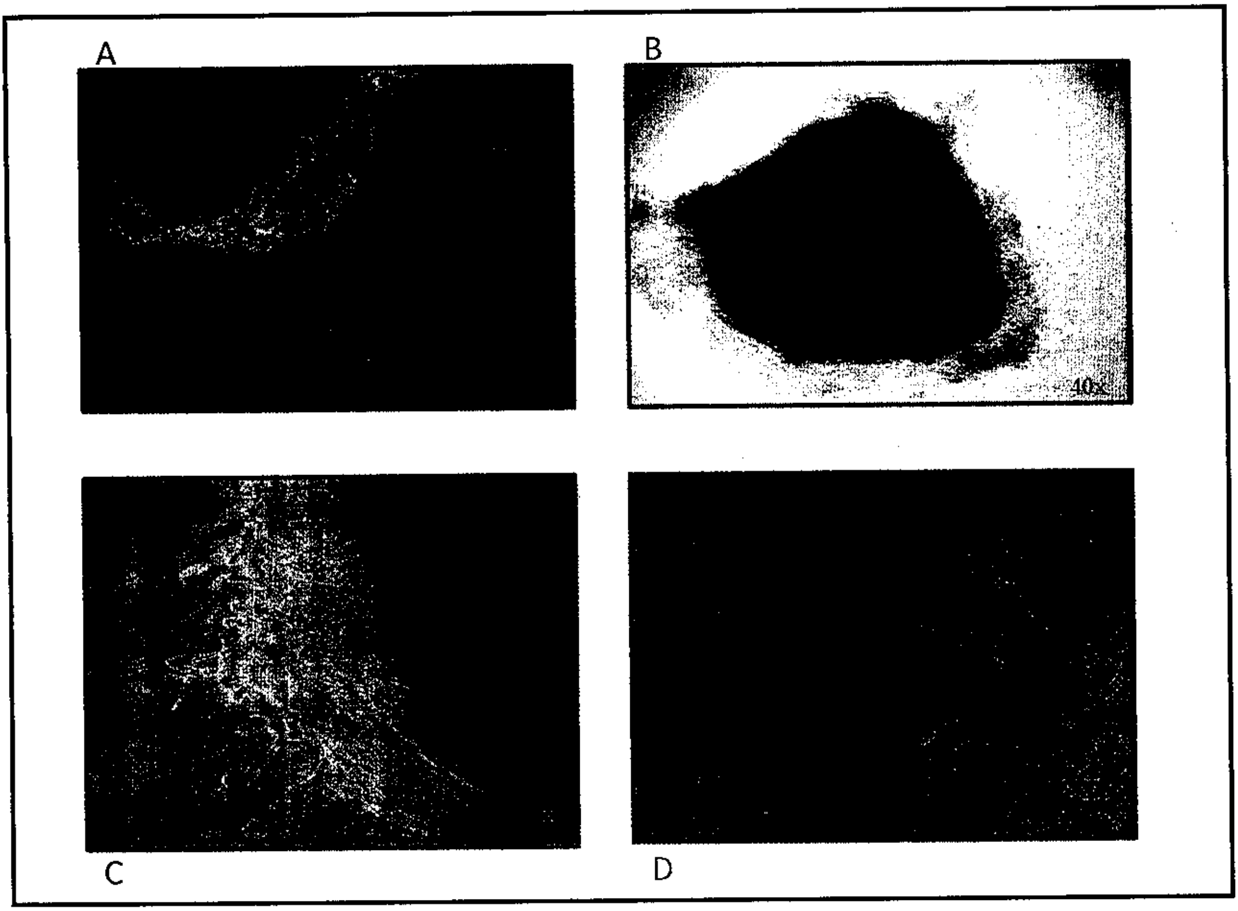
4.2 Tissue growth using corneal medium:

Following standardization of the growth of skin, fresh explants culture from the same subjects was carried out in corneal medium. The corneal medium contained several growth factors and additional supplements (as described in the materials section). Growth of the skin explants in corneal medium was photographed and was found to be satisfactory. The cells reached confluency in 22 days and the stages of their growth are presented in figure 4.2

The growth of explants followed a similar pattern in corneal medium as the skin growth medium. The explants displayed outgrowths at the end of 48 hours. At the end of four days significant growth of cells surrounding each explants was observed. Decolourisation and disintegration of explants began to occur at the end of 14 days and complete confluency was attained at the end of 22 days. The concoction of supplements is seen to provide the microenvironment observed in the cornea thus aiding the growth of explants.

Sudha *et al* (2008) employed the same combination of media supplements and reported satisfactory growth of limbus on feeder layer for the regeneration of cornea. This is supported by the results reported by Blazejewska *et al* (2008) suggesting that corneal microenvironment aids in the differentiation of stem cells into cornea.

Figure 4.2: The stages of growth of explants in corneal medium



The figure shows the growth of skin tissue in corneal growth medium after 48hrs (A), 4 days (B), 14 days (C) and 22 days (D).

4.3 Trypan blue assay:

The Trypan blue assay was carried out in order to estimate the extent of viable cells present in the culture. The process was performed at random intervals and the percentage of viability was measured. The number of viable cells obtained in the skin culture and in corneal culture, which was performed as a control, is listed in Table 4.1

The percentage of viable cells was calculated to determine the efficiency of the culture method. This was calculated using the formula:

$$\text{Percentage of viable cells} = \frac{\text{Total no. of viable cells per ml of aliquot}}{\text{Total no. of cells per ml of aliquot}} \times 100$$

The percentage of viable cells in both skin and cornea are listed in Table 4.2. Also, a graph was plotted to display the viability of the skin and cornea and are shown in figure 4.3 and figure 4.4.

Skin, being a more compact epithelium than the cornea, displays lower percentage of viability. Inatomi *et al* (2006) reported higher percentage of viability in oral mucosa when compared to corneal viability.

Table 4.1: Number of viable cells of skin and cornea

No. of Days	Number of viable cells	
	Skin	Cornea
2	54	57
6	220	237
10	410	534
16	480	680
22	670	766

Figure 4.3: A plot of the number of viable cells against the number of days of growth of skin tissue

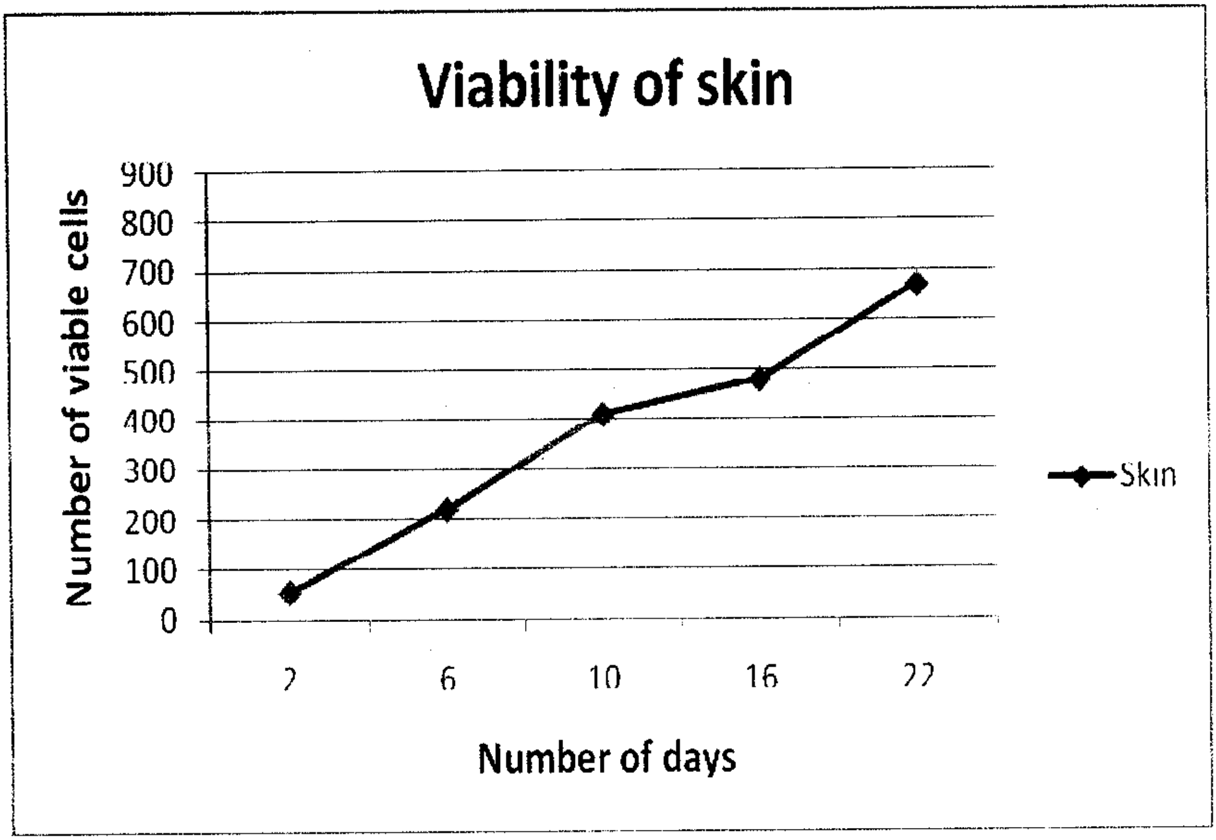


Figure 4.4: A plot of the number of viable cells of cornea grown from limbus for the duration of growth.

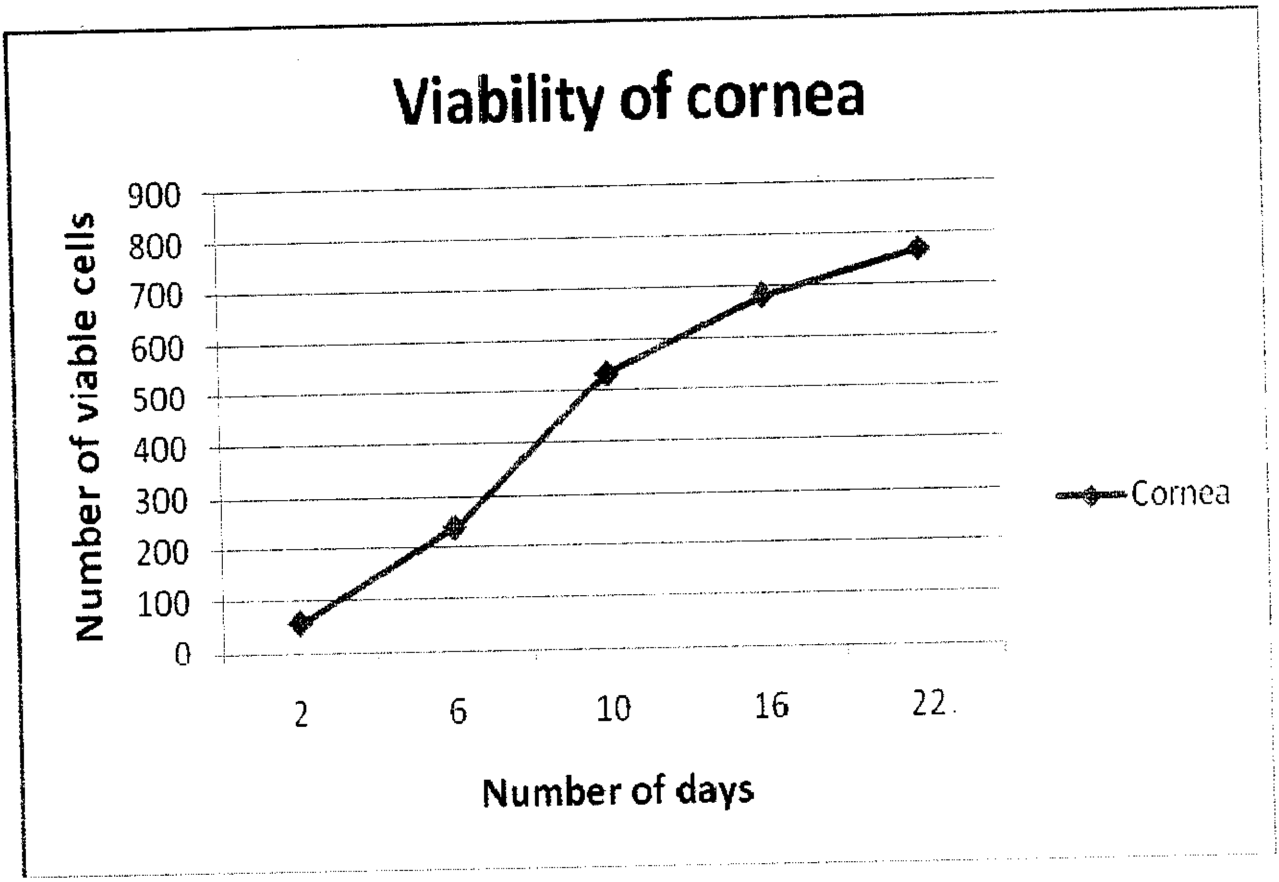


Table 4.2: Percentage of viable cells in skin and cornea

No. of Days	Percentage of viable cells	
	Skin	Cornea
2	10.70	10.90
6	32.02	32.55
10	52.23	63.57
16	65.63	72.26
22	70.29	74.51

4.4 RNA isolation:

RNA isolation from the cells was carried out in order to detect the presence of markers confirming the transdifferentiation of skin into cornea. RNA was extracted from the cells according to the procedure described. 5µl of the extracted RNA was used and run on a 1% agarose gel by electrophoresis. The picture taken of the gel observed under ultraviolet radiation is shown in figure 4.5.

4.5 Marker gene expression:

The study of marker genes was carried out following the conversion of RNA to cDNA by RT PCR. The cDNA obtained was analysed for the presence of markers such as ABCG2, p63 and K3/K12 with GAPDH as internal control.

The PCR products for GAPDH marker expression were analysed by electrophoresis on a 2% agarose gel. The gel pictures are shown in figure 4.6. GAPDH gene is constitutively expressed in many tissues and has been extensively reported to be a useful internal control in PCR product analyses. (http://www.ambion.com/techlib/tb/tb_151.html). It is highly useful as a confirmation of the conversion of RNA to cDNA.

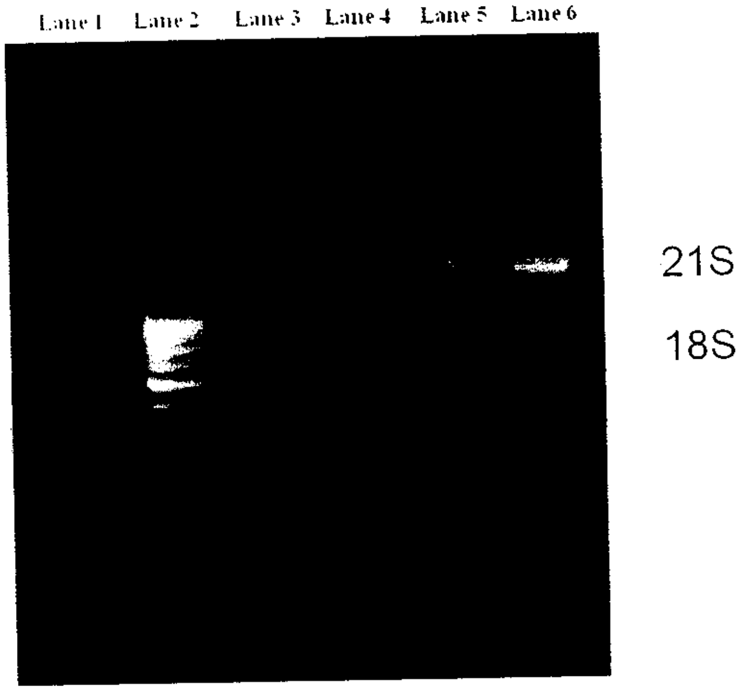
The cDNA obtained was analysed for ABCG2 expression which is a stem cell marker (Chen *et al*, 2004). The absence of the gene expressing ABCG2 in both cornea and the transifferentiated skin indicated that the cultured cells had been differentiated and did not possess the property of stemness. Balasubramanian *et al*(2008) obtained results suggesting the course taken during differentiation of limbus into cornea by reporting the presence of ABCG2 in the initial period of culture, when stemness could be observed, and its subsequent absence when differentiation was complete. The gel picture showing the absence of ABCG2 is given in figure 4.7.

The positive expression of p63 which is a keratinocyte marker, in the cultured skin tissue indicates that the skin transdifferentiated but retained its property of being a keratinocyte. Several isoforms of p63 have been studied and their presence in cornea has been reported (Balasubramanian *et al*, 2008). p63 has also been detected in limbal basal epithelium from which corneal regeneration takes place (Chen *et al*, 2004). The gel picture indicating the presence of p63 is shown in figure 4.8.

Corneal phenotype markers such as K3/K12 were detected in the differentiated tissue at the end of 22 days (Figure 4.9). These markers are exclusive to cornea and indicate the presence of corneal tissue. Similar results using K3 K12 as a marker have been reported by Balasubramanian *et al* (2008) and Yang *et* al (2008) confirming the conversion of skin into cornea.

Other markers such as integrin α, connexin 43 and the like have been used to study transdifferentiation. Markers such as PAX 6 have been reported to be upregulated and expressed in cultured corneal tissue (Yang *et al*, 2008)

Figure 4.5: Total RNA isolated on agarose gel



Lane 1: Empty lane

Lane 2: Ladder (100 bp)

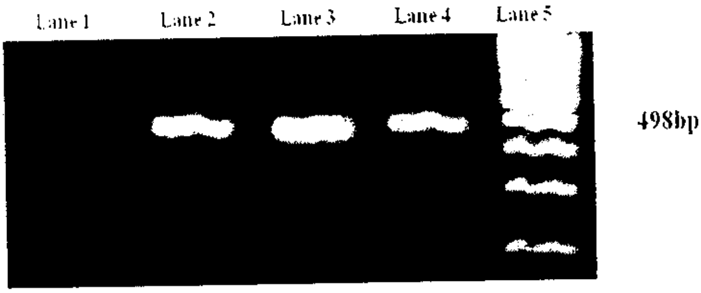
Lane 3: Negative control

Lane 4: Negative control

Lane 5: Transdifferentiated skin

Lane 6: Cornea

Figure 4.6: Agarose gel electrophoresis picture of GAPDH expression



Lane 1: Negative control

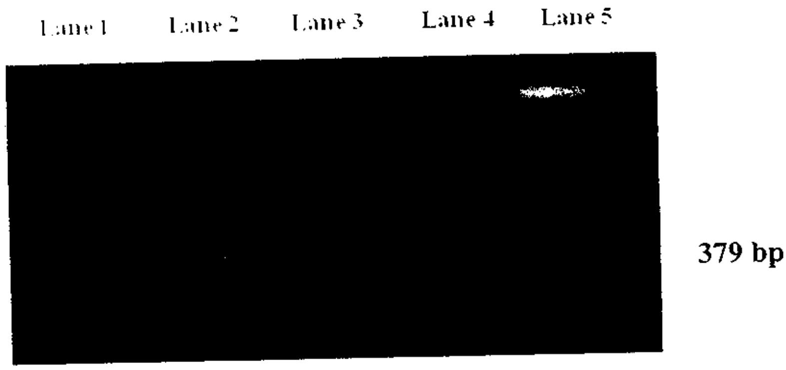
Lane 2: Positive control

Lane 3: Transdifferentiated skin

Lane 4: Cornea

Lane 5: Ladder (100 bp)

Figure 4.7: Agarose gel electrophoresis picture of ABCG2 expression



The lanes on the gel are as follows:

Lane 1: Negative control

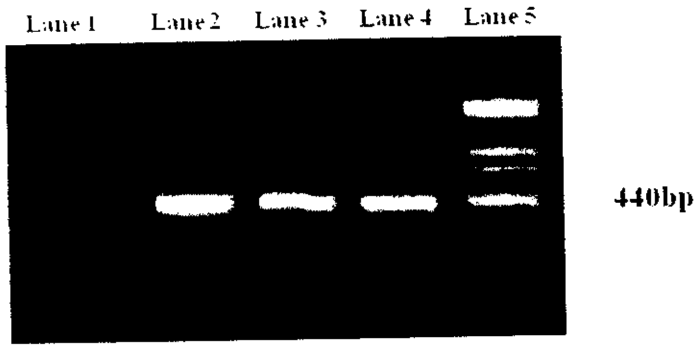
Lane 2: Positive control

Lane 3: Differentiated skin

Lane 4: Cornea

Lane 5: Ladder (100 bp)

Figure 4.8: Agarose gel electrophoresis picture of p63 expression



Lane 1: Negative control

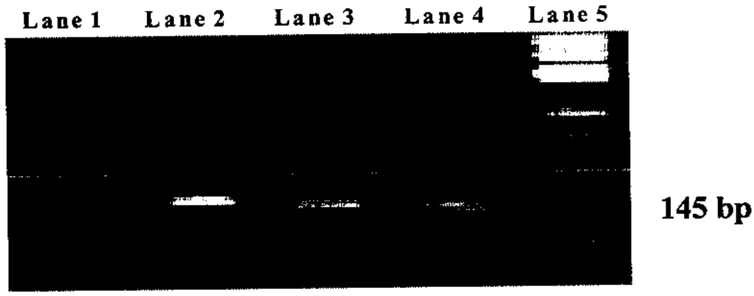
Lane 2: Positive control

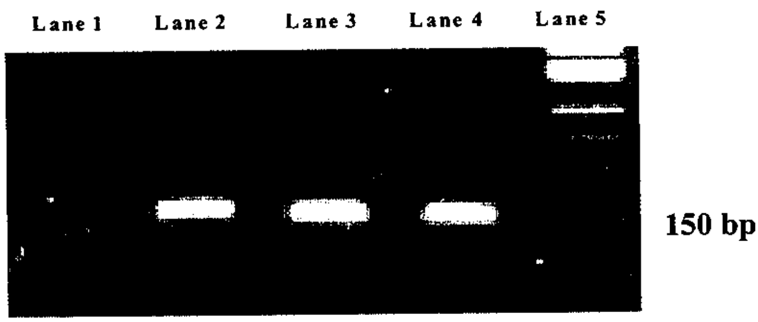
Lane 3: Transdifferentiated skin

Lane 4: Cornea

Lane 5: Ladder (100 bp)

Figure 4.9: Agarose gel electrophoresis picture of K3/K12 expression





Lane 1: Negative control

Lane 2: Positive control

Lane 3: Cornea

Lane 4: Transdifferentiated skin

Lane 5: Ladder (100 bp)

CONCLUSION

5. CONCLUSION:

Bilateral limbal stem cell deficiency is effectively treated by transplantation. For this purpose several sources of tissue, other than the limbus, which may be used for corneal growth have been studied. This study supports the use of skin as an alternative based on the following findings:

- Considerable viability was observed in the skin tissue that was grown in corneal medium and was comparable to the viability observed in corneal growth from limbus.
- The absence of the ABCG2 gene, which is considered a putative stem cell marker, indicated that the skin tissue had undergone transdifferentiation at the end of 22 days. ABCG2 expression occurs in a variety of normal tissues and is relatively limited to primitive stem cells.
- The gene encoding for the production of p63, which is a keratinocyte stem cell marker, was detected. It is known that p63 is a well recognized marker to determine the presence of keratinocyte stem cells and its presence in the cultured cells is indicative of the fact that differentiation from skin to cornea, which are both keratinocytes, through a phase of stemness occurs.
- The presence of the genes expressing the marker K3/K12 was observed. K3/K12 is a corneal phenotype marker whose expression confirms the presence of corneal cells. This indicates that the skin has undergone transdifferentiation expressing corneal phenotype.

Our study concludes that human skin is a viable alternative for the generation of corneal tissue. Other sources such as limbus and conjunctiva are crippled by the disadvantage that it is low in quantity and hence not as available as skin tissue. Skin has emerged a promising source for growth of corneal epithelium and its capacity to transdifferentiate opens up new avenues in transplantation research. Further study in this area is sure to throw more light on the use of skin in the treatment of limbal stem cell deficiency.

REFERENCES

7. REFERENCES:

- Arnold HL Jr. RB Odom and WD James, (1990). The skin: basic structure and function. Andrews Disease of the Skin, Elsevier, 1–13.

- Balasubramanian Sudha, Guruswamy Sitalakshmi, Geetha Krishnan Iyer & Subramanian Krishnakumar, (2008). Putative stem cell markers in limbal epithelial cells cultured on intact & denuded human amniotic membrane. Indian Journal of Medical Research 128 ; 149-156.

- Blazejewska EA, U S Tzer-Schrehardt, M Zenkel, B Bachmann, E Chankiewitz. C Jacobi and FE Kruse, (2008). Corneal Limbal Microenvironment Can Induce Transdifferentiation of Hair Follicle Stem Cells into Corneal Epithelial-like Cells. Stemcells, 27:642-652.

- Chen Z, CS de Paiva. L Luo. FL Kretzer, SC Pflugfelder and DQ Li, (2004). Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells, 22: 355–366.

- Cogan DG. T Kuwabara, DD Donaldson, E Collins, (1974). Microcystic dystrophy of the cornea. Archives of Ophthalmology, 92 (6): 470-474.

- Cotsarelis G. SZ Cheng, G Dong, T-T Sun and RM Lavker, (1989). .Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell, 57:201-209.

- Daniels JT. JK Dart. SJ Tuft and PT Khaw, (2001). Corneal stem cells in review. Wound Repair and Regeneration, 9(6):483-94.

- Davanger M and A Evensen, (1971). Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature, 229: 560-561.

- De Paiva CS. Z Chen, RM Corrales, SC Pflugfelder and D-Q Li, (2004). ABCG2 Transporter identifies a population of clonogenic human limbal epithelial cells. Stemcells, 23: 63 – 73.
- Dua HS, JS Saini, A Azuarza-Blanco and P Gupta, (2000). Limbal stem cell deficiency: Concept, aetiology, clinical presentation, diagnosis and management. Indian Journal of Ophthalmology, 48:83-92.
- Freshney RI. (1994). Culture of animal cells: A manual of basic techniques. 3 edition Wiley – Liss.
- Friedenwald JS. W Bushcke and ME Morris, (1944). Mitotic activity and wound healing in the corneal epithelium of vitamin A deficient rats. The Journal of Nutrition. 299-308.
- Gao N. Z Wang, B Huang, J Ge, R Lu, K Zhang, Z Fan , L Lu, Z Peng, G Cui, (2007). Putative epidermal stem cell convert into corneal epithelium-like cell under corneal tissue in vitro. Science China Life Sciences, 50:101–110.
- Goldberg MF and AJ Bron, (1982). Limbal palisades of Vogt. Transactions of the American Ophthalmology Society, 80:155-71.
- Gruterich M .EM Espana and SCG Tseng, (2003). Ex vivo expansion of limbal Epithelial stem cells-Amniotic membrane serving as a stem cell Niche. Survey Ophthalmology, 48 (6): 631-46.
- Hayashida Y, K Nishida, M Yamato, K Watanabe, N Maeda, H Watanabe, A Kikuchi, T Okano and Y Tano, (2005). Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. Investigative Ophthalmology & Visual Science, 46: 1632-1639.

- Hogan MJ, JA Alvarado, JE Weddell (1971). Histology of the human eye. Philadelphia: W.B. Saunders, 79-83.

- Huang B, ZC Wang, J Ge , XG Chen, JB Liu, ZG Fan, J Tao, BQ Liu and FF Guo, (2004). A pilot study on transdifferentiation of skin stem cell in reconstructing corneal epithelium. Zhonghua Yi Xue Za Zhi. 84(10):838-42.

- Inatomi T, T Nakamura, N Koizumi, C Sotozono, N Yokoi, S Kinoshita, (2006). Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. American Journal of Ophthalmology, 141:267–275.

- Koizumi N, LJ Cooper, NJ Fullwood, T Nakamura, K Inoki, M Tsuzuki and S Kinoshita, (2002). An Evaluation of Cultivated Corneal Limbal Epithelial Cells, Using Cell-Suspension Culture. Investigative Ophthalmology and Visual Science, 43:2114-2121.

- Li W, Y Hayashida, Y Chen and SCG Tseng, (2007). Niche regulation of corneal epithelial stem cells at the limbus. Cell Research , 17: 26–36.

- Liu J, Z Bian, AM Kuijpers-Jagtman and JW Von den Hoff, (2010). Skin and oral mucosa equivalents-construction and performance. Orthodontics and Craniofacial Research, 13:11–20.

- Maumenee AE (1964). Repair in cornea. Advanced Biology Skin 5, 208215.

- Michel M, N Török, M-J Godbout, M Lussier, P Gaudreau, A Royal and L Germain, (1996). Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. Journal of Cell Science, 109: 1017-1028.

- Nakamura T and S Kinoshita, (2003). Ocular surface reconstruction using cultivated mucosal epithelial stem cells. Cornea, 22(7): 75–80.
- Nishida K. M Yamato and Y Hayashida, (2004). Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. New England Journal of Medicine, 351:1187–1196.
- Ono K. S Yokoo, T Mimura et al, (2007). Autologous transplantation of conjunctival epithelial cells cultured on amniotic membrane in a rabbit model. Molecular Vision, 13:1138–1143.
- Pizzarello LD and FA Jakobiec, (1978). Bowman's disease of the conjunctiva. Ocular and adnexal tumors, 553-571.
- Puangsricharern V and SCG Tseng, (1995). Cytologic evidence of corneal diseases with limbal stem cell deficiency. Ophthalmology, 102:1476-1485.
- Schermer A. S Galvin, and T-T Sun, (1986). Differentiation-related Expression of a Major 64K Corneal Keratin In Vivo and In Culture Suggests Limbal Location of Corneal Epithelial Stem Cells. The Journal of Cell Biology, 103: 49-62.
- Schrader S. M Notara, M Beaconsfield, SJ Tuft, JT Daniels, and G Geerling, (2009). Tissue Engineering for Conjunctival Reconstruction: Established Methods and Future Outlooks. Current Eye Research, 34(11): 913–924.

- Schwab IR, M Reyes and RR Isseroff, (2000). Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea, 19:421-6.

- Sudha Balasubramanian, Srilatha Jasty, Guruswamy Sitalakshmi, H.N. Madhavan & Subramanian Krishnakumar, (2008). Influence of feeder layer on the expression of stem cell markers in cultured limbal corneal epithelial cells. Indian Journal of Medical Research 128; 616-622.

- Tanioka H, S Kawasaki, K Yamasaki, LPK Ang, N Koizumi, T Nakamura, N Yokoi, A Komuro, T Inatomi and Shigeru Kinoshita, (2006). Establishment of a cultivated human conjunctival epithelium as an alternative tissue source for autologous corneal epithelial transplantation. Investigative Ophthalmology and Visual Sciences, 47:3820–3827.

- Ti S-E, M Grueterich, E Espana, A Touhami, DF Anderson and SCG Tseng. (2004). Correlation of long term phenotypic and clinical outcomes following limbal epithelial transplantation cultivated on amniotic membrane in rabbits. British Journal of Ophthalmology, 88:422-427.

- Tosh D and JMW Slack, (2003). How cells change their phenotype. Nature Reviews Molecular Cell Biology, 3:187-194.

- Townsend WM, (1991). The limbal palisades of Vogt. Transactions of the American Ophthalmology Society, 89: 721 -56.

- Wang D-Y, C-C Cheng, M-H Kao, I-J Hsueh, DHK Ma and J-K Chen, (2005). Regulation of Limbal Keratinocyte Proliferation and Differentiation by TAp63 and Np63 Transcription Factors. Investigative Ophthalmology and Visual Science, 46:3102-3108.

- Yang X, L Qu, X Wang, M Zhao, W Li, J Hua, M Shi, N Moldovan, H Wang, Z Dou, (2007). Plasticity of epidermal adult stem cells derived from adult goat ear skin. Molecular Reproduction and Development, 74:386–396.
- Yang X, NI Moldovan, Q Zhao, S Mi, Z Zhou, D Chen, Z Gao, D Tong and Z Dou, (2008). Reconstruction of damaged cornea by autologous transplantation of epidermal adult stem cells. Molecular Vision, 14:1064-1074.
- Zhou S, JD Schuetz, KD Bunting, A Colapietro, J Sampath, JJ Morris, I Lagutina, GC Grosveld, M Osawa, H Nakauchi and BP Sorrentino, (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature Medicine, 7: 1028–34.

LIST OF WEBSITES

- http://en.wikipedia.org/wiki/Human_eye
- http://www.medhelp.org/gov/cornea.htm
- http://wuphysicians.wustl.edu/dept.aspx?pageID=17&ID=6
- http://www.lasikcomplications.com/images/CornealStructure.jpg
- http://www.medrounds.org/ocular-pathology-study-guide/2006/03/what-are-landmarks-of-limbus.html
- http://fc.units.it/ppb/neurobiol/Neuroscienze%20per%20tutti/skin.gif
- http://www.fishersci.com/wps/downloads/segment/Scientific/pdf/HyClone_Protocol_3.pdf
- http://en.wikipedia.org/wiki/Cornea
- http://www.ambion.com/techlib/tb/tb_151.html